

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

TEMPUS AI, INC.,
Petitioner,
v.

GUARDANT HEALTH, INC.,
Patent Owner.

Case IPR2025-01435

U.S. Patent No. **10,793,916**

**DECLARATION OF SYLVIA D. HALL-ELLIS, Ph.D.,
IN SUPPORT OF PETITION FOR *INTER PARTES* REVIEW
OF U.S. PATENT NO. 10,793,916**

I, Sylvia D. Hall-Ellis, declare as follows:

I. INTRODUCTION

1. My name is Sylvia D. Hall-Ellis. I have been retained by Tempus AI, Inc. (“Tempus” or “Petitioner”) as an expert in this proceeding before the United States Patent and Trademark Office (“PTO”) Patent Trial and Appeal Board (“Board”) regarding U.S. Patent No. 10,793,916 (“the ’916 Patent”) (the “Challenged Patent”).

2. I have written this Declaration at the request of Petitioner to provide my expert opinion regarding the authenticity and public availability of several publications relevant to the concurrently filed petition for *inter partes* review of the ’916 Patent. This Declaration sets forth my opinions in detail and provides the basis for my opinions.

3. I reserve the right to supplement or amend my opinions, and bases for them, in response to any additional evidence, testimony, discovery, argument, and/or other additional information that may be provided to me after the date of this declaration.

4. I am being compensated for my time spent working on this matter at my normal consulting rate, plus reimbursement for any additional reasonable expenses. My compensation is not in any way tied to the content of this declaration,

the substance of my opinions, or the outcome of this proceeding. I have no other interests in this proceeding or with any of the parties.

5. All of the materials that I considered and relied upon are discussed explicitly in this declaration.

II. QUALIFICATIONS

6. I am currently an Adjunct Professor in the School of Information at San José State University in San José, California. I obtained a Master of Library Science from the University of North Texas in 1972 and a Ph.D. in Library and Information Science from the University of Pittsburgh in 1985. Over the last sixty years, I have held various positions in the field of library and information resources. I was first employed as a librarian in 1966 and have been involved in the field of library sciences since, holding numerous positions.

7. I am a member of the American Library Association (ALA) and its Association for Library Collections & Technical Services (ALCTS) Division, and I served on the Committee on Cataloging: Resource and Description (which wrote the new cataloging rules) and as the chair of the Committee for Education and Training of Catalogers and the Competencies and Education for a Career in Cataloging Interest Group. I also served as the founding Chair of the ALCTS Division's Task Force on Competencies and Education for a Career in Cataloging. Additionally, I have served as the Chair for the ALA Office of Diversity's Committee on Diversity,

as a member of the REFORMA National Board of Directors, as a member of the Editorial Board for the ALCTS premier cataloging journal, *Library Resources and Technical Services*, and as a Co-Chair for the Library Research Round Table of the American Library Association. Currently I serve as a member of the CORE Margaret Mann Citation Committee and the CORE Awards & Scholarships Coordination Committee.

8. I have also given over one hundred presentations in the field, including several on library cataloging systems and Machine-Readable Cataloging (“MARC”) standards. My current research interests include library cataloging systems, metadata, and organization of electronic resources.

9. I have been deposed thirty-nine times: (1) *Symantec Corp. v. Finjan, Inc.*, Petition for *Inter Partes Review* of U.S. Patent No. 7,613,926, IPR 2015-01892, May 26, 2016, on behalf of Symantec Corp.; (2) *Symantec Corp. v. Finjan, Inc.*, 14-cv-299-HSG (N.D. Cal.), on behalf of Symantec Corp., September 14, 2017; (3) one deposition for ten matters: *Intellectual Ventures I LLC v. AT&T Mobility LLC*; *AT&T Mobility II LLC*, New Cingular Wireless Services, Inc., SBC Internet Services, Inc., Wayport, Inc., and Cricket Wireless LLC, C.A. No. 12-193 (LPS); *Intellectual Ventures II LLC v. AT&T Mobility LLC*; *AT&T Mobility II LLC*, New Cingular Wireless Services, Inc., SBC Internet Services, Inc., Wayport, Inc., and Cricket Wireless LLC, C.A. No. 13-1631 (LPS);

Intellectual Ventures I LLC v. T-Mobile USA, Inc. and T-Mobile US, Inc., C.A. No. 13-1632 (LPS); *Intellectual Ventures II LLC v. T-Mobile USA, Inc. and T-Mobile US, Inc.*, C.A. No. 13-1633 (LPS); *Intellectual Ventures I LLC, v. Nextel Operations, Inc., Sprint Spectrum L.P., Boost Mobile, LLC and Virgin Mobile USA, L.P.*, C.A. No. 13-1634 (LPS); *Intellectual Ventures II LLC v. Nextel Operations, Inc., Sprint Spectrum L.P., Boost Mobile, LLC and Virgin Mobile USA, L.P.*, C.A. No. 13-1635 (LPS); *Intellectual Ventures I LLC, v. United States Cellular Corporation*, C.A. No. 13-1636 (LPS); *Intellectual Ventures I LLC v. United States Cellular Corporation*, C.A. No. 13-1637 (LPS); *Intellectual Ventures II LLC v. AT&T Mobility LLC, AT&T Mobility II LLC, New Cingular Wireless Services, Inc.*, C.A. No. 15-799 (LPS); *Intellectual Ventures I LLC v. T-Mobile USA, Inc. and T-Mobile US, Inc.*, C.A. No. 15-800 (LPS), on behalf of AT&T Mobility LLC; AT&T Mobility II LLC, Boost Mobile, LLC Cricket Wireless LLC, Nextel Operations, Inc., New Cingular Wireless Services, Inc., SBC Internet Services, Inc., Sprint Spectrum L.P., T-Mobile USA, Inc., T-Mobile US, Inc., United States Cellular Corporation Virgin Mobile USA, L.P., and Wayport, Inc., November 15, 2016; (4) *Hitachi Maxell, LTD., v. Top Victory Electronics (Taiwan) Co. Ltd., et al.*, 2:14-cv-1121 JRG-RSP (E.D. Texas), on behalf of Top Victory Electronics (Taiwan) Co. LTD, *et. al.*, January 20, 2016; (5) *Sprint Spectrum, L.P. v. General Access Solutions, Ltd.*, Petition for *Inter Partes Review* of U.S. Patent No. 7,173,916, IPR 2017-01885, on behalf of

Sprint Spectrum L.P., July 13, 2018; (6) *Nichia Corporation v. Vizio, Inc.*, 8:16-cv-00545; on behalf of Vizio, Inc., October 12, 2018; (7) *Intellectual Ventures I LLC, v. T-Mobile USA, Inc., T-Mobile US, Inc., Ericsson Inc., and Telefonaktiebolaget LM Ericsson*, 2:17-cv-00557 (JRG), on behalf of T-Mobile USA, Inc., T-Mobile US, Inc., Ericsson Inc., and Telefonaktiebolaget LM Ericsson, October 19, 2018; (8) *Pfizer, Inc. v. Biogen, Inc.*, Petition for *Inter Partes Review* of U.S. Patent No. 8,821,873, IPR 2017-01168, on behalf of Pfizer, November 3, 2018; (9) *Finjan, Inc. v. ESET, LLC and ESET SPOL. S.R.O.*, 3:17-cv-00183-CAB-BGS, on behalf of ESET, January 15, 2019; (10) *Finjan, Inc. v. Cisco Systems, Inc.*, 5:17-cv-00072-BLF-SVK, on behalf of Cisco Systems, Inc., September 6, 2019; (11) *Facebook, Inc., Instagram, LLC and Whatsapp Inc. v. Blackberry Limited* for *Inter Partes Review* of U.S. Patent No. 9,349,120 B2, on behalf of Facebook, Inc., Instagram, LLC and Whatsapp Inc., December 20, 2019; (12) *3Shape A/S and 3Shape Inc. v. Align Technology, Inc.*, Petition for *Inter Partes Review* of U.S. Patent No. 7,156,661, IPR 2020-00222 and IPR 2020-00223, August 10, 2020, on behalf of 3Shape A/S and 3Shape Inc.; (13) *Finjan Inc. v. Rapid7, Inc. and Rapid7 LLC*, District of Delaware, 1:18-cv-01519-MN, September 15, 2020, on behalf of Rapid7 Inc. and Rapid7 LLC; (14) *VLSI Technology LLC v. Intel Corporation*, Western District of Texas, 6:19-cv-00254, 6:19-cv-00255, 6:19-cv-00256, September 23, 2020, on behalf of the Intel

Corporation; (15) *Finjan Inc. v. Sonicwall, Inc.*, Northern District of California, 5:17-cv-04467-BLF-HRL, October 27, 2020, on behalf of Sonicwall, Inc.; (16) *VLSI Technology, LLC v. Intel Corporation*, District of Delaware, 1:18-cv-00966-CFC-CJB, February 5, 2021, on behalf of the Intel Corporation; (17) *Unified Patents, LLC v. 3BCOM, LLC*, Petition for *Inter Partes Review* of U.S. Patent 7,127,210, February 11, 2021, on behalf of Unified Patents; (18) *Finjan, Inc. v. Qualsys, Inc.*, Northern District of California, 4:18-cv-07229-YGR, March 1, 2021, on behalf of Qualsys, Inc.; (19) *Qualcomm, Inc. v. Monterey Research LLC*, Petition for *Inter Partes Review* of U.S. Patent 6,534,805, May 6, 2021, on behalf of Qualcomm, Inc.; (20) *Hulu, LLC v. Sound View Innovations, LLC*, Petition for *Inter Partes Review* of U.S. Patent 5,806,062, May 14, 2021, on behalf of Hulu, LLC; and, (21) *VLSI Technology LLC v. Intel Corporation*, Western District of Texas, 6:19-cv-00254, 6:19-cv-00255 and 6:19-cv-00256, August 3, 2021, on behalf of Intel Corporation; (22) *Liquidia Technologies, Inc. v. United Therapeutics Corporation*, Petition for *Inter Partes Review* of U.S. Patent 10,716,793 B2, October 20, 2021, on behalf of Liquidia Technologies, Inc.; (23) *EcoFactor, Inc. v. Google, Inc.*, Western District of Texas, 6:20-cv-00075 (ADA), 6:20-0078-ADA, and 6:20-cv-00080 ADA, October 27, 2021, on behalf of Google, Inc.; (24) *Liquidia Technologies, Inc. v. United Therapeutics Corporation*, Petition for *Inter Partes Review* of U.S. Patent 10,716,793 B2, March

11, 2022, on behalf of Liquidia Technologies, Inc.; (25) *Juniper Networks, Inc. v. Swarm Technology LLC*, Petition for *Inter Partes* Review of U.S. Patent 9,852,004, May 5, 2022, on behalf of Juniper Networks, Inc.; and, (26) *ParkerVision, Inc. v. Intel Corporation*, Western District of Texas, 6:20-cv-108 (ADA), October 26, 2022, on behalf of the Intel Corporation; (27) *CommScope v. TQ Delta*, Petition for *Inter Partes* Review of U. S. Patents 9,094,348 and 10,833,809, February 16, 2023, on behalf of CommScope; (28) *WSOU Investments, LLC d/b/a Brazos Licensing and Development v. Salesforce, Inc.*, Western District of Texas, 6:20-cv-01164-ADA, April 18, 2023, on behalf of Salesforce, Inc.; (29) *Regents of the University of Minnesota v. AT&T Mobility LLC*, District of Minnesota, 14:cv-4999-JRT-TNL & 14-cv-4669-JRT-TNL, May 18, 2023, on behalf of AT & T Mobility LLC; (30) *VLSI Technology LLC v. Intel Corporation*, Northern District of California, 5:17-cv-0571-BLF-NC, July 11, 2023, on behalf of Intel Corporation; (31) *Trustees of Purdue University v. ST Microelectronics*, Western District of Texas, 6:21-cv-00727-ADA, July 27, 2023, on behalf of ST Microelectronics; (32) *Advanced Micro Devices, Inc. v. Realtek Semiconductor Corp.*, Petition for *Inter Partes* Review of U. S. Patents 7,936,245 & 9,590,582, August 10, 2023, on behalf of Advanced Micro Devices, Inc.; (33) *Motif Foodworks, Inc. v. Impossible Foods, Inc.*, Petition for *Inter Partes* Review of U. S. Patent 9,943,096, August 24, 2023, on behalf of Motif Foodworks, Inc.; (34) *Network System Technologies, LLC v.*

Texas Instruments, Inc., Eastern District of Texas, 2:22-cv-00482, March 20, 2024, on behalf of Texas Instruments, Inc.; (35) *Network System Technologies, LLC v. Texas Instruments, Inc.*, Eastern District of Texas, 2:22-cv-00482, May 22, 2024, on behalf of Texas Instruments, Inc.; (36) *Pardalis Technology Licensing, LLC v. International Business Machines, Corporation*, Eastern District of Texas, 2:22-cv-00452, May 23, 2024, on behalf of International Business Machines, Corporation; (37) *Meta Platforms, InstaGram, Inc., Whatsapp LLC, Facebook Technologies LLC, and Giphy, Inc. v. VL Collective IP LLC*, Petition for *Inter Partes Review* of U. S. Patent 7,436,980, July 18, 2024, on behalf of Meta Platforms, InstaGram, Inc., Whatsapp LLC, Facebook Technologies LLC, and Giphy, Inc.; (38) *LCY Biotechnology Holding, Inc. v. RadiciChimica S.p.A.*, Petition for *Inter Partes Review* of U. S. Patent 11,781,148, August 6, 2024, on behalf of LCY Biotechnology Holding, Inc.; and, (39) *SAP America, Inc. v. Valtrus Innovations Ltd., Key Patent Innovations Ltd., and Patent Platform Services, LLC*, Petition for *Inter Partes Review* of U. S. Patent 7,313,575, June 18, 2025, on behalf of SAP America, Inc.

10. My full curriculum vitae is provided as Attachment A to this declaration.

III. PRELIMINARIES

A. Scope of Declaration and Legal Standards

11. I am not an attorney and will not offer opinions on the law. I am, however, rendering my expert opinion on the authenticity of the documents referenced herein and on when and how each of these documents was disseminated or otherwise made available to the extent that persons interested and ordinarily skilled in the subject matter or art, exercising ordinary diligence, could have located the documents before September 4, 2012.

12. I am informed by counsel that a printed publication qualifies as publicly accessible as of the date it was disseminated or otherwise made available such that a person interested in and ordinarily skilled in the relevant subject matter could locate it through the exercise of ordinary diligence.

13. While I understand that the determination of public accessibility under the foregoing standard rests on a case-by-case analysis of the facts particular to an individual publication, I also understand that a printed publication is rendered “publicly accessible” if it is cataloged and indexed by a library such that a person interested in the relevant subject matter could locate it (*i.e.*, I understand that cataloging and indexing by a library is sufficient, though there are other ways that a printed publication may qualify as publicly accessible). One manner of sufficient indexing is indexing according to subject matter category. I understand that the

cataloging and indexing by a single library of a single instance of a particular printed publication is sufficient, even if the single library is in a foreign country. I understand that, even if access to a library is restricted, a printed publication that has been cataloged and indexed therein is publicly accessible so long as a presumption is raised that the portion of the public concerned with the relevant subject matter would know of the printed publication. I also understand that the cataloging and indexing of information that would guide a person interested in the relevant subject matter to the printed publication, such as the cataloging and indexing of an abstract for the printed publication, is sufficient to render the printed publication publicly accessible.

14. I understand that routine business practices, such as general library cataloging and indexing practices, can be used to establish an approximate date on which a printed publication became publicly accessible.

B. Persons of ordinary skill in the art.

15. I am told by counsel that the subject matter of this proceeding relates to DNA sequencing technology.

16. I have been informed by counsel that a “person of ordinary skill in the art at the time of the invention” (“POSITA”) is a hypothetical person who is presumed to be familiar with the relevant field and its literature at the time of the inventions. This hypothetical person is also a person of ordinary creativity, capable of understanding the scientific principles applicable to the pertinent field.

17. I am told by counsel that persons of ordinary skill in this subject matter or art would have a Ph.D. in bioinformatics, genetics, molecular biology or a related field, and at least five years of research in an academic or industry setting, including at least two to three years of research experience in the field of cancer genomics. I am also told by counsel that persons of ordinary skill in this subject matter or art would have had knowledge of DNA sequencing, including NGS and related sequencing methods, and related sample preparation techniques; bioinformatics methods for analyzing sequence reads and mapping sequence reads onto genomes; and methods for identifying genetic variants in a sample. I have been further informed by counsel that a person of ordinary skill in the art would have been familiar with and able to understand the information known in the art relating to these fields, including the publications discussed in this declaration.

18. It is my opinion that such a person would have been engaged in research, learning, study, and practice in the field, and possibly formal instruction so that bibliographic resources relevant to his or her research would be familiar. By not later than early 2012, such a person would have had access to a vast array of long-established print resources as well as to a rich set of online resources providing indexing information, abstracts, and full text services for publications relevant to the field of this dispute.

IV. LIBRARY PROFESSIONAL PRACTICES

19. In preparing this declaration, I used authoritative databases, such as the OCLC bibliographic database, the Library of Congress Online Catalog, and the Library of Congress Subject Authorities, to confirm citation details of the various publications discussed. Unless I note otherwise below in reference to a specific serial publication, it is my expert opinion that this standard protocol was followed for the serial publication discussed below.

20. *Indexing.* A researcher may discover material relevant to his or her topic in a variety of ways. One common means of discovery is to search for relevant information in an index of periodicals and other publications. Having found relevant material, the researcher will then normally obtain it online, look for it in libraries, or purchase it from the publisher, a bookstore, a document delivery service, or other provider. Sometimes, the date of a document's public accessibility will involve both indexing and library date information. However, date information for indexing entries is often unavailable. This is especially true for online indices.

21. Indexing services use a wide variety of controlled vocabularies to provide subject access and other means of discovering the content of documents. The Library of Congress Subject Authorities includes standard forms of terms and cross references that are included in bibliographic records. Subject headings are terms that an individual seeking a document regardless of format can enter in the

search bar of the online catalog. Subjects also connect an authenticated term (one included in the Library of Congress subject headings list) with related, broader, and narrower terms. The formats in which these access terms are presented vary from service to service.

22. Online indexing services commonly provide bibliographic information, abstracts, and full-text copies of the indexed publications, along with a list of the documents cited in the indexed publication. These services also often provide lists of publications that cite a given document. A citation of a document is evidence that the document was publicly available and in use by researchers no later than the publication date of the citing document. Prominent indexing services include *Scopus*, the *IEEE Xplore* database, the *ACM Digital Library*, *Google Scholar*, and the *Internet Archive*.

23. A major firm known for the breadth of subjects and comprehensive treatment in the preparation of index volumes, the H. W. Wilson Company offered these reference resources since the firm was founded in 1898. The *Reader's Guide to Periodical Literature* is one of the best-known titles available from H. W. Wilson. Each volume includes a comprehensive index for 300 of the most popular and important periodicals published in the United States and Canada. Information seekers have subject access expressed in plain language terminology, author access, and cross references to find the desired results from their searches. The family of

index titles included *Science & Technology Index*, *Business Periodicals*, *Applied Science & Technology Index*, *Humanities Index*, *Biological & Agricultural Index*, and *Industrial Arts Index*. These printed indices have been superseded by digital database offerings available to information seekers through Ebsco.

24. Information seekers also used printed versions of *Chemical Abstracts* and *Index Medicus* to locate articles, scientific declarations, and research papers. *Chemical Abstracts* began publication in 1907 and by 2007 its databases “contained more than 27 million records of journal and patent literature.”¹ In 2010, *Chemical Abstracts* discontinued the print index. Access is now available through two electronic databases: *CAplus* and *Registry*.² *Index Medicus* is a bibliographic index to medical science information, started in 1879.³ Currently, *PubMed* includes

¹ Chemical Abstracts Service, “CAS History,” *available at* <https://www.cas.org/about/cas-history> (last accessed August 12, 2025); American Chemical Society, “Chemical Abstracts Service,” *available at* <https://www.acs.org/content/acs/en/education/whatischemistry/landmarks/cas.html> (last accessed August 12, 2025).

² American Chemical Society, “Chemical Abstracts Service,” *available at* <https://www.acs.org/content/acs/en/education/whatischemistry/landmarks/cas.html> (last accessed August 12, 2025).

³ Stephen Greenberg and Patricia Gallagher, “The great contribution: *Index Medicus*, *Index-Catalogue*, and *IndexCast*,” *Journal of Medical Library Association*, April 2009, available at

the content that had been published as *Index Medicus*.⁴

25. Established in 1836 as part of the Surgeon General of the Army's Office, the National Library of Medicine (NLM) has been instrumental in the development of access to medical books, journals, and research publications. In the 1970s, NLM introduced MEDLINE, followed by "the establishment of the National Center for Biotechnology Information in 1988, the introduction of free MEDLINE in 1997, the creation of consumer-friendly MedlinePlus in 1998, and the introduction of ClinicalTrials.gov in 2000."⁵

26. Online indexing services such as Google Scholar⁶ or ScienceDirect⁷ commonly provide bibliographic information, abstracts, and full-text copies of the indexed publications, along with a list of the documents cited in the indexed publication. These services also often provide lists of publications that cite a given

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2670211/> (last accessed August 12, 2025).

⁴ U.S. National Library of Medicine, "List of All Journals Cited in PubMed®," https://www.nlm.nih.gov/bsd/serfile_addedinfo.html (last accessed August 12, 2025).

⁵ U.S. National Library of Medicine, "A Brief History of NLM," available at <https://www.nlm.nih.gov/about/briefhistory.html>.

⁶ <https://scholar.google.com/>.

⁷ <https://www.sciencedirect.com/>.

document.

27. A citation of a document by another is evidence that the document was publicly available and in use no later than the publication date of the citing document.

A. MARC RECORDS AND THE ONLINE LIBRARY CATALOG

28. I am fully familiar with the library cataloging standard known as the MARC standard, which is an industry-wide standard method of storing and organizing library catalog information.⁸ MARC was first developed in the 1960's by the Library of Congress. A MARC-compatible library is one that has a catalog consisting of individual MARC records for each of its items. Today, MARC is the primary communications protocol for the transfer and storage of bibliographic metadata in libraries.⁹

⁸ The full text of the standard is available from the Library of Congress at <http://www.loc.gov/marc/bibliographic/> (last visited August 12, 2025).

⁹ Almost every major library in the world is MARC-compatible. *See, e.g., MARC Frequently Asked Questions (FAQ)*, Library of Congress, <https://www.loc.gov/marc/faq.html> (last visited August 21, 2025) (“MARC is the acronym for Machine-Readable Cataloging. It defines a data format that emerged from a Library of Congress-led initiative that began nearly fifty years ago. It provides the mechanism by which computers exchange, use, and interpret bibliographic information, and its data elements make up the foundation of most

29. Since at least the early 1970s and continuing to the present day, MARC has been the primary communications protocol for the transfer and storage of bibliographic metadata in libraries.¹⁰ As explained by the Library of Congress:

You could devise your own method of organizing the bibliographic information, but you would be isolating your library, limiting its options, and creating much more work for yourself. Using the MARC standard prevents duplication of work and allows libraries to better share bibliographic resources. Choosing to use MARC enables libraries to acquire cataloging data that is predictable and reliable. If a library were to develop a “home-grown” system that did not use MARC records, it would not be taking advantage of an industry-wide standard whose primary purpose is to foster communication of information.

Using the MARC standard also enables libraries to make use of commercially available library automation systems to manage library operations. Many systems are available for libraries of all sizes and are designed to work with the MARC format. Systems are maintained and improved by the vendor so that libraries can benefit from the latest

library catalogs used today.”). MARC is the ANSI/NISO Z39.2-1994 (reaffirmed 2016) standard for Information Interchange Format.

¹⁰ A complete history of the development of MARC can be found in *MARC: Its History and Implications* by Henrietta D. Avram (Washington, DC: Library of Congress, 1975) and available online from the Hathi Trust (<https://babel.hathitrust.org/cgi/pt?id=mdp.39015034388556;view=1up;seq=1>; last visited August 12, 2025).

advances in computer technology. The MARC standard also allows libraries to replace one system with another with the assurance that their data will still be compatible.

Why Is a MARC Record Necessary? LIBRARY OF CONGRESS.¹¹

30. Thus, almost every major library in the world is MARC-compatible. See, e.g., *MARC Frequently Asked Questions (FAQ)*, LIBRARY OF CONGRESS.¹² (“MARC is the acronym for MACHine-Readable Cataloging. It defines a data format that emerged from a Library of Congress-led initiative that began nearly fifty years ago. It provides the mechanism by which computers exchange, use, and interpret bibliographic information, and its data elements make up the foundation of most library catalogs used today.”). MARC is the ANSI/NISO Z39.2-1994 standard (reaffirmed in 2016) for Information Interchange Format. The full text of the standard is available from the Library of Congress.¹³

B. MARC RECORDS

31. A MARC record comprises several fields, each of which contains specific data about the work. Each field is identified by a standardized, unique, three-digit code corresponding to the type of data that follow. For example, a work’s title

¹¹ <http://www.loc.gov/marc/umb/um01to06.html#part2>.

¹² <https://www.loc.gov/marc/faq.html>.

¹³ <http://www.loc.gov/marc/bibliographic/>.

is recorded in Field 245; the primary author of the work is transcribed in Field 100; an item's International Standard Book Number ("ISBN") consisting of ten or thirteen digits is transcribed in Field 020; an item's International Standard Serial Number ("ISSN") is transcribed in Field 022; the Library of Congress classification notation is recorded in Field 050; and the publication date is recorded in Field 260 under the subfield "c." If a work is a periodical, then its publication frequency is recorded in Field 310, and the publication dates (e.g., the first and last publication) are recorded in Field 362, which is also referred to as the enumeration/chronology field.¹⁴

32. The library that created the record is recorded in Field 040 in subfield "a" with a unique library code. When viewing the MARC record online via Online Computer Library Center's ("OCLC") bibliographic database, hovering over this code with the mouse reveals the full name of the library. I used this method of "mousing over" the library codes in the OCLC database to identify the originating library for the MARC records discussed in this declaration. Where this "mouse over" option was not available, I consulted the Directory of OCLC Libraries in order to identify the institution that created the MARC record.¹⁵

¹⁴ <http://www.loc.gov/marc/bibliographic/bd3xx.html>.

¹⁵ <https://www.oclc.org/en/contacts/libraries.html>.

33. MARC records also include several fields that include subject matter classification information. An overview of MARC record fields is available through the Library of Congress.¹⁶ For example, 6XX fields are termed “Subject Access Fields.”¹⁷ Among these, for example, is the 650 field; this is the “Subject Added Entry – Topical Term” field.¹⁸ The 650 field is a “[s]ubject added entry in which the entry element is a topical term.” These entries “are assigned to a bibliographic record to provide access according to generally accepted thesaurus-building rules (e.g., *Library of Congress Subject Headings* (LCSH), *Medical Subject Headings* (MeSH)).” *Id.* Further, MARC records include call numbers, which themselves include a classification number. For example, the 050 field is the “Library of Congress Call Number.”¹⁹ A defined portion of the Library of Congress Call (LCC) Number is the classification number, and “source of the classification number is *Library of Congress Classification* and the *LC Classification-Additions and Changes*.” Thus, included in the 050 field is a subject matter classification. Further, the 082 field is the “Dewey Decimal Call Number.”²⁰ A defined portion of the

¹⁶ <http://www.loc.gov/marc/bibliographic/>.

¹⁷ <http://www.loc.gov/marc/bibliographic/bd6xx.html>.

¹⁸ <http://www.loc.gov/marc/bibliographic/bd650.html>.

¹⁹ <http://www.loc.gov/marc/bibliographic/bd050.html>.

²⁰ <http://www.loc.gov/marc/bibliographic/bd082.html>.

Dewey Decimal Call (DDC) Number is the classification number, and “source of the classification number is the *Dewey Decimal Classification and Relative Index*.” Thus, included in the 082 field is a subject matter classification. Each item in a library has a single classification number. A library selects a classification scheme (*e.g.*, the Library of Congress classification scheme just described or a similar scheme such as the Dewey Decimal classification scheme) and uses it consistently. When the Library of Congress assigns the LCC classification number, it appears as part of the 050 field. When the Library of Congress assigns the DDC classification number, it appears as part of the 082 field. If a local library assigns the classification number, it appears in a 090 field. In either scenario, the MARC record includes a classification number that represents a subject matter classification.

C. OCLC

34. The OCLC was created “to establish, maintain and operate a computerized library network and to promote the evolution of library use, of libraries themselves, and of librarianship, and to provide processes and products for the benefit of library users and libraries, including such objectives as increasing availability of library resources to individual library patrons and reducing the rate of rise of library per-unit costs, all for the fundamental public purpose of furthering ease of access to and use of the ever-expanding body of worldwide scientific, literary

and educational knowledge and information.”²¹ Among other services, OCLC and its members are responsible for maintaining the WorldCat database (<http://www.worldcat.org/>), used by independent and institutional libraries throughout the world.

35. OCLC also provides its members online access to MARC records through its OCLC bibliographic database. When an OCLC member institution acquires a work, it creates a MARC record for this work in its computer catalog system in the ordinary course of its business. MARC records created at the Library of Congress are tape-loaded into the OCLC database through a subscription to MARC Distribution Services daily or weekly. Once the MARC record is created by a cataloger at an OCLC member institution or is tape-loaded from the Library of Congress, the MARC record is then made available to any other OCLC members online, and therefore made available to the public. Accordingly, once the MARC record is created by a cataloger at an OCLC member institution or is tape-loaded from the Library of Congress or another library anywhere in the world, any publication corresponding to the MARC record has been cataloged and indexed according to its subject matter such that a person interested in that subject matter

²¹ Third Article, Amended Articles of Incorporation of OCLC Online Computer Library Center, Incorporated (available at <https://www.oclc.org/content/dam/oclc/membership/articles-of-incorporation.pdf>).

could, with reasonable diligence, locate and access the publication through any library with access to the OCLC bibliographic database or through the Library of Congress.

36. When an OCLC member institution creates a new MARC record, OCLC automatically supplies the date of creation for that record. The date of creation for the MARC record appears in the fixed Field (008), characters 00 through 05. The MARC record creation date reflects the date on which, or shortly after which, the item was first acquired or cataloged. Initially, Field 005 of the MARC record is automatically populated with the date the MARC record was created in year, month, day format (YYYYMMDD) (some of the newer library catalog systems also include hour, minute, second (HHMMSS)). Thereafter, the library's computer system may automatically update the date in Field 005 every time the library updates the MARC record (*e.g.*, to reflect that an item has been moved to a different shelving location within the library). Field 005 is visible when viewing a MARC record via an appropriate computerized interface and shows the date upon which the most recent update occurred.

37. Once one library has cataloged and indexed a publication by creating a MARC record for that publication, other libraries that receive the publication do not create additional MARC records—the other libraries instead rely on the original MARC record. They may update or revise the MARC record to ensure accuracy, but

they do not replace or duplicate it. This practice does more than save libraries from duplicating labor. It also enhances the accuracy of MARC records. Further, it allows librarians around the world to know that a particular MARC record is authoritative (in contrast, a hypothetical system wherein duplicative records were created would result in confusion as to which record is authoritative).

38. Catalogers can create MARC records for all types of print, online, and digital resources. The date of creation of the MARC record by a cataloger at an OCLC member institution reflects when the underlying item is accessible to the public. Upwards of two-thirds to three-quarters of book sales to libraries come from a jobber or wholesaler for online and print resources. These resellers make it their business to provide books to their customers as fast as possible, often providing turnaround times of only a single day after publication. Libraries purchase a significant portion of the balance of their books directly from publishers themselves, which provide delivery on a similarly expedited schedule. In general, libraries make these purchases throughout the year as the books are published and shelve the books as soon thereafter as possible in order to make the books available to their patrons. Thus, books are generally available at libraries across the country within just a few days of publication.

D. JOURNALS

39. Catalogers can create MARC records for all types of print, online, and

digital resources. For example, MARC records cover serial publications, including both serially-published monographs and journals. OCLC hosts MARC records for more than 320 million serial publications. Serial publications are those publications that have the same collective title but are intended to be continued indefinitely with enumeration such as a volume or issue number (*e.g.*, magazines, journals, etc.). In the OCLC bibliographic database, the first issue of the serial publication is typically cataloged (*i.e.*, a corresponding MARC record is created), but the date is left open-ended with the use of a punctuation mark such as a dash. OCLC serial publication MARC records represent the entire run of the serial title. With knowledge of the first issue published, future issues can be predicted based on the information provided in the MARC record, for example in field 362. In my extensive professional experience, is it highly unusual for a library to stop collecting and shelving a serial publication prior to the end of its publication run. If a subscription to a serial publication ends its run or is cancelled before the end of its run, the library will denote that it has stopped receiving new volumes by filling in the end date in the MARC record.

40. The handling of printed journal subscriptions is shown on the cover or the first few pages of individual issues. As was the best practice among libraries, issues arrived at a central facility and were immediately received, verified as part of a subscription, checked in, and stamped or labeled with the institution's name and

date. Determining that the issue was part of the library subscription ensured that the entire set of publications for the year had been received so that they could be professionally bound and retained. This process also verified that all of the published issues arrived so that the library staff did not have to request or claim an issue that did not arrive as expected. In large public libraries with branches and multi-campus libraries within academic institutions, the journals were sorted and delivered to the subscribing unit. The new issue was placed in the public area; the older issue was stored so that it remained available.

41. The foregoing process has been standard library practice longer than I have been working in the profession. I first learned the steps in the process in the late 1970s and later supervised it. Although the checking in process has become automated and now links electronically to holdings records for the MARC record for each serial title, the manual placement of a stamp or label and placing the issue in a public area has not changed for 50 years. Unless I note otherwise below in reference to a specific serial publication, it is my expert opinion that this standard protocol was followed for the serial publications discussed below.

V. PUBLICATIONS

A. PUBLICATION 1: Exhibit 1010 (“FORSHEW”)

42. Attached hereto as Exhibit 1010 is a copy of the journal article titled “Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep

Sequencing of Plasma DNA” by Tim Forshew, Muhammed Murtaza, Christine Parkinson, Davina Gale, Dana W. Y. Tsui, Fiona Kaper, Sarah-Jane Dawson, Anna M. Piskorz, Mercedes Jimenez-Linan, David Bentley, James Hadfield, Andrew P. May, Carlos Caldas, James D. Brenton, and Nitzan Rosenfeld (hereafter “Forshew”). The Forshew article was published in Volume 4, Issue 136 of the open access journal *Science Translational Medicine* and was found in the National Library of Medicine (Bethesda, Maryland). The Forshew article appears beginning on page ra68 of this issue dated May 30, 2012. Exhibit 1010 is a true and correct copy of the Forshew article (34 pages) that I understand is being submitted as an exhibit in this proceeding. Specifically, the text of the article is complete; no pages are missing, and the text on each page appears to flow seamlessly from one page to the next; further, there are no visible alterations to the document. Exhibit 1010 was found within the custody of a library – a place where an authentic copy of this open access journal would likely be. Exhibit 1010 is a true and correct copy in a condition that creates no suspicion about its authenticity

43. The Forshew article is available from the publisher,²² the American Association for the Advancement of Science, and through the National Library of

²² <https://www.science.org/doi/10.1126/scitranslmed.3003726>.

Medicine.²³

44. Attached hereto as Attachment 1A is a true and correct copy of the MARC record for the open access journal *Science Translational Medicine* in the National Library of Medicine online catalog. The library continues to update and enhance this MARC record to meet current cataloging rules. The most recent enhancement to Attachment 1A occurred on February 27, 2025, as shown in field 005 (“20250227”). I personally identified and retrieved the MARC record that is Attachment 1A.

45. As previously noted, the library that created the record is recorded in field 040 with a unique library code. For Attachment 1A, that library code is “NSD,” which means that the MARC record for this serial was cataloged as part of the National Serials Data Program at the Library of Congress. As can be seen in field 008 (“Date entered on file”) in the MARC record for this exhibit, a cataloger at the Library of Congress created OCLC record number 299570656 on January 26, 2009, (“090126”). The “BLvl” entry in Attachment 1A is “s,” which indicates that the open access journal *Science Translational Medicine* is a serial publication. Field 310 of Attachment 1A reads “Monthly.” As noted in the holdings information (362 field), the National Library of Medicine has received the open access journal *Science*

²³ <https://pubmed.ncbi.nlm.nih.gov/22649089/>.

Translational Medicine since October 2009 and continues to receive the publication. Accordingly, the MARC record for Exhibit 1010 corresponds to the open access journal *Science Translational Medicine* from the time the serial title began publication and includes the date of the Forshew article.

46. Attachment 1A includes an entry in field 060 (“W1 \$b SC781Q”)—as described above, a subject matter classification number consistent with the National Library of Medicine classification system (analogous to the Dewey Decimal classification system). Attachment 1A further includes a descriptor term reading “Translational Research, Biomedical \$v periodical” (see Attachment 1B, National Library of Medicine subject heading D057170) in the 650 field. Thus, as of its cataloging, the publication corresponding to the MARC record attached hereto as Attachment 1A was indexed according to its subject matter by virtue of the title (field 245), the classification notation (field 060), and the subject heading (field 650).

47. As of January 26, 2009, the MARC record attached hereto as Attachment 1A was accessible through any library with access to the OCLC bibliographic database or the online catalog at a library that subscribed to the open access journal, which means that the corresponding publication was publicly available on or before that same date through any library with access to the OCLC bibliographic database or through an individual library.

48. WorldCat indicates that the open access journal *Science Translational*

Medicine as cataloged at the Library of Congress is currently available from 213 libraries.²⁴ In view of the above, this issue of the open access journal *Science Translational Medicine* was publicly available on or shortly after May 30, 2012, because by that date it had been cataloged and indexed at the Library of Congress, made part of the OCLC bibliographic database, and received in the National Library of Medicine. For these reasons, it is my opinion that the Forshew article (Exhibit 1010) published in the open access journal *Science Translational Medicine* was publicly available on or shortly after May 30, 2012, as indicated on the publisher's website.

B. PUBLICATION 2: Exhibit 1077 (“VAN LIER”)

49. Attached hereto as Exhibit 1077 is a copy of the journal article titled “A Review on the Molecular Diagnostics of Lynch Syndrome: A Central Role for the Pathology Laboratory” by Margot G. F. van Lier, Anja Wagner, Monique E. van Leerdam, Katharina Biermann, Ernst J. Kuipers, Ewout W. Steyerberg, Hendrikus Jan Dubbink, and Winand N. M. Dinjens (hereafter “van Lier”). The van Lier article was published in Volume 14, Numbers 1-2 of the open access *Journal of Cellular and Molecular Medicine* and was found in the National Library of Medicine (Bethesda, Maryland). The van Lier article appears beginning on page 181 of this

²⁴ <https://search.worldcat.org/title/299570656>.

issue dated November 19, 2009. Exhibit 1077 is a true and correct copy of the van Lier article (pages 181-197) that I understand is being submitted as an exhibit in this proceeding. Specifically, the text of the article is complete; no pages are missing, and the text on each page appears to flow seamlessly from one page to the next; further, there are no visible alterations to the document. Exhibit 1077 was found within the custody of a library – a place where an authentic copy of this open access journal would likely be. Exhibit 1077 is a true and correct copy in a condition that creates no suspicion about its authenticity

50. The van Lier article is available from the publisher,²⁵ the Wiley Online Library, through the National Library of Medicine,²⁶ and the digital repository *ResearchGate*.²⁷

51. Attached hereto as Attachment 2A is a true and correct copy of the MARC record for the open access *Journal of Cellular and Molecular Medicine* in the National Library of Medicine online catalog. The library continues to update and enhance this MARC record to meet current cataloging rules. The most recent

²⁵ <https://onlinelibrary.wiley.com/doi/10.1111/j.1582-4934.2009.00977.x>.

²⁶ <https://pmc.ncbi.nlm.nih.gov/articles/PMC3837620/>.

²⁷

https://www.researchgate.net/publication/40023407_A_review_on_the_molecular_diagnostics_of_Lynch_syndrome_A_central_role_for_the_pathology_laboratory.

enhancement to Attachment 2A occurred on February 27, 2025, as shown in field 005 (“20250227”). I personally identified and retrieved the MARC record that is Attachment 2A.

52. As previously noted, the library that created the record is recorded in field 040 with a unique library code. For Attachment 2A, that library code is “DNLM,” which means that the MARC record for this serial was cataloged at the National Library of Medicine. As can be seen in field 008 (“Date entered on file”) in the MARC record for this exhibit, a cataloger at the National Library of Medicine created OCLC record number 46362607 on December 27, 2000, (“001227”). The “BLvl” entry in Attachment 2A is “s,” which indicates that the open access *Journal of Cellular and Molecular Medicine* is a serial publication. Field 310 of Attachment 2A reads “Quarterly.” As noted in the holdings information (362 field), the National Library of Medicine has received the open access *Journal of Cellular and Molecular Medicine* since July 2000 and continues to receive the publication. Accordingly, the MARC record for Exhibit 1077 corresponds to the open access *Journal of Cellular and Molecular Medicine* from the time the serial title began publication and includes the date of the van Lier article.

53. Attachment 2A includes an entry in field 060 (“W1 \$b JO7575”)—as described above, a subject matter classification number consistent with the National Library of Medicine classification system (analogous to the Dewey Decimal

classification system). Attachment 2A further includes three descriptor terms reading “Cell Biology, Biomedical \$v periodical” (see Attachment 2B, National Library of Medicine subject heading D003585), “Medicine \$v periodical” (see Attachment 2C, National Library of Medicine subject heading D008511), and “Molecular Biology \$v periodical” (see Attachment 2D, National Library of Medicine subject heading D008967) in the 650 fields. Thus, as of its cataloging, the publication corresponding to the MARC record attached hereto as Attachment 2A was indexed according to its subject matter by virtue of the title (field 245), the classification notation (field 060), and the subject headings (field 650).

54. As of December 27, 2000, the MARC record attached hereto as Attachment 2A was accessible through any library with access to the OCLC bibliographic database or the online catalog at a library that subscribed to the open access journal, which means that the corresponding publication was publicly available on or before that same date through any library with access to the OCLC bibliographic database or through an individual library.

55. WorldCat indicates that the open access *Journal of Cellular and Molecular Medicine* as cataloged at the National Library of Medicine is currently available from 1,168 libraries.²⁸ In view of the above, this issue of the open access

²⁸ <https://search.worldcat.org/title/46362607>.

Journal of Cellular and Molecular Medicine was publicly available on or shortly after November 19, 2009, because by that date it had been received, cataloged, and indexed at the National Library of Medicine and made part of the OCLC bibliographic database. For these reasons, it is my opinion that the van Lier article (Exhibit 1077) published in the open access *Journal of Cellular and Molecular Medicine* was publicly available on or shortly after November 19, 2009, as indicated on the publisher's website.

C. PUBLICATION 3: Exhibit 1078 (“BOLAND-1”)

56. Attached hereto as Exhibit 1078 is a copy of the journal article titled “Microsatellite Instability in Colorectal Cancer” by C. Richard Boland and Ajay Goel (hereafter “Boland-1”). The Boland-1 article was published in Volume 138, Issue 6 of the open access journal *Gastroenterology* and was found in the National Library of Medicine (Bethesda, Maryland). Boland-1 was downloaded from the PubMed website at the National Library of Medicine. The Boland-1 article appears beginning on page 2073 of this issue dated June 2010. Exhibit 1078 is a true and correct copy of the Boland-1 article (pages 2073-2087) that I understand is being submitted as an exhibit in this proceeding. Specifically, the text of the article is complete; no pages are missing, and the text on each page appears to flow seamlessly from one page to the next; further, there are no visible alterations to the document. Exhibit 1078 was found within the custody of a library – a place where an authentic

copy of this open access journal would likely be. Exhibit 1078 is a true and correct copy in a condition that creates no suspicion about its authenticity

57. The Boland-1 article is available from the publisher,²⁹ Elsevier, through the National Library of Medicine,³⁰ and the American Gastroenterological Association website.³¹

58. Attached hereto as Attachment 3A is a true and correct copy of the MARC record for the open access journal *Gastroenterology* in the National Library of Medicine online catalog. The library continues to update and enhance this MARC record to meet current cataloging rules. The most recent enhancement to Attachment 3A occurred on February 27, 2025, as shown in field 005 (“20250227”). I personally identified and retrieved the MARC record that is Attachment 3A.

59. As previously noted, the library that created the record is recorded in field 040 with a unique library code. For Attachment 3A, that library code is “DNLM,” which means that the MARC record for this serial was cataloged at the National Library of Medicine. As can be seen in field 008 (“Date entered on file”) in the MARC record for this exhibit, a cataloger at the National Library of Medicine

²⁹ <https://www.sciencedirect.com/science/article/abs/pii/S0016508510001691>.

³⁰ <https://pubmed.ncbi.nlm.nih.gov/20420947/>.

³¹ [https://www.gastrojournal.org/article/S0016-5085\(10\)00169-1/fulltext?referrer=https%3A%2F%2Fpubmed.ncbi.nlm.nih.gov%2F](https://www.gastrojournal.org/article/S0016-5085(10)00169-1/fulltext?referrer=https%3A%2F%2Fpubmed.ncbi.nlm.nih.gov%2F).

created OCLC record number 1570457 on November 13, 1973, (“731113”). The “BLvl” entry in Attachment 3A is “s,” which indicates that the open access journal *Gastroenterology* is a serial publication. Field 310 of Attachment 3A reads “Monthly.” As noted in the holdings information (362 field), the National Library of Medicine has received the open access journal *Gastroenterology* since January 1943 and continues to receive the publication. Accordingly, the MARC record for Exhibit 1078 corresponds to the open access journal *Gastroenterology* from the time the serial title began publication and includes the date of the Boland-1 article.

60. Attachment 3A includes an entry in field 060 (“W1 \$b GA458”)—as described above, a subject matter classification number consistent with the National Library of Medicine classification system (analogous to the Dewey Decimal classification system). Attachment 3A further includes a descriptor term reading “Gastroenterology \$v periodical” (see Attachment 3B, National Library of Medicine subject heading D005762) in the 650 field. Thus, as of its cataloging, the publication corresponding to the MARC record attached hereto as Attachment 3A was indexed according to its subject matter by virtue of the title (field 245), the classification notation (field 060), and the subject heading (field 650).

61. As of November 13, 1973, the MARC record attached hereto as Attachment 3A was accessible through any library with access to the OCLC bibliographic database or the online catalog at a library that subscribed to the open

access journal, which means that the corresponding publication was publicly available on or before that same date through any library with access to the OCLC bibliographic database or through an individual library.

62. WorldCat indicates that the open access journal *Gastroenterology* as cataloged at the National Library of Medicine is currently available from 1,423 libraries.³² In view of the above, this issue of the open access journal *Gastroenterology* was publicly available on or shortly after April 24, 2010, because by that date it had been received, cataloged, and indexed at the National Library of Medicine and made part of the OCLC bibliographic database. For these reasons, it is my opinion that the Boland-1 article (Exhibit 1078) published in the open access journal *Gastroenterology* was publicly available on or shortly after April 24, 2010, as indicated on the publisher's website.³³

D. PUBLICATION 4: Exhibit 1079 (“BOLAND-2”)

63. Attached hereto as Exhibit 1079 is a copy of the journal article titled “A National Cancer Institute Workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition: Development of International Criteria for the Determination of Microsatellite Instability in Colorectal Cancer” by C. Richard Boland, Stephen N. Thibodeau, Stanley R. Hamilton, David Sidransky, James R.

³² <https://search.worldcat.org/title/1570457>.

³³ <https://www.sciencedirect.com/science/article/abs/pii/S0016508510001691>.

Eshleman, Randall W. Burt, Stephen J. Meltzer, Miguel A. Rodriguez-Bigas, Riccardo Fodde, G. Nadia Ranzani, and Sudhir Srivastava (hereafter “Boland-2”). The Boland-2 article was published in Volume 58, Issue 22 of the journal *Cancer Research* and was found in the National Library of Medicine (Bethesda, Maryland). The Boland-2 article appears beginning on page 5248 of this issue dated November 15, 1998. Exhibit 1079 is a true and correct copy of the Boland-2 article (pages 5248-5257) that I understand is being submitted as an exhibit in this proceeding. Specifically, the text of the article is complete; no pages are missing, and the text on each page appears to flow seamlessly from one page to the next; further, there are no visible alterations to the document. Exhibit 1079 was found within the custody of a library – a place where an authentic copy of this journal would likely be. Exhibit 1079 is a true and correct copy in a condition that creates no suspicion about its authenticity

64. The Boland-2 article is available from the publisher,³⁴ through the

³⁴

<https://www.google.com/url?sa=i&url=https%3A%2F%2Faacrjournals.org%2Fcan- cerres%2Farticle- pdf%2F58%2F22%2F5248%2F2468117%2Fcr0580225248.pdf&psig=AOvVaw3 OThBqVOgMVmXr2CEj9WrU&ust=1755209088818000&source=images&cd=vf e&opi=89978449&ved=0CAQQn5wMahcKEwiop6DD5YiPAxUAAAAAHQAA AAAQBA>

National Library of Medicine,³⁵ and the digital repositories *ResearchGate*³⁶ and *Semantic Scholar*.³⁷

65. Attached hereto as Attachment 4A is a true and correct copy of the MARC record for the journal *Cancer Research* in the National Library of Medicine online catalog. The library continues to update and enhance this MARC record to meet current cataloging rules. The most recent enhancement to Attachment 4A occurred on March 31, 2025, as shown in field 005 (“20250331”). I personally identified and retrieved the MARC record that is Attachment 4A.

66. As previously noted, the library that created the record is recorded in field 040 with a unique library code. For Attachment 4A, that library code is “DNLM,” which means that the MARC record for this serial was cataloged at the National Library of Medicine. As can be seen in field 008 (“Date entered on file”) in the MARC record for this exhibit, a cataloger at the National Library of Medicine created OCLC record number 1553285 on July 21, 1984, (“840721”). The “BLvl”

³⁵ <https://pubmed.ncbi.nlm.nih.gov/9823339/>.

³⁶

https://www.researchgate.net/publication/13464047_Boland_CR_Thibodeau_SN_Hamilton_SR_Sidransky_D_Eshleman_JR_Burt_RW_Meltzer_SJ_Rodriguez-Bigas_MA_Fodde_R_Ranzani_GN_and_Srivastava_S_A_National_Cancer_Institute_Workshop_on_Microsatellite_Instability_.

³⁷ <https://www.semanticscholar.org/paper/A-National-Cancer-Institute-Workshop-on-Instability-Boland-Thibodeau/d5b0760c94f06b5f1473e63c124d2360892d747d>.

entry in Attachment 4A is “s,” which indicates that the journal *Cancer Research* is a serial publication. Field 310 of Attachment 4A reads “Semimonthly \$b <Jan. 1, 1987->.” As noted in the holdings information (362 field), the National Library of Medicine has received the journal *Cancer Research* since January 1941 and continues to receive the publication. Accordingly, the MARC record for Exhibit 1079 corresponds to the journal *Cancer Research* from the time the serial title began publication and includes the date of the Boland-2 article.

67. Attachment 4A includes an entry in field 060 (“W1 \$b CA688”)—as described above, a subject matter classification number consistent with the National Library of Medicine classification system (analogous to the Dewey Decimal classification system). Attachment 4A further includes two descriptor terms reading “Neoplasms \$v periodical” (see Attachment 4B, National Library of Medicine subject heading D009369) and “Neoplasms, Experimental \$v periodical” (see Attachment 4C, National Library of Medicine subject heading D009374) in the 650 fields. Thus, as of its cataloging, the publication corresponding to the MARC record attached hereto as Attachment 4A was indexed according to its subject matter by virtue of the title (field 245), the classification notation (field 060), and the subject heading (field 650).

68. As of July 21, 1974, the MARC record attached hereto as Attachment 4A was accessible through any library with access to the OCLC bibliographic

database or the online catalog at a library that subscribed to the open access journal, which means that the corresponding publication was publicly available on or before that same date through any library with access to the OCLC bibliographic database or through an individual library.

69. WorldCat indicates that the journal *Cancer Research* as cataloged at the National Library of Medicine is currently available from 935 libraries.³⁸ In view of the above, this issue of the journal *Cancer Research* was publicly available on or shortly after November 15, 1998, because by that date it had been received, cataloged, and indexed at the National Library of Medicine and made part of the OCLC bibliographic database. For these reasons, it is my opinion that the Boland-2 article (Exhibit 1079) published in the journal *Cancer Research* was publicly available on or shortly after November 15, 1998.

E. PUBLICATION 5: Exhibit 1080 (“SINICROPE”)

70. Attached hereto as Exhibit 1080 is a copy of the journal article titled “Clinical Implications of Microsatellite Instability in Sporadic Colon Cancers” by Frank A. Sinicrope and Daniel J. Sargent (hereafter “Sinicrope”). The Sinicrope article was published in Volume 21, Number 4 of the journal *Current Opinion in Oncology* and was found in the National Library of Medicine (Bethesda, Maryland).

³⁸ <https://search.worldcat.org/title/1553285>.

The Sinicrope article appears beginning on page 369 of this issue dated July 2009. Exhibit 1080 is a true and correct copy of the Sinicrope article (pages 369-373) that I understand is being submitted as an exhibit in this proceeding. Specifically, the text of the article is complete; no pages are missing, and the text on each page appears to flow seamlessly from one page to the next; further, there are no visible alterations to the document. Exhibit 1080 was found within the custody of a library – a place where an authentic copy of this journal would likely be. Exhibit 1080 is a true and correct copy in a condition that creates no suspicion about its authenticity

71. The Sinicrope article is available from the publisher,³⁹ Wolters Kluwers Health, and through the National Library of Medicine.⁴⁰

72. Attached hereto as Attachment 5A is a true and correct copy of the MARC record for the journal *Current Opinion in Oncology* in the National Library of Medicine online catalog. The library continues to update and enhance this MARC record to meet current cataloging rules. The most recent enhancement to Attachment 5A occurred on February 27, 2025, as shown in field 005 (“20250227”). I personally identified and retrieved the MARC record that is Attachment 5A.

73. As previously noted, the library that created the record is recorded in

³⁹ https://journals.lww.com/co-oncology/abstract/2009/07000/clinical_implications_of_microsatellite.14.aspx.

⁴⁰ <https://pubmed.ncbi.nlm.nih.gov/19444104/>.

field 040 with a unique library code. For Attachment 5A, that library code is “DNLM,” which means that the MARC record for this serial was cataloged at the National Library of Medicine. As can be seen in field 008 (“Date entered on file”) in the MARC record for this exhibit, a cataloger at the National Library of Medicine created OCLC record number 18553548 on May 25, 1990, (“900525”). The “BLvl” entry in Attachment 5A is “s,” which indicates that the journal *Current Opinion in Oncology* is a serial publication. Field 310 of Attachment 5A reads “Bimonthly.” As noted in the holdings information (362 field), the National Library of Medicine has received the journal *Current Opinion in Oncology* since October 1989 and continues to receive the publication. Accordingly, the MARC record for Exhibit 1080 corresponds to the journal *Current Opinion in Oncology* from the time the serial title began publication and includes the date of the Sinicrope article.

74. Attachment 5A includes an entry in field 060 (“W1 \$b CU799GGBL”)—as described above, a subject matter classification number consistent with the National Library of Medicine classification system (analogous to the Dewey Decimal classification system). Attachment 5A further includes a descriptor term reading “Neoplasms \$v periodical” (see Attachment 5B, National Library of Medicine subject heading D009369) in the 650 field. Thus, as of its cataloging, the publication corresponding to the MARC record attached hereto as Attachment 5A was indexed according to its subject matter by virtue of the title (field

245), the classification notation (field 060), and the subject heading (field 650).

75. As of May 25, 1990, the MARC record attached hereto as Attachment 5A was accessible through any library with access to the OCLC bibliographic database or the online catalog at a library that subscribed to the journal, which means that the corresponding publication was publicly available on or before that same date through any library with access to the OCLC bibliographic database or through an individual library.

76. WorldCat indicates that the journal *Current Opinion in Oncology* as cataloged at the National Library of Medicine is currently available from 500 libraries.⁴¹ In view of the above, this issue of the journal *Current Opinion in Oncology* was publicly available on or shortly after July 31, 2009, because by that date it had been received, cataloged, and indexed at the National Library of Medicine and made part of the OCLC bibliographic database. For these reasons, it is my opinion that the Sinicrope article (Exhibit 1080) published in the journal *Current Opinion in Oncology* was publicly available on or shortly after July 31, 2009.⁴²

⁴¹ <https://search.worldcat.org/title/18553648>.

⁴² The *Current Opinion in Oncology* was published bimonthly and, therefore, new issues were available approximately 60 days. Based on the frequency of publication (see Attachment 5A, field 310) and my years of experience working

F. PUBLICATION 6: Exhibit 1081 (“LI”)

77. Attached hereto as Exhibit 1081 is a copy of the journal article titled “Mechanisms and Functions of DNA Mismatch Repair” by Guo-Min Li (hereafter “Li”). The Li article was published in Volume 18, Number 1 of the open access journal *Cell Research* and was found in the National Library of Medicine (Bethesda, Maryland). The Li article appears beginning on page 85 of this issue dated January 2008. Exhibit 1081 is a true and correct copy of the Li article (pages 85-98) that I understand is being submitted as an exhibit in this proceeding. Specifically, the text of the article is complete; no pages are missing, and the text on each page appears to flow seamlessly from one page to the next; further, there are no visible alterations to the document. Exhibit 1081 was found within the custody of a library – a place where an authentic copy of this open access journal would likely be. Exhibit 1081 is a true and correct copy in a condition that creates no suspicion about its authenticity

78. The Li article is available from the publisher,⁴³ SpringerNature, and through the National Library of Medicine.⁴⁴

with serials, it is my opinion that issues would be published by the end of the named month. Therefore, a public availability date of July 31, 2009, is reasonable.

⁴³ <https://www.nature.com/articles/cr2007115>.

⁴⁴ <https://pubmed.ncbi.nlm.nih.gov/18157157/>.

79. Attached hereto as Attachment 6A is a true and correct copy of the MARC record for the open access journal *Cell Research* in the National Library of Medicine online catalog. The library continues to update and enhance this MARC record to meet current cataloging rules. The most recent enhancement to Attachment 6A occurred on February 27, 2025, as shown in field 005 (“20250227”). I personally identified and retrieved the MARC record that is Attachment 6A.

80. As previously noted, the library that created the record is recorded in field 040 with a unique library code. For Attachment 6A, that library code is “DNLM,” which means that the MARC record for this serial was cataloged at the National Library of Medicine. As can be seen in field 008 (“Date entered on file”) in the MARC record for this exhibit, a cataloger at the National Library of Medicine created OCLC record number 52535380 on August 1, 1996, (“960801”). The “BLvl” entry in Attachment 6A is “s,” which indicates that the open access journal *Cell Research* is a serial publication. Field 310 of Attachment 6A reads “Monthly \$b 2005-.” As noted in the holdings information (362 field), the National Library of Medicine has received the open access journal *Cell Research* since March 1990 and continues to receive the publication. Accordingly, the MARC record for Exhibit 1081 corresponds to the open access journal *Cell Research* from the time the serial title began publication and includes the date of the Li article.

81. Attachment 6A includes an entry in field 060 (“W1 \$b CE128WD”)—

as described above, a subject matter classification number consistent with the National Library of Medicine classification system (analogous to the Dewey Decimal classification system). Attachment 6A further includes a descriptor term reading “Cells \$v periodical” (see Attachment 6B, National Library of Medicine subject heading D002477) in the 650 field. Thus, as of its cataloging, the publication corresponding to the MARC record attached hereto as Attachment 6A was indexed according to its subject matter by virtue of the title (field 245), the classification notation (field 060), and the subject heading (field 650).

82. As of August 1, 1996, the MARC record attached hereto as Attachment 6A was accessible through any library with access to the OCLC bibliographic database or the online catalog at a library that subscribed to the open access journal, which means that the corresponding publication was publicly available on or before that same date through any library with access to the OCLC bibliographic database or through an individual library.

83. WorldCat indicates that the open access journal *Cell Research* as cataloged at the National Library of Medicine is currently available from 1,182 libraries.⁴⁵ In view of the above, this issue of the open access journal *Cell Research* was publicly available on or shortly after December 24, 2007, because by that date

⁴⁵ <https://search.worldcat.org/title/52535380>.

it had been received, cataloged, and indexed at the National Library of Medicine and made part of the OCLC bibliographic database. For these reasons, it is my opinion that the Li article (Exhibit 1081) published in the open access journal *Cell Research* was publicly available on or shortly after December 24, 2007, as indicated on the publisher's website.⁴⁶

G. PUBLICATION 7: Exhibit 1012 (“SHIROGUCHI”)

84. Attached hereto as Exhibit 1012 is a copy of the journal article titled “Digital RNA Sequencing Minimizes Sequence-Dependent Bias and Amplification Noise with Optimized Single-Molecule Barcodes” by Katsuyuki Shiroguchi, Tony Z. Jia, Peter A. Sims, and X. Sunney Xie (hereafter “Shiroguchi”). The Shiroguchi article was published in Volume 109, Number 4 of the journal *Proceedings of the National Academy of Science of the United States of America (PNAS)* and was found in the National Library of Medicine (Bethesda, Maryland). The Shiroguchi article appears beginning on page 1347 of this issue dated January 24, 2012. Exhibit 1012 is a true and correct copy of the Shiroguchi article (pages 1347-1352) that I understand is being submitted as an exhibit in this proceeding. Specifically, the text of the article is complete; no pages are missing, and the text on each page appears to flow seamlessly from one page to the next; further, there are no visible alterations to

⁴⁶ <https://www.nature.com/articles/cr2007115>.

the document. Exhibit 1012 was found within the custody of a library – a place where an authentic copy of this journal would likely be. Exhibit 1012 is a true and correct copy in a condition that creates no suspicion about its authenticity

85. The Shiroguchi article is available from the publisher,⁴⁷ the National Academy of Science of the United States of America, through the National Library of Medicine,⁴⁸ and the digital repository *ResearchGate*.⁴⁹

86. Attached hereto as Attachment 7A is a true and correct copy of the MARC record for the journal *PNAS* in the National Library of Medicine online catalog. The library continues to update and enhance this MARC record to meet current cataloging rules. The most recent enhancement to Attachment 7A occurred on February 27, 2025, as shown in field 005 (“20250227”). I personally identified and retrieved the MARC record that is Attachment 7A.

87. As previously noted, the library that created the record is recorded in field 040 with a unique library code. For Attachment 7A, that library code is

⁴⁷ <https://www.pnas.org/doi/10.1073/pnas.1118018109>.

⁴⁸ <https://pubmed.ncbi.nlm.nih.gov/22232676/>.

⁴⁹

https://www.researchgate.net/publication/221736129_Digital_RNA_sequencing_minimizes_sequence-dependent_bias_and_amplification_noise_with_optimized_single-molecule_barcode.

“DNLM,” which means that the MARC record for this serial was cataloged at the National Library of Medicine. As can be seen in field 008 (“Date entered on file”) in the MARC record for this exhibit, a cataloger at the National Library of Medicine created OCLC record number 1607201 on June 26, 1975, (“750626”). The “BLvl” entry in Attachment 7A is “s,” which indicates that the journal *PNAS* is a serial publication. Field 310 of Attachment 7A reads “Weekly \$b 2004-.” As noted in the holdings information (362 field), the National Library of Medicine has received the journal *PNAS* since January 1915 and continues to receive the publication. Accordingly, the MARC record for Exhibit 1012 corresponds to the journal *PNAS* from the time the serial title began publication and includes the date of the Shiroguchi article.

88. Attachment 7A includes an entry in field 060 (“W1 \$b PR586A”)—as described above, a subject matter classification number consistent with the National Library of Medicine classification system (analogous to the Dewey Decimal classification system). Attachment 7A further includes a descriptor term reading “Science \$v periodical” (see Attachment 7B, National Library of Medicine subject heading D012586) in the 650 field. Thus, as of its cataloging, the publication corresponding to the MARC record attached hereto as Attachment 7A was indexed according to its subject matter by virtue of the title (field 245), the classification notation (field 060), and the subject heading (field 650).

89. As of June 26, 1975, the MARC record attached hereto as Attachment 7A was accessible through any library with access to the OCLC bibliographic database or the online catalog at a library that subscribed to the journal, which means that the corresponding publication was publicly available on or before that same date through any library with access to the OCLC bibliographic database or through an individual library.

90. WorldCat indicates that the journal *PNAS* as cataloged at the National Library of Medicine is currently available from 1,927 libraries.⁵⁰ In view of the above, this issue of the journal *PNAS* was publicly available on or shortly after January 9, 2012, because by that date it had been received, cataloged, and indexed at the National Library of Medicine and made part of the OCLC bibliographic database. For these reasons, it is my opinion that the Shiroguchi article (Exhibit 1012) published in the journal *PNAS* was publicly available on or shortly after January 9, 2012, as indicated on the publisher's website.⁵¹

VI. SUMMARY OF OPINIONS

91. In view of the foregoing, it is my opinion that the publications described above were publicly available no later than the corresponding date listed in the table below:

⁵⁰ <https://search.worldcat.org/title/1607201>.

⁵¹ <https://www.pnas.org/doi/10.1073/pnas.1118018109>.

Exhibit	Publication	Publicly Available On or Shortly After
1010	Forshew, Tim, Muhammed Murtaza, Christine Parkinson, Davina Gale, Dana W. Y. Tsui, Fiona Kaper, Sarah-Jane Dawson, Anna M. Piskorz, Mercedes Jimenez-Linan, David Bentley, James Hadfield, Andrew P. May, Carlos Caldas, James D. Brenton, and Nitzan Rosenfeld. “Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA.” <i>Science Translational Medicine</i> , vol. 4, issue 136 (30 May 2012): ra68.	May 30, 2012
1077	van Lier, Margot G. F., Anja Wagner, Monique E. van Leerdam, Katharina Biermann, Ernst J. Kuipers, Ewout W. Steyerberg, Hendrikus Jan Dubbink, and Winand N. M. Dinjens. “A Review on the Molecular Diagnostics of Lynch Syndrome: A Central Role for the Pathology Laboratory.” <i>Journal of Cellular and Molecular Medicine</i> , vol. 14, nos. 1-2 (19 November 2009): 181-197.	November 19, 2009
1078	Boland, C. Richard, and Ajay Goel. “Microsatellite Instability in Colorectal Cancer.” <i>Gastroenterology</i> , vol. 138, issue 6 (June 2010): 2073–2087	April 24, 2010
1079	Boland, C. Richard, Stephen N. Thibodeau, Stanley R. Hamilton, David Sidransky, James R. Eshleman, Randall W. Burt, Stephen J. Meltzer, Miguel A. Rodriguez-Bigas, Riccardo Fodde, G. Nadia Ranzani, and Sudhir Srivastava. “A National Cancer Institute Workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition: Development of International Criteria for the Determination of Microsatellite Instability in Colorectal	November 15, 1998

Exhibit	Publication	Publicly Available On or Shortly After
	Cancer.” <i>Cancer Research</i> , vol. 58, issue 22 (15 November 1998): 5248–5257.	
1080	Sinicrope, Frank A., and Daniel J. Sargent. “Clinical Implications of Microsatellite Instability in Sporadic Colon Cancers.” <i>Current Opinion in Oncology</i> , vol. 21, no.4 (July 2009): 369–373.	July 31, 2009
1081	Li, Guo-Min. “Mechanisms and Functions of DNA Mismatch Repair.” <i>Cell Research</i> , vol. 18, no. 1 (January 2008): 85-98.	December 24, 2007
1012	Shiroguchi, Katsuyuki, Tony Z. Jia, Peter A. Sims, and X. Sunney Xie. “Digital RNA Sequencing Minimizes Sequence-Dependent Bias and Amplification Noise with Optimized Single-Molecule Barcodes.” <i>Proceedings of the National Academy of Science of the United States of America (PNAS)</i> , vol. 109, no. 4 (24 January 2012): 1347–1352.	January 9, 2012

92. In signing this Declaration, I recognize that the Declaration will be filed as evidence in a case before the Patent Trial and Appeal Board of the United States Patent and Trademark Office. I also recognize that I may be subject to cross-examination in the case and that cross-examination will take place within the United States. If cross-examination is required of me, I will appear for cross-examination within the United States during the time allotted for cross-examination.

93. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

DATED: August 15, 2025

By: *Sylvia D. Hall-Ellis*
Sylvia D. Hall-Ellis, Ph.D.

Exhibit A

CURRICULUM VITAE SYLVIA D. HALL-ELLIS

EDUCATION

Ph.D., University of Pittsburgh, Pittsburgh, Pennsylvania, 1985
M.P.S., University of Denver, Denver, Colorado, 2014
Post Graduate Studies, University of Texas – San Antonio, Texas, 1975-1976
M.L.S., University of North Texas, Denton, Texas, 1972
B.A., Rockford University, Rockford, Illinois, 1971

PROFESSIONAL EXPERIENCE

- 1981- Consultant for higher education, non-profit organizations, and corporations.**
- 2002- Adjunct Professor, School of Information, San José State University, San José, California.** Serve as part-time faculty member teaching graduate students in technical services (cataloging, bibliographic control, classification), “core courses,” and special topics.
- 2014-2016 Director, Grants and Resource Development, Colorado Community College System.**
Provided leadership and vision to foster the continued growth of rigorous scholarship, innovative projects, and creative work for statewide system, 13 campuses, and 50 teaching sites serving 155,000 students. Responsible for leadership and ensured efficient functioning of contract and grants in compliance with state & federal requirements and successful implementation and management. Served as a subject matter expert and liaison for college Grant Directors for all issues relating to grants and subcontracts.
- 2010-2014 Senior Grant Administrator, Morgridge College of Education, University of Denver (Colorado).** Provided leadership and vision to foster continued growth of rigorous scholarship, innovative research, and creative work in the Morgridge College of Education. Ensure that contract and grants processes function effectively and efficiently for 60 faculty and researchers with a focus on the successful progression and efficient management of grants totaling \$13M. Worked effectively and collegially with Department Chairs and Program Coordinators on operational grant-related management activities and with a broad range of internal and external constituencies. Supported the dissemination and promotion of faculty research and scholarship to outside constituents at conferences and through publications. Assisted Principal Investigators and grant project teams by coaching, mentoring, and financial management.
- 2011-2013 Interim Director & Assistant Dean, Westminster Law Library, Sturm College of Law, University of Denver.** Planned, organized, and directed all administrative activities for the library serving students, faculty, and alumni; oversaw the employment, retention, promotion, transfer and termination of library personnel; represented the library at professional conferences and public meetings; created and promoted a climate and culture of acceptance for new programs and services, a positive high-quality image of the law library, and that reflect the organization’s values, encourage excellent performance, and reward high productivity and innovation; provided leadership and set strategic direction of the organization; ensured that the library provided excellent customer service through solution-oriented staff response to patron needs and by responsiveness and continuous improvement of the organization; promoted, developed, and maintained positive working relationships with colleagues and customers including key stakeholders and groups, higher education institutions, the legal community, other regional libraries and districts statewide, and national library organizations.

- 2007-2014** **Associate Professor, Library & Information Science, Morgridge College of Education, University of Denver (Colorado).** Served in leadership role and worked collaboratively in program, college, campus and community environments. Advised and supervised students, taught core and specialized courses at the graduate level in an integrative, student-centered learning environment. Served on LIS, College, and University committees, and maintained working relationships with colleagues in other academic units and information professionals in the Rocky Mountain region and beyond. Served on and chair doctoral student dissertation committees. Oversaw and facilitated the College and LIS graduate student association.
- 2002-2007** **Assistant Professor, Library & Information Science, College of Education, University of Denver (Colorado).** Served as tenure-track faculty member teaching graduate students in “core courses,” resource description and access, service learning, and independent studies. Advised graduate students, participate on LIS and College committees, and serve on doctoral student dissertation committees. Oversaw and facilitated the LIS graduate student association and alumni association.
- 2000-2002** **Affiliate Faculty, Library & Information Science, College of Education, University of Denver (Colorado).** Served as part-time faculty member teaching graduate students in technical services (cataloging, bibliographic control, classification), “core courses,” and special topics. Oversaw and facilitated the LIS graduate student association and alumni association.
- 2000-2001** **Special Assistant to the Secretary’s Regional Representative, U.S. Department of Education, Region VIII, Denver, Colorado.** Served as the principal advisor and representative of the U. S. Secretary of Education’s Regional Representative (SRR). Ensured the implementation of major goals of the SRR and the Secretary. Provided leadership on behalf of the SRR in contacts with high-level officials in Region VIII requiring sensitive policy interpretation in communication with senior Department officials to solve problems and resolve issues raised by State and local education officials. Served as the primary contact for School-to-Work/Career, Children’s Health Insurance Program, and Safe and Drug-Free Schools. Delivered technical assistance to local education agencies and institutions of higher education in technology, professional development, and school construction.
- 1999-2000** **Catalog Librarian, Jefferson County Public Library, Lakewood, Colorado.** Performed original, copy cataloging and classification of library materials (English and Spanish) using standard library protocols; completed original descriptive cataloging and subject analysis; enhanced brief catalog and authority records in III.
- 1997-1999** **Development Officer, McREL International, Aurora, Colorado.** Served as senior member of corporate management team in strategic planning, development of proposals and contracts, implementation, and evaluation of new services, products, and programs for educational agencies. Provided creative leadership to corporate committees to solicit ideas, identify goals and objectives, plan, develop, present, and evaluate professional development opportunities.
- 1995-1997** **Education Specialist, Education Service Center, Region One, Edinburg, Texas.** Served as member of Administrative Cabinet team in strategic planning, development of proposals and contracts, implementation, and evaluation of telecommunications capabilities, services, products, and programs for 40 school districts serving 283,000 students in 7 counties. Provided creative leadership to regional and state committees to solicit ideas, identify strategic goals and objectives, plan, develop, present, and evaluate funding opportunities and professional development for 400 librarians.

- 1993-1996** **Assistant Professor of Library Science, Sam Houston State University, Huntsville, Texas.** Served as a faculty member teaching 400 graduate students in technical services (cataloging, bibliographic control, classification), automation, and networking. Participated in distance education program and coordinated annual conference. Conducted university and Texas Library Association-funded field research focused on library collection development and academic achievement.
- 1992-1993** **Head Librarian, Rocky Mountain College of Art & Design, Denver, Colorado.** Responsible for the daily operation, selection and acquisition of materials, formulation of policies for library operations, media center, and photography/slides archives. Designed and implemented library automation and delivery of electronic resources to college community.
- 1981-1985** **Development Officer, PRLC, Inc., Pittsburgh, Pennsylvania.** Served as senior member of corporate management team in strategic planning, development of proposals and contracts, implementation, and evaluation of new services, products, and programs for 100 institutional member organizations. Coordinated the development of proposals and contracts totaling \$4,000,000 annually. Provided creative leadership to corporate committees to solicit ideas, identify goals and objectives, plan, develop, present, and evaluate professional development opportunities.
- 1981** **Director of Library Development, Pennsylvania Department of Education, Harrisburg, Pennsylvania.** Responsible for statewide development, technical assistance, professional development, resource sharing, children's services, institutional library services, networking, and state aid program for all libraries throughout the Commonwealth. Functioned as liaison to Governor's Advisory Council, LSCA Advisory Council, District Administrators, private colleges, universities, consortia managers, and network directors. Supervised \$14,000,000 formula-based state aid program and \$3,000,000 grant awards to individual libraries, consortia, and networks.
- 1978-1981** **Assistant Director, Southern Tier Library System, Corning, New York.** Coordinated operation of system-wide programs (technical assistance, professional development, resource sharing, technical services, outreach) to 40 public libraries in 5 counties serving 500,000 residents. Solicited ideas, identified goals, sponsored, and evaluated professional development opportunities and technical assistance sessions.
- 1976-1978** **Division Librarian for Technical Services, Corpus Christi Public Libraries, Corpus Christi, Texas.** Provided leadership in acquisitions, cataloging, serials control, and processing for main library and 4 branches serving 250,000 residents. Participated as senior member of library management team. Compiled and prepared technical evaluations, reports, and statistical analyses of Division operations to measure the achievement and cost of annual goals, objectives, and staff performance.
- 1975-1976** **System Coordinator, San Antonio Major Resource Center, San Antonio, Texas.** Served as senior member of the management team for District X Office, charged to provide technical assistance, resource sharing, media services, and professional development to librarians and staff representing 30 public library jurisdictions in 21 counties serving 1,500,000 residents. Functioned as liaison to System Director, staff, and members of governing bodies with the System Board of Directors and the Texas State Library and Historical Commission. Prepared LSCA grant applications and monitored awards totaling \$1,100,000 annually.

- 1973-1975** **Bilingual Branch Librarian, San Antonio Public Library, San Antonio, Texas.** Worked as librarian providing reference, information, and readers' advisory services in branch serving 50,000 Spanish-speaking residents in southwest San Antonio. Participated in collection development and resource acquisition activities, specializing in children's work, Spanish language resources, and multicultural studies.
- 1972-1973** **Librarian, Holding Institute, Laredo, Texas.** Worked as high school librarian serving 500 boarding students in Spanish-speaking environment of private school. Provided reference, research assistance, and library instruction to students and 35 faculty members.
- 1966-1971** **Rockford Public Library, Rockford, Illinois.** Worked in branches as part-time as a Library Assistant, Clerk, and Page in city library serving 150,000 residents.

PUBLICATIONS

Editor-reviewed Monographs (Completed and in Progress)

Hall-Ellis, Sylvia D., and Mary Bolin. *Contemporary Cataloging in an RDA Environment: A Handbook for Students and Practitioners*. Chicago, IL: American Library Association. Under contract & In development.

RDA Testing: Lessons Learned and Challenges Revealed. Sylvia D. Hall-Ellis and Robert O. Ellett, Jr., eds. Binghamton, N.Y.: Haworth, 2012. 128 p.

Hall-Ellis, Sylvia D., Stacey L. Bowers, Christopher D. Hudson, and M. Claire Williamson. *Librarian's Handbook for Seeking, Writing, and Managing Grants*. Santa Barbara, Calif.: Libraries Unlimited, 2011. 315 p.

Hall-Ellis, Sylvia D., with Ann Jerabek, and Merrie W. Valliant. *Contemporary Cataloging: A Handbook for Practitioners and Students*. Open access text. Athens, GA: University of Georgia System Regents, 2011. 767 p.

Grealy, Deborah S. and Sylvia D. Hall-Ellis. *From Research to Practice: The Scholarship of Teaching and Learning in LIS Education*. Westport, Conn.: Libraries Unlimited, 2009. 175 p.

Hall-Ellis, Sylvia D. with J. Ann Jerabek. *Grants for School Libraries*. Westport, Conn.: Libraries Unlimited, 2003. 197 p.

Hall-Ellis, Sylvia D., Doris Meyer, Frank W. Hoffmann, with J. Ann Jerabek. *Grant Writing for Small Libraries and School Library Media Centers*. Boulder, Colo.: NetLibrary, 2001. 173 p.

Hall-Ellis, Sylvia D., Doris Meyer, Frank W. Hoffmann, with J. Ann Jerabek. *Grant Writing for Small Libraries and School Library Media Centers*. Englewood, Colo.: Libraries Unlimited, 2000. 173 p.

Editor-reviewed Chapters (Completed and In Progress)

Hall-Ellis, Sylvia D. "Prepared to Lead: Talent, Skills, and Competencies." In *Telling the Technical Services Story*. Chicago, IL: American Library Association, 2020. Program Presentation for Re-Accreditation by the American Library Association, School of Information, San Jose State University, 2022.

Hall-Ellis, Sylvia D. "Grant Writing and Sponsored Research Funding for Academic Librarians." In *The New Librarianship*. Vol. 4. Bradford Lee Eden, ed. New York: Scarecrow Press, 2015. (pp. 163-174)

Hall-Ellis, Sylvia D. "Organizing Information: Technical Services." In *Information Services Today: An Introduction*. Sandra Hirsch, ed. Lanham, Md.: Rowman and Littlefield, 2015. (pp. 139-148)

Hall-Ellis, Sylvia D. "Metadata, MARC, and More." In *Rethinking Technical Services, Considering Our Profession and Ourselves: What's the Future of Our Profession?* Mary Beth Weber, ed. Lanham, Md.: Rowman and Littlefield, 2015. (pp. 29-55)

Hall-Ellis, Sylvia D., ed. "Contingent Faculty: Non-Tenure Track Faculty Series." In the *Faculty Personnel Guidelines Relating to Appointment, Promotion, and Tenure*. November 2011. Denver, Colo.: University of Denver, 2011. 42 p.

Hall-Ellis, Sylvia D., ed. "Standard VII: Information Resources." In the *Sturm College of Law Self-Study Presentation for Accreditation by the American Bar Association*. Denver, Colo.: University of Denver, Sturm College of Law, 2011. 20 p.

Hall-Ellis, Sylvia D. "Applying for Grants from Foundations, Corporations, or Government." In *The Volunteers' Guide to Fundraising: Raise Money for Your School Team, Library or Community Group*. 1st ed. Ilona M. Bray, ed. Berkeley, Calif.: Nolo, 2011. (pp. 1-38 on accompanying disc)

Hall-Ellis, Sylvia D., ed. "Standard III: The Faculty." In the *Library and Information Science Program Self-Study Document for Accreditation by the American Library Association*. Denver, Colo.: University of Denver, Morgridge College of Education, 2010. 22 p.

Hall-Ellis, Sylvia D. "Library and Information Science Programs and Education for Catalogers and Metadata Specialists: Challenges for the Twenty-first Century." In *Conversations with Catalogers in the Twenty-First Century*. Elaine R. Sanchez, ed. Santa Barbara, Calif.: ABC-CLIO, 2010. (pp. 226-254)

Hall-Ellis, Sylvia D., ed. "Standard III: The Faculty." In the *Library and Information Science Program Self-Study Document for Accreditation by the American Library Association*. Denver, Colo.: University of Denver, College of Education, 2003. 15 p.

Lesesne, Teri S. and Sylvia D. Hall-Ellis. "The Selection, Evaluation, and Integration of Culturally Authentic Texts: A Case for Making the Online Catalog Reflect Parallel Cultures." In *Literacy: Traditional, Cultural, Technological*. Pittsburgh, Pa.: International Association of School Librarianship, 1995. (pp. 110-113)

Lesesne, Teri S. and Sylvia D. Hall-Ellis. *The Selection, Evaluation, and Integration of Culturally Authentic Texts: A Case for Making the Online Catalog Reflect Parallel Cultures*. In *Conference Proceedings of the 23rd Annual International Association of School Librarianship*, Pittsburgh, Pennsylvania, July 17-22, 1994. ERIC Document ED374816. 17 p.

Hall-Ellis, Sylvia D. "Curriculum Folio for School Library Media Specialist Programs." In the *National Council for Accreditation of Teacher Education Self-Study for Sam Houston State University*. Huntsville, Tex.: Sam Houston State University, College of Education and Applied Science, 1994. 25 p.

Bruntjen, Scott and Sylvia D. Hall. "Attempting to Automate: Lessons Learned Over Five Years." In *Advances in Library Administration*. Volume 4. Weston, Conn.: JAI Press, 1985. (pp. 177-192)

Peer-Reviewed Journal Articles (Completed and In Progress)

Hall-Ellis-Sylvia D. "Job Design for Cataloging and Metadata Librarians." Submitted to *Journal of Library Administration*. In progress.

Hall-Ellis, Sylvia D. "The Relationship of Situational Leadership and the Dreyfus Model of Skill Acquisition for Supervisors in Cataloging and Metadata Services." To be submitted to *Library Resources and Technical Services*. In progress.

Hall-Ellis, Sylvia D. *The Relationship of Core Competencies on Learning Outcomes and Employers' Expectations for Catalog Librarians and Metadata Specialists*. To be submitted to the *Journal of Education for Library and Information Science*. In progress.

Hall-Ellis, Sylvia D. "Building Cataloger Competencies: The Dreyfus Model as a Prototype for the Education and Professional Development of Catalog Librarians and Metadata Specialists in Bibliographic Control." To be submitted to the *Cataloging & Classification Quarterly*. In progress.

Hall-Ellis, Sylvia D. "Descriptive Impressions of Entry-Level Cataloger and Metadata Specialists Positions, 2000-2016: Reflections and Trends." To be submitted to *Library Resources and Technical Services*. In progress.

- Hall-Ellis, Sylvia D. "Competencies for Metadata and Cataloging Leaders: What Employers Expect as Reflected in Position Descriptions, 2000-2016." *Cataloging & Classification Quarterly*. In progress.
- Hall-Ellis, Sylvia D. "Stackable Micro-credentials – A Framework for the Future." *The Bottom Line* 29, no. 4 (April 2016), <http://www.emeraldinsight.com/doi/pdfplus/10.1108/BL-02-2016-0006>
- Ellis, Sylvia D. "Succession Planning and Staff Development – A Winning Combination." *The Bottom Line*, 28 no. 3 (May 2015): 95-98.
- Hall-Ellis, Sylvia D. "Succession Planning and Staff Development – A Winning Combination." *The Informed Librarian Online* (September 2015), http://www.informedlibrarian.com/featuredArticle.cfm?FILE=succession_1509.pdf
- Hall-Ellis, Sylvia D. "Nudges and Decision Making: A Winning Combination." *The Bottom Line* 28, no. 4 (July 2015): 133-136.
- Hall-Ellis, Sylvia D. "Succession Planning and Staff Development – A Winning Combination." *The Bottom Line* 28, no. 3 (May 2015): 95-98.
- Hall-Ellis, Sylvia D. "Metadata Competencies for Entry-Level Positions: What Employers Expect as Reflected in Position Descriptions, 2000-2013." *Journal of Library Metadata* 15, no. 2 (June 2015): 102-134.
- Hall-Ellis, Sylvia D. "Onboarding to Improve Library Retention and Productivity." *The Bottom Line* 27, no. 4 (October 2014).
- Hall-Ellis, Sylvia D. "Accept, Coach, and Inspire: A Formula for Success." *The Bottom Line* 27, no. 3 (July 2014): 103-106.
- Hall-Ellis, Sylvia D. *Reward Systems for Staff in Higher Education Institutions: Case Studies of 15 Higher Education Institutions in Colorado*. Capstone Thesis. Denver, Colo.: University of Denver, University College, 2014.
- Hall-Ellis, Sylvia D. "Reward Systems Promote High-Performance Work Teams Achieving Library Mission." *The Bottom Line* 27, no. 2 (April 2014): 66-69.
- Hall-Ellis, Sylvia D. "Investment in Staff Development for Seeking External Grant Funds." *The Bottom Line* 27, no. 1 (January 2014): 22-25.
- Hall-Ellis, Sylvia D. "Staff Investments for High Performance Teams." *The Bottom Line* 26, no. 4 (October 2013): 149-152.
- Hall-Ellis, Sylvia D. and Deborah S. Greal. "Service Learning in LIS Education: A Case for Holistic Assessment." *Catholic Library World* 82, no. 3 (March 2012): 178-187.
- Hall-Ellis, Sylvia D., and Deborah S. Greal. "The Dreyfus Model of Skill Acquisition: A Career Development Framework for Succession Planning and Management in Academic Libraries." *College and Research Libraries* (June 2012). <http://crl.acrl.org/content/early/2012/06/19/crl12-349.full.pdf+html>
- Hall-Ellis, Sylvia D. "Cataloger Competencies...What Do Employers Require?" *Cataloging & Classification Quarterly* 46, no. 3 (2008): 305-330. <http://ccq.haworthpress.com/doi:10.1080/01639370802034565>

Hall-Ellis, Sylvia D. "Subject Access for Readers' Advisory Services: Their Impact on Contemporary Spanish Fiction in Selected Public Library Collections." *Public Library Quarterly* 27, no.1 (2007): 1-18.

<http://plq.haworthpress.com/doi:10.1080/01616840802122377>

Hall-Ellis, Sylvia D. "Language Proficiencies among Catalogers and Technical Services Librarians." *Technical Services Quarterly* 25, no. 2 (2007) 31-47. http://tsq.haworthpress.com/doi:10.1300/J124v25n02_03

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- Hall-Ellis, Sylvia D. *Planning for Educational Technology: the Distance Education Implementation Phase: An Investigation of Technology and Telecommunication Needs for Region One Education Service Center, South Texas Community College, TCI Cablevision of South Texas, Inc., Tech Prep of the Rio Grande Valley, Inc., Texas State Technical College, Centro Cultural en el Valle de Texas, McAllen Independent School District, Harlingen Consolidated Independent School District, Donna Independent School District, Edinburg Consolidated Independent School District, La Joya Independent School District, Mercedes Independent School District, Pharr-San Juan-Alamo Independent School District, Sharyland Independent School District, Easter Seal Society of the Rio Grande Valley, Inc., and McAllen Memorial Library*. Harlingen, Tex.: Tech Prep of the Rio Grande Valley, 1996.
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Bruntjen, Scott and Sylvia D. Hall. *Discovering Resource Sharing Treasures: Accessing Pennsylvania's High School Library Collections*. Harrisburg, Pa.: State Library of Pennsylvania, Bureau of School Library Media Resources, 1985.

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- Bruntjen, Scott and Sylvia D. Hall. *Zebras: Bar Codes, Standards, and Library Automation*. Central City, Colo.: The Blue Bear Group, Inc., 1985.
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- Hall-Ellis, Sylvia D., and Robert O. Ellett, Jr., guest editors. *RDA Testing: Lessons Learned and Challenges Revealed: A Special Issue of Cataloging & Classification Quarterly* 49, no. 7-8 (November 2011).
- Hall-Ellis, Sylvia D. and Robert O. Ellett, Jr. *Fundamentals of Cataloging I: An Online ALCTS Course*. Chicago, Ill.: American Library Association, 2009.
- Hall-Ellis, Sylvia D., Angela J. Kinney and Martha Ann Bace, editors. *Best Practices in Cataloging Education Identified at the 2007 ALCTS Pre-conference: A Special Issue of the Journal for Library and Information Science Education* 49, no. 2 (Spring 2008).

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- Hall-Ellis, Sylvia D., ed. *Library and Information Science Program Self-Study Document for Accreditation by the American Library Association*. Denver, Colo.: University of Denver, College of Education, 2003. 125 p. with CD-ROM.
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- Developing a Compensation Plan for Your Library*. Paula M. Singer and Laura L. Francisco, comps. 2nd ed. Chicago, IL: American Library Association, 2009. 978-0-8389-0985-0, \$57.00 [*Colorado Libraries*, March 2012]
- Frugal Librarian*. Carol Smallwood, ed. Chicago, IL: American Library Association, 2011. 978-0-83891-075-7, \$42.00 [*Colorado Libraries*, February 2012]

- Dowlin, Ken. *Getting the Money: How to Succeed in Fundraising for Public and Nonprofit Libraries*. Westport, Conn.: Libraries Unlimited, 2008. 978-1-59158-597-X, \$50.00 [*Colorado Libraries*, February 2009]
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- Bonelli, Florencia. *Marlene*, by Florencia Bonelli. Buenos Aires: Aguilar, Altea, Taurus, Alfaguara, 2008. 978-987-04-0995-3, \$24.95 [*Críticas*, October 2008]
- Keret, Etgar. Traducción de Ana María Bejarano. *Pizzería Kamikaze y Otras Relatos*. México, D.F.: Editorial Sexto Piso, 2008. 968-5679-29-0, no price given [*Críticas*, September 2008]
- Littauer, Marita, and Florence Littauer. *Enriquece tu Comunicación. Miami, Fla.: Editorial Unilit, 2008. 978-0-7899-1521-4, \$11.99 [*Críticas*, August 2008]*
- Martínez, Guillermo. *La Muerte Lenta de Luciana B.* New York: Rayo Planeta, 2008. 978-0-06-156551-9, \$14.95 [*Críticas*, May 2008]
- Matthews, Joseph R. *The Evaluation and Measurement of Library Services*. Westport, Conn.: Libraries Unlimited, 2007. 978-1-59158-532-9, \$50.00 [*Colorado Libraries*, April 2008]
- Gómez-Jurado, Juan. *A Masacre de Virginia Tech: Anatomía de una Mente Torturada*. Barcelona: Ediciones El Andén, 2007. 978-84-935789-4-7, €16.50 [*Críticas*, February, 2008]
- Matthews, Joseph R. *Library Assessment in Higher Education*. Westport, Conn.: Libraries Unlimited, 2007. 978-1-59158-531-2, \$45.00 [*Colorado Libraries*, February 2008]
- Chacón, Inma. *La Princesa India: Cuando el Viento Azul*. México: Alfaguara, 2006. 970-770398-9, \$19.95 [*Críticas*, June 2006]
- Vargas Llosa, Mario. *Israel/Palestina: Paz o Guerra Santa*. fotogs. by Morgana Vargas Llosa. Spain/U.S.: Aguilar: Santillana, 2006. 84-03-09691-7, paper, \$19.95 [*Críticas*, May 2006]
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- Pequeñas Resistencias 4: Antología del Nuevo Cuento Norteamericano y Caribeño*. Menéndez, Ronaldo, Ignacio Padilla & Enrique del Risco, eds. Madrid: Páginas de Espuma, 2005. 84-95642-59-X, paper, \$36.95 [*Críticas*, April 2006]
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- Becerra, Ángela. *El Penúltimo Sueño*. 1st ed. Barcelona: Planeta, 2005. 84-08-05795-2, \$25.95. [*Críticas*, January 2006]
- Aguilar Camín, Héctor. *La Conspiración de la Fortuna*. 1st ed. México: Planeta, 2005. 970-37-0368-2, \$19.95 [*Críticas*, October 2005]
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- Abraham Lincoln*. Colección Grandes Biografías. Madrid: Edimat Libros, 2003. 84-8403-858-0, €4.95 [*Críticas*, 2004]
- Alponte, Juan María. *Colón: el Hombre, el Navegante, la Leyenda*. México, D.F.: Aguilar, 2003. 968-19-1260-8, \$16.95 [*Críticas*, 2004]
- Olcese Salvatecci, Alfieri. *Cómo Estudiar con Éxito: Técnicas y Hábitos Para Aprender Mejor*. México, D.F.: Alfaomega Grupo Editor, 2002. 970-15-0764-9 [*Críticas*, 2003]
- Mujeres Como Islas: Antología de Narradoras Cubanas, Dominicanas, Puertorriqueñas*. Edición: Olga Marta Pérez, Thelma Jiménez, Andrés Blanco D., eds. Santo Domingo, República Dominicana: Ediciones Ferilibro, 2002. 959-209-419-5 [*Críticas*, 2003]
- Tibol, Raquel. *Los Murales de Diego Rivera: Universidad Autónoma Chapingo*. México, D.F.: Editorial RM, Universidad Autónoma Chapingo, 2002. 968-5208-08-5 [*Críticas*, 2003]
- Lindsay-Poland, John. *Emperors in the Jungle: the Hidden History of the U.S. in Panama*. Durham, N.C.: Duke University Press, 2003. 0-8223-3098-9, \$18.95 [*Críticas*, 2003]
- Kaplan, Allison G. and Ann Marlowe Riedling. *Catalog It! A Guide to Cataloging School Library Materials*. Worthington, Ohio: Linworth Pub., 2002. 1-58683-014-7, \$44.95 [*Colorado Libraries*, 2002]
- Bardach, Ann Louise. *Cuba Confidential: Love and Vengeance in Miami and Havana*. New York: Random, 2002. 0-375-50489-3, \$25.95 [*Library Journal*, 2002]
- Swan, James. *Fundraising for Libraries: 25 Proven Ways to Get More Money for Your Library*. New York: Neal Schuman, 2002. 1-55570-433-6, \$69.95 [*Colorado Libraries*, 2002]
- Puig de Lange, Victoria. *Sol Con Agua*. Nashville, Tenn.: Editorial Vistazo/Ediciones Reio Negro, 2002. 0-9724506-0-2, \$17.75 [*Críticas*, 2002]
- The Encyclopedia of Latin American Politics*. Edited by Diana Kapiszewski; assistant editor, Alexander Kazan. Westport, Conn.: Oryx Press, 2002. 1-57356-306-4, \$74.95 [*Library Journal*, 2002]
- Vargas Lizano, Isabel. *Y Si Quieres Saber de Mi Pasado*. Con la colaboración de J.C. Vales. Madrid: Santillana Ediciones Generales, S.L., 2002. 84-03-09278-4, \$18.95 [*Críticas*, 2002]
- Ruy Sánchez, Alberto. *Los Jardines Secretos de Mogador: Voces de Tierra*. México, D.F.: Alfaguara, S.A., 2001. 968-19-0879-1, \$16.95 [*Críticas*, 2002]
- Chávez, Ricardo and Celso Santajuliana. *El Final de las Nubes*. Barcelona: RBA Libros, S.A., 2001. 84-7901-760-0, \$11.95 [*Críticas*, 2002]

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Martínez, Rubén. *Crossing Over: A Mexican Family on the Migrant Trail.* Metropolitan: Holt, 2001. 0-8050-4908-8, \$26.00 [*Library Journal*, 2001]

Guevara, Ernesto "Che." *The African Dream: the Diaries of the Revolutionary War in the Congo.* Translated from the Spanish by Patrick Camiller. With an introduction by Richard Gott and a foreword by Aleida Guevara March. New York: Grove Press, 2001. 0-8021-3834-9, \$13.00 [*Library Journal*, 2001]

Cooper, Gail and Garry Cooper. *New Virtual Field Trips.* Englewood, Colo.: Libraries Unlimited, 2001. 1-56308-887-8, \$27.50 [*Colorado Libraries*, 2001]

NATIONAL SERVICE & PROFESSIONAL AFFILIATIONS

American Library Association, Life Member

Association for Library Collections & Technical Services (now CORE)
Cataloging, Classification, and Metadata Section
Awards & Scholarships Coordination Committee, 2025-
Margaret Mann Citation Committee, 2023-
ALCTS Editorial Board, Member, 2015-2019
Committee on Cataloging: Description and Access, Member, 2008-2012
Test Site for RDA, Manager, 2009-2011
Nominating Committee, Member, 2007-2008
Committee for Education and Training of Catalogers, Chair, 2006-2007, Member, 2003-2007
Competencies & Education for a Career in Cataloging Interest Group, Chair, 2010-2012; Member, 2010-
President's Program Committee, 2015-2016
Education Committee, Member, 2005-2007
Task Force on Competencies and Education for a Career in Cataloging, Chair, 2007-2009
2007 Annual Meeting Pre-conference, "What They Don't Teach in Library School: Competencies,
Education and Employer Expectations for a Career in Cataloging," Steering Committee Chair
Fundraising Committee, Member, 2004-2006
Office of Diversity
Committee on Diversity, Chair, 2010-2011
Diversity Research Advisory Committee, Member, 2009-2010
Spectrum Scholar Mentor, 2009
Diversity Grants Review Committee, Member, 2007, 2010, 2012
Office of Statistics and Research
Research and Statistics Committee, Member, 2005-2007; Intern, 2003-2005
Library Research Round Table
Nominating Committee, 2022-2023
Membership Committee, 2019-2021
Board of Directors, Member At-Large, 2009-2012
Office of Accreditation
Accreditation Panel Member (training completed 2004)
Accreditation Review Panel Member (training completed 2004)

REFORMA, Life Member

National Board of Directors, 2005-2013
National Fundraising Chair, 2005-2013
National Recruiting and Mentoring Committee, 2008-2010
Colorado Chapter, Secretary, 2004-2005
Colorado Chapter Liaison to National Board of Directors, 2004-2013

American Association of Law Libraries (AALL), Life Member Online Audiovisual Catalogers Association (OLAC) Colorado Association of Law Librarians (CoALL)

American Association of University Women Association for Library and Information Science Education (ALISE)

University of Denver ALISE Representative, 2003-2008; 2010-2011
Membership Advisory Committee, 2007-2010
Technical Services Education Special Interest Group, 2003-
Garfield Doctoral Dissertation Award Reviewer, 2012
Garfield Doctoral Dissertation Scholarship Reviewer, 2014

**American Association of University Professors (AAUP), 2006-
American Grant Writers Association, 2014- Grant Professionals Institute, 2016- National Grants
Management Association, 2014-**

Grant Reviewer (National and Regional Team Leader), U.S. Department of Education, 1998- Grant
Reviewer, Broadband Technologies Opportunity Program, U.S. Department of Commerce, 2009
Grant Reviewer, Institute of Museum and Library Services, 1998-2000
Grant Reviewer, Colorado Department of Education, 2014-
Grant Reviewer, Colorado State Library, 1998-
Grant Reviewer, American Association of Community Colleges *Working Connections* program, 1999
Peer Reviewer, *Journal for Library and Information Science Education*, 2005-
Peer Reviewer, *Journal of Library Metadata*, 2009
Peer Reviewer, *International Journal of Library and Information Science*, 2011
Regular Columnist, *The Bottom Line*, 2013-2016
Book Reviewer, *Library Journal*, 2001
Book Reviewer, *Críticas*, 2002-2009
Book Reviewer, *Colorado Libraries*, 2000-2012

REGIONAL SERVICE & PROFESSIONAL AFFILIATIONS

Mountain Plains Library Association

Professional Development Grants Committee, Member, 2005-2006
Professional Development Policy and Guidelines Sub-committee Chair, 2006

Colorado Association of Libraries

“Student Voices” Column Editor, *Colorado Libraries*, 2005-2006
Conference Planning Committee, Member, 2002
Technical Services and Automation Division, Chair, 2002-2003; Member, 2000-
Academic Libraries Division, Peer Review Conference Papers Committee, Member, 2007-2009
Education Committee, Member, 1988-1993
Diverse Populations Committee, Member, 2009-2013

SERVICE TO THE UNIVERSITY OF DENVER

Center for Teaching and Learning Faculty Advisory Board, 2007-2008; 2010-2012
Center for Community Engagement and Service Learning Advisory Board, 2007-2012
Faculty Senate
Executive Committee, 2008-2013
Nominations, Rules & Credentials Committee, Chair, 2008-2013; Member 2007-2013
Appointment, Promotion, and Tenure Revision Committee, 2010-2012
Grievance Policy Committee Member, 2007-2010
Law Library Director Search Committee Chair, 2012-2013
PROF Grant Review, College Representative to the University Review Team, 2006, 2008
Project Homeless Connect Evaluations, Principal Investigator, 2006-2009
University Technology Council, 2007-2009
University of Denver Hyde Interviews for Incoming Freshmen, 2003-2012

SERVICE TO THE MORGRIDGE COLLEGE OF EDUCATION

Appointment, Tenure & Review Committee
Member, voting, 2003-2005; 2007- 2009
Chair, Clinical Faculty Promotion & Tenure Policy Subcommittee, 2008-2009
Member, Community Engagement Subcommittee, 2007-2009
Member, Tenure Review Panel, 2003
Advancement and Alumni Relations Committee, Chair, 2003-2007; Member, 2002-2007

College Building Committee Member, 2004-2010
College of Education Student Association, Faculty Advisor, 2004-2007; 2009-2010
Faculty Senator, 2007-2013
Research and Scholarship Committee, Chair, 2008-2009; Member, 2002-2003, 2008-2009
Research and Grant Mentoring Committee, Chair, 2009-2010
Research Task Force Member, 2010-2012
Search Committee Member, Assistant Professor for Curriculum & Instruction, 2010-2011
Workload Task Force Member, 2010-2011

SERVICE TO THE LIBRARY & INFORMATION SCIENCE PROGRAM

Library and Information Science Student and Alumni Association, Faculty Advisor, 2002-2008
ALA Student Chapter, Faculty Advisor, 2005-2008
Beta Phi Mu Phi Chapter, Faculty Advisor, 2004-2014
Steering Committee Member, Accreditation by the American Library Association, 2001-2004
Search Committee Member, Associate Professor for LIS, 2006
Search Committee Chair, Assistant Professors for LIS, 2003, 2005, 2006, 2007
Search Committee Ex-Officio Member, Director for LIS, 2004

SERVICE TO SAM HOUSTON STATE UNIVERSITY

Rio Roundup: South Texas Literature Conference, Conference Coordinator, 1993
External Relations, Fund Raising, Grants Committee, Chair, 1993-1995
Advisory Council Committee, Chair, 1993-1995
Students, Admissions, and Advisement Committee, Chair, 1994
Institutional Effectiveness Committee, Member, 1993-1995

SERVICE TO THE COLLEGE OF EDUCATION AND APPLIED SCIENCE

Faculty Affairs Committee, Member, 1993-1995
Curriculum Committee, Member, 1993-1995
Continuing Education Committee, Member, 1993-1995

SERVICE TO THE COMMUNITY

French Teacher & Student Exchange with Laon, France, Monarch High School, Boulder Valley School District (Boulder, Colorado), 2017-
Denver/Boulder Games 2022, Board of Directors, Secretary-Treasurer, 2015-2018
United Way Campaign Committee, College of Education, 2006
Arapahoe County, Election Judge for the County Clerk and Recorder, 2000-2005
Arthritis Foundation, Rocky Mountain Chapter, Certified Educator & Trainer, 1999-2008
Bonfils Blood Center, Silver Level Donor, 2000-2016
Denver Museum of Natural History, Docent, 1992
Tech Prep of the Lower Rio Grande Valley, Inc. (Harlingen, Texas) Board of Directors, 1995-1997
Executive Committee, 1996-1997; Chair, Development Committee, 1995-1997; Chair, Fiscal Agency Committee, 1995-1996; Chair, Colleges and Universities Committee, 1996-1997
Gilpin County (Colorado) Public Library Board of Trustees, 1986-1989; Vice President, 1987-1989
City of Central (Colorado) Economic Development Committee, 1987-1989
Columbine Family Health Centers, Inc. (Nederland and Black Hawk, Colorado) Board of Directors, 1988-1989

CERTIFICATION

Permanent Public Librarian Certificate - Pennsylvania, New York, Texas
Westlaw Expert Witness, 2008-
Certified Grant Writer®, 2016-

AWARDS AND HONORS

Advanced Practitioner for Service Learning and Community Engagement, University of Denver, 2011
 Platinum Star Alumnae, College of Information, Library Science & Technologies, University of North Texas, 2009
 Commendation for Integration of Technologies in Teaching & Learning Environment, University of Denver, 2006
 Outstanding Adjunct Faculty Member Award, College of Education, University of Denver, 2002
 Beta Phi Mu, Pi, University of Pittsburgh, 1985
 Alpha Lambda Sigma, University of North Texas, 1972
Albert Nelson Marquis Lifetime Achievement Award, Who's Who in America
Who's Who in American Women
Who's Who of Women Executives
Dictionary of International Biography
Who's Who in the East
Who's Who in the South and Southwest
Who's Who in the World
Who's Who of Online Professionals
Who's Who in Library and Information Science
2,000 Notable Women
Who's Who of Emerging Leaders
Who's Who in Professional and Executive Women
Who's Who in American Education
International Who's Who of Professional and Business Women
International Leaders in Achievement
International Educator of the Year
Who's Who in Finance and Industry
Who's Who in Finance and Business

Invited International and National Conference Presentations

Hall-Ellis, Sylvia D. *Presentation of the Robert O. Ellett Endowed Scholarship*. Presentation delivered to the School of Information at San Jose State University Alumni & Student Program, American Library Annal Conference, San Diego, Calif., June 29, 2024.

Driscoll, Margaret, Christy Confetti Higgins, Scott Brown, and Sylvia D. Hall-Ellis. *A View from Within: Open House Tour of Three Canvas Core Courses*. Presentation to be delivered at the School of Information, San José State University Professional Development Seminar, San José, California, October 17, 2019.

Hall-Ellis, Sylvia D. *Invest in Me -- I'm Your Future: Succession Planning for Libraries*. Keynote presentation delivered at the ALCTS President's Symposium, Boston, Mass., January 8, 2016.

Seidel, Kent E. and Sylvia D. Hall-Ellis. *Making Grants Work for You: Strategies for Doctoral Students and Early Career Scholars*. Presentation delivered at the University Council for Educational Administration, Early Career Scholars Session, Indianapolis, Ind., November 9, 2013.

Hall-Ellis, Sylvia D. *So You Want to be a Manager; Leadership Skills and Competencies for Technical Services Managers and Administrators*. Presentation delivered at the 138th Annual Conference, American Library Association, Chicago, Ill., June 29, 2013.

Seidel, Kent E., Karen S. Riley, Lyndsay Agans, Susan Korach, and Sylvia D. Hall-Ellis. *Making Grants Work for You (Instead of Just Working for Grants)*. A panel discussion delivered at the University Council for Educational Administration, Early Career Scholars Session, Denver, Colo., November 17, 2012.

Hall-Ellis, Sylvia D. *After the Great TS Reorganization: The Westminster Law Library*. Presentation delivered at the 137th Annual Conference, American Library Association, Anaheim, Calif., June 23, 2012.

Hall-Ellis, Sylvia D. *Conversations with Catalogers in the 21st Century*. A panel discussion sponsored by the ALCTS Competencies for a Career in Cataloging Interest Group, delivered at the 137th Annual Conference, American Library Association, Anaheim, Calif., June 22, 2012.

Hall-Ellis, Sylvia D., moderator. *Mid-Career Leaders Program*. A panel discussion sponsored by the ALA Committee on Diversity delivered at the 136th Annual Conference, American Library Association, New Orleans, La., June 26, 2011.

Hall-Ellis, Sylvia D., moderator. *Diversity Town Hall*. A community conversation sponsored by the ALA Committee on Diversity delivered at the 136th Annual Conference, American Library Association, New Orleans, La., June 24, 2011.

LaBarre, Kathryn, Sylvia D. Hall-Ellis, Karen Anderson, Rick Hasenyager, Christopher Cronin, and Penny Baker. *Briefings from RDA Test Participants*. A panel discussion delivered at the Midwinter Conference, American Library Association, San Diego, Calif., January 7, 2011.

Miksa, Shawne, Marjorie Bloss, and Sylvia D. Hall-Ellis. *Educating the Next Generation of Catalogers: Teaching RDA*. A panel discussion delivered at the 97th Annual Conference, Association for Library and Information Science Education, San Diego, Calif., January 7, 2011.

Hall-Ellis, Sylvia D., Robert Maxwell, John Hostage, and George Prager. *RDA Panel: What Cataloging Managers Need to Know*. Presentation delivered at the 103rd Annual Conference, American Association of Law Librarians, Denver, Colo., July 12, 2010.

Hall-Ellis, Sylvia D. and Stacey L. Bowers. *Catalogers in the RDA Environment: Skill Sets, Expectations and Challenges*. Presentation delivered at the 103rd Annual Conference, American Association of Law Librarians, Denver, Colo., July 11, 2010.

Hall-Ellis, Sylvia D. *Comfortable in Your Cataloging and Metadata Specialist Skin? Or, So You Want to Hire a Cataloger*. Presentation delivered to the ALCTS Research Group at the 134th Annual Conference, American Library Association, Chicago, Ill., July 11, 2009.

Perez, Megan, Sylvia D. Hall-Ellis, and Denise Anthony. *From Novice to Expert: Collaboration for Succession Planning*. A "hot topic" presentation delivered at the 14th ACRL Conference, Seattle, Wash., March 13, 2009.

Hall-Ellis, Sylvia D. *Cataloging in the RDA Environment: Skill Sets, Expectations and Challenges*. Presentation delivered to the ALCTS Research and Publications Committee at the Midwinter Conference, American Library Association, Denver, Colo., January 24, 2009.

Hall-Ellis, Sylvia D. *LIS Cataloging Education for the 21st Century: Expectations and Challenges*. A panel discussion held at the 95th Annual Conference, Association for Library and Information Science Education, Denver, Colo., January 23, 2009.

Chu, Clara, Sylvia D. Hall-Ellis, and Mark Winston. *The Doctoral Degree & Building a Career*. A panel discussion delivered at the ALA Office of Diversity Spectrum Doctoral Fellows E.J. Josey Leadership Institute, Midwinter Conference, American Library Association, Denver, Colo., January 20, 2009.

- Hall-Ellis, Sylvia D. and Robert O. Ellett, Jr. *Fundamentals of Cataloging Course: An Overview of the ALCTS Online Course*. Presentation delivered to the ALCTS Big Heads Group, 133rd Annual Conference, American Library Association, Anaheim, Calif., June 30, 2008.
- Hall-Ellis, Sylvia D. *Employers' Expectations for Technical Services Librarians: What We Don't Know*. Presentation delivered to the ALCTS Research and Publications Committee Program, 133rd Annual Conference, American Library Association, Anaheim, Calif., June 28, 2008.
- Hall-Ellis, Sylvia D., Virginia R. Maloney, and Mary Stansbury. *Institutional Responses to Engaged Scholarship: The Carnegie Foundation Engaged University Classification at Two Universities*. Presentation delivered to the 94th Annual Conference, Association for Library and Information Science Education, Philadelphia, Pa., January 11, 2008.
- Hall-Ellis, Sylvia D. *Puzzles, Problems, and Predicaments*. Presentation delivered to the ALCTS Research Discussion Group, 132nd Annual Conference, American Library Association, Washington, D.C., June 23, 2007.
- Hall-Ellis, Sylvia D. *Cataloging Education: A New Emphasis for the Library and Information Science Curriculum*. Presentation delivered to the ALCTS Pre-conference, 132nd Annual Conference, American Library Association, Washington, D.C., June 22, 2007. <http://www.loc.gov/catdir/cpsocareer.html>
- Ellett, Jr., Robert O. and Sylvia D. Hall-Ellis. *Copy Cataloging Done Smarter*. Presentation delivered to the International Conference on Interdisciplinary Information Sciences and Technologies (InSciT2006), October 25-28, 2006.
- Hall-Ellis, Sylvia D. and Robert O. Ellett, Jr. *Cooperative Cataloging: Challenges and Opportunities for Defense Libraries*. Presentation delivered to the 1st Annual Conference of Defense Libraries, Spanish Ministry of Defense, Madrid, Spain, July 7, 2006.
- Hall-Ellis, Sylvia D. *Cataloger Competencies: Do the Employers Require What the Professors Teach?* Presentation delivered to the ALCTS CCS Heads of Cataloging Discussion Group, 131st Annual Conference, American Library Association, New Orleans, La., June 26, 2006.
- Greal, Deborah S. and Sylvia D. Hall-Ellis. *From Research to Practice: The Scholarship of Teaching and Learning in LIS Education*. Presentation delivered to the at the 92nd Annual Conference, Association for Library and Information Science Education, San Antonio, Tex., January 18, 2006.
- Hall-Ellis, Sylvia D. *Employers' Expectations for Entry-Level Catalogers: What Position Announcement Data Indicate*. Research paper delivered to the Technical Services Special Interests Group, 91st Annual Conference, Association for Library and Information Science Education, Boston, Mass., January 12, 2005. <http://dlist.sir.arizona.edu/>
- Hall-Ellis, Sylvia D. *Common Errors in MARC Records Prepared by LIS Students: What Does It Mean?* Research paper delivered to the ALCTS CCS Cataloging Norms Discussion Group, Mid-Winter Conference, 90th American Library Association, San Diego, Calif., January 10, 2004.
- Hall-Ellis, Sylvia D. *Visual Arts as Foundation for Successful Library Automation: The Rocky Mountain College of Art & Design Experience*. Paper delivered at the 6th Annual Conference of Higher Education, Charleston, S. C., 1993.
- Hall, Sylvia D. *Design Elements for Bibliographic Databases: An Overview*. Paper delivered at the 14th Online National Conference, New York, 1983.

Invited Regional Conference Presentations

Hall-Ellis, Sylvia D., Hudson, Christopher D., Brittany Cronin, and Kathryn Michaels. "The Colorado Law Project: Meeting the Public's Need for Legal Information." Panel discussion delivered at the Mountain Plains Library Association Conference, Billings, Mont., April 9, 2011.

Grealy, Deborah S. and Sylvia D. Hall-Ellis. *Education for Information Professionals in New Mexico: Library & Information Science Graduate Education at the University of Denver*. Presentation delivered at the New Mexico Library Education Summit, Las Vegas, N.M., September 26, 2005.

Hall-Ellis, Sylvia D. *Public Library-School Library Partnerships*. Presentation delivered at the 3rd Annual Colorado Association of Libraries Conference with the Mountain Plains Library Association, Denver, Colo., October 22, 2004.

Hall-Ellis, Sylvia D. *Learn All You Can – Educational Partnership Opportunities for the Lewis and Clark Bicentennial Commemoration*. Paper delivered at the 3rd Annual Lewis and Clark Bicentennial Council National Planning Conference, Bismarck, N.D., April 26, 1998.

Invited State Conference Presentations

Grealy, Deborah S. and Sylvia D. Hall-Ellis. *Academic Library Leadership Changes: Using Succession Planning and Mentoring*. Presentation delivered at the Minnesota Library Education Conference, St. Cloud, Minn., October 10, 2013.

Hall-Ellis, Sylvia D., Merrie Valliant, and Melissa Powell. *RDA: What Is It and What Do You Need To Do With It At Your Library?* Presentation delivered at the Colorado Library Consortium Spring Conference, Ft. Morgan, Colo., April 26, 2013.

Hall-Ellis, Sylvia D. *Service Learning and the Library & Information Science Graduate Education at the University of Denver*. Presentation delivered at the 4th LEADS Scholars Orientation, Denver, Colo., August 5, 2009.

Hall-Ellis, Sylvia D. *Service Learning: Enhancement to Library & Information Science Graduate Education at the University of Denver*. Presentation delivered at the 3rd LEADS Scholars Orientation, Denver, Colo., June 2008.

Hall-Ellis, Sylvia D. *Law Librarianship: A Community Conversation*. Sponsored by the Colorado Association of Law Libraries. Paper delivered at the Colorado Supreme Court Library, Denver, Colo., May 14, 2008.

Hall-Ellis, Sylvia D. *Opportunities and Challenges in Law Librarianship: A Community Conversation*. Presentation delivered at the Sturm College of Law, Denver, Colo., November 7, 2007.

Ellis, Sylvia D. *Grant Writing Resources for Nursing Professionals*. Presentation delivered at the Presbyterian / St. Luke's Health One Medical Center 1st Annual Research Symposium, Denver, Colo., October 17, 2007.

Hall-Ellis, Sylvia D. *Project Homeless Connect 4 Event Evaluation – Insights and Lessons Learned*. Presentation delivered at the Homelessness Research Symposium: What is DU Doing about Homelessness in Denver, Denver, Colo., September 14, 2007.

Hall-Ellis, Sylvia D. *Service Learning and the Library & Information Science Graduate Education at the University of Denver*. Presentation delivered at the 2nd LEADS Scholars Orientation, Denver, Colo., August 10, 2007.

- Hall-Ellis, Sylvia D. *Education for Information Professionals in a Digital Environment: Library & Information Science Graduate Education at the University of Denver*. Presentation delivered at the 15th Spring Mountains and Plains Parapros Conference, Denver, Colo., February 24, 2007.
- Hall-Ellis, Sylvia D. *Public Library Service to Spanish-Speaking and Latino Residents in Denver: A Case Study*. Presentation delivered at the 4th Annual Colorado Association of Libraries Conference, Denver, Colo., November 10, 2005.
- Hall-Ellis, Sylvia D. *Education for Information Professionals in a Digital Environment: Library & Information Science Graduate Education at the University of Denver*. Presentation delivered at the 14th Annual Mountains and Plains Parapros Conference, Aurora, Colo., July 29, 2005.
- Hall-Ellis, Sylvia D. *Educational Opportunities: Library & Information Science Graduate Education at the University of Denver*. Presentation delivered at the 13th Annual Mountains and Plains Parapros Conference, Centennial, Colo., August 6, 2004.
- Hall-Ellis, Sylvia D. *Library Education & Training: Focus on the West: An LIS Faculty Member's Personal Response*. Presentation delivered at the 1st Annual Colorado Association of Libraries Conference, Keystone, Colo., October 18, 2002.
- Hall-Ellis, Sylvia D. *Grant Writing for School Librarians*. Presentation delivered at the 2002 Annual Colorado Education Media Association Conference, Colorado Springs, Colo., February 15, 2002.
- Hall-Ellis, Sylvia D. *Grants – Opportunities for the Future*. Paper delivered at the Southeast Regional Accountability Annual Conference, Lamar, Colo., November 12, 1998.
- Hall-Ellis, Sylvia D. *The Texas Library Connection and Interlibrary Loan: An Experiment in Resource Sharing*. Paper delivered at the Texas Computer Educators' Association Annual Conference, Austin, Tex., February 6, 1997.
- Hall-Ellis, Sylvia D. *Finding Grant Sources on the Internet: A Guide for Librarians*. Paper delivered at the 2nd Annual Institute for School Library Personnel, Pharr-San Juan-Alamo North High School, Pharr, Tex., July 29, 1996.
- Hall-Ellis, Sylvia D. *Mathematical and Logical Thinking: A Critical Intelligence*. Paper delivered at the 3rd Annual Paraprofessional Conference at the University of Texas - Pan American, Edinburg, Tex., March 8, 1996.
- Hall-Ellis, Sylvia D. *Cataloging Trends and Issues: Update Session*. Paper delivered at the 1st Annual Institute for School Library Personnel, South Texas Community College, McAllen, Tex., July 19, 1995.
- Hall-Ellis, Sylvia D. *Grant Writing: Tips and Encouragement for School Librarians*. Paper delivered at the 1st Annual Institute for School Library Personnel, South Texas Community College, McAllen, Tex., July 18 and 19, 1995.
- Hall-Ellis, Sylvia D. *How to Become an Expert Grant Writer*. Paper delivered at the 3rd Annual High School Principals' Academy, South Padre Island, Port Isabel, Tex., June 19, 1995.
- Texas Library Study: Results from Regions I and II*. Paper delivered at the 3rd Annual Technology Conference, Texas A&M University, College Station, Tex., November 18, 1994.
- Hall-Ellis, Sylvia D. *Academic Achievement and Middle School Students*. Paper delivered at the 8th Annual Young Adult Conference, Sam Houston State University, Huntsville, Tex., November 5, 1994.
- Hall-Ellis, Sylvia D. *Multimedia Resources for Library Leaders*. Paper delivered at the Institute for Librarians in A Multicultural Environment, Sam Houston State University, Huntsville, Tex., June 10, 1994.

Hall-Ellis, Sylvia D. *Finding the Resource: Empowering the User, or, the Case for Curriculum Based Subject Access to Learning Resource Center Collections*. Paper delivered at the 81st Annual Texas Library Association Conference, Corpus Christi, Tex., April 12-16, 1994.

Hall-Ellis, Sylvia D. and William H. Pichette. *Sam Houston State University Makes Use of OCLC/AMIGOS Collection Analysis CD*. Paper delivered at the 81st Annual Texas Library Association Conference, Corpus Christi, Tex., April 12-16, 1994.

Hall, Sylvia D. *Funding and Library Development in Pennsylvania: A Symbiotic Relationship*. Paper delivered at the Annual Graduate Student Colloquia, University of Pittsburgh, School of Library and Information Science, 1982.

Hall, Sylvia D. *Leadership for Public Library Trustees*. Paper delivered for the Trustees Division, Pennsylvania Library Association Annual Conference, Lancaster, Penn., 1981.

Seminar and Professional Development Presentations

Taylor, Meredith, and Sylvia D. Hall-Ellis. *Talent Management and Succession Planning*. ALCTS eForum, held March 22, 2017.

Hirsh, Sandra, Heather O'Brien, Michelle Holschuh Simmons, Michael Krasulski, and Sylvia D. Hall-Ellis. *Information Services Today: An Introduction. Part 3: Information Services: Roles in the Digital Age*. Rowan and Littlefield in partnership with Library Journal webinar, recorded February 5, 2015.

Hall-Ellis, Sylvia D. and Jennifer Sweda. *Copy Cataloging in an RDA Environment*. ALCTS eForum, held May 14 and 15, 2013.

Hall-Ellis, Sylvia D. *Law Librarianship: A Community Conversation*. Sponsored by the Colorado Association of Law Libraries, presented at the Colorado Supreme Court Library, Denver, Colo., May 14, 2008.

Hall-Ellis, Sylvia D. and Beatrice Z. Gerrish. *Reading and Libraries: Recent Research in Reading*. Presentation at the Ricks Center for Gifted Education, Denver, Colo., March 4, 2008.

Hall-Ellis, Sylvia D. *Cataloger Competencies: Do the Employers Require What the Professors Teach?* Presentation for the School of Library and Information Science, San José State University, February 12, 2008.

Hall-Ellis, Sylvia D. and Robert O. Ellett, Jr. *Cooperative Cataloging: Rules, Tools, and Conventions for Building a Multi-institutional Catalog*. Sponsored for the Spanish Ministry of Defense, Madrid, Spain, July 10, 2006.

Hall-Ellis, Sylvia D. *Cash for Kids: Grant Writing Opportunities for Youth Services Librarians*. Sponsored by the Colorado Young Adult Librarians; presented at Bemis Memorial Library, Littleton, Colo., November 13, 2002.

Hall-Ellis, Sylvia D. *MARC Records and Authority Control: Planning for Bibliographic Database Migration*. Sponsored and held at the Douglas County Public Library, Castle Rock, Colo., May 28, 2002.

Grant Writing: A Refresher for Librarians. Sponsored by Library and Information Science Program, College of Education, University of Denver; presented at University Center at Chaparral, August 12, 2000.

Hall-Ellis, Sylvia D. *Shaking the Money Tree – Basic Grant Writing for Colorado Educators*. Sponsored by the Office of Educational Telecommunications of the Colorado Department of Education.

Pikes Peak Community College, Colorado Springs, Colo., November 16, 1998.

Pueblo School District 60, Pueblo, Colo., November 12, 1998.

University of Northern Colorado, Greeley, Colo., November 10, 1998.

United Technology Educational Partnership, Grand Junction, Colo., November 9, 1998.

Hall-Ellis, Sylvia D. *Cataloging Multimedia, Kits, Globes and Map Materials in USMARC*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., April 24, 1997 and December 12, 1996.

Hall-Ellis, Sylvia D. *New Standards for School Library Media Centers*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., April 15, 1997.

Hall-Ellis, Sylvia D. *The School Library Media Specialist in the 21st Century: Visions for the Future*. Sponsored and hosted by Pharr-San Juan-Alamo Independent School District, Pharr, Tex., April 4, 1997.

Hall-Ellis, Sylvia D. *Cataloging Sound Recordings and Audio Materials in USMARC*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., March 20, 1997 and November 21, 1996.

Hall-Ellis, Sylvia D. *Evaluating and Selecting CD-ROMS for School Library Media Collections*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., March 18, 1997.

Hall-Ellis, Sylvia D. *Cataloging Audiovisual Materials in USMARC*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., February 27, 1997, November 7, 1996 and October 24, 1996.

Hall-Ellis, Sylvia D. *Introduction to Dialog: Basic Searching Strategies*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., February 14, 1997.

Hall-Ellis, Sylvia D. *Cataloging Books in USMARC*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., January 30, 1997, October 10, 1996, September 26, 1996 and August 8, 1996.

Hall-Ellis, Sylvia D. *Advanced Internet Searching Techniques for Librarians*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., January 21, 1997.

Hall-Ellis, Sylvia D. *Texas Library Connection Full-Text Searching*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., December 13, 1996 and October 25, 1996.

Hall-Ellis, Sylvia D. *Developing Evaluation Strategies for Grants and Proposals*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., December 10, 1996.

Hall-Ellis, Sylvia D. *Developing Needs Assessment for Grants and Proposals*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., November 12, 1996.

Hall-Ellis, Sylvia D. *Texas Library Connection Union Catalog Searching*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., November 8, 1996 and September 6, 1996.

Hall-Ellis, Sylvia D. *Grant Resources on the Internet*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., October 23, 1996.

Hall-Ellis, Sylvia D. *Preparing a Response to the Telecommunications Infrastructure Fund Board: Needs Assessment, Professional Development Framework, and Evaluation Strategies*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., October 16, 1996.

Hall-Ellis, Sylvia D. *Texas Library Connection: Building the Districtwide Bibliographic Database*. Sponsored and hosted by Mercedes Independent School District, Mercedes, Tex., September 13, 1996 and August 8, 1996. Sponsored and hosted by Los Fresnos Consolidated Independent School District, Los Fresnos, Tex., August 12, 1996.

Hall-Ellis, Sylvia D. *School-to-Work and Special Education: An Inclusive Partnership for Success*. Sponsored and hosted by the Office of Special Education, Region One Education Service Center, Edinburg, Tex., September 11, 1996.

Hall-Ellis, Sylvia D. *Telecommunications Infrastructure Fund Board: An Overview of Funding for Secondary Schools*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., August 31, 1996.

Hall-Ellis, Sylvia D. *Cataloging Books, Multimedia, and Realia in USMARC*. Sponsored and hosted by Edinburg Consolidated Independent School District, Edinburg, Tex., August 6, 1996.

Hall-Ellis, Sylvia D. *Internet Resources for Grant Writers*. Sponsored and hosted by Region One Education Service Center, Edinburg, Tex., April 20, 1996.

Hall-Ellis, Sylvia D. *Enhanced Grant Writing Skills for Mathematics Educators: Writing Skills for Campus Teams*. Sponsored and hosted by the Office of General Education, Region One Education Service Center, Edinburg, Tex., March 22, 1996.

Hall-Ellis, Sylvia D. *Internet Resources for Grant Writers*. Sponsored and hosted by the College of Education and Applied Science, Sam Houston State University, Huntsville, Tex., March 9, 1996.

Hall-Ellis, Sylvia D. *Grant Writing for Mathematics Educators: A Development Process for Campus Teams*. Sponsored and hosted by the Office of General Education, Region One Education Service Center, Edinburg, Tex., March 4, 1996.

Hall-Ellis, Sylvia D. *Shaking the Money Tree: Preparing Successful Technology Grant Applications*. Sponsored and hosted by the Office of Technology and Media Services, Region One Education Service Center, Edinburg, Tex., February 29, 1996 and April 29, 1996.

Hall-Ellis, Sylvia D. *District-wide Technology Planning: Technical Assistance for the Texas Education Agency Initiative*. Sponsored and hosted by the Office of Technology and Media, Region One Education Service Center, Edinburg, Tex., February 2, 1996.

Hall-Ellis, Sylvia D. *Enhanced Grant Writing Skills*. Sponsored and hosted by Pharr-San Juan-Alamo Independent School District, Pharr, Tex., January 27, 1996.

Hall-Ellis, Sylvia D. *United States Copyright Act of 1976, Video Transmissions, Computer Software and the Internet*. Sponsored and hosted by the Office of Technology and Media, Region One Education Service Center, Edinburg, Tex., January 24, 1996.

Hall-Ellis, Sylvia D. *The Grant Writing Development Process*. Sponsored and hosted by Pharr-San Juan-Alamo Independent School District, Pharr, Tex., January 20, 1996.

Hall-Ellis, Sylvia D. *MARC Cataloging of Materials for Library Media Centers*. Sponsored and hosted by the Office of Media Services, Region One Education Service Center for the Donna Independent School District, Donna, Tex., August 17, 1995.

Hall-Ellis, Sylvia D. *Classification*. Sponsored by AJ Seminars, Rockville, Maryland; presented at University Hilton Hotel, Houston, Tex., May 10, 1995.

Hall-Ellis, Sylvia D. *Library Technical Services*. Sponsored by AJ Seminars, Rockville, Maryland; presented at University Hilton Hotel, Houston, Tex., April 26, 1995.

Hall-Ellis, Sylvia D. *Grant Writing: An Introduction for Public School Administrators*. Sponsored and hosted by the Office of Administrative Services, Region One Education Service Center, Edinburg, Tex., April 19, 1995.

Hall-Ellis, Sylvia D. *The School Library Media Specialist in the 21st Century: Visions for the Future*. Sponsored and hosted by the United Independent School District, Laredo, Tex., March 31, 1995.

Hall-Ellis, Sylvia D. *Using USMARC*. Sponsored by AJ Seminars, Rockville, Maryland; presented at University Hilton Hotel, Houston, Tex., March 29, 1995.

Hall-Ellis, Sylvia D. *Library Media Center Policies and Guidelines: How to Prepare for School Board Adoption*. Sponsored and hosted by the Office of Technology and Media Services, La Joya Independent School District, La Joya, Tex., March 10, 1995.

Hall-Ellis, Sylvia D. *Developing District-wide Policies and Guidelines for Library Media Centers*. Sponsored and hosted by the Office of Technology and Media Services, La Joya Independent School District, La Joya, Tex., January 10, 1995.

Hall-Ellis, Sylvia D. *MARC Cataloging of Audiovisual Materials for Library Media Centers*. Sponsored and hosted by the Office of Library Media and Technology Services, Cypress-Fairbanks Independent School District, Houston, Tex., December 1, 1994.

Hall-Ellis, Sylvia D. *The School Library Media Specialist in the 21st Century: Visions for the Future*. Sponsored and hosted by United Independent School District, Laredo, Tex., October 14, 1994.

Hall-Ellis, Sylvia D. *The School Library Media Specialist in the 21st Century: Visions for the Future*. Sponsored and hosted by the Laredo Independent School District, Laredo, Tex., October 7, 1994.

Hall-Ellis, Sylvia D. *MARC Cataloging for Library Media Centers*. Sponsored by the Office of Library Media and Technology Services, Cypress-Fairbanks Independent School District, Houston, Tex., October 5, 1994.

Hall-Ellis, Sylvia D. *Jump to the Head of the Class: Undergraduate Library Resources Available at Sam Houston State University*. Sponsored by the Office of the Associate Vice President for Student Services, Sam Houston State University, Huntsville, Tex., October 4, 1994.

Hall-Ellis, Sylvia D. *CD-ROMs - 1994's Newest and the Best for Secondary Level Media Centers*. Sponsored and hosted by Clear Lake Independent School District, Houston, Tex., June 3, 1994.

Hall-Ellis, Sylvia D. *Introduction to Classification*. Sponsored by AJ Seminars, Rockville, Maryland; presented at Holiday Inn, Market Center, Dallas, Tex., May 18, 1994.

Hall-Ellis, Sylvia D. *Basic Descriptive Cataloging*. Sponsored by AJ Seminars, Rockville, Maryland; presented at University Hilton Hotel, Houston, Tex., May 4, 1994.

Hall-Ellis, Sylvia D. *Shaking the Money Tree - Part II: Writing Successful Grant Applications*. Sponsored by Donna Independent School District, Donna, Texas, and Region One Education Service Center Edinburg, Texas; presented at South Texas Community College Library, McAllen, Tex., April 29, 1994.

Hall-Ellis, Sylvia D. *Using MARC*. Sponsored by AJ Seminars, Rockville, Maryland; presented at Holiday Inn, Market Center, Dallas, Tex., April 6, 1994.

Hall-Ellis, Sylvia D. *Automated Authority Control*. Sponsored by AJ Seminars, Rockville, Maryland; presented at University Hilton Hotel, Houston, Tex., March 23, 1994.

Hall-Ellis, Sylvia D. *Automating the District School Library Media Centers: Choices and Opportunities*. Sponsored and hosted by the Office of Technology and Library Media Services, Fort Bend Independent School District, Sugar Land, Tex., March 4, 1994.

Hall-Ellis, Sylvia D. *Shaking the Money Tree - Part I: Preparing Successful Grant Applications*. Sponsored and hosted by the Office of Library Media Services, Donna Independent School District, Donna, Tex., February 4, 1994.

GRADUATE COURSES TAUGHT

San José State University, School of Library and Information Science

- INFO 249 – Advanced Cataloging and Classification (Fall 2015, 2016; Summer 2016)
- INFO 287 – Special Topics in Cataloging and Classification (Spring 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026; Fall 2020, 2021, 2022, 2023, 2024, 2025, 2026)
- LIBR 248 – Beginning Cataloging and Classification (Summer 2002, 2003, 2004, 2005, 2006)
- LIBR 249 – Advanced Cataloging and Classification (Summer 2003; Fall 2014; Summer 2015)

University of Denver, University College

- Grant Writing Certificate Seminar for Non-Profit Organizations (Fall 2020, 2021; Spring 2021)

University of Denver, Morgridge College of Education HED

- 5991 – Grant Writing in Higher Education (Spring 2011)
- LIS 4010 – Organization of Information (Fall 2002-2009; Winter 2005; Spring 2004-2006)
- LIS 4020 – Professional Principles and Ethics (Summer 2000)
- LIS 4040 – Management of Libraries and Information Centers (Fall 2010; Spring 2003, 2009 (DS); Winter 2005)
- LIS 4070 – Cataloging and Classification (Winter 2008, 2009, 2010, 2011, 2012; Fall 2009 (DS)) LIS 4321 – Collection Management (Spring 2005)
- LIS 4326 – LIS Research (Winter 2009 (DS); Spring 2009 (DS))
- LIS 4350 – Adult Materials and Services (Summer 2006, 2009)
- LIS 4379 – Social Sciences Resources (Spring 2009)
- LIS 4400 – Cataloging and Classification (Spring 2000, 2001; Winter 2002-2009; Summer 2005, 2006 – course renumbered LIS 4070, September 2007)
- LIS 4401 – Descriptive Cataloging (Winter 2001; Spring 2002, 2003, 2006-2009; Summer 2005)
- LIS 4402 – Subject Cataloging (Spring 2001, 2009; Summer 2005, 2006, 2007, 2008)
- LIS 4403 – Classification Schemes (Fall 2007, 2008, 2009 (DS)) LIS
- LIS 4405 – Authority Control (Winter 2009)
- LIS 4510 – Materials and Services for Children (Winter 2004; Summer 2005)
- LIS 4620 – Grant Writing and Fundraising (Summer 2000, 2002, 2004, 2010; Winter 2006; Fall 2006-2009)
- LIS 4700 – Seminar in Technical Services (Fall 2001)
- LIS 4700 – Seminar in Public Libraries (Summer 2002)
- LIS 4804 – Management of Electronic Records (Spring 2004; Fall 2005)
- LIS 4902 – Capstone Projects (Winter 2003, Spring 2008, 2009, 2011)
- LIS 4910 – Independent Study (every quarter Winter 2000 through Spring 2011)
- LIS 4920 – Service Learning (every quarter Summer 2004 through Spring 2011)
- RMS 4954 – Grant Writing (Summer 2013, 2014)
- RMS 4959 – Content Analysis Methodology (Spring 2015)
- QRM 4978 – Grant Writing (Summer 2011, 2012)

Rutgers University, School of Communication and Information

- SC&I 522 – Cataloging and Classification (Summer 2013)

University of Arizona, College of Behavioral and Social Sciences

- LIS 602 – Cataloging and Classification (Summer 1995)
- LIS 612 – Advanced Online Search and Retrieval (Summer 1995)

Sam Houston State University, College of Education & Applied Science

- LIS 532 - Cataloging and Classification (Fall 1993, 1994, 1995)
- LIS 563 - Advanced Cataloging and Classification (Summer 1994)
- LIS 567 - Research Methods (Spring 1994, Spring 1995)
- LIS 591 - Educational Technology (Spring 1994, 1995)
- LIS 596 - Networking and Computer Technologies in Education (Fall 1993, Summer 1994, Fall 1995)

Dissertations at the University of Denver

- Bowers, Stacey L. *Library Anxiety of Law School Students: A Study Utilizing the Multidimensional Library Anxiety Scale*. Chair, May 5, 2010.
- Fattor, Melissa M. *Student Engagement Differences by Ethnicity and Scale for Ninth Grade Students*. Chair, November 1, 2010.
- Fulton, Roseanne. *A Case Study of Culturally Responsive Teaching in Middle School Mathematics*. Kent Seidel, Chair, June 18, 2009, Outside Chair.
- Grealy, Deborah S. *Tribes and Territories in Library and Information Studies Education*. Bruce Uhrmacher, Chair, June 10, 2008, Committee Member.
- McCord, J. Michael. *Developing a Standard of Care for Educational Malpractice*. Chair, April 15, 2011.
- Priebe, Sarah J. *Distinguishing Effects of Domain and General Knowledge on Passage Fluency and Comprehension*. Jan Keenan, Chair. July 21, 2011, Outside Chair.
- Snyder-Mondragon, Sandra M. *Institutional Factors that Impact the Retention of Graduate Students of Color in Schools of Library and Information Science: A Metaregression of Accredited Library School Statistics on Student Retention and Graduation Rates*. Kathy Green, Chair, July 24, 2009, Committee Member.
- Taylor, Karen Pickles. *Effective Teaching*. Elinor Katz, Chair, July 15, 2009, Outside Chair.
- Thompson, Jennifer. *Distinguishing a Western Women's College: A History of the Curriculum and Student Experience at Colorado Women's College*. Edith W. King, Chair, July 16, 2010, Committee Member.
- Walker, Emelda. *Influence of Organizational Factors on Job Satisfaction of Disability Service Providers at Postsecondary Institutions*. Chair, April 29, 2010.

Dissertation Proposals at the University of Denver

- Bowers, Stacey L. *Library Anxiety of Law School Students: A Study Utilizing the Multidimensional Library Anxiety Scale*. Chair, November 5, 2009.
- McCord, J. Michael. *Developing a Standard of Care for Educational Malpractice*. Edith W. King, Chair. April 23, 2010.
- Thompson, Jennifer. *History of Colorado Women's College*. Edith W. King, Chair, October 30, 2008. Committee Member.
- Walker, Emelda. *Influence of Organizational Factors on Job Satisfaction of Disability Service Providers at Postsecondary Institutions*. Chair, July 21, 2009.

Dissertations at Other Institutions

- Rodríguez-Mori, Howard. *The Information Behavior of Puerto Rican Immigrants to Central Florida, 2003-2009: Grounded Analysis of Six Case Studies Use of Social Networks during the Migration Process*. Kathleen Burnett, Chair, April 10, 2009, Florida State University, Committee Member.
- Schwartz, Brian *More than a Look-up Skill: Medical Information Literacy Education in Osteopathic Medical Schools*. D. Mirah Dow, Chair, July 18, 2017. Emporia State University, Committee Member.
- Snow, Karen. *A Study of the Perception of Cataloging Quality among Catalogers*. Shawne D. Miksa, Chair, August 1, 2011. University of North Texas, Committee Member.

Dissertation Proposals at Other Institutions

Schwartz, Brian *More than a Look-up Skill: Medical Information Literacy Education in Osteopathic Medical Schools*. D. Mirah Dow, Chair, December 4, 2015. Emporia State University, Committee Member.

Snow, Karen. *A Study of the Perception of Cataloging Quality among Catalogers*. Shawne D. Miksa, Chair, May 11, 2010. University of North Texas, Committee Member.

Master's Thesis at the University of Denver

Hemingson, Jeff. *Recital Paper*. Lamont School of Music, February 2010, Outside Chair.

Capstones at the University of Denver

Anthony, Alisa. *Correlation of Library and Information Science Program outcomes and Vacant Position Qualifications Listed on the Colorado State Library Jobline by Employers During the Period September 1, 2000 through August 31, 2002*. Chair, Winter Quarter, 2003.

Borden, Donna M. *Improving Emergency Communications Systems: Is a Radio Communications Network the Answer?* Fall Quarter, 2014.

Bowden, Heather L.M. *Exploring Biological Models for Long-term Data Preservation*. Chair, Spring Quarter, 2008.

Casenada, Cassandra Y. *Challenges to the Recruitment, Education, and Retention of Librarians of Color*. Chair, Spring Quarter, 2008.

Chang, Jennifer C. *Legal Research Practice and Preference: A Law Firm Perspective*. Chair, Spring Quarter 2011.

Ellis, Megan S. Fitzgerald. *Design of a Public Library Adult Volunteer Recruitment Program and Training Curriculum*. Chair, Winter Quarter, 2003.

Kircher, Kathy. *Development of a Library Pathfinder for Exobiology and Posting it on the Internet*. Chair, Winter Quarter, 2003.

Melhado, Loretta. *Design of St. John's Episcopal Church Library*. Chair, Spring Quarter, 2008.

Radcliff, Kathy. *Original Cataloging of Archival materials in the HERS Collection in Penrose Library*. Chair, Winter Quarter, 2003.

Sass, Carol Ann. *ACT Periodical Index: Access to Catholic Thinking Periodical Index: Web Index to Select Catholic Periodicals*. Spring Quarter 2000.

Stone, Sergio D. *Conducting Community Analysis for the Bemis Public Library (Littleton, Colorado) Using 2002 U.S. Census Data and the Online Outcome-Based Evaluation Toolkit*. Chair, Winter Quarter, 2003.

Tureson, Tamara. *Design and Use of an Information Audit Tool for Use in a Law Library*. Chair, Winter Quarter, 2003.

Tweed, Beth. *Evaluation of Email Reference Service in a Consumer Health Library Environment*. Chair, Winter Quarter, 2003.

GRANTS AND CONTRACTS

Student Learning in Agriculture STEM through Teacher Professional Development. Research team: Stanton Gartin (PI) and Cyndi Hofmeister, Northeast Junior College; Jeff Cash, Cheryl Sánchez, Anne-Marie Crampton, Lamar Community College; Suzanna Spears, Morgan Community College; Jack Wiley, Kerry Gabrielson, Trinidad State Junior College; Michael J. Miller, Colorado State University; Michael Womochil (Co-PI), Casey Sacks, and Sylvia D. Hall-Ellis, Colorado Community College System. U.S. Department of Agriculture, Agriculture and Food Research Initiative, Food, Agriculture, Natural Resources and Human Sciences Education and Literacy Initiative, \$404,460 (2017-2020)

Leading and Achieving: the Colorado Agriculture Regional Consortium. Research team: Jack Wiley (PI), Kerry Gabrielson, Trinidad State Junior College; Jeff Cash (PI), Cheryl Sánchez, Anne-Marie Crampton, Lamar Community College; Suzanna Spears, Morgan Community College; Cyndi Hofmeister, Northeast Junior College; Michael Womochil (Co-PI), Casey Sacks, and Sylvia D. Hall-Ellis, Colorado Community College System. U.S. Department of Agriculture, Hispanic Serving Institutions Education Grants Program, \$504,414 (2017-2020)

Cyber Prep Program Planning Grant. Research team: Debbie Sagen (PI), Brenda Lauer, Pikes Peak Community College; Gretchen Martin, Koiosa Insights; and, Sylvia D. Hall-Ellis, Colorado Community College System. U.S. Department of Commerce, Regional Alliances and Multi-stakeholder Partnerships to Stimulate (RAMPS) Cybersecurity Education and Workforce Development, \$199,681 (2016-2018)

Career and Technical Education in Colorado: Pathways to Education and Employment. Research team: Heather McKay (PI), Rutgers University; Sarah Heath, Casey Sacks, Sylvia D. Hall-Ellis, Colorado Community College System. U.S. Department of Education, Institute of Education Sciences, \$1,400,000 (2017-2021)

Pre-Alliance Planning Grant Colorado Community College Alliance. Research team: Victor Vialpondo (PI) and Janel Highfill, Community College of Aurora; Heidi Loshbaugh (Co-PI), Community College of Denver; Rick Reeves, Bill McGreevy, Liz Cox, and Kristin Aslin, Red Rocks Community College; Cathy Pellish, Front Range Community College; Samuel DeVries, Arapahoe Community College; Sylvia D. Hall-Ellis, Colorado Community College System. National Science Foundation, Louis Stokes Alliance for Minority Participation (LSAMP), \$86,817 (2016-2017)

Towards Scalable Differentiated Instruction Using Technology-enabled, Competency-based, Dynamic Scaffolding. Research team: Karen Wilcox (PI) and Vijay Kumar, Center for Computational Engineering, Massachusetts Institute of Technology; Flora McMartin, Broad-based Knowledge; Quinsigamond Community College; and, Casey Sacks, and Sylvia D. Hall-Ellis, Colorado Community College System. U.S. Department of Education, First in the World Developmental Grant, \$2,891,882 (2015-2019)

Colorado Strategic Partnerships Emergency Grant. Research team: Elise Lowe-Vaughn (PI), Amy Hodson, Celia Hardin, Barbara McBride, James Newby, Christopher Dewhurst, Nina Holland, Kate Anderson, MaryAnn Roe, Chrystalynn Chrystalynn, Elaine Edon, Mona Barnes, Tom Morgan, Rob Hanni, and Marie Valenzuela, Colorado Department of Labor and Employment; Steve Anton, Joelle Brouner, and, Katie Griego, Colorado Department of Human Services; Rebecca Holmes, Judith Martinez, and Jennifer Jirous, Colorado Department of Education; Emily Templin Lesh, Colorado Workforce Development Council; Cory Everett, Colorado Department of Regulatory Agencies; and, Casey Sacks and Sylvia D. Hall-Ellis, Colorado Community College System. U.S. Department of Labor, Strategic Partnerships Emergency Grant, \$5,000,000 (2015-2017)

CAEL Jump Start Program: Competency-Based Education. Research team: Jerry Migler (PI), Casey Sacks, Debra Cohn, Thomas Hartman, and Sylvia D. Hall-Ellis, Colorado Community College System; Matt Jamison, Front Range Community College; Mike Coste, Red Rocks Community College; Amanda Corum, Pueblo Community College; Janet Colvin, Pikes Peak Community College; and, MaryAnn Matheny, Community College of Denver. Council for Adult and Experiential Learning, \$15,000 (2015)

Summit on the Redesign of Developmental Education. Research team: Jerry Migler (PI), Casey Sacks, and Sylvia D. Hall-Ellis, Colorado Community College System; Chip Nava, Pueblo Community College; Kim Moultny, Arapahoe Community College; Debbie Ulibarri, Trinidad State Junior College; and, Kris Bernard, Front Range Community College. American Association of Community Colleges, \$15,000 (2015-2016)

Equity in Excellence at Colorado Community Colleges. Research team: Keith Howard (PI) and Sylvia D. Hall-Ellis, Colorado Community College System; Estela Mata Bensimon, Center for Urban Education, University of Southern California; and Kerry Gabrielson, Trinidad State Junior College. Colorado Department of Higher Education, Colorado Opportunity Scholarship Program, \$150,000 (2015-2016)

MBA High School of Business in Colorado. Research team: Laurie Urich (PI) and Sylvia D. Hall-Ellis, Colorado Community College System; Rudolph Sumpter and Beatrice Gerrish, Boulder Valley Schools; Keith Curry Lance, RSL Research Group. Colorado Department of Higher Education, Colorado Opportunity Scholarship Program, \$501,295 (2015-2016)

Fullbridge Program in Colorado. Research team: Keith Howard (PI) and Sylvia D. Hall-Ellis, Colorado Community College System; Suzanna Spears, Fort Morgan Community College; and, Cheryl Sánchez and Anne-Marie Compton, Lamar Community College. Colorado Department of Higher Education, Colorado Opportunity Scholarship Program, \$300,000 (2015-2017)

Colorado Community College System Alternative Credit Program. Research team: Jerry Migler (PI), Keith Howard, and Sylvia D. Hall-Ellis, Colorado Community College System. American Council on Education, \$13,000 (2015)

Internationalization in Higher Education. Researcher: Samuel D. Museus. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Internationalization, \$8,000 (2014-2015)

International Perspectives on Bilingual Education. Researcher: Sharolyn Pollard-Durodola. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Internationalization, \$3,700 (2014-2015)

Cultivating Culturally Relevant and Responsive Curriculum and Pedagogy in College. Researcher: Samuel Museus. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, iRise Grant, \$5,000 (2014-2015)

Project EMERGE (Educational Model for Evaluation and Replicability in Gifted Environments). Research team: Norma Hafenstein (PI) and Bruce Uhrmacher. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Lynde and Harry Bradley Foundation, \$235,000 (2014-2015)

Collecting Asian American Refugee Stories. Researcher: Samuel Museus. Technical reviewer for MCE: Sylvia D. Hall-Ellis. American Educational Research Association, Research Grant, \$5,000 (2014-2015)

Developing Expertise in Teaching K-5 Mathematics. Research team: Julie Sarama (PI) and Douglas H. Clements (Co-PI), in partnership with the School of Education at the University of Michigan. Technical reviewer for MCE: Sylvia D. Hall-Ellis. National Science Foundation, \$130,344 (2013-2015)

Investigation of the Long-Term Outcomes for Special Education Students. Researcher: Antonio Olmos-Gallos. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Jefferson County School District, Office of Assessment, \$40,000 (2014)

Refugee Community Collaboration. Researcher: Vicki Tomlin (PI) in partnership with the African Community Center and Jewish Family Service. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Center for Community Engagement and Service Learning, \$14,854 (2014-2015)

Access to Mathematics for All. Research team: Richard S. Kitchen (PI), Nicole M. Russell (Co-PI), and Terrence Blackman (Co-PI), Curriculum Studies and Teaching Program, Morgridge College of Education; Álvaro Árias (Co-PI, Department of Mathematics); and, James Gray (Co-PI), Department of Mathematics, Community College of Aurora. Technical reviewer for MCE: Sylvia D. Hall-Ellis. National Science Foundation, The Robert Noyce Scholarship Program, Capacity Building Project, \$349,926 (2014-2016)

Cognitive Test Battery for Intellectual Disabilities. Research team: Karen Riley (PI), Lyndsay Agans, Jessica Lerner, and Karin Dittrick-Nathan in partnership with David Hessel (PI), The MIND Institute at the University of California – Davis, and Elizabeth Berry-Kravis, Rush University Medical Center. Technical reviewer for MCE: Sylvia D. Hall-Ellis. National Institutes of Health, Outcome Measures for Use in Treatment Trials for Individuals with Intellectual and Developmental Disabilities (R01), \$2,499,996 (\$588,672 at DU) (2014- 2019)

Broadening Participation in Engineering among Women and Latino/as: A Longitudinal, Multi-Site Study. Researcher: Patton Garriott (PI) in partnership with the University of North Dakota and the University of Missouri. Technical reviewer for MCE: Sylvia D. Hall-Ellis. National Science Foundation, HER Core Research, \$677,390 (\$69,992 at DU) (2014-2019)

Designing a Teacher Evaluation System to Improve Teacher Effectiveness for Culturally and Linguistically Diverse Learners. Research team: María del Carmen Salazar (PI), Jessica Lerner, and Kathy Green. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Professional Research Opportunities for Faculty, \$29,988 (2014-2016)

Developing a College-Going Culture in Latina/O Families: Exploring the Influence of Funds of Knowledge on Family Outreach Programs. Researcher: Judy Marquez Kiyama. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Professional Research Opportunities for Faculty, \$18,720 (2014-2016)

Assessment of Quality of Life in Neural Implantation Surgery for the Treatment of Parkinson's Disease. Researcher: Cynthia McRae. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Internationalization, \$3,967 (2014-2015)

Pura Vida: Cloud Forest, Curriculum and Cross-Cultural Study. Research team: Norma Hafenstein (PI) and Bruce Uhrmacher (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Internationalization, \$8,000 (2014-2015)

Online Course Development for Curriculum and Instruction. Researcher: Ruth Chao. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Teaching and Learning, \$3,000 (2014)

Online Course Development for Curriculum and Instruction. Researcher: María del Carmen Salazar. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Teaching and Learning, \$3,000 (2014)

Online Course Development for Curriculum and Instruction. Researcher: Jessica Lerner. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Teaching and Learning, \$3,000 (2014)

Online Course Development for Curriculum and Instruction. Researcher: Duan Zhang. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Teaching and Learning, \$3,000 (2014)

The Mathematics Education of African Americans, 1866 – 1954. Researcher: Nicole M. Russell. Technical reviewer for MCE: Sylvia D. Hall-Ellis. National Academy of Education, Spencer Foundation Postdoctoral Fellowship, \$55,000 (2014-2016)

Early Childhood Care and Education Study. Research team: Carrie Germeroth (PI), Melissa Mincic, and Douglas H. Clements. Technical reviewer for MCE: Sylvia D. Hall-Ellis. State of North Dakota, Department of Public Instruction, \$73,500 (2013-2014)

Graduate Level Specialty in Addiction Counselor Training with Emphasis on Integration of Native American Specific Content. Research team: Ruth Chao (PI) and Michael J. Faragher (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. The Galena Foundation, \$289,732 (2013-2016)

The Collecting Asian American and Pacific Islander Refugee Stories (CARS) Project. Researcher: Samuel Museus. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Center for Community Engagement and Service Learning, \$15,000 (2014)

Parents in Transition: A Multiple Case Study of Parent and Family Orientation Programs. Researcher: Judy Marqu  z Kiyama. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Faculty Research Fund, \$1,500 (2014-2015)

The Sistah Network: Black Women Graduate Students Supporting and Retaining Each Other. Researcher: Nicole M. Russell. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Faculty Research Fund, \$2,708 (2014-2015)

Evaluation of the Northeast Denver Babies Ready for College Program. Research team: Carrie Germeroth (PI), Melissa Mincic, and Douglas H. Clements. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Mile High Montessori, \$9,922 (2013-2014)

Developing Teaching Expertise in K-5 Mathematics. Research team: Julie Sarama (PI) and Douglas Clements (University of Denver), in partnership with Timothy Boerst (PI), Meghan Shaughnessy, Deborah Ball, Hyman Bass (School of Education at the University of Michigan). Technical reviewer for MCE: Sylvia D. Hall-Ellis. National Science Foundation, \$449,827 (\$130,344 at the University of Denver) (2013-2015)

Early Learning Care and Education Study Program Grant for the State of North Dakota. Research team: Carrie Germeroth (PI), Melissa Mincic, and Sheridan Green. Technical reviewer for MCE: Sylvia D. Hall-Ellis. State of North Dakota Department of Public Instruction, \$73,500 (2013-2014)

Local Professional Learning Community for School Leaders. Research team: Susan Korach (PI), Kristina Hesbol, and Rebecca McClure. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Education Development Center, \$10,950 (2013)

Healthy Eaters, Lifelong Movers 2: Implementing Evidence-Based School Environment, Policy, and Curricular Changes to Increase Opportunities for Healthy Eating and Physical Activity in Low Income, Rural Colorado. Research team: Elaine Berlansky, University of Colorado at Denver (PI), Nicholas Cutforth, University of Denver (Co-PI), and Allison Reeds. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Colorado Health Foundation, \$3,103,108 (2013-2017)

Hughes Rare Books Library Room Renovation in the Sturm College of Law. Project team: Patti H. Marks, Sylvia D. Hall-Ellis, and Leigh Elliott. Mabel T. Hughes Charitable Trust, \$34,000 (2013-2014)

The Promise Center Partnership with the Marsico Institute for Early Learning and Literacy and the City and County of Denver. Research team: Karen Riley (PI), Douglas H. Clements (Co-PI), and Sheridan Green. Technical reviewer for MCE: Sylvia D. Hall-Ellis. The Piton Foundation, \$223,468 (2013-2014)

Center of Excellence for Problem Gambling. Research team: Ruth Chao (PI) and J. Mike Faragher. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Colorado Department of Behavioral Health, \$68,021 (2012-2013)

United Way Implementation and Validation Review. Research team: Douglas H. Clements (PI), Amanda Moreno, and Sheridan Green. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Mile High United Way, \$19,737 (2013)

Math/Science Partnership in Rural Districts. Research team: Kristen Bunn (PI, Eagle County Schools), Paul Michalec (Co-PI), and Alegra Reiber. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Colorado Department of Education, Colorado's Mathematics and Science Partnership Program, \$750,000 (2013-2014)

Mathematics and Science Education of African Americans. Research team: Nicole Russell (PI), Sylvia D. Hall-Ellis, Steve Fisher (Penrose Library). Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Professional Research Opportunities for Faculty, \$29,994 (2013-2015)

Online Course Development for Curriculum and Instruction. Researcher: Nicole Russell. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Teaching and Learning, \$3,000 (2013)

Discourse and Opportunity: Undocumented Students and Higher Education Policy. Researcher: Ryan Gildersleeve. Technical reviewer for MCE: Sylvia D. Hall-Ellis. National Academy of Education, Spencer Foundation Postdoctoral Fellowship, \$55,000 (2012-2014)

An Anthropological Study of the Latino Graduation Ceremony. Researcher: Ryan Gildersleeve. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Spencer Foundation, \$39,900 (2012-2013)

Fragile-X and Pharmaceutical Company: Clinical Trial of AFQ056. University of California – Davis Children's Hospital MIND Institute (Sacramento), Children's Hospital Denver, and Rush Children's Hospital at Rush University Medical Center (Chicago). Karen Riley, PI. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Novartis Pharmaceuticals Corporation, \$394,206 (2012-2014)

Project Engage, Phase 2, a DAPRA Grant in partnership with Total Immersion Systems, Inc., and Texas A&M University. Research team: Karen Riley (PI) and Lyndsay Agans (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. U.S. Department of Defense, \$60,000 (2011-2013)

International School Psychology Practicum Exchange. Researcher: Gloria L. Miller. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Center for Community Engagement and Service Learning, \$14,951 (2012-2013)

Online Course Development for Curriculum and Instruction. Research team: Bruce Uhrmacher (PI) and Norma Hafenstein (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Teaching and Learning, \$18,193 (2012-2014)

Writers in the Schools. Research team: Karen Riley (PI) and Amanda Moreno (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. Colorado Humanities, \$4,965 (2012-2013)

Learning Ecosystem Validation Grant. Research team: Karen Riley (PI), Lyndsay Agans, Kent Seidel, and Shimelis Assefa. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Bill & Melinda Gates Foundation, \$315,000 (2012-2013)

Project Words of Oral Reading and Language Development (WORLD). Research team: Jorge E. Gonzalez (PI), Texas A&M University; Laura Saenz (Co-PI), University of Texas – Pan American; and, Sharolyn Pollard-Durodola (Co-PI), University of Denver. Technical reviewer for MCE: Sylvia D. Hall-Ellis. U.S. Department of Education, Institute of Education Sciences, \$53,354 (2012-2015); award \$640,718, transfer from Texas A&M University

Increasing the Efficacy of an Early Mathematics Curriculum with Scaffolding Designed to Promote Self-Regulation. Research team: Douglas H. Clements (PI) and Julie Sarama (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. U.S. Department of Education, Institute of Education Sciences, \$1,445,315 (2008-2014); award \$4,541,975, transfer from University at Buffalo, The State University of New York

Using Rule Space and Poset-based Adaptive Testing Methodologies to Identify Ability Patterns in Early Mathematics and Create a Comprehensive Mathematics Ability Test. Research team: Douglas H. Clements (PI) and Julie Sarama (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. National Science Foundation, \$323,791 (2010-2014); award \$1,194,944, transfer from University at Buffalo, The State University of New York

Comprehensive Postdoctoral Training in Scientific Education Research. Researcher: Julie Sarama (PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. U.S. Department of Education, Institute of Education Sciences, \$133,458 (2010-2014); award \$613,353, transfer from University at Buffalo, The State University of New York

Longitudinal Study of a Successful Scaling Up Project: Extending TRIAD. Research team: Douglas H. Clements (PI), Julie Sarama (Co-PI), and Abt Associates (Carolyn Layzer, Fatih Unlu, Laurie Bozzi, Lily Fesler, Alina Martinez, Cristofer Price, James van Orden). Technical reviewer for MCE: Sylvia D. Hall-Ellis. Institute of Education Sciences, \$384,940 (2011-2015; award \$1,250,286, transfer from University at Buffalo, The State University of New York

Early Childhood Education in the Context of Mathematics, Science, and Literacy. Research team: Julie Sarama (PI), Douglas H. Clements (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. National Science Foundation, \$990,020 (2010-2014; award \$2,285,228, transfer from University at Buffalo, The State University of New York

Early Childhood Clearinghouse Information Center Redesign. Research team: Karen Riley (PI) and Amanda Moreno (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. Office of the Lt. Governor of Colorado, \$12,000 (2012-2013)

Morgridge Rural Educational Leadership Initiative. Research team: Lyndsay Agans (PI), Linda Brookhart (Co-PI), Susan Korach, and Rebecca McClure. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Morgridge Family Foundation, \$100,000 (2012-2014)

Center of Excellence for Problem Gambling. Research team: Patrick Sherry (PI) and J. Mike Faragher. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Colorado Department of Behavioral Health, \$55,000 (2012-2013)

Intermodal Transportation Institute Research Initiatives. Researcher: Patrick Sherry. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University Transportation Centers Program, Research and Innovative Technology Administration, U.S. Department of Transportation. \$600,000 (2012-2014)

Mile High United Way Social Innovation Fund Early Literacy Initiative. Gloria L. Miller, PI, Amanda Moreano, Kim Hartnett-Edwards, and Sheridan Green. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Mile High United Way, \$89,992 (2012-2013)

International School Psychology Practicum Exchange. Researcher: Gloria L. Miller. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Center for Community Engagement and Service Learning, \$1,000 (2012-2013)

Refugee Student Art Outreach. Researcher: Karin Ditttrick-Nathan. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Faculty Research Fund, \$3,000 (2012-2013)

Resistance, Resilience, and Reciprocity: Centering the Voices of Black Doctoral Women with Faculty Aspirations. Researcher: Nicole M. Russell. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Faculty Research Fund, \$2,965 (2012-2013)

Assessing Learning through Student Notebooks. Research team: Keith Miller, Nancy Sasaki, and Kathy Green.

Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Professional Research Opportunities for Faculty, \$30,000 (2012-2014)

International School Psychology Practicum Exchange. Researcher: Gloria L. Miller. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Internationalization, \$2,000 (2012-2013)

ELO in Colorado. Research team: Cynthia Hazel and Duan Zhang. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Colorado Legacy Foundation, \$150,000 (2012-2013)

Creating Online LIS Courses. Research team: Mary C. Stansbury (PI), Shimelis Assefa, Denise Anthony, Xiao Hu, and Krystyna Matusiak. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Center for Teaching and Learning, \$15,000 (2011-2013)

Maritime Piracy Seminar. Researcher: Ved Nanda, The Nanda Center, Sturm College of Law. Technical reviewer for Sturm College of Law: Sylvia D. Hall-Ellis. Arsenault Family Foundation, \$15,000 (2012-2013).

Early Childhood Librarianship: An Interdisciplinary, Experiential Learning MLIS. Researcher: Mary C. Stansbury (PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. Laura Bush's 21st Century Librarians Program, Institute for Museums and Library Services, \$249,066 (2012-2014)

Learning Ecosystem Planning Grant. Research team: Karen Riley (PI), Lyndsay Agans, Kent Seidel, and Shimelis Assefa. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Bill & Melinda Gates Foundation, \$281,217 (2011-2012)

Advanced Service Learning Practitioner Faculty Grant, Sylvia D. Hall-Ellis, University of Denver, Center for Community Engagement and Service Learning, \$400 (2011)

Evaluating and Enhancing the EspeciallyMe Program. Research team: Lori D. Patton and Nicole Russell. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Center for Community Engagement and Service Learning, Public Good Fellowship Grant, \$24,780 (2012)

Choosing Excellence: Let Every Child Bloom. Research team: Shimelis Assefa (PI) and Mary C. Stansbury (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Center for Community Engagement and Service Learning, Public Good Grant, \$7,657 (2012)

K-8 STEM Content Specific Professional Development to Improve Elementary Student Achievement. Research team: Kent Seidel (PI), Nicole Russell (Co-PI), Kimberly Hartnett-Edwards, Paul Michalec, Jeff Farmer, Keith Miller, Alegra Reiber, and Nancy Sasaki. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Colorado Department of Education, Improving Teacher Quality Grant, (Title II ESEA), \$307,299 (2011-2012)

Building a Better Principal Pipeline to Boost Student Achievement. Researcher: Susan Korach. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Denver Public Schools; a subcontract from the Wallace Foundation Grant, \$170,000 (2011-2017)

Center of Excellence for Problem Gambling. Research team: J. Mike Faragher (Co-PI) and Bobbie Vollmer (PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. Colorado Department of Behavioral Health, \$79,999 (2011-2012)

An Exploration of Novice Teachers' Core Competencies: Impacts on Student Achievement, and Effectiveness of Preparation. Research team: Kent Seidel (PI), Kathy Green (Co-PI), Kimberly Hartnett-Edwards, and Duan Zhang. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Institute of Education Sciences, Effective Teachers and Effective Teaching, \$990,987 (2012-2015)

Project Engage, a DAPRA Grant in partnership with Total Immersion Systems, Inc., and Texas A&M University.

Research team: Karen Riley (PI) and Lyndsay Agans (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. U.S. Department of Defense, \$49,964 (2011-2014)

User-centered Evaluation of Music Search Engines. Researcher: Xiao Hu. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Faculty Research Fund, \$2,931 (2011-2012)

Educational Practicum in Vietnam and China to Promote the Inclusion of Young Children with Disabilities.

Researcher: Gloria L. Miller. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Office of Internationalization, \$5,000 (2011-2012)

Evaluation of Colorado's Enhancing Quality in Infant-Toddler (EQIT) Initiative. Research team: Virginia R.

Maloney and Amanda Moreno. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Buell Foundation, \$395,884 (2011-2013)

Creating Engaging Environments to Teach Pre-Algebra Mathematics to Elementary Students. Research team:

Álvaro Arias, Mario López, María del Carmen Salazar, and Lyndsay Agans. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver Interdisciplinary Grant, \$60,000 (2011-2012)

Morgridge Education Technology Accessible (META) Resource Project. Research team: Lyndsay Agans (PI) and

Shimelis Assefa (Co-PI). Technical reviewer for MCE: Sylvia D. Hall-Ellis. Morgridge Family Foundation, \$36,000 (2011)

MCE Connect: A 21st Century Framework for Faculty Development. Research team: Bruce Uhrmacher (PI),

Shimelis Assefa (Co-PI), Lyndsay Agans (Co-PI), Kimberly Hartnett-Edwards, Norma Hafenstein, Xiao Hu, Paul Michalec (Co-PI), María del Carmen Salazar (Co-PI), and Sandra Snyder-Mondragon. Technical reviewer for MCE: Sylvia D. Hall-Ellis. University of Denver, Center for Teaching and Learning, \$22,355 (2011-2012)

Parent Education and Parent Leadership and Advocacy. Research team: Virginia Maloney (PI) and Amanda

Moreno, Marsico Institute for Early Learning and Literacy. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Colorado Health Foundation, \$27,358 (2010-2011)

Intentional School Culture. Researcher: Cynthia Hazel. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Denver Public Schools, \$38,040 (2010-2011)

Healthy Eaters, Lifelong Movers: Implementing Evidence-Based School Environment, Policy, and Curricular Changes to Increase Opportunities for Healthy Eating and Physical Activity in Low Income, Rural Colorado.

Research team: Elaine Berlansky, University of Colorado at Denver (PI), Nicholas Cutforth, University of Denver (Co-PI), and Allison Reeds. Technical reviewer for MCE: Sylvia D. Hall-Ellis. Colorado Health Foundation, \$1,683,277 (2011-2014)

Second Life Learning Community. Research team: Don McCubbrey (PI), Sylvia D. Hall-Ellis (Co-PI), Walter

LaMendola, and Paul Novak. University of Denver, Center for Teaching and Learning, \$13,000 (2010-2011)

Reintroducing the Value of Law Librarians to Academic and Public Librarians in Colorado through the

Identification and Use of Emerging Technologies. Research team: Sylvia D. Hall-Ellis (Co-PI), Stacey L.

Bowers (PI), and Christopher Hudson, in partnership with Denver Public Library, Arapahoe Library District, and the Colorado State Supreme Court Library. University of Denver, Center for Community Engagement and Service Learning, \$5,773 (2010-11)

Reintroducing the Value of Law Librarians to Public Librarians through the Identification and Use of Emerging

Technologies and Resources. Research team: Stacey Bowers (PI) and Sylvia D. Hall-Ellis (Co-PI). American Association of Libraries, Wolters Kluwer Law & Business Grant Program, \$2,725 (2010-2011)

Faculty Service Learning Pod. Research team: María del Carmen Salazar and Nicholas Cutforth (Curriculum and Instruction Program), Frank Tuitt (Higher Education Program), Cynthia Hazel and Gloria Miller (Child, Family, and School Psychology Program), and Sylvia D. Hall-Ellis (Library and Information Science Program). University of Denver, Center for Community Engagement and Service Learning, \$8,000 (2010-2011)

Lincoln Collaborative. Research team: Antonio Esquibel (Principal, Lincoln High School), María del Carmen Salazar (Curriculum and Instruction Program), and Sylvia D. Hall-Ellis. School Improvement Grant, Denver Public Schools, \$375,000 (2010-2012)

Future LEADers of America: Leaders III. Research team: Denver Public Library (Kristen Svendsen, PI), the University of Denver (Sylvia D. Hall-Ellis), the Colorado Chapter of REFORMA (Orlando Archibeque), and the Colorado Association of Libraries (Martin Garner). Laura Bush's Recruiting Librarians for the 21st Century Program, Institute for Museums and Library Services, \$988,366 (2009-2012)

Teaching for Success in the Library Environment: LIS 4030 in Library 2.0. Research team: Deborah S. Greal (PI) and Sylvia D. Hall-Ellis (Co-PI). University of Denver, Center for Teaching and Learning, \$ 9,350 (2009-10)

Writing Group Faculty Grant, Sylvia D. Hall-Ellis, University of Denver, Center for Community Engagement and Service Learning, \$750 (2008-2009)

Collaborative Learning Faculty Grant, Sylvia D. Hall-Ellis, University of Denver, Center for Teaching and Learning, \$1,500 (2008)

Connecting Information Literacy to Learning. Research team: Lori Micho, Merrie Valliant, Amanda Samland, and Sylvia D. Hall-Ellis. Colorado State Library, LSTA Discretionary Grant Program, \$19,548 (2008-2009)

Law Librarianship Fellows Program. Research team: Sylvia D. Hall-Ellis (PI), Stacey Bowers (Co-PI), and Christopher Hudson, Westminster Law Library. Laura Bush's 21st Century Librarians Program, Institute for Museums and Library Services, \$999,370 (2008-2012)

Grant Writing in a Cooperative Learning Environment. Sylvia D. Hall-Ellis. University of Denver, Center for Teaching and Learning, \$2,000 (2008-2009)

Project Homeless Connect 6 Event Evaluation. Research team: Sylvia D. Hall-Ellis and Duan Zhang. University of Denver Center for Community Engagement and Service Learning, \$9,785 (2008)

Project Ecuador: International Learning Service Libraries – A Faculty Development Experience in Ecuador. Sylvia D. Hall-Ellis, Office of Internationalization, University of Denver, \$600 (2007)

Project Ecuador: International Learning Service Libraries – A Faculty Development Experience in Ecuador. Sylvia D. Hall-Ellis, International Service Learning Office, University of Denver, \$400 (2007)

Project Homeless Connect 5 Event Evaluation. Research team: Sylvia D. Hall-Ellis and Duan Zhang. University of Denver Center for Community Engagement and Service Learning, \$1,000 (2007)

Project Homeless Connect 4 Event Evaluation. Research team: Sylvia D. Hall-Ellis and Duan Zhang. University of Denver Center for Community Engagement and Service Learning, \$4,595 (2007)

Future LEADers of America: Leaders II. Research team: Denver Public Library (Kristen Svendsen, PI), the University of Denver (Sylvia D. Hall-Ellis), and the Colorado Chapter of REFORMA (Orlando Archibeque). Laura Bush's Recruiting Librarians for the 21st Century Program, Institute for Museums and Library Services, \$988,518 (2007-2010)

Strategic Planning Assistance for the Denver Medical Library. Sylvia D. Hall-Ellis. Denver Medical Library, Inc., \$30,000 (2006-2007)

Destiny Software for JMAC Student Lab. Sylvia D. Hall-Ellis, gift from the Sagebrush Corporation, Minneapolis, Minnesota, \$10,000 (2006)

Libraries: Tools for Education and Development Worldwide: A Faculty Development Experience in France. Sylvia D. Hall-Ellis, Office of Internationalization, University of Denver, \$800 (2005)

Denver Public Library: Opportunities for Change. Sylvia D. Hall-Ellis, Colorado Community Based Research Network, \$2,000 (2005)

Future LEADers of America. Research team: Denver Public Library (Letty Icolari and Steve Taylor, PIs), Emporia State University (Jim Agee), and the University of Denver (Sylvia D. Hall-Ellis). Recruiting Librarians for the 21st Century Program, Institute for Museums and Library Services, \$670,315 (2005-2008)

American Association of University Professors Summer Institute Professional Development Grant. Sylvia D. Hall-Ellis, American Association of University Professors, \$300 (2005)

Beta Phi Mu Alumni Tea. Sylvia D. Hall-Ellis, gift from the Office of Alumni and Parent and Relations, University of Denver, \$2,000 (2005)

Spectrum Software for LIS Student Lab. Sylvia D. Hall-Ellis, gift from the Sagebrush Corporation, Minneapolis, Minnesota, \$5,000 (2005)

Developing Research Capacity in Community Organizations and Residents through Training and Technical Assistance. Nicholas J. Cutforth (PI) and Sylvia D. Hall-Ellis. Piton Foundation, \$25,000 (2005-2006)

Libraries: Tools for Education and Development Worldwide: A Faculty Development Experience in Argentina. Sylvia D. Hall-Ellis, Office of Internationalization, University of Denver, \$500 (2004)

Developing Research Capacity in Community Organizations and Residents through Training and Technical Assistance. Research team: Nicholas J. Cutforth (PI), Gary Lichtenstein and Sylvia D. Hall-Ellis, Piton Foundation, \$25,000 (2003-2004)

Increasing Spanish-Speaking and Hispanic Diversity among Library and Information Science Students at the University of Denver: Development of a Student Recruitment Model. Researcher: Sylvia D. Hall-Ellis (PI), Office of Multicultural Excellence, University of Denver, \$2,750 (2003-2004)

Collection Development Enrichment to Support the Cataloging & Classification Specialization and School Librarianship within the Library & Information Science Program at the University of Denver. Research team: Sylvia D. Hall-Ellis (PI) and Deborah S. Grealy. Women's Library Association, University of Denver, \$4,000 (2003)

Collection Development Enrichment to Support the Library & Information Science Program at the University of Denver. Research team: Deborah S. Grealy (PI) and Sylvia D. Hall-Ellis. Women's Library Association, University of Denver, \$4,000 (2001)

Wireless LAN for Library Education. Research team: Deborah S. Grealy (PI) and Sylvia Hall-Ellis. University of Denver, Center for Teaching and Learning, \$24,000 (2001-2002)

Upward Bound – A Program for South Texas Youth. Research team: Monte Churchill, Executive Director of Community Relations; Gary R. Saucedo, Outreach Coordinator; Raymond Hernandez, (PI) Associate Dean for Student Success; and Sylvia D. Hall-Ellis, South Texas Community College (McAllen, Texas). U.S. Department of Education, Office of Postsecondary Education, \$2,672,003 (1999-2003)

Write Now! Improving Elementary Students' Writing Skills. Research team: Caryl G. Thomason, Assistant Superintendent (PI); Karen Tankersley, Principal; and, Hal Anderson, Director of Technology, Cheyenne Mountain School District 12 (Colorado Springs, Colorado); and Sylvia D. Hall-Ellis. Colorado Department of Education, Educational Telecommunications Unit, \$189,453 (1999-2001)

Operation Quick Start -- Distance Learning in Rural Colorado. Research team: Randal Weigum, Technology Coordinator (PI); Bonnie Barns, Director of Federal Programs and Staff Development; and, Adam "Joe" Raskop, Executive Director, Southeastern Board of Cooperative Educational Services (Lamar, Colorado); and Sylvia D. Hall-Ellis. Colorado Department of Education, Educational Telecommunications Unit, \$400,000 (1999-2001)

Advanced Technological Training for Information Professionals for the 21st Century. Research team: Mario Reyna, Division of Business Director (PI) and Sylvia D. Hall-Ellis, South Texas Community College (McAllen, Texas). Phi Delta Kappa and the National Science Foundation, \$250,000 (1999-2001)

Working Connections – Training Information Technologies Professionals for the 21st Century. Research team: Mario Reyna, Division of Business Director (PI) and Sylvia D. Hall-Ellis, South Texas Community College (McAllen, Texas). Microsoft Corporation and the American Association of Community Colleges, \$1,147,775 (1999-2001)

The ROAD (Research Oriented Amplification of Development to Literacy) Program. Research team: Deborah J. Leong (PI), Metropolitan State University; Elena Bodrova, Metropolitan State University; Dmitri Semenov, Robert J. Marzano, and Sylvia D. Hall-Ellis, Mid-continent Regional Educational Laboratory (Aurora, Colorado). Hewlett-Packard Foundation, \$25,000 (1998-1999)

Press to Literacy. Research team: Deborah J. Leong (PI), Metropolitan State University; Elena Bodrova, Metropolitan State University; Dmitri Semenov, Robert J. Marzano, and Sylvia D. Hall-Ellis, Mid-continent Regional Educational Laboratory (Aurora, Colorado). The Denver Post and the Robert S. McCormick Foundation, \$49,818 (1998-1999)

International Telementor Center. Research team: David Neils, David B. Frost (PI), and Sylvia D. Hall-Ellis, Mid-continent Regional Educational Laboratory (Aurora, Colorado). Hewlett-Packard Philanthropy, \$100,000 (1998-1999)

America Reads: Providing Tutor Training for the America Reads Challenge. Research team: Louis F. Cicchinelli (PI) and Sylvia D. Hall-Ellis, Mid-continent Regional Educational Laboratory (Aurora, Colorado). U.S. Department of Education, Office of Educational Research and Improvement, \$306,000 (1998-1999)

Comprehensive School Reform. Research team: Louis F. Cicchinelli (PI), J. Timothy Waters, and Sylvia D. Hall-Ellis, Mid-continent Regional Educational Laboratory (Aurora, Colorado). U.S. Department of Education, Office of Educational Research and Improvement, \$285,000 (1998-1999)

Sustainable Energy Education (SEE) Program for Grades 4-8: Preparing Today's Youth for Lifelong Learning and Responsible Actions in Energy Conservation. Research team: Mary Gromko, Colorado Department of Education; Gene McCarthy, Rocky Flats Field Office, U.S. Department of Energy; Gina Kissell, National Renewable Energy Laboratory; and Barbara L. McCombs (PI), Janet L. Bishop, and Sylvia D. Hall-Ellis, Mid-continent Regional Educational Laboratory (Aurora, Colorado). State of Colorado Governor's Office for Energy Conservation, \$499,936 (1997-1999)

Review of Kansas Curriculum Standards in Mathematics and Language Arts. Research team: Robert J. Marzano (PI), John S. Kendall, David B. Frost, and Sylvia D. Hall-Ellis, Mid-continent Regional Educational Laboratory (Aurora, Colorado). Submitted to the Kansas State Department of Education, \$25,903 (1998)

International Telementor Center. Research team: David Neils, John Kuglin, Chris Rapp, David B. Frost (PI), and Sylvia D. Hall-Ellis, Mid-continent Regional Educational Laboratory (Aurora, Colorado). Hewlett-Packard Company, \$54,752 (1998)

Genesis Mission: Education Public Outreach. Developed in partnership by the Jet Propulsion Laboratory, California Institute of Technology, Los Alamos National Laboratory, Lockheed Martin Astronautics, and the Mid-continent Regional Educational Laboratory (Aurora, Colorado). Research team: John T. Sutton (PI), Alice Krueger, Martha Henry, Greg Rawls, Shae Isaacs, Jeff Johnson, Deb Jordan, Arlene Mitchell, David B. Frost, Jana Caldwell, J. Timothy Waters, and Sylvia D. Hall-Ellis. National Aeronautics and Space Administration, \$139,700,000; subcontract award, \$4,750,000 (1997-2007)

North Dakota Mathematics Assessment Project. Developed in partnership by the North Dakota Department of Education and Mid-continent Regional Educational Laboratory (Mid-continent Regional Educational Laboratory). Research team: Ann Clapper, Clarence Bina, Greg Gallagher, North Dakota Department of Education; Don Burger (PI), Hillary Michaels, and Sylvia D. Hall-Ellis, Mid-continent Regional Educational Laboratory (Aurora, Colorado). U.S. Office of Education, \$1,618,214; subcontract award, \$389,076 (1997-2001)

Pacific Resources for Education and Learning Distance Education: Project Evaluation. Developed in partnership by the Pacific Educational Community, the Pacific Resources for Education and Learning (PREL), and Mid-continent Regional Educational Laboratory (McREL). Research team: John W. Kofel (PI), Executive Director, PREL; J. Timothy Waters, Executive Director; Joan Buttram, Robert Keller, and Sylvia D. Hall-Ellis, Mid-continent Regional Educational Laboratory (Aurora, Colorado). U.S. Office of Education, Star Schools Program, \$10,000,000; subcontract award, \$500,000 (1997-2002)

Identification of Bilingual Gifted and Talented Children: A Comprehensive School Grants for Bilingual Education. Developed in partnership by Hidalgo (Texas) ISD, Los Fresnos (Texas) CISD, Progreso (Texas) ISD, University of Texas – Pan American (Edinburg, Texas), and Education Service Center, Region One (Edinburg, Texas). Research team: Hilda Medrano, Dean, College of Education, University of Texas – Pan American; Linda Phemister (PI), Janie Navarro, and Sylvia D. Hall-Ellis, Education Service Center, Region One. U.S. Department of Education, Office of Bilingual Education and Minority Languages Affairs, \$1,670,633 (1997-2002)

Academics 2000: First Things First -- The Texas Goals 2000. Developed for Jim Hogg (Hebbronville, Texas) County ISD, Mirando City (Texas) ISD, and San Isidro (Texas) ISD. Research team: Hilda Medrano, Dean, College of Education, University of Texas - Pan American (Edinburg, Texas); Angie Lehmann, Amy Mares, Ellen Gonzalez (PI), and Sylvia D. Hall-Ellis, Education Service Center, Region One (Edinburg, Texas). Texas Education Agency, \$339,987 (1997-2000)

Southwestern Bell's Learning Communities Initiative. Developed for Tech Prep of the Rio Grande Valley, Inc. (Harlingen, Texas); the Center for Professional Teacher Development, University of Texas – Brownsville; Teach for America – Rio Grande Valley (McAllen, Texas); and Education Service Center, Region One (Edinburg, Texas). Research team: Patricia G. Bubb, Executive Director (PI), Tech Prep of the Rio Grande Valley; Martin Winchester, Executive Director, Teach for America – Rio Grande Valley; Aileen Johnson, Director, School of Education, University of Texas – Brownsville; and Sylvia D. Hall-Ellis. Southwestern Bell Foundation, \$100,000 (1997-1998)

Texas School to Work: Regional Implementation. Developed for Tech Prep of the Rio Grande Valley, Inc. (Harlingen), South Texas Community College (McAllen), Texas State Technical College (Harlingen), Texas Southmost College (Brownsville), Empowerment Zone of the Rio Grande Valley (Mercedes), Project VIDA (Weslaco), Youth Fair Chance (McAllen), and Education Service Center, Region One (Edinburg). Research team: Patricia G. Bubb (PI), Tech Prep of the Rio Grande Valley; Stephen Vassberg, Texas State Technical College; Ellen Trevino, Youth Fair Chance; Wanda Garza, Project VIDA; Leonardo Olivares, University of Texas – Pan American; Michael Bell, South Texas Community College; and Sylvia D. Hall-Ellis. Texas Workforce Commission, \$4,250,000 (1997-2002).

Comprehensive Bilingual Education Grant for Hidalgo ISD and Roma ISD. Developed for Hidalgo (Texas) ISD, Roma (Texas) ISD, College of Education, University of Texas – Pan American (Edinburg, Texas), and Education Service Center, Region One (Edinburg, Texas). Research team: Tomas Thomas (PI), Director, Office of Bilingual Education and Sylvia D. Hall-Ellis. U.S. Department of Education, Office of Bilingual Education and Minority Languages Affairs, \$1,531,361 (1997-2002)

Lopez High School, Porter High School, Rivera High School, Central Middle School, and Perkins Middle School, Brownsville (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$1,473,611 (1997-1998)

Donna High School, Todd Middle School, and Solis Middle School, Donna (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$704,052 (1997-1998)

Memorial Middle School and Nellie Schunior Middle School, La Joya Independent School District (Texas). Telecommunications Infrastructure Fund Board, \$293,641 (1997-1998)

Martin High School, Christen Middle School, Cigarroa Middle School, Lamar Middle School, Laredo (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$1,200,000 (1997-1998).

Lasara Middle School, Lasara (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$246,210 (1997-1998)

Los Fresnos High School and Resaca Middle School, Los Fresnos (Texas) Consolidated Independent School District. Telecommunications Infrastructure Fund Board, \$500,000 (1997-1998)

Lyford Junior High School, Lyford (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$354,997 (1997-1998)

Travis Middle School, McAllen (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$269,139 (1997-1998)

Mission High School, Mission (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$297,010 (1997-1998)

Progreso High School, Progreso (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$300,000 (1997-1998)

San Perlita High School, San Perlita (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$293,490 (1997-1998)

Myra Green Junior High School, Raymondville (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$225,000 (1997-1998)

Rio Grande City High School, Ringgold Middle School, Gruella Middle School, Rio Grande City (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$809,934 (1997-1998)

Rio Hondo Junior High School, Rio Hondo (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$297,024 (1997-1998)

Roma High School, Roma (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$333,178 (1997-1998)

San Benito High School, Miller Jordan Junior High School, San Benito (Texas) Consolidated Independent School District. Telecommunications Infrastructure Fund Board, \$547,000 (1997-1998)

Santa Maria High School and Santa Maria Middle School, Santa Maria (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$400,000 (1997-1998)

Salvador High School, United Independent School District (Laredo, Texas). Telecommunications Infrastructure Fund Board, \$250,879 (1997-1998)

Weslaco High School, Cabaza Middle School, Cuellar Middle School, Weslaco (Texas) Independent School District. Telecommunications Infrastructure Fund Board, \$874,242 (1997-1998)

Principals' Assessment and Training Center. Developed for Education Service Center, Region One (Edinburg, Texas). Research team: Roberto Zamora, Leonel Barrera, William H. Parry, and Sylvia D. Hall-Ellis. Texas Principals' Leadership Initiative, Texas Association of Secondary School Principals, Texas Association of Elementary School Principals, and the Sid Richardson Foundation. \$461,439 (1996-1998)

The Texas Library Connection -- Integrating and Sharing Resources. Developed for Hidalgo County Library System (McAllen), Cameron County Library System (Brownsville), South Texas Community College (McAllen), the University of Texas - Pan American (Edinburg), University of Texas - Brownsville, and Education Service Center, Region One (Edinburg). Research team: William R. McGee, Coordinator, Hidalgo County Library System; Joe Garcia, Director, Cameron County Library System; Michael D. Bell, Library Director, South Texas Community College; Eleanor Folger Foster, University Library, University of Texas - Pan American; Jaime Vela (PI), Director of Instructional Technology and Media Services, Ron Pontius, Instructional Technology, and Fabiola Fuentes, Media Services, Education Service Center, Region One; and Sylvia D. Hall-Ellis. Office of Library Media Services, Technology Services, Texas Education Agency. \$8,000 (1996-1997)

Developing Leadership Communities for Improving Algebra I for All Students. Developed for a partnership of the Region One Statewide Systemic Initiative Team. Research team: Noel Villarreal (PI), Eduardo Cancino, and Sylvia D. Hall-Ellis, Education Service Center, Region One (Edinburg). Texas Statewide Systemic Initiative for Reform in Mathematics, Science and Technology Education to The Charles A. Dana Center for Mathematics and Science Education, The University of Texas at Austin. \$25,000 (1996-1997)

Academics 2000: First Things First -- The Texas Goals 2000. Developed for Edinburg (Texas) CISD. Research team: Hilda Medrano, Dean, College of Education, University of Texas - Pan American (Edinburg); Helen Jones, Director (PI), Gifted and Talented Education, Edinburg CISD; and Sylvia D. Hall-Ellis, Education Service Center, Region One (Edinburg). Texas Education Agency, \$750,000 (1996-2001)

Texas Teachers Empowered for Achievement in Mathematics (TEXTEAM) Institute for Algebra I. Developed for Hidalgo ISD, Jim Hogg (Hebbronville) County ISD, La Joya ISD, La Villa ISD, Lyford ISD, Mission CISD, Pharr-San Juan-Alamo ISD, Rio Hondo ISD, San Isidro ISD, San Perlita ISD, Santa Maria ISD, Santa Rosa, Sharyland ISD, Valley View ISD, Webb (Laredo) CISD, Weslaco ISD, and Zapata County (Zapata) ISD. Research team: Noel Villarreal (PI), Chuck McInteer, Ellen M. Gonzalez, Education Service Center, Region One (Edinburg); and Sylvia D. Hall-Ellis. Charles A. Dana Center for Mathematics and Science Education, University of Texas - Austin, \$15,975 (1996)

Community Learning Center for La Villa, Texas. Developed for Edcouch-Elsa Independent School District. Research team: Noe Gonzalez (PI), Assistant Superintendent, Edcouch-Elsa Independent School District, and Sylvia D. Hall-Ellis. Delta Region Subzone, Rio Grande Valley (Texas) Rural Empowerment Zone, \$325,000 (1996-1997)

Monte Alto (Texas) Community Learning Center. Developed for Monte Alto Independent School District. Research team: Homero A. Diaz (PI), Superintendent, Monte Alto Independent School District, and Sylvia D. Hall-Ellis. Delta Region Subzone, Rio Grande Valley (Texas) Rural Empowerment Zone, \$300,000 (1996-1997)

Community Learning Center for La Villa, Texas. Developed for La Villa Independent School District. Research team: Sam Gonzalez (PI), Assistant Superintendent, La Villa Independent School District; and Sylvia D. Hall-Ellis. Delta Region Subzone, Rio Grande Valley (Texas) Rural Empowerment Zone, \$325,000 (1996-1997)

Building Project for Taylor Elementary. Developed for Mercedes (Texas) Independent School District. Research team: Mrs. Denise Rivera, Librarian, and Eduardo Infante, Principal, Taylor Elementary School; Ismael S. Cantu (PI), Federal Programs Director, Mercedes Independent School District; and Sylvia D. Hall-Ellis. Delta Region Subzone, Rio Grande Valley (Texas) Rural Empowerment Zone, \$375,000 (1996-1997)

Border Education Network (BEN) - Distance Education through Cable Television. Developed for Edinburg (Texas) Consolidated Independent School District. Research team: Noe Torres (PI), Library Media Specialist, Magdalena Rosas, Library Media and Technology Coordinator, Edinburg Consolidated Independent School District; and Sylvia D. Hall-Ellis, Education Service Center, Region One (Edinburg). Edinburg (Texas) Consolidated School District, \$250,000 (1996-1997)

Multiservice One-Stop Open Enrollment Charter School. Developed for the Information Referral Resource Assistance, Inc. (McAllen, Texas). Research team: Pablo Perez and Aguié Pena (PI), Executive Director, Information Referral Resource Assistance, Inc.; Roberto Zamora and Sylvia D. Hall-Ellis, Education Service Center, Region One (Edinburg). Texas Education Agency, \$3,066,000 (1996-2001)

Project OK: A Community Youth Opportunities Grant for Summer, 1996. Developed for McAllen (Texas) Independent School District, St. Joseph the Worker Catholic Church, and the Office of Adult Education, Education Service Center, Region One. Research team: Father Bart Flatt, St. Joseph the Worker Catholic Church; Noe Calvillo (PI), Office of Adult Education; Maria Louisa Garcia, McAllen Independent School District; and Sylvia D. Hall-Ellis, Education Service Center, Region One (Edinburg). Texas Protective and Regulatory Agency through the Office of the Mayor, City of McAllen, \$350,000 (1996-2001).

Early Childhood: a Time of Discovery. Developed for a partnership of Lasara ISD, Rio Hondo ISD, San Perlita ISD, the School of Education, University of Texas - Brownsville, and Education Service Center, Region One (Edinburg). Research team: Hugo Rodriguez, Dean, School of Education, University of Texas - Pan American; Leonel Barrera and Jack Damron, Field Service Agents, Ellen M. Gonzalez (PI), Administrator for Student Instructional Services, Ruth Solis, Education Specialist in Special Education, and Sylvia D. Hall-Ellis, Education Service Center, Region One (Edinburg). Texas Education Agency, \$722,090 (1996-2001)

Innovative Gifted and Talented Programs for Early Childhood and Elementary Education Students. Developed for a partnership of Edinburg (Texas) CISD, the School of Education, University of Texas - Pan American (Edinburg), and Education Service Center, Region One (Edinburg). Research team: Hilda Medrano, Dean, School of Education, University of Texas - Pan American; Helen de la Garza (PI), Director of Elementary Curriculum and Instruction, Edinburg CISD; and Sylvia D. Hall-Ellis. Texas Education Agency, \$750,000 (1996-2001)

Reading Recovery in Early Elementary Grades. Developed for a partnership of La Villa (Texas) ISD the School of Education, University of Texas - Pan American (Edinburg), and Education Service Center, Region One (Edinburg). Research team: Hilda Medrano, Dean, School of Education, University of Texas - Pan American; Marcario Salinas (PI), Supervisor of Elementary Curriculum and Instruction, La Villa ISD; and Sylvia D. Hall-Ellis, Education Service Center, Region One. Texas Education Agency, \$750,000 (1996-2001)

Connected Mathematics Project for Middle and Junior High Students. Developed for a partnership of Michigan State University, The Charles A. Dana Center for Mathematics and Science Education, The University of Texas at Austin and Region One Statewide Systemic Initiative Team. Research team: Jack Damron, Chuck McInteer, Noel Villarreal (PI), and Sylvia D. Hall-Ellis, Education Service Center, Region One (Edinburg). National Science Foundation through Michigan State University to the Charles A. Dana Center at the University of Texas - Austin, \$539,610 (1996-1999)

Sharing Resources: Testing the Interlibrary Loan Potential of the Texas Library Connection -- Integrating Media Resources. Developed under the sponsorship of the Hidalgo County Library System (McAllen) and Education Service Center, Region One (Edinburg). Research team: William H. McGee, Hidalgo County System Coordinator; Fabiola Fuentes, Library Media; Ronald Pontius (PI), Instructional Technology, Education Service Center, Region One; and Sylvia D. Hall-Ellis. Library Media Services Program, Office of Technology Services, Texas Education Agency, \$25,000 (1996-1997)

State and Federal Adult Education JOBS Program. Developed for Education Service Center, Region One (Edinburg). Research team: Noe Calvillo (PI), Director, Adult Education Program, and Sylvia D. Hall-Ellis. Adult Education Program, Texas Education Agency, \$532,846 (1995-1996)

Creating Safe and Drug-Free Schools and Communities. Developed for a partnership of the Region One Consortium for Safe and Drug-Free Education Environments. Research team: Clara Contreras (PI), Health Specialist, and Sylvia D. Hall-Ellis, Education Service Center, Region One (Edinburg). Texas Education Agency, \$150,000 (1995-1996)

Developing Leadership Communities for Improving Mathematics Performance for All Students on Title I Campuses. Developed for a partnership of the Region One Statewide Systemic Initiative Team. Research team: Jack Damron, Chuck McInteer, Noel Villarreal (PI), and Sylvia D. Hall-Ellis, Education Service Center, Region One (Edinburg). Texas Statewide Systemic Initiative for Reform in Mathematics, Science and Technology Education to The Charles A. Dana Center for Mathematics and Science Education, The University of Texas at Austin, \$50,669 (1995-1996)

The Impact of Library Resource Centers on Academic Achievement in Selected Public Schools in South Texas. Sylvia D. Hall-Ellis (PI). Developed under the sponsorship of the Department of Library Science, College of Education and Applied Science, Sam Houston State University (Huntsville). Texas Association of School Librarians, Children's Services Round Table, and Young Adults Round Table (Austin, Texas), \$2,500 (1995)

School Library Media Specialists Fellowship Program. Sylvia D. Hall-Ellis (PI). Developed under the sponsorship of the Department of Library Science, College of Education and Applied Science, Sam Houston State University (Huntsville, Texas). U.S. Department of Education, HEA Title II-B, Library Education and Human Resource Development Program, \$44,000 (1995-1996)

Planning for Educational Technology. Research team: Ruth Ann Riggins (PI), Director of Library Media and Technology Services, Donna (Texas) Independent School District; Noe Torres, Education Service Center, Region One; Patricia G. Bubb, Tech Prep of the Rio Grande Valley, Inc; Michael D. Bell, South Texas Community College; and Sylvia D. Hall-Ellis, Sam Houston State University. Developed under the sponsorship of Donna (Texas) Independent School District, Education Service Center, Region One (Edinburg), Tech Prep of the Rio Grande Valley, Inc. (Harlingen), South Texas Community College (McAllen), and the Department of Library Science, Sam Houston State University (Huntsville). Funded through Infusion of Educational Technology Planning Grant Program (H.B. 18: Models). Texas Education Agency, \$18,000 (1993-1994)

The Impact of Library Resource Centers on Academic Achievement in Selected Public Schools in South Texas.

Sylvia D. Hall-Ellis (PI) and Mary Ann Berry. Developed under the sponsorship of the Department of Library Science, College of Education and Applied Science, Sam Houston State University (Huntsville). Sam Houston State University Research Enhancement Fund, \$7,500 (1993-1994)

Electronic Mail Resource Sharing System: Management and Operation of the Iowa Computer-Assisted Network. State Library of Iowa, \$294,000 (1986-1993)

The Iowa Locator: A CD-ROM Resource Sharing Tool. State Library of Iowa, \$567,000 (1986-1992)

Statewide Database Development: An OCLC Tape Analysis To Determine Feasibility. State Library of South Dakota, \$15,000 (1987)

The Iowa Locator: A Feasibility Study. State Library of Iowa, \$50,000 (1986)

Electronic Mail Resource Sharing System: A Feasibility Study for Libraries in the State of Iowa. State Library of Iowa, \$5,000 (1985)

Access Pennsylvania: A Feasibility Study. State Library of Pennsylvania, \$50,000 (1984)

Automating Library Administrative and Management Tasks: Procurement and Distribution of Microcomputer Systems for District Library Centers in the Commonwealth of Pennsylvania. State Library of Pennsylvania, \$495,000 (1982)

Sharing Serial Titles Resources: Procurement and Distribution of Microfiche Readers for 550 Libraries in the Commonwealth of Pennsylvania. State Library of Pennsylvania, \$500,000 (1982)

Electronic Mail System: A Pilot Project for Libraries throughout the Commonwealth of Pennsylvania. State Library of Pennsylvania, \$125,000 (1982)

Literacy Program for Adults in Rural Upstate New York State: Program Outreach and Evaluation - Phase 3. Appalachian Regional Commission, \$50,000 (1981)

Database Building: A Cooperative Project of the Finger Lakes Library System (Ithaca), Four County Library System (Binghamton), and the Southern Tier Library System (Corning). New York State Library, \$450,000 (1980-1985)

Faces of the Southern Tier: A Professional Photographer-in-Residence. New York State National Endowment for the Humanities, \$25,000 (1980)

Literacy Program for Adults in Rural Upstate New York State: Program Implementation - Phase 2. Appalachian Regional Commission, \$50,000 (1980)

Small Business Resources Center: A Pilot Project for Rural Public Libraries. New York State Library, \$50,000 (1980)

Media Programming: A Professional Development Program for Public Librarians in Upstate New York: A Program in Allegheny, Chemung, Schuyler, Steuben, and Yates Counties. New York State Library, \$35,000 (1979)

Information and Library Resources for Inmates and Prisoners in Selected Upstate New York Facilities: A Cooperative Program in Allegheny, Chemung, Schuyler, Steuben, and Yates Counties. New York State Library, \$25,000 (1979)

Library Resources for Homebound Adults in Upstate New York: An Outreach Program in Allegheny, Chemung, Schuyler, Steuben, and Yates Counties. New York State Library, \$40,000 (1979)

Literacy Program for Adults in Rural Upstate New York State: Program Initiation and Establishment - Phase 1. Funded by the Appalachian Regional Commission. Awarded to the Corning (New York) Public Library, \$50,000 (1979)

Information Reference Services to Homebound Adults. New York State Library, \$31,000 (1978)

Books-By-Mail Services to Homebound Adults. New York State Library, \$31,000 (1978)

System Headquarters Services and Programs for Public Libraries in District 10. Funded through Library Services and Construction Act Title I. Texas State Library and Historical Commission, \$880,000 (1976)

County Library Development Grant for Atascosita County. Funded through Library Services and Construction Act Title I. Texas State Library and Historical Commission, \$15,000 (1976)

Spanish Language Materials Collection Development Program. Texas State Library and Historical Commission, \$40,000 (1975)

Establishment of System Headquarters for Alamo Regional Library System (District 10) Headquartered at the San Antonio (Texas) Public Library. Texas State Library and Historical Commission, \$800,000 (1975)

SELECTED CONSULTANTSHIPS

Project Strategic Planning & Funding Proposal Development

Walden University, 2015.
Pikes Peak Library District, 2014.
Douglas County Public Libraries, 2011-2012.
Denver School for Science and Technology, 2008-2011.
Johnson & Wales University, Denver Campus, 2007-2009.
Challenges, Choices, and Images K-12 Charter School, Denver Public Schools, 2007.
Denver Medical Library, Inc., 2006-2010.
Bemis Public Library (Littleton), Ergonomic Design and Facilities Enhancement Consultant, 2003.
Curtis Park Community Center (Denver), Community Technology Center Evaluation and Proposal Development Consultant, 2003.
Colorado Community Based Research Network (Denver), Funding Research Associate, 2002.
Our Lady of the Rosary Academy (Edgewater), Learning Resource Center Development Project Consultant, 2001-2003
University of Southern Colorado (Pueblo). Technology Integration and Curriculum Enhancement into Higher Education Learning Environment. Proposal Development Consultant, 2000.
Jefferson County Library System (Lakewood), 1999-2000.
Southeastern BOCES (Lamar, CO). Distance Learning Curriculum Content Development Project. Proposal Development Consultant, 1998-2004.
Cheyenne Mountain School District 12 (Colorado Springs, CO). Technology Integration into Elementary Writing Curriculum Project. Proposal Development Consultant, 1998.
Telecommunications Infrastructure Fund Board Round #2 Application, 1997. Technical assistance to the following: La Villa (Texas) Independent School District; Mirando City (Texas) Independent School District; Monte Alto (Texas) Independent School District; San Isidro (Texas) Independent School District.
Telecommunications Infrastructure Fund Board Round #1, 1996. Technical assistance to the following: Brownsville (Texas) Independent School District; Donna (Texas) Independent School District; Edinburg (Texas) Consolidated Independent School District; Harlingen (Texas) Consolidated Independent School District; Jim Hogg County Independent School District (Hebbronville, Texas); La Feria (Texas) Independent School District; La Joya (Texas) Independent School District; La Villa (Texas) Independent School District; Los Fresnos (Texas) Consolidated Independent School District; Progreso (Texas) Independent School District; Raymondville (Texas) Independent School District; Rio Hondo (Texas) Independent School District; San Benito (Texas) Independent School District; Sharyland Independent School District (Mission, Texas); Weslaco (Texas) Independent School District; Zapata (Texas) County Independent School District.
South Texas Community College (McAllen). Strategy Development to Meet the Technology Instructional Needs for Vocational, School-To-Work and Academic Programs, 1994-2000. Development Consultant.
Donna (Texas) Independent School District. Planning for the Infusion of Technology in Middle Schools Serving High At-Risk Students, 1994-1995. Research Associate.
Pettus (Texas) Independent School District. Planning for District-wide Automation in School Library Media Centers: Preparation of Data, and CD-ROM Hardware/Software Configuration Evaluation, 1994. Principal Investigator.
Fort Bend Independent School District (Sugar Land, TX). Planning for District-wide Automation in School Library Media Centers, Preparation of Data, and CD-ROM Hardware/Software Configuration Evaluation, 1994-1995. Principal Investigator.
Northern Waters Library Services (Ashland, WI). Professional Consulting Services to Make Recommendations on the Development of System-Wide Efforts of the Northern Waters Library Service, 1988-1989. Research Associate.
State Library of Iowa (Des Moines). The Iowa Locator Compact Disc to Support Multi-Type Libraries Resource Sharing including Database Design, Building, Production, and Distribution, 1986-1991. Project Director.
State Library of Iowa (Des Moines). Iowa Computer-Assisted Network Development, Enhancement, and Operation, 1985-1992. Technical Director.
State Library of South Dakota (Pierre). Statistical Sampling and Analysis of Multi-Institutional OCLC Archive Tape for Statewide Online Database Building, 1985-1986. Research Associate.
District of Columbia Public Library (Washington, DC). Planning Document for the Retrospective Conversion of Bibliographic Records and Automation Issues in the 1990's, 1984-1985. Research Associate.

State Library of Pennsylvania (Harrisburg). Database Development for High School Libraries, 1984-1985. Research Associate.

MINITEX (Minneapolis, MN). Workshops for Retrospective Conversion, Bar Coding, Library Statistics, and OCLC Serials Format, 1985. Presenter and Research Associate.

ABC Film Consortium (Altoona, Bellefonte, Johnstown, PA). Database Building, System Design, and Implementation of Online Film Booking System, 1983-1985. Database Manager.

Library Automation -- System Design & Implementation

Douglas County Library District (Castle Rock, CO). Library Automation Technical and System Performance Specifications, 2001.

Pharr-San Juan-Alamo Independent School District (Pharr, TX). Library Automation Technical and System Performance Specifications, 1997. Project Development Consultant.

Harlingen (Texas) Independent School District. Planning for District-wide Automation in Junior High School Library Media Centers: Preparation of Data, and CD-ROM Hardware/Software Configuration Evaluation, 1996. Principal Investigator.

Sharyland (Texas) Independent School District. Design and Construction of New Elementary School Library and Technology Resources Center, 1995-1996. Principal Investigator.

South Texas Independent School District (Mercedes, TX). Design and Construction of a New High School Library and Technology Resources Center, 1995-1996. Principal Investigator.

Citizens' Library (Washington, PA). Online Community Resources Files: Design and Implementation, 1984. Research Associate.

Altoona (Pennsylvania) Hospital. Integrated Online System Upgrade Study, 1984. Research Associate.

State Library of South Dakota (Pierre). Automation Plan and State Database Development, 1982-1983. Principal Investigator.

State Library of Pennsylvania (Harrisburg). COM Production and Preparation of the Technical Specifications Document, Procurement, and Distribution of Microcomputers in the Commonwealth of Pennsylvania, 1983-1984. Research Associate.

Altoona (Pennsylvania) Area Public Library. Integrated System (Circulation/Online Catalog) Study, 1982-1983. Principal Investigator.

COPSCAULD (Council of Pennsylvania State College and University Library Directors, Edinboro, PA). Design of Online Media Catalog, 1982. Principal Investigator.

Erie (Pennsylvania) County Library System. Operations Research and Design for Automated Circulation, Hardware Upgrade and Re-Retrospective Conversion, 1982. Research Associate.

Michigan Library Consortium (Lansing, MI). Tape Management System Design and Implementation, 1982-1984. Research Associate.

State Library of Pennsylvania (Harrisburg). Preparation of the Technical Specifications Document, Procurement and Distribution of Microfiche Readers in the Commonwealth of Pennsylvania, 1982. Research Associate.

Community College of Allegheny County, Allegheny Campus (Pittsburgh, PA). Integrated Systems Study, 1981. Principal Investigator.

Community College of Allegheny County, South Campus (West Mifflin, PA). Retrospective Conversion Training Program for Handicapped Students, 1981. Technical Director.

Southern Tier Library System (Corning, NY). OCLC Acquisitions Subsystem Evaluation, 1981. Principal Investigator.

South Central Reference and Research Council (Ithaca, NY). South Central Regional Delivery System, 1980. Principal Investigator.

Southern Tier Library System (Corning, NY). Newspapers on Microfilm in the Chemung-Southern Tier Library System, 1979-1981. Principal Investigator.

Cataloging & Bibliographic Database Building

Challenges, Choices, and Images K-12 Charter School, Denver Public Schools, 2007.

Denver Medical Library, Inc., 2006-2010.

Ricks Gifted and Talented School Library, 2005-

American Humane Society (Englewood), 2004-2006.

Fisher Early Learning Center Library, 2004-

Colorado Community Based Research Network (Denver), 2004-
Boulder Valley School District (Boulder), 2002-2003.
Douglas Public Library District (Castle Rock), 2001-2002.

Bibliographic Center for Research, Inc. (Denver). Original Cataloging, 2001-2003 & 1992-1993. Professional Cataloger. Libraries include Arapahoe Library District (Centennial); Clarke College (Clarke, IA); Kaiser Permanente Center for Health Research (Portland, KS); Douglas County Library System (Carson City, NV); Grinnell College (Grinnell, IA); Kansas State University (Manhattan, KS); Kansas State Library (Topeka, KS); McPherson College (McPherson, KS); Newman University (Wichita, KS); Montana Technical University (Helena, MT); Montana State Library (Helena, MT); University of Texas Southwest Medical Center Library (Dallas, TX); Community College of Southern Nevada (Las Vegas, NV); Pikes Peak Library District (Colorado Springs); Mesa Community College (Grand Junction); Coe College (Cedar Rapids, IA); Briar Cliff College (Sioux City, IA); Tri-Care Health Systems (Aurora); The Penrose-St. Francis Healthcare System (Colorado Springs); Westminster Public Library (Westminster); Lutheran Medical Center Medical Library (Wheat Ridge); American Heritage Center, University of Wyoming (Laramie, WY); National Wildlife Research Center Library (Lakewood); University of Colorado Health Sciences Center, Denison Memorial Library (Denver); Water Resources Library, Denver District Office, Bureau of Land Management (Lakewood); Mesa State Community College Learning Resources Center (Pueblo); Front Range Community College Learning Resources Center (Westminster).

Colorado Historical Society Library (Denver). Original Cataloging of Serials for Multi-Institutional Union Listing Project, 1993. Technical Services Associate. Institutional partners: Denver Art Museum, Denver Museum of Natural History Library, and Denver Botanical Gardens Library.

Arapahoe Library District (Centennial). Original Cataloging of Major Media, 1991.

Original Cataloging Projects: Davis and Elkins College (Elkins, WV), 1989-1990; Waldorf College (Forest City, IA), 1989-1990; Kennametal Corporation (Latrobe, PA), 1984-1985; Pennsylvania Public Libraries Film Center (Harrisburg), 1982-1985.

Retrospective Conversion Projects: North Central Regional Library Service (Mason City, IA), 1987-1988; Southeastern Library Services (Davenport, IA), 1986-1987; Virginia Theological Seminary (Alexandria), 1984-1985; California University of Pennsylvania (California, PA), 1984-1985; Kennametal Corporation (Latrobe, PA), 1984-1985; West Virginia University (Morgantown), 1983-1985; Altoona Area Public Library (Altoona, PA), 1983-1984; George Washington University (Washington, DC), 1983-1984; Indiana University of Pennsylvania (Indiana, PA), 1983-1985; Health Education Center (Pittsburgh), 1983-1984; Central Pennsylvania District Library Center (Bellefonte, PA), 1982-1985; Duquesne University Law School Library (Pittsburgh), 1982-1983; Allegheny County Law Library (Pittsburgh), 1982-1984; Calgon Technical Information Center (Pittsburgh), 1982; Dow Corning Corporation, Technical Information Center (Midland, MI), 1982-1983; Tri-System Public Library Retrospective Conversion Project (Binghamton, Corning, and Ithaca, NY), 1978-1981, Technical Project Director.

Updated July 2025

CANCER GENOMICS

Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA

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Plasma of cancer patients contains cell-free tumor DNA that carries information on tumor mutations and tumor burden. Individual mutations have been probed using allele-specific assays, but sequencing of entire genes to detect cancer mutations in circulating DNA has not been demonstrated. We developed a method for tagged-amplicon deep sequencing (TAm-Seq) and screened 5995 genomic bases for low-frequency mutations. Using this method, we identified cancer mutations present in circulating DNA at allele frequencies as low as 2%, with sensitivity and specificity of >97%. We identified mutations throughout the tumor suppressor gene *TP53* in circulating DNA from 46 plasma samples of advanced ovarian cancer patients. We demonstrated use of TAm-Seq to noninvasively identify the origin of metastatic relapse in a patient with multiple primary tumors. In another case, we identified in plasma an *EGFR* mutation not found in an initial ovarian biopsy. We further used TAm-Seq to monitor tumor dynamics, and tracked 10 concomitant mutations in plasma of a metastatic breast cancer patient over 16 months. This low-cost, high-throughput method could facilitate analysis of circulating DNA as a noninvasive “liquid biopsy” for personalized cancer genomics.

INTRODUCTION

Circulating cell-free DNA extracted from plasma or other body fluids has potentially transformative applications in cancer management (1–7). Characterization of tumor mutation profiles is required for informed choice of therapy, given that biological agents target specific pathways and effectiveness may be modulated by specific mutations (8–11). Yet, mutation profiles in different metastatic clones can differ significantly from each other or from the parent primary tumor (12, 13). Evolutionary changes within the cancer can alter the mutational spectrum of the disease and its responsiveness to therapies, which may necessitate repeat biopsies (14–17). Biopsies are invasive and costly and only provide a snapshot of mutations present at a given time and location. For some applications, mutation detection in plasma DNA as a “liquid biopsy” could potentially replace invasive biopsies as a means to assess tumor genetic characteristics (2–7). Sensitive methods for detecting cancer mutations in plasma may find use in early detection screening (1), prognosis, monitoring tumor dynamics over time, or detection of minimal residual disease (3, 18, 19). In high-grade serous

ovarian carcinomas (HGSOC), mutations in the tumor suppressor gene *TP53* have been observed in 97% of cases (20, 21), but these are located throughout the gene and are difficult to assay. A cost-effective method that could detect and measure allele frequency (AF) of *TP53* mutations in plasma may be highly applicable as a biomarker for HGSOC (22).

Circulating DNA is fragmented to an average length of 140 to 170 base pairs (bp) and is present in only a few thousand amplifiable copies per milliliter of blood, of which only a fraction may be diagnostically relevant (2, 3, 23–25). Recent advances in noninvasive prenatal diagnostics highlight the clinical potential of circulating DNA (25–28), but also the challenges involved in analysis of circulating tumor DNA (ctDNA), where mutated loci and AFs may be more variable. Various methods have been optimized to detect extremely rare alleles (1, 2, 6, 7, 29–31), and can assay for predefined or hotspot mutations. These methods, however, interrogate individual or few loci and have limited ability to identify mutations in genes that lack mutation hotspots, such as the *TP53* and *PTEN* tumor suppressor genes (32). In patients with more advanced cancers, ctDNA can comprise as much as 1% to 10% or more of circulating DNA (2), presenting an opportunity for more extensive genomic analysis. Targeted resequencing has been recently used to identify mutations in selected genes at AFs as low as 5% (33–35). However, identifying mutations across sizeable genomic regions spanning entire genes at an AF as low as 2%, or in few nanograms of fragmented template from circulating DNA, has been more challenging.

In response, we describe a tool for noninvasive mutation analysis on the basis of tagged-amplicon deep sequencing (TAm-Seq), which allows amplification and deep sequencing of genomic regions spanning thousands of bases from as little as individual copies of fragmented DNA. We applied this technique for detection of both abundant and

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rare mutations in circulating DNA from blood plasma of ovarian and breast cancer patients. This sequencing approach allowed us to monitor changes in tumor burden by sampling only patient plasma over time. Combined with faster, more accurate sequencing technologies or rare allele amplification strategies, this approach could potentially be used for personalized medicine at point of care.

RESULTS

Targeted deep sequencing of fragmented DNA by TAM-Seq

To amplify and sequence fragmented DNA, we designed primers to generate amplicons that tile regions of interest in short segments of about 150 to 200 bases (Fig. 1A and table S1), incorporating universal

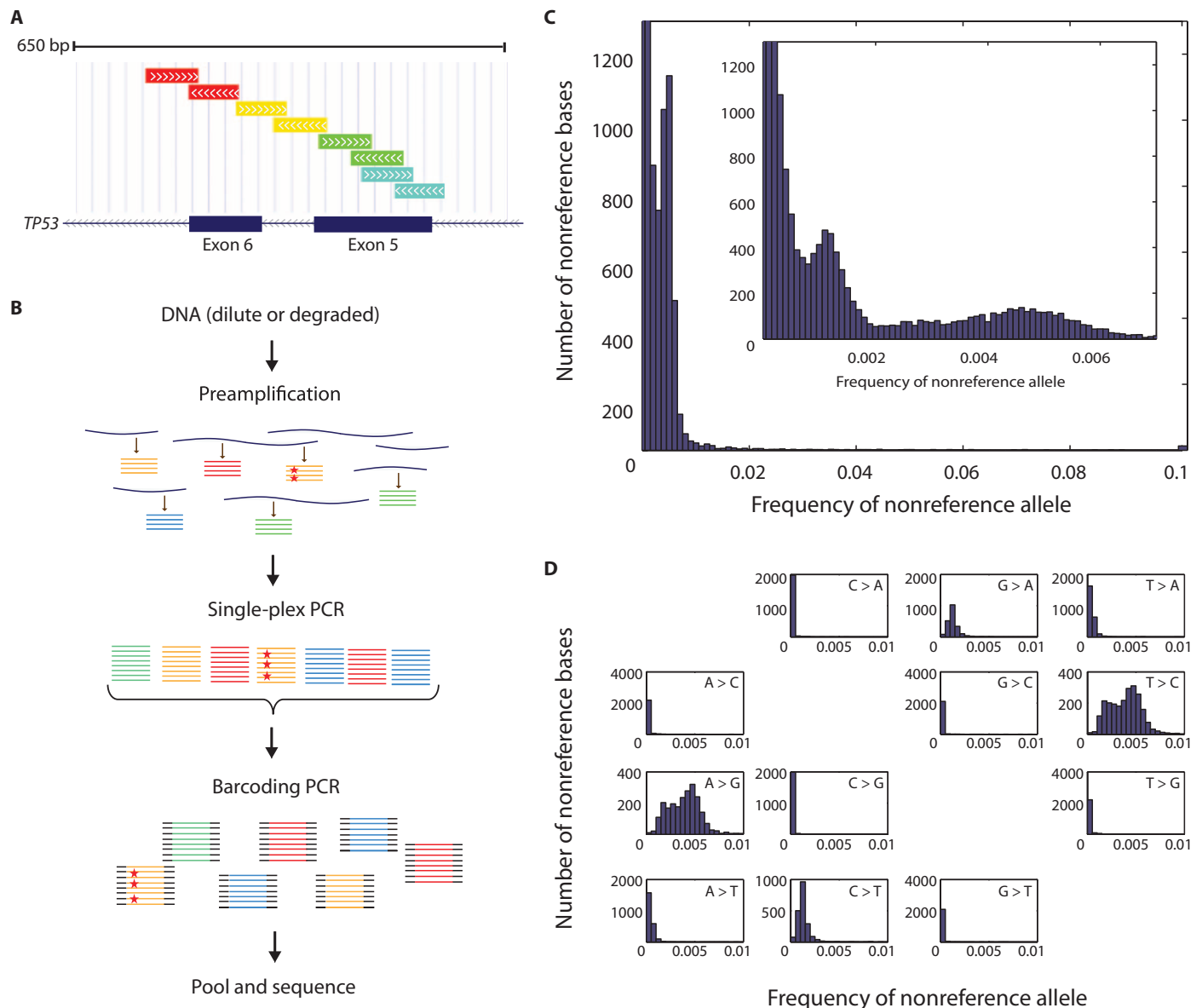


Fig. 1. Overview of tagged amplicon sequencing (TAM-Seq). **(A)** Illustration of amplicon design. Primers were designed to amplify regions of interest in overlapping short amplicons (table S1). Amplicon design is illustrated for a region covering exons 5 to 6 of *TP53*. Colored bars, segmented into forward and reverse reads, show regions covered by different amplicons (excluding primer regions). Sequencing adaptors are attached at either end, such that a single-end read generates separate sets of forward and reverse reads (fig. S1). Because amplicons are mostly shorter than 200 bp, the forward and reverse reads also partially overlap. Figure adapted from University of California, Santa Cruz, Genome Browser (<http://genome.ucsc.edu/>). **(B)** Workflow overview. Multiple regions were amplified in parallel. An initial preamplification step was

performed for 15 cycles using a pool of the target-specific primer pairs to preserve representation of all alleles in the template material. The schematic diagram shows DNA molecules that carry mutations (red stars) being amplified alongside wild-type molecules. Regions of interest in the preamplified material were then selectively amplified in individual (single-plex) PCR, thus excluding nonspecific products. Finally, sequencing adaptors and sample-specific barcodes were attached to the harvested amplicons in a further PCR. **(C)** Distribution of observed nonreference read frequencies, averaged over 47 FFPE samples, across all loci and all nonreference bases. Inset expands the low-frequency range. **(D)** Distribution of the observed background nonreference read frequencies averaged over 47 FFPE samples for the 12 different A/C/G/T base substitutions.

adaptors at 5' ends (fig. S1). Performing single-plex amplification with each of these primer pairs would require dispersing the initial sample into many separate reactions, considerably increasing the probability of sampling errors and allelic loss. Multiplex amplification using a large set of primers could result in nonspecific amplification products and biased coverage. We therefore applied a two-step amplification process: a limited-cycle preamplification step where all primer sets were used together to capture the starting molecules present in the template, followed by individual amplification to purify and select for intended targets (Fig. 1B) (Supplementary Methods). The final concentration of each primer in the preamplification reaction was 50 nM, reducing the potential for interprimer interactions, and 15 cycles of long-extension (4 min) polymerase chain reaction (PCR) were used to remain in the exponential phase of amplification. We used a microfluidic system (Access Array, Fluidigm) to perform parallel single-plex amplification from multiple preamplified samples using multiple primer sets. An additional PCR step attached sequencing adaptors (fig. S1) and tagged each sample by a unique molecular identifier or "barcode" (table S2). Sequencing adaptors were separately attached at either end and the products mixed together, such that single-end sequencing generated separate sets of forward and reverse reads. We performed 100-base single-end sequencing (GAIIx sequencer, Illumina), with an additional 10 cycles using the barcode sequencing primer, generating ~30 million reads per lane. This produced an average read depth of 3250 for each of 96 barcoded samples for 48 amplicons read in two possible orientations.

Validation and sensitivity for mutation identification in ovarian tumor samples

We designed a set of 48 primer pairs to amplify 5995 bases of genomic sequence covering coding regions (exons and exon junctions) of *TP53* and *PTEN*, and selected regions in *EGFR*, *BRAF*, *KRAS*, and *PIK3CA* (table S1) by overlapping short amplicons (Fig. 1A). The sequenced regions cover mutations that account for 38% of all point mutations in the COSMIC database (v55) (32). We used TAM-Seq to sequence DNA extracted from 47 formalin-fixed, paraffin-embedded (FFPE) tumor specimens of ovarian cancers (table S3), which were also sequenced for *TP53* by Sanger sequencing (36) (Supplementary Methods). DNA extracted from FFPE samples is generally degraded and fragmented as a result of fixation and long-term ambient storage. We amplified DNA from each sample in duplicate, tagging each replicate with a different barcode. Using a single lane of sequencing, we generated 3.5 gigabases of data passing signal purity filters, producing mean read depth of 3200 above Q30 for each of the 9024 expected read groups (48 amplicons $\times$ 2 directions $\times$ 94 barcoded samples). Background frequencies of nonreference reads were ~0.1% (median, 0.03%; mean, 0.2%; in keeping with Q30 quality threshold applied), yet varied substantially between loci and base substitutions (Fig. 1C) and showed a clear bias toward purine/pyrimidine conservation (Fig. 1D). Sixty-six percent of loci had mean background rate of <0.1%, and 96% of loci had background rate of <0.6%.

The data set interrogated nearly 18,000 possible single-base substitutions for each sample, which introduces a risk of false detection. To control for sporadic PCR errors and reduce false positives, we called point mutations in a sample only if nonreference AFs were above the respective substitution-specific background distribution at a high confidence margin (0.9995 or greater), and ranked high in the list of nonreference AFs, in both replicates (Supplementary Methods). Duplicate

sequencing data were obtained for 44 samples, and 43 single-base substitutions were called (table S3). These matched 100% of mutations identified by Sanger sequencing and included three additional mutations at low AFs that were below detection thresholds of Sanger sequencing (fig. S2). The upper bound of AFs that may have been missed was estimated (Supplementary Methods) at <5% for 36 of 44 FFPE samples (82%) and <10% for 42 of 44 samples (95%), with median value of 1.3% and mean value of 2.7%. Mutant AFs were highly reproducible in duplicate samples. For 42 of 43 mutations called, the difference in measured frequency between duplicates was less than 0.08, and the relative difference was 25% or less (Fig. 2A). Mutant AFs correlated significantly with tumor cellularity in the FFPE block (correlation coefficient = 0.422; $P = 0.0049$, t test) (Fig. 2B).

In a separate run, we sequenced libraries prepared from six different diluted mixtures of six FFPE samples, with a different known point mutation in *TP53* in each, to mean read depth of 5600. Of more than 100,000 possible non-SNP (single-nucleotide polymorphism) substitutions, we identified all 33 expected point mutations present at AF >1%, including 6 mutations present at AF <2%, with one false-positive called with AF = 1.9%. Using less stringent parameters (Supplementary Methods), we identified three additional mutations present at AF = 0.6% (Fig. 2C), with no additional false positives. Thus, we obtained 100% sensitivity, identifying mutations at AFs as low as 0.6%. A positive predictive value (PPV) of 100% was calculated for mutations at AF >2%, and a PPV of 90% for mutations identified at AF <2% (Fig. 2D).

Quantitative limitations of mutation detection

When applying TAM-Seq to measure a predefined mutation (as opposed to screening thousands of possible substitutions), the frequency of the mutant allele can be read out directly from the data at the desired locus. False detection is less likely, and criteria for confident mutation detection for a predefined substitution can be less stringent than those described above for de novo mutation identification (Supplementary Methods). The minimal nonreference AFs that could be detected depend on the read depth and background rates of nonreference reads, which vary per locus and substitution type. Minimal detectable frequencies increase when higher confidence margins are used (Supplementary Methods) and had a median value of 0.14% at confidence margin of 0.95 and 0.18% at confidence margin of 0.99 (fig. S3). The minimal detectable frequency would also be limited if a minimal number of reads is applied for confident mutation detection; for example, a minimum of 10 reads implies that sequencing depth of 5000 would be required to detect mutations at AF as low as 0.2%. For alleles present at ~10 or fewer copies in the starting template, reproducibility would also be limited by sampling noise, because these alleles may be over- or underrepresented in any particular reaction.

To characterize the quantitative accuracy of TAM-Seq as applied to circulating DNA, we simulated rare circulating tumor mutations by mixing plasma DNA from two healthy individuals. Using the same set of primers as used for the FFPE experiment, we identified that these two individuals differed at five known SNP loci (table S4). Total amplifiable copies in both plasma DNA samples were determined by digital PCR and mixed to obtain minor AFs ranging from 0.16% to 40% (Supplementary Methods). We sequenced diluted templates containing between 250 and <1 expected copy of the minor allele (table S5). The coefficient of variation (CV) of the observed AFs was equal on average to the inverse square root ($1/\sqrt{n}$) of the expected number of copies of the rare allele (Fig. 3A), which is the theoretical

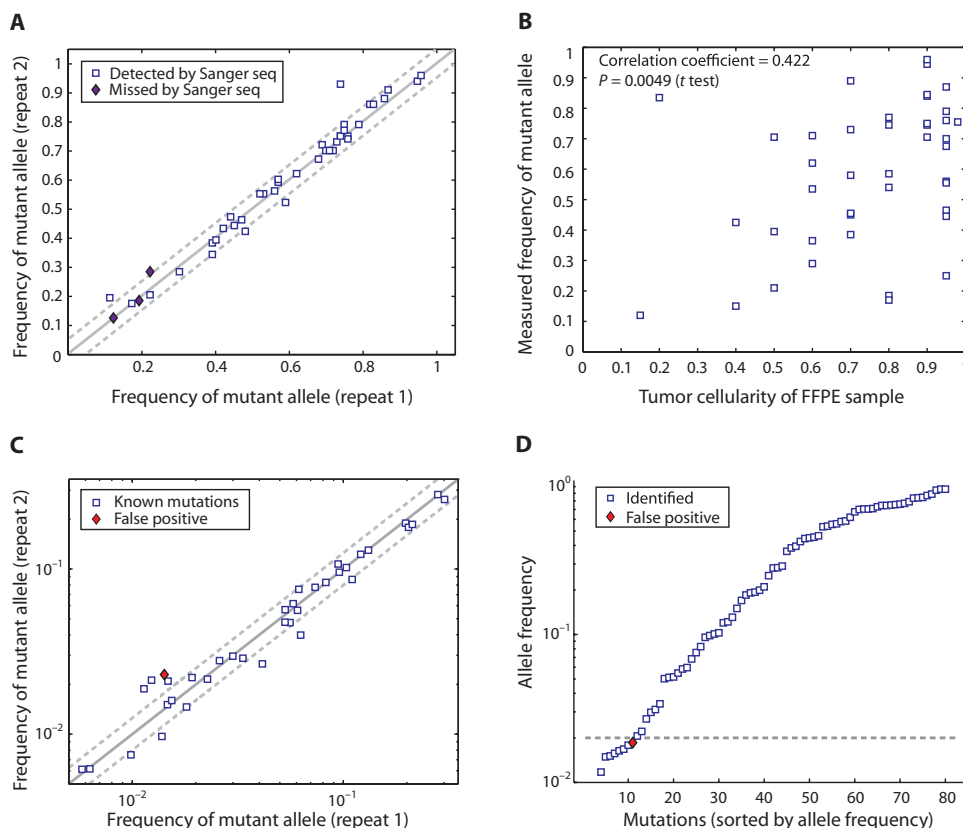


Fig. 2. Identification of mutations in ovarian cancer FFPE samples by TAM-Seq. **(A)** Concordance between duplicate measurements of AFs of mutations identified in fragmented DNA extracted from FFPE samples. The mutation frequency in each library was calculated as the fraction of reads with the mutant (nonreference) base. Solid line indicates equality. Dotted lines indicate a difference in AF of 0.05. **(B)** Correlation of AF with FFPE tumor cellularity. The measured mutant AF (average of both repeats) correlated significantly with the cellularity, estimated from histology (table S3). **(C)** Concordance between duplicate measurements of AFs of mutations identified in a mixture of DNA extracted from different FFPE samples. **(D)** Summary of mutations called in FFPE using TAM-Seq, sorted by increasing AF. Dotted line indicates AF of 2%.

limit of accuracy set by the Poisson distribution for independently segregating molecules. We compared the observed AF to the expected AF for cases where more than six copies of the minor allele were expected. Of 24 such cases, the root mean square (RMS) relative error between the expected and the observed frequency was 14%, with only 2 of 24 cases exhibiting more than 20% discrepancy. For samples with expected minor AF of 0.025, the RMS error was 23% (Fig. 3B).

Noninvasive identification of cancer mutations in plasma circulating DNA

We applied TAM-Seq to directly identify mutations in plasma of cancer patients. We studied a cohort of samples from individuals with HGSOc. These samples were first analyzed for tumor-specific mutations using digital PCR (Supplementary Methods), a method that is highly accurate (2, 3, 7, 37) but requires design and validation of a different assay for every mutation screened and relies on previous identification of mutations in tumor samples from the same patients (2, 3). We initially selected for analysis seven cases that had relatively high levels of circulating mutant *TP53* DNA in the plasma (as assessed by digital PCR). Using the equivalent amount of DNA present in 30

to 120 μ l of plasma, we performed duplicate preamplification reactions for each sample. For all seven patients, *TP53* tumor mutations were identified in the circulating DNA at frequencies of 4% to 44% (Table 1). In one plasma sample collected from an ovarian cancer patient at relapse, we also identified a de novo mutation in the tyrosine kinase domain of *EGFR* (exon 21), at AF of 6% (patient 27, Table 1). We subsequently validated the presence of this mutation in plasma by performing replicate Sanger sequencing reactions of highly diluted template (Supplementary Methods), and 4 of 91 wells that were successfully Sanger-sequenced contained the *EGFR* mutation (fig. S4). We further validated the presence of this mutation by designing a sequence-specific TaqMan probe targeting this mutation and performing digital PCR (Table 1). The mutation was also identified by TAM-Seq in additional plasma collected from the same individual (sample 16, Table 2). This mutation in *EGFR* was not found in the ovarian mass removed by interval debulking surgery 15 months before the blood sample was collected, although the same sample did contain the concomitant *TP53* mutation found in the same patient's plasma, at AF of 85% (patient 27, table S3). We subsequently used TAM-Seq to sequence seven additional samples collected at the time of initial surgery including deposits in right and left ovaries and omentum. The *EGFR* mutation was detected in the two omental samples above the 0.99 confidence margin (fig. S3) at AF of 0.7%, but

was not detected in the six ovarian samples (below the 0.8 confidence margin). Without previous identification in plasma, this mutation would not have been directly identified on screening those samples using high-specificity mutation identification criteria owing to its low AF. In contrast, the *TP53* mutation was identifiable in all biopsy and plasma samples (Fig. 4A). The frequency of mutant alleles in the relapsed tumor could not be directly assessed because a biopsy at relapse was not available.

We validated the TAM-Seq method on a larger panel of plasma samples in which levels of tumor-specific mutations were measured in parallel using patient-specific digital PCR assays. DNA extracted from 62 additional plasma samples collected at different time points from 37 patients with advanced HGSOc was amplified in duplicate (table S6), using DNA present in ~ 0.15 ml of plasma per reaction (range, 0.06 to 0.2 ml). Amplicon libraries were tagged and pooled together for sequencing with libraries prepared from 24 control samples. This generated an average sequencing depth of 650 for 62 plasma samples, sufficient to detect mutations present at AFs of 1% to 2%. Of >1.5 million possible substitutions, 42 mutations were called using the parameters previously optimized for FFPE analysis (table S6).

Fig. 3. Noninvasive identification and quantification of cancer mutations in plasma DNA by Tam-Seq. **(A)** Sampling noise in sequencing of sparse DNA using dilutions of plasma DNA from healthy individuals. CV of triplicate AF readings was calculated for each of the five SNPs in each of the mixes, which had varying numbers of copies of the minor allele (*n*) (blue dots). Bin averages (red diamonds) are the mean CVs calculated for each bin (bin edges denoted by the dotted vertical lines). A linear fit to the log₂ of the mean CV as a function of the log₂ expected copy number was calculated (black line). Two data points, with (*n* = 100, CV = 0.0064) and (*n* = 32, CV = 0.0185), were omitted from the figure for enhanced scaling. Three data points with minor allele copies of <0.8 were omitted from the analysis (*n* = 0.51, CV = 0.62; *n* = 0.41, CV = 0.86; *n* = 0.20, CV = 0.99). **(B)** Expected versus observed frequency of rare alleles in a dilution series of circulating DNA. Mean observed frequency was calculated for each of five SNPs for samples, where expected initial number of minor allele copies was greater than 6. Expected frequencies were calculated on the basis of quantification by digital PCR. Dotted lines represent 20% deviation from the expected frequencies. Inset highlights cases with expected minor AF <0.025. **(C)** Mutations identified in 62 plasma samples from patients with advanced HGSOC using Tam-Seq. AFs are based on digital PCR measurement for confirmed mutations (identified or missed by Tam-Seq), and on Tam-Seq for the false positives called using parameters optimized for analysis

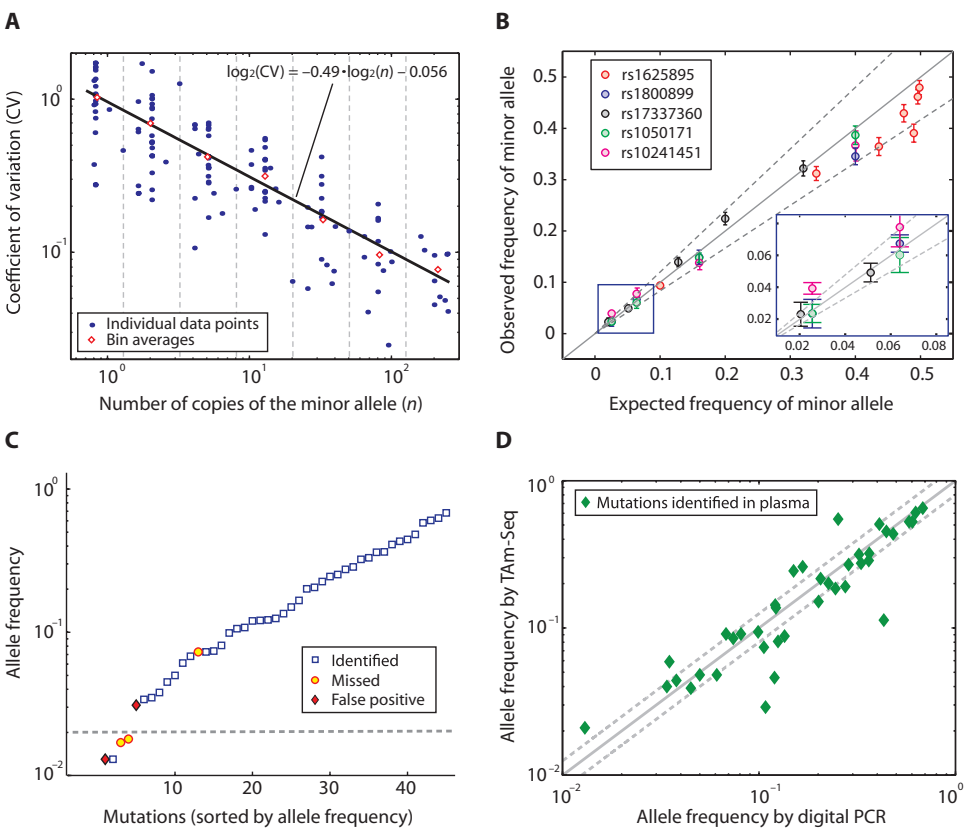


Table 1. Mutations identified by Tam-Seq in plasma samples from seven ovarian cancer patients. Tam-Seq was used to sequence DNA extracted from plasma of subjects with HGSOC (stage III/IV at diagnosis). Plasma was collected when patients presented with relapse disease, before initiation of chemotherapy. For patient 46, DNA from a formalin-fixed, paraffin-

embedded (FFPE) sample was not included in the Tam-Seq set and the mutation was validated in FFPE by Sanger sequencing. CA125 was measured at time of plasma collection. Mean depth of coverage at the mutation locus in the Tam-Seq data was averaged over the repeats (RMS deviation = 850). AF, allele frequency; N, no; Y, yes.

Patient ID	Age at diagnosis	Time elapsed since surgery (months); number of previous lines of chemotherapy	CA125 (U/ml)	Plasma per amplification reaction (μl)	Gene	Mutation and base change (genome build hg19)	Protein change	Detected in FFPE	Mean depth (sequencing reads)	Mean AF using Tam-Seq	Mean AF using digital PCR
8	60	13; 1	2122	50	TP53	17:7577120 C>T	p.R273H	Y	5000	0.09	0.10
12	62	27; 3	365	50	TP53	17:7577579 G>T	p.Y234*	Y	5000	0.10	0.08
14	58	50; 3	260	120	TP53	17:7578212 G>A	p.R213*	Y	5800	0.15	0.12
25	61	9; 1	944	110	TP53	17:7578404 A>T	p.C176S	Y	4800	0.04	0.08
27 [†]	68	15; 1	1051	90	TP53	17:7578262 C>G	p.R196P	Y	7700	0.06	0.14
					EGFR	7:55259437 G>A	p.R832H	N	5700	0.06	0.05
31	64	12; 1	313	30	TP53	17:7578406 C>T	p.R175H	Y	4500	0.44	0.56
46	56	30; 2	1509	30	TP53	17:7578406 C>T	p.R175H	Y	4200	0.23	0.30

*Indicates stop codon. [†]Both a TP53 and an EGFR mutation were identified in this sample (Fig. 4A).

Table 2. Mutations identified by TAm-Seq in a set of 62 plasma samples from ovarian cancer patients. Forty mutations were identified by TAm-Seq using stringent parameters for mutation calling. Plasma sam-

ples described in this table are distinct from those in Table 1, but patients included overlap. Additional data on patients and mutations are provided in table S6.

Sample number	Plasma volume per amplification reaction (μl)	DNA amount per amplification reaction (ng)	Gene	Protein change	Mean depth (sequencing reads)	Mean AF using TAm-Seq	Mean AF using digital PCR
1	70	0.9	<i>TP53</i>	p.R273C	640	0.260	0.167
2	160	4.2	<i>TP53</i>	p.R248Q	340	0.244	0.150
3	160	5.7	<i>TP53</i>	p.R248Q	640	0.507	0.410
4	120	9.9	<i>TP53</i>	p.R213X	810	0.059	0.035
5	120	1.4	<i>TP53</i>	p.C141Y	680	0.021	0.013
6	120	2.1	<i>TP53</i>	p.C141Y	720	0.044	0.038
7	190	17.9	<i>TP53</i>	p.I195N	800	0.091	0.081
8	160	14.8	<i>TP53</i>	p.R175H	510	0.608	0.627
9	160	10.7	<i>TP53</i>	p.R175H	550	0.526	0.604
10	160	6.1	<i>TP53</i>	p.R175H	530	0.651	0.682
11	160	4.9	<i>TP53</i>	p.R175H	490	0.526	0.581
13	160	2.8	<i>TP53</i>	p.C135R	480	0.039	0.045
14	160	2.5	<i>TP53</i>	p.C135R	610	0.046	0.120
15	160	3.0	<i>TP53</i>	p.C135R	470	0.091	0.068
16 [†]	130	3.7	<i>TP53</i>	p.R196P	1070	0.088	0.135
			<i>EGFR</i>	p.R832H	614	0.048	0.050
17	160	4.2	<i>TP53</i>	p.C176S	580	0.113	0.432
18	160	4.4	<i>TP53</i>	p.C176S	620	0.029	0.108
20	140	5.2	<i>TP53</i>	p.R175H	650	0.201	0.226
21	140	3.6	<i>TP53</i>	p.R175H	650	0.085	0.074
22	140	4.1	<i>TP53</i>	p.R175H	630	0.081	0.125
23	140	3.7	<i>TP53</i>	p.R175H	710	0.074	0.106
24	140	7.1	<i>TP53</i>	p.R175H	760	0.269	0.286
25	130	3.9	<i>TP53</i>	p.R273H	750	0.094	0.099
26	160	5.7	<i>TP53</i>	p.R282W	640	0.048	0.061
27	150	3.6	<i>TP53</i>	p.C141Y	480	0.321	0.364
29	150	9.5	<i>TP53</i>	p.E258K	190	0.548	0.253
31	160	3.6	<i>TP53</i>	p.C135Y	620	0.040	0.034
32	140	2.4	<i>TP53</i>	p.E56X	1480	0.137	0.122
33	160	13.2	<i>TP53</i>	p.K132N	740	0.216	0.206
34	60	5.3	<i>TP53</i>	p.K132N	570	0.151	0.201
36	160	5.8	<i>TP53</i>	p.K132N	620	0.191	0.275
37	160	9.4	<i>TP53</i>	p.K132N	530	0.287	0.362
38	160	10.1	<i>TP53</i>	p.K132N	590	0.275	0.331
39	160	16.4	<i>TP53</i>	p.K132N	700	0.315	0.323
40	160	19.7	<i>TP53</i>	p.K132N	830	0.435	0.482
41	160	15.0	<i>TP53</i>	p.K132N	730	0.452	0.445
42	160	8.5	<i>TP53</i>	p.K132N	560	0.185	0.245
43	150	3.6	<i>TP53</i>	Splicing	680	0.143	0.121
44 [‡]	170	5.2	<i>TP53</i>	p.C238R	1543	0.071	0.073

[†]Both a *TP53* and an *EGFR* mutation were identified in this sample, collected from patient 27 (Table 1), 25 months after initial surgery (Fig. 4A).
amplification in this sample in the initial experiment and was identified successfully in repeat analysis.

[‡]The amplicon containing the mutation failed

Thirty-nine of these matched mutations detected by digital PCR in those samples (Fig. 3C). Three potential false positives were called, at AF of 3.1%, 1.3%, and 0.7% (the latter in a control sample). Using higher-

stringency parameters for mutation identification (Supplementary Methods), we retained only the 39 validated mutations called, with no false positives (Table 2).

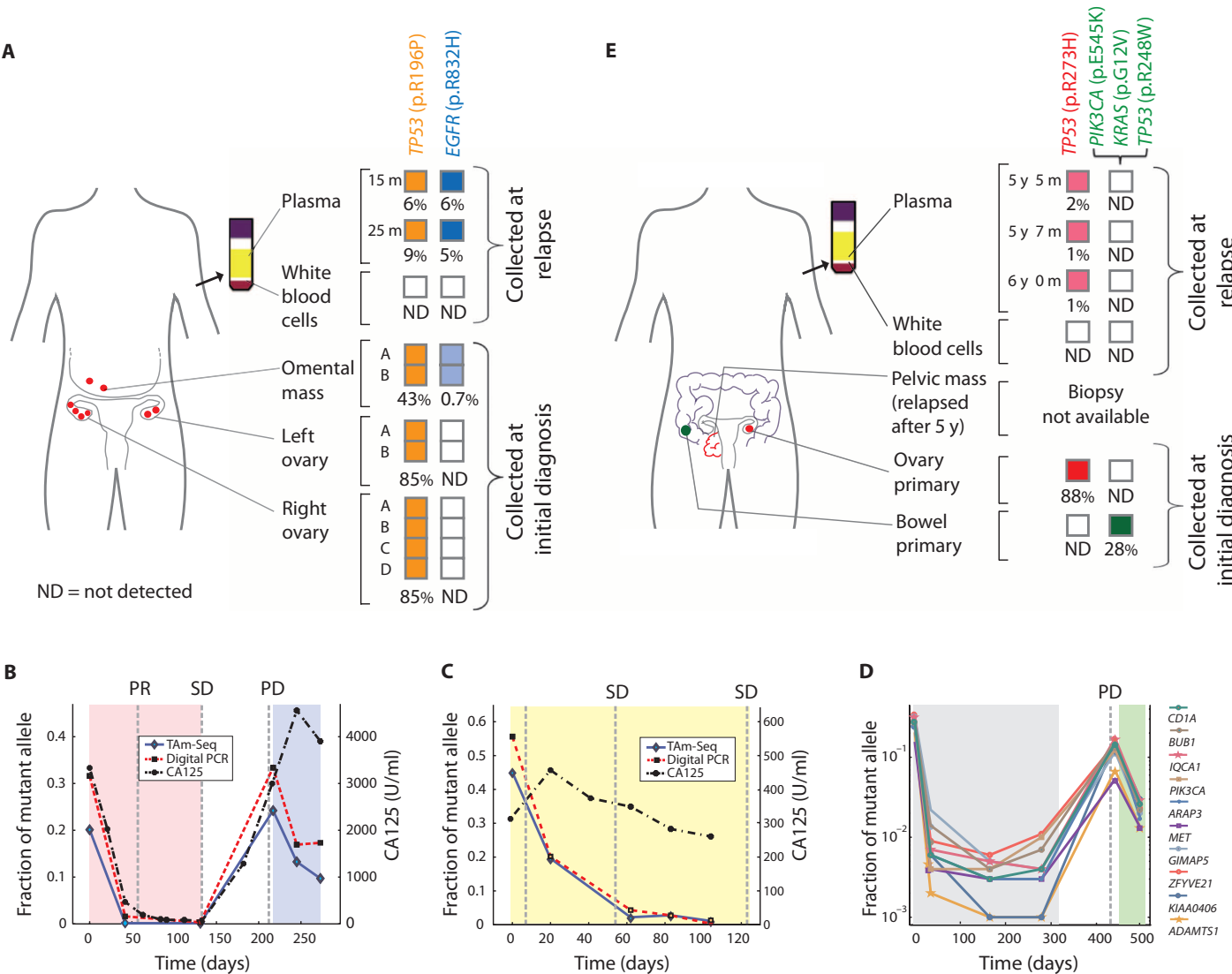


Fig. 4. Clinically relevant applications of plasma DNA sequencing using TAM-Seq. **(A)** Retrospective analysis by TAM-Seq of plasma samples collected during patient follow-up and biopsy specimens collected at initial surgery. We identified a mutation in exon 21 of *EGFR* (dark blue boxes) in two separate plasma samples, collected 15 and 25 months after initial surgery from patient 27 (Tables 1 and 2). This mutation was not directly identified in eight tumor biopsy specimens collected at the time of initial surgery (two from omental mass, two from left ovary, and four from right ovary). Having identified the mutation in the plasma samples, we examined this mutation using the lower-specificity criteria defined for mutation detection (Supplementary Methods) and detected the mutation in the two specimens that had been collected from the omentum at the time of surgery (light blue boxes) but not in the six ovarian specimens. A mutation in *TP53* was identified in all tumor and plasma samples collected from this patient (Tables 1 and 2 and table S3), but not in white blood cells (buffy coat). Percentages indicate mutant AFs. Empty boxes and “ND” indicate samples where a mutation was not identified or detected (below 0.8 confidence margin). **(B)** Monitoring frequency

of mutant DNA in plasma of an ovarian cancer patient (patient 46) over time using TAM-Seq and digital PCR. TAM-Seq results are reported as the mean frequency of duplicate analyses. Parallel data are shown for digital PCR and serum CA125. Shaded regions indicate periods of chemotherapy, and vertical dashed lines indicate radiological assessment of patient responses: PR, partial response; SD, stable disease; PD, progressive disease. **(C)** Monitoring frequency of mutant DNA in plasma of an ovarian cancer patient (patient 31) over time. **(D)** Dynamics of 10 tumor-specific mutations in plasma of a breast cancer patient (not included in the other sets of samples analyzed). **(E)** Retrospective analysis of samples from synchronous primary tumors (bowel and ovarian) collected at the time of initial surgery and three plasma samples collected at relapse. In primary tumors from this patient (not included in the other sets of samples analyzed), a *TP53* mutation was identified in the ovarian cancer (red box), and mutations in *PIK3CA*, *KRAS*, and *TP53* were identified in the bowel cancer (green box). At relapse, a biopsy was not performed on the pelvic mass. The *TP53* mutation that was identified in the ovarian primary tumor (p.R273H) was detected in plasma, whereas the bowel-associated mutations were not detected.

Of 40 point mutations detected at AF >2% by digital PCR, 38 (95%) were identified by TAm-Seq in a single experiment (Fig. 3C). One additional mutation was located in an amplicon that failed in that sample and was identified in repeated analysis; the other was likely missed by TAm-Seq owing to sampling noise, because it was found in one of the duplicate preamplified libraries but not the other (table S6). One of three mutations detected by digital PCR at 1% < AF < 2% was identified by TAm-Seq (Fig. 3C). Eleven additional point mutations detected by digital PCR at AF <1% were not detected by TAm-Seq at these settings. TAm-Seq and digital PCR measurements of AF had excellent agreement, with correlation coefficient of 0.90, increasing to 0.97 when discarding the two strongest outliers (Fig. 3D). Thus, we screened 62 samples across sizeable genomic stretches, using minute amounts of plasma DNA (median, 4 ng), and obtained 97.5% sensitivity with PPV of 100% for identifying mutations at AF >2% in plasma by TAm-Seq. Using parameters optimized for FFPE samples, one potential false positive was called at AF >2%, reducing the PPV to 97.5% (Table 3).

Monitoring levels of ctDNA

Various methods have been suggested to monitor changes in mutation load in plasma. These can have enhanced sensitivity compared to TAm-Seq for tracking individual mutations, but require design of personalized assays (3, 18, 19). None of these methods have been widely adopted. We therefore applied TAm-Seq as a generic tool to measure changes in the frequency of ctDNA over time. We studied serial plasma

samples collected during follow-up and treatment of two patients with relapsed HGSOc, collected during 104 and 273 days of follow-up and treatment, respectively. Frequencies of mutant *TP53* alleles were measured by TAm-Seq and in parallel by digital PCR using a mutation-specific probe. The two methods of quantification had excellent agreement. Mutant AFs in plasma of ovarian cancer patients reflected well the clinical course of the disease compared to the serum marker CA125, showed marked decrease when systemic treatment was initiated, and increased in parallel to disease progression. In the first case (Fig. 4B), a 56-year-old woman with relapsed ovarian cancer (patient 46) was treated with fourth-line carboplatin + paclitaxel chemotherapy for six cycles (pink-shaded region). Radiology showed partial response on mid-treatment computed tomography (CT) scan. End-of-treatment CT showed stable disease. Twelve weeks from the end of her fourth-line treatment, the patient developed progressive disease. The patient then initiated fifth-line chemotherapy with liposomal doxorubicin (purple-shaded region). In the second case (Fig. 4C), a 64-year-old woman with relapsed ovarian cancer (patient 31) was treated with second-line ECX (epirubicin, cisplatin, and capecitabine) chemotherapy for six cycles. Radiology showed stable disease on mid- and end-of-treatment CT scans. The patient then remained off treatment, until she progressed 3 months later.

TAm-Seq can be flexibly adapted to sequence different genomic regions by designing primers to amplify regions of interest. We used this capability to study dynamics of multiple mutations in parallel.

Table 3. Summary of mutations identified in 69 plasma samples of ovarian cancer patients. Samples were analyzed by TAm-Seq and in parallel by digital PCR. Using parameters optimized for plasma DNA, false-positive calls were lost, whereas all confirmed calls were retained, resulting in specificity and PPV of 100%.

First set of plasma samples	
Plasma samples analyzed	7
Point mutations originally detected by digital PCR, using patient-specific assays targeting mutations identified in tumor samples	7
Point mutations identified directly in plasma by TAm-Seq	8
De novo mutations identified by TAm-Seq only, subsequently confirmed by digital PCR	1
Second set of plasma samples	
Plasma samples analyzed	62
Point mutations detected by digital PCR at AF >2%	40
Point mutations with AF >2% (by digital PCR) identified by TAm-Seq	39
Point mutations missed by TAm-Seq due to sampling error	1
Sensitivity of TAm-Seq for identifying mutations at AF >2%	97.5%
PPV of mutations called by TAm-Seq with AF >2%	97.5%*
ctDNA in ovarian cancer	
Advanced ovarian cancer patients in both sets†	38
Patients where TAm-Seq identified cancer mutations	20

*One unconfirmed substitution was called at AF >2% using parameters optimized for FFPE material. †The first set included 7 patients (Table 1), and the second set included 37 patients (table S6), 6 of whom overlap.

Whole-genome sequencing of tumor material was used to identify tumor mutations in a patient with metastatic breast cancer undergoing two phases of chemotherapy. Ten mutations were selected, and short amplicons (<120 bp) were designed to cover the mutation loci (table S7). Serial plasma samples were collected over the course of 497 days, both before and after treatment. We performed TAm-Seq in duplicate, using DNA from 0.08 ml of plasma per amplification, and tracked dynamics of all mutations in parallel (Fig. 4D). The patient was treated with single-agent epirubicin (gray-shaded region). After 4 months off treatment, a CT scan showed progressive disease and the patient commenced further treatment with paclitaxel chemotherapy. The 10 mutations followed a common pattern of sharp decline in AF upon onset of therapy and an increase in AF upon disease progression after termination of therapy (Fig. 4D).

Finally, we used TAm-Seq to study plasma from a patient who had a history of two synchronous primary cancers, bowel and ovarian, which were resected simultaneously. After a 5-year remission, a pelvic mass of uncertain origin was detected. A biopsy was considered to guide selection of therapy but was not performed owing to risk of complications and comorbidities. The patient commenced empirically on an ovarian cancer chemotherapy regimen, to which she responded. Retrospective analysis by TAm-Seq of FFPE from the primary tumors collected at initial surgery, and three plasma samples collected serially at the time of relapse (5 years and 5 months, 5 years and 7 months, and 6 years after initial surgery), showed that the patient's plasma at relapse contained the *TP53* (p.R273H) mutation identified in the ovarian primary tumor (exceeding the 0.98, 0.93, and 0.97 confidence margins, respectively), but not the *PIK3CA* (p.E545K), *KRAS* (p.G12V), or *TP53* (p.R248W) mutations identified in the primary bowel cancer (below the 0.8 confidence margin) (Fig. 4E). Had these results been available, uncertainty and treatment delays may have been avoided, as well as the risk of prescribing chemotherapy for an inappropriate tumor site. An alternative possible outcome may have involved a finding of the *PIK3CA* or *KRAS* mutations (present in the primary bowel cancer) in the patient's plasma at the time of relapse. Such a finding, if available to clinicians at the time, may not only have led to alternate chemotherapy being offered but may have also opened the possibility of enrolment into a trial for targeted therapy with mammalian target of rapamycin (mTOR), phosphatidylinositol 3-kinase (PI3K), or mitogen-activated protein kinase kinase (MEK) inhibitors (11).

DISCUSSION

Detection of rare mutations in circulating DNA has long been pursued owing to its potentially transformative impact on cancer diagnosis and management. Important progress has been made using sequence-specific assays that target predefined mutations and that detect extremely rare alleles. Assays such as PCR (6, 7), ligation (5), and primer extension/mass spectrometry (27) can identify specific, predefined mutations in plasma samples. Enhanced detection down to 1 mutant allele in 10,000 or more wild-type alleles can be obtained using a variety of methods, such as peptide nucleic acid and primer extension ("PPEM") (38), ligation followed by quantitative PCR ("LigAmp") (39), bead-based digital PCR in emulsions ("BEAMing") (2, 3), microfluidic-based (7) or droplet-based digital PCR (40), or microinsertion/deletion/indel-activated pyrophosphorolysis ("MAP") (29). Nonetheless, identification of rare mutations in tumor suppressor genes such as *TP53*,

which are widely mutated in cancers but lack a well-defined hotspot region, remains an elusive goal.

In patients with advanced cancers, mutant alleles can reach a sizeable fraction of DNA. For example, Dukes' D colorectal cancers have median 8% mutant AF (2). Screening of entire genes for mutations would therefore be useful for some applications, even if analytical selectivity is limited to a few percent. Advances in massively parallel sequencing make new approaches possible. These have largely focused on large-scale analyses, including whole-genome or whole-exome sequencing (41). This generates a large amount of data on genomic regions that do not, at present, inform clinical decisions. Moreover, the depth of coverage for clinically significant loci is not sufficient to detect changes that occur at low frequency (<5%). Such approaches have recently been complemented by methods for examination of individual amplicons at great depth (30).

The intermediate scale of sequencing is most likely to have immediate impact on clinical genomics. Targeted sequencing has been applied for tumor DNA (34, 35) and cyst fluid (33) to detect mutations down to 5% AF, but has not been applied for analysis of circulating tumor nucleic acids. Here, we demonstrate noninvasive identification of mutant alleles in plasma, at AFs as low as 2%, by targeted deep sequencing of circulating DNA. Our TAm-Seq method uses a combination of short amplicons, two-step amplification, sample barcodes, and high-throughput PCR. Because the amplicons are short, this method effectively amplifies even small amounts of fragmented DNA such as are present in circulating DNA. The two-step amplification permits extensive primer multiplexing that enables the amplification and sequencing of sizeable genomic regions by tiling short amplicons without loss of fidelity or efficiency. Duplicate sequencing of each sample is used to avoid false positives stemming from PCR errors. Sample barcodes and high-throughput PCR reduce the per-sample costs to a range where this may be widely applicable. Preparing TAm-Seq libraries for sequencing from 48 samples takes less than 24 hours and involves only a few hours of hands-on time. New platforms for massively parallel sequencing allow for fast turnaround times, which make this approach practical in a clinical setting.

The sensitivity presently achieved can provide useful diagnostic information in certain advanced cancers. We studied a cohort of subjects with advanced HGSOc in which the tumor suppressor gene *TP53* is a driver mutation (20). Of the 69 plasma samples collected from 38 different individuals with advanced HGSOc, we identified mutations in *TP53* in 46 samples (67%) from 20 of the cases (53%). In contrast, a previous study using a ligase detection reaction with bespoke primers found mutated *TP53* sequences in plasma for only 30% of advanced ovarian cancer patients (5), and a study using single-strand conformation polymorphism found no ctDNA in preoperative plasma samples from high-grade serous cancer patients (42).

Targeted agents, such as inhibitors of poly(adenosine diphosphate-ribose) polymerase (PARP), or tyrosine kinase inhibitors targeting epidermal growth factor receptor (EGFR), may be applicable for systemic treatment of advanced HGSOc (8, 10, 22). In a recent study of 203 HGSOc tumors, *EGFR* was found to be the most frequently mutated oncogene and was mutated in nearly 10% of cases (10). In one case, we identified in plasma a de novo mutation in the tyrosine kinase domain (exon 21) of *EGFR*, located 26 amino acids upstream of the L858R activating mutation widely documented for lung cancer. In a subset of tumor samples collected from the same patient 15 months earlier, this mutation was detected at AF of 0.7%, but could not have

been identified by analysis of those samples alone without previous knowledge of the mutation identified in plasma (Fig. 4A). In a clinical setting, identification of such a mutation could potentially guide treatment with alternative molecularly targeted therapy (10). Current clinical recommendations in lung adenocarcinoma suggest mutation assessment in exons 18 to 21 of *EGFR* (a region of ~560 bp) in the tumor tissue to identify patients eligible for treatment with gefitinib or erlotinib (9). Using a commercial PCR-based in vitro diagnostic kit (Qiagen), 28 different *EGFR* variants can be assayed (not including the mutation we identified), but the sample needs to be subdivided into seven different reactions. When sample is limited or mutant alleles are rare, this could introduce sampling errors.

Using standard amplification primers tailored to the mutation loci, we also used TAm-Seq to monitor the dynamics of 10 mutations in plasma DNA of a single patient with metastatic breast cancer, using minute amounts of input DNA. Previous studies have followed up to two mutations in any individual patient (3, 19). Tracking multiple mutations can provide insight into clonal evolution and, at the same time, increases the robustness for tumor monitoring by compensating for effects of sampling noise or mutational drift. For example, if a patient has only five copies of a mutant allele per milliliter of plasma (on average), there is a 37% probability that this mutation will not be present in a 0.2-ml sample, and even a perfect assay will fail to detect residual tumor, whereas a method that measures multiple mutations in parallel can have a low likelihood of a false-negative result even if the detection rate for each mutation is less than 50%.

A current limitation of TAm-Seq is the detection limit compared to assays that target individual loci (2, 3, 7, 40), which have been shown to detect two to three orders of magnitude lower frequencies. Our approach may be sufficient for analyzing plasma from patients with certain advanced cancers, but further improvement may be necessary before this method can be more widely used in the clinic. Higher read depth or fidelity, additional replicates, or improved algorithms could allow for enhanced mutation detection without change to protocols. An alternative strategy is through rare allele enrichment, for example, by combining TAm-Seq with protocols such as COLD-PCR (co-amplification at lower denaturation temperature PCR) (31).

Previously proposed methods for personalized monitoring of tumor dynamics relied on expensive custom-designed probes (3) or identification of rearrangements using whole-genome sequencing (18, 19). These have better analytical sensitivity than currently achieved by TAm-Seq, but are difficult to implement on a routine basis. TAm-Seq strikes a balance between sensitivity and ease of use and could facilitate study and application of circulating DNA. Using TAm-Seq, we identified cancer mutations in the plasma of most advanced ovarian cancer patients and tracked dynamics of *TP53* mutations without requiring any specially designed probes. In summary, TAm-Seq is a flexible and cost-effective platform for applications in noninvasive cancer genomics and diagnostics. We have shown that this method can be used for high-throughput sequencing of plasma samples to identify and monitor levels of multiple cancer mutations in circulating DNA. This could also be applied to screen for rare mutations in a variety of heterogeneous sample types such as low-cellularity tumor specimens, cytological samples, or circulating tumor cells (16). With further developments, this and derivative methods may be applied in molecular screening for earlier detection or for differential diagnosis of cancer from benign masses. For genetic analysis of FFPE or small biopsy samples, TAm-Seq can be applied as is, as a cost-effective clinical aid.

MATERIALS AND METHODS

Sample collection

FFPE blocks were obtained from the pathology archives at Addenbrooke's Hospital (Cambridge, UK). Plasma samples were collected upon disease relapse, before and during chemotherapy treatment. Sample collection for this study was approved by Cambridgeshire Research Ethics Committee (REC 08/H0306/61 and 07/Q0106/63). Peripheral blood samples were collected into EDTA tubes and centrifuged at 820g for 10 min within 1 hour of collection to limit degradation of cell-free DNA and leukocyte lysis. Aliquots (1 ml) of plasma were centrifuged in a bench-top microfuge at 14,000 rpm for 10 min. The supernatant was transferred to sterile 1.5-ml tubes and stored at -80°C before extraction.

Extraction of DNA from FFPE and blood plasma

Paraffin blocks were cut as 8-μm sections on plain glass slides. Targeted regions for sampling were marked on adjacent hematoxylin and eosin sections by the study pathologist and recovered by scrape macrodissection. Between 3 and 20 sections were macrodissected depending on the tissue sample's size. DNA from FFPE sections was extracted with QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions.

Circulating DNA was extracted from between 0.85 and 2.2 ml of plasma with the QIAamp Circulating Nucleic Acid kit (Qiagen), following the manufacturer's instructions, and with the QIAvac 24 Plus vacuum manifold. Carrier RNA was added to ACL lysis buffer to enhance binding of nucleic acids to the QIAamp membrane with the aim to enhance yields.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/4/136/136ra68/DC1

Methods

Fig. S1. PCR strategy and primer design.

Fig. S2. Sanger traces for mutations identified by tagged-amplicon sequencing.

Fig. S3. Background frequencies and detection limits for base substitutions.

Fig. S4. Replicate dilute Sanger sequencing of a mutation identified in plasma.

Table S1. Target-specific primers.

Table S2. Unique sequencing barcodes.

Table S3. Mutations identified in FFPE samples.

Table S4. SNPs identified in circulating DNA from two plasma control samples.

Table S5. Frequency of SNP alleles in dilution series of DNA from control plasma.

Table S6. Additional data for Table 2 for mutations identified in plasma samples.

Table S7. Mutations and amplicons studied in one breast cancer patient.

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Acknowledgments: We thank H. Biggs, C. Hodgkin, S. Richardson, and L. Jones for assistance in sample collection, S. Aldridge for assistance in genomic analysis, and S. Tavaré, B. Davis, and M. Dunning for assistance in data analysis. **Funding:** We acknowledge the support of Cancer Research UK, the University of Cambridge, National Institute for Health Research Cambridge Biomedical Research Centre, Cambridge Experimental Cancer Medicine Centre, and Hutchison Whampoa Limited. C.P. was supported in part by the Academy of Medical Sciences, Wellcome Trust, British Heart Foundation, and Arthritis Research UK. **Author contributions:** T.F., M.M., C.P., D.G., D.W.Y.T., C.C., J.D.B., and N.R. designed the study. T.F., M.M., D.W.Y.T., F.K., J.H., A.P.M.,

and N.R. developed methods. T.F., D.G., D.W.Y.T., A.M.P., and S.-J.D. collected the data. T.F., M.M., and N.R. analyzed TAm-Seq data. C.P., S.-J.D., C.C., and J.D.B. designed clinical studies and collected samples and clinical data. M.J.-L. performed pathological analysis. D.B. contributed sequencing data. T.F., M.M., C.P., D.G., D.W.Y.T., J.H., A.P.M., J.D.B., and N.R. interpreted the data. T.F., M.M., and N.R. wrote the manuscript with assistance from C.P., D.G., D.W.Y.T., A.P.M., J.D.B., and other authors. All authors approved the final manuscript. **Competing interests:** A.P.M. and F.K. hold equity in Fluidigm and may stand to gain by publication of these findings. D.B. and F.K. hold equity in Illumina and may stand to gain by publication of these findings.

Submitted 24 October 2011
Accepted 18 April 2012
Published 30 May 2012
10.1126/scitranslmed.3003726

Citation: T. Forshew, M. Murtaza, C. Parkinson, D. Gale, D. W. Y. Tsui, F. Kaper, S.-J. Dawson, A. M. Piskorz, M. Jimenez-Linan, D. Bentley, J. Hadfield, A. P. May, C. Caldas, J. D. Brenton, N. Rosenfeld, Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci. Transl. Med.* **4**, 136ra68 (2012).

Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA

Tim Forshew, Muhammed Murtaza, Christine Parkinson, Davina Gale, Dana W. Y. Tsui, Fiona Kaper, Sarah-Jane Dawson, Anna M. Piskorz, Mercedes Jimenez-Linan, David Bentley, James Hadfield, Andrew P. May, Carlos Caldas, James D. Brenton and Nitzan Rosenfeld

Sci Transl Med 4, 136ra68136ra68.
DOI: 10.1126/scitranslmed.3003726

Deep Sequencing Tumor DNA in Plasma

Five liters of circulating blood contain millions of copies of the genome, broken into short fragments; in cancer patients, a small fraction is circulating tumor DNA (ctDNA). An even smaller number harbor mutations that affect cancer outcome. Looking for diagnostic answers in circulating DNA is a challenge, but Forshew, Murtaza, and colleagues have risen to the occasion by developing a tagged-amplicon deep sequencing (TAm-Seq) method that can amplify and sequence large genomic regions from even single copies of ctDNA. By sequencing such large regions, the authors were able to identify low-level mutations in the plasma of patients with high-grade serous ovarian carcinomas.

Forshew *et al.* designed primers to amplify 5995 bases that covered select regions of cancer-related genes, including *TP53*, *EGFR*, *BRAF*, and *KRAS*. In plasma obtained from 38 patients with high levels of ctDNA, the authors were able to identify mutations in *TP53* at allelic frequencies of 2% to 65%. In plasma samples from one patient, they also identified a de novo mutation in *EGFR* that had not been detected 15 months prior in the tumor mass itself. Finally, the TAm-Seq approach was used to sequence ctDNA in plasma samples collected from two women with ovarian cancer and one woman with breast cancer at different time points, tracking as many as 10 mutations in parallel. Forshew and coauthors showed that levels of mutant alleles reflected the clinical course of the disease and its treatment—for example, stabilized disease was associated with low allelic frequency, whereas patients at relapse exhibited a rise in frequency.

Through several experiments, the authors were able to show that TAm-Seq is a viable method for sequencing large regions of ctDNA. Although this provides a new way to noninvasively identify gene mutations in our blood, TAm-Seq will need to achieve a more sensitive detection limit (<2% allele frequency) to identify mutations in the plasma of patients with less advanced cancers. Nevertheless, once optimized, this "liquid biopsy" approach will be amenable to personalized genomics, where the level and type of mutations in ctDNA would inform clinical decision-making on an individual basis.

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Supplementary Materials for

Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA

Tim Forshew, Muhammed Murtaza, Christine Parkinson, Davina Gale, Dana W. Y. Tsui, Fiona Kaper, Sarah-Jane Dawson, Anna M. Piskorz, Mercedes Jimenez-Linan, David Bentley, James Hadfield, Andrew P. May, Carlos Caldas, James D. Brenton,* Nitzan Rosenfeld*

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Published 30 May 2012, *Sci. Transl. Med.* **4**, 136ra68 (2012)
DOI: 10.1126/scitranslmed.3003726

The PDF file includes:

Methods

Fig. S1. PCR strategy and primer design.

Fig. S2. Sanger traces for mutations identified by tagged-amplicon sequencing.

Fig. S3. Background frequencies and detection limits for base substitutions.

Fig. S4. Replicate dilute Sanger sequencing of a mutation identified in plasma.

Table S1. Target-specific primers.

Table S2. Unique sequencing barcodes.

Table S3. Mutations identified in FFPE samples.

Table S4. SNPs identified in circulating DNA from two plasma control samples.

Table S5. Frequency of SNP alleles in dilution series of DNA from control plasma.

Table S6. Additional data for Table 2 for mutations identified in plasma samples.

Table S7. Mutations and amplicons studied in one breast cancer patient.

SUPPLEMENTARY METHODS

Sanger sequencing

Coding sequences of the *TP53* gene (exons 2 to 11) were amplified as described previously (36) with the following modifications: PCR reactions were performed in 25 µl, universal primers M13 forward and M13 reverse were tagged onto both primers pairs and used to sequence in both the forward and reverse directions. To sequence exon 7, TP53-7F (CAGGTCTCCCCAAGGCGCAC) was used owing to a poly A tract downstream of the exon 7 forward primer described in (36): CATCCTGGCTAACGGTGAAAC. PCR products were sequenced on an ABI 3730 (Applied Biosystem). Mutational analysis was performed using Mutation Surveyor Software version 3.97 (SoftGenetics), using default settings (mutation score=5.00, mutation height=500, overlapping factor=0.20, dropping factor=0.20, SnRatio=1.00, mobility shift=imbedded algorithm). All exons were sequenced for 26 samples. For 12 samples, sequencing was performed for selected exons only.

Digital PCR

The digital PCR method and the use of the BioMark system for digital PCR analysis have been described (7, 37). Samples were mixed with sequence-specific TaqMan probes, Master Mix, and 20× GE Sample Loading Reagent (Fluidigm). This mixture was then added to the 12.765 Digital Array Chip (Fluidigm) where it was partitioned into 765 separate PCR reactions. These were PCR cycled on the Fluidigm BioMark and all positive reaction chambers counted. Amplifiable copies were calculated from the number of positive wells using a Poisson correction.

Estimation of input DNA amounts

DNA amount was calculated as total amplifiable copies, including both mutant and germline alleles, as determined by digital PCR (median amplicon size 84 bp, range 58-177 bp), multiplied by the estimated amount of DNA in 1 haploid genome (3.3 pg). Amplifiable copies in control healthy plasma samples were estimated using the *FTH1* TaqMan assay Hs01694011_s1 (Life Technologies), which has an amplicon size of 180 bp.

Dilution of plasma DNA samples to determine allele quantification accuracy

After estimation of amplifiable copy numbers using digital PCR, both samples were diluted to 100 amplifiable copies per µl. A 5-µl mix was prepared containing 500 amplifiable copies, of which 400 copies were contributed by plasmaDna1 and 100 copies were contributed by plasmaDna2 (table s4). This was serially diluted 2.5× 6 times (reaching 244× dilution) in plasmaDna2. Each of the 7 mixes were then diluted 2.5× in water a further six times until we had an expected 2 total amplifiable copies of DNA. Twenty-eight of these mixes (table s5) were amplified using the described primer set (table S1) in triplicate, barcoded, and sequenced. Minor allele frequencies expected for the dilutions can be calculated using heterozygosity status for each SNP in either sample, an example that applies to 3 of the 5 SNPs (rs1800899, rs1050171 and rs10241451) we studied is given in table s5.

Replicate dilute Sanger sequencing

We performed replicate dilute Sanger Sequencing using the primers EGFR_A_F (CAGCAGGGTCTTCTCTGTTTCA) and EGFR_A_R (GGTGTTTTTCACCAGTACGTTCT). A PCR mastermix was made containing 0.05 U/μl FastStart High Fidelity Enzyme Blend (Roche), 1× FastStart High Fidelity Enzyme Buffer, 200 μM each of dNTPs, 4.5 mM MgCl₂, 5% DMSO, 50 nM of each primer, and 1X EvaGreen DNA binding dye (Biotium). Plasma DNA was quantified using digital PCR and diluted such that when partitioned in a 384-well plate, each reaction well would contain on average less than 1 amplifiable template. Forty-five cycles of PCR (50°C 2 min, 70°C 20 min, 95°C 10 min, 45 cycles of 95°C 15 s, 60°C 30 s, 72°C 1 min) were performed on a 7900HT Fast Real-Time PCR machine (Applied Biosystems).

TAm-Seq

Primer design for TAm-Seq

Target-specific primers were designed with universal primer sequences (termed CS1 and CS2) appended at the 5'-end (table S1). Primers were designed using Primer3 with a T_m range of 58-62°C, allowing homopolymer stretches no longer than 3 and keeping primer %GC less than 65%. Primer designs were screened against the hg19 reference genome to prevent cross-product generation, and were screened to prevent primer-dimer interactions and formation of intramolecular secondary structure. Primers were tested by amplifying from 10 ng human genomic DNA (GM17317, Coriell) in 5 μl reaction volumes containing 0.05 U/μl FastStart High Fidelity Enzyme Blend (Roche), 1× FastStart High Fidelity Enzyme Buffer, 200 μM each of dNTPs, 4.5 mM MgCl₂, 5% DMSO, 50 nM of each primer, and 1× Access Array sample loading solution (Fluidigm) using 35 cycles of amplification: 50°C 2 min, 70°C 20 min, 95°C 10 min, 10 cycles of 95°C 15 s, 60°C 30 s, 72°C 60 s, 2 cycles of 95°C 15 s, 80°C 30 s, 60°C 30 s, 72°C 60 s, 8 cycles of 95°C 15 s, 60°C 30 s, 72°C 60 s, 2 cycles of 95°C 15 s, 80°C 30 s, 60°C 30 s, 72°C 60 s, 8 cycles of 95°C 15 s, 60°C 30 s, 72°C 60 s, 5 cycles of 95°C 15 s, 80°C 30 s, 60°C 30 s, 72°C 60 s, 1 cycle of 72°C for 3 min.

Barcode primers (table S2) comprised either the PE1 or PE2 sequences for Illumina cluster generation, a 10-bp barcode, followed by either CS1 or CS2 adaptor sequences. For example, one pair of barcode primers comprised 5'-PE1-CS1-3' + 5'-PE2-BC-CS2-3', the second pair comprised 5'-PE1-CS2-3' + 5'-PE2-BC-CS1-3'.

The sequencing reagent for read1 contained custom sequencing primers designed to anneal to both CS1 and CS2. The sequencing reagent for the index read contained custom sequencing primers designed to anneal to the reverse complements of CS1 and CS2.

Preamplification for TAm-Seq

Preamplification reactions were carried out in 10-μl reaction volumes containing 50 nM of each forward and reverse target-specific primer. Target DNA (1 to 5 μl) was added to a mastermix containing 0.5 U FastStart High Fidelity Enzyme Blend (Roche), 1× FastStart High Fidelity Enzyme Buffer, 200 μM each of dNTPs, 4.5 mM MgCl₂, and 5% DMSO. Reactions were subjected to 15 cycles of amplification (95°C 10 min, 15 cycles of 95°C 15 s, 60°C 4 min). Following preamplification, 4 μl Exo-SAP-it (Affymetrix) was added to each reaction and incubated for 15 min at 37°C, then for 15 min at 80°C. The preamplified samples were diluted 5-fold in PCR-grade water prior to amplification on the Access Array IFC system.

Target-specific amplification on the Access Array microfluidic system

Individual primer pairs were loaded into the primer inlets of the Access Array IFC (Fluidigm) at a final concentration of 1 μ M (20 $\times$ the final concentration of 50 nM required for the PCR reaction) to account for the volume ratio of sample and primer chambers (20:1) in the Access Array IFC. One μ l of each preamplified sample was added to 4 μ l pre-sample mix containing 0.05 U/ μ l FastStart High Fidelity Enzyme Blend (Roche), 1 $\times$ FastStart High Fidelity Enzyme Buffer, 200 μ M each of dNTPs, 4.5 mM MgCl₂, 5% DMSO, and 1 $\times$ Access Array sample loading solution (Fluidigm). Samples and primers were loaded into the IFC using an IFC-AX controller (Fluidigm). The volume of each sample chamber within the Integrated Fluidic Circuit (IFC) was 33 nl, containing 0.7% of the input reaction volume (5 μ l). The IFC was then subjected to thermal cycling using a Biomark system (Fluidigm): 35 cycles of amplification (50°C 2 min, 70°C 20 min, 95°C 10 min, 10 cycles of 95°C 15 s, 60°C 30 s, 72°C 60 s, 2 cycles of 95°C 15 s, 80°C 30 s, 60°C 30 s, 72°C 60 s, 8 cycles of 95°C 15 s, 60°C 30 s, 72°C 60 s, 2 cycles of 95°C 15 s, 80°C 30 s, 60°C 30 s, 72°C 60 s, 8 cycles of 95°C 15 s, 60°C 30 s, 72°C 60 s, 5 cycles of 95°C 15 s, 80°C 30 s, 60°C 30 s, 72°C 60 s, 1 cycle of 72°C for 3 min). Harvesting solution (0.05% Tween-20) was loaded onto the IFC prior to harvesting on an IFC-AX controller. One μ l of harvested product was then transferred to a clean PCR plate containing 99 μ l PCR-free water.

Sequencing adaptor and barcode primer addition

For each sample, 1 μ l of the 100-fold diluted PCR products was added to each of two PCR plates containing 15 μ l pre-sample mastermix containing 0.05 U/ μ l FastStart High Fidelity Enzyme Blend (Roche), 1 $\times$ FastStart High Fidelity Enzyme Buffer, 200 μ M each of dNTPs, 4.5 mM MgCl₂, and 5% DMSO. In the first plate, 4 μ l of one pair of primers containing an individual 10-base barcode (BC) sequence, and sequence tags for reading in one direction (PE1-BC-CS1 + PE2-CS2) were added to each well. In the second plate, 4 μ l of primers containing (PE1-BC-CS2 + PE2-CS1) were added to each well. The corresponding wells in both plates contained primers with the same barcode sequence (e.g. plate 1, well A1 = Barcode FLD0001, plate 2, well A1 = barcode FLD0001). Reaction products in plates were amplified for 15 cycles: 95°C 10 min, 15 cycles of 95°C 15 s, 60°C 30 s, 72°C 4 min, 1 cycle of 72°C for 3 min.

Quantification and clean up of DNA library

After PCR products were barcoded, they were analyzed using Agilent 2100 BioAnalyzer to ensure expected insert size (~180 bp) was obtained. They were then pooled together and purified using AMPure XP beads using a bead to amplicon ratio of 1.8:1. The library was quantified by Agilent BioAnalyzer and subjected to Illumina cluster generation. Single-end sequencing of 100 bases was performed on an Illumina GAIIx sequencer followed by a 10-base indexing (barcode) read, using custom sequencing primers targeted to the CS1 and CS2 tags for both read1 and the index read according to manufacturer's recommendations.

Analysis of sequencing data

De-multiplexing and alignment. Reads generated by the primary Illumina pipeline were demultiplexed using a known list of barcodes (table S2), allowing one base mismatch out of 10 bases. Each set of reads was aligned independently to the hg19 reference genome using bwa-short in the single-end mode. Using expected genomic positions, each set of aligned reads was separated further into its constituent amplicons. A pileup was generated for each amplicon using samtools v1.12a. Using a base quality and a mapping quality cut-off of 30, observed frequencies of non-reference alleles for every sequenced locus across all amplicons and barcodes were calculated.

Mutation identification. For each locus and base, the distribution of non-reference background allele frequencies/reads was modeled as a Normal distribution and as a Poisson distribution, fitting to a Normal distribution and using the mean number of observed non-reference reads in a set of representative barcodes as the parameter for the Poisson distribution. For each barcode, the probability of obtaining the observed frequency/number of reads (or greater) was calculated in both models, and the lower value was retained. Putative substitutions that passed a probability cut-off (confidence margin) of 0.9995 were kept for further analysis as candidate mutations.

For each barcode, all loci that passed the probability cut-off were ranked by observed frequency, corrected for the median frequency (over all samples) for each locus/base. Known SNPs obtained from the 1000 Genomes project and regions covering amplification primers were discarded, leaving 17,934 possible base calls per sample. Indels are not analyzed in the current calling algorithm. A point mutation was called in a sample if it ranked among the 6 loci with highest non-reference frequency in both duplicates, and was represented by at least 10 reads in each. If a mutation was called, it was also discarded from the pool and the process was repeated by testing the 6 highest remaining frequencies. Mutations that occurred at low frequency may have fallen outside the list of top-ranked loci, especially for more noisy samples, and may have therefore been missed. For each sample, we estimated the threshold for mutation calling by the highest background-corrected non-reference allele frequency that did not get into the top-ranked list and may have been missed by the calling algorithm. For mutations called in multiple (overlapping) amplicons, the amplicon showing best concordance between duplicates was retained.

For the sequencing data obtained from a mix of FFPE samples, we performed an additional lower-stringency analysis using a probability cut-off of 0.9995 keeping the 12 loci with highest non-reference frequency to enhance sensitivity. For the data obtained from plasma sequencing, we performed an additional higher-stringency analysis using a probability cut-off of 0.9999 keeping the 6 loci with highest non-reference frequency to enhance sensitivity.

Mutation detection. When applying TAM-Seq to measure a pre-defined mutation (as opposed to screening nearly 18,000 possible substitutions), the frequency of the mutant allele can be read out directly from the data at the desired locus. We used confidence margins of ~ 0.95 using a Normal distribution model for each examined substitution, and requiring a minimum of 10 representative reads, in at least one amplified library per sample. When a mutation was positively detected, its allele frequency was estimated by averaging the allele frequencies in all replicates after subtracting substitution-specific background frequencies.

SUPPLEMENTARY FIGURES

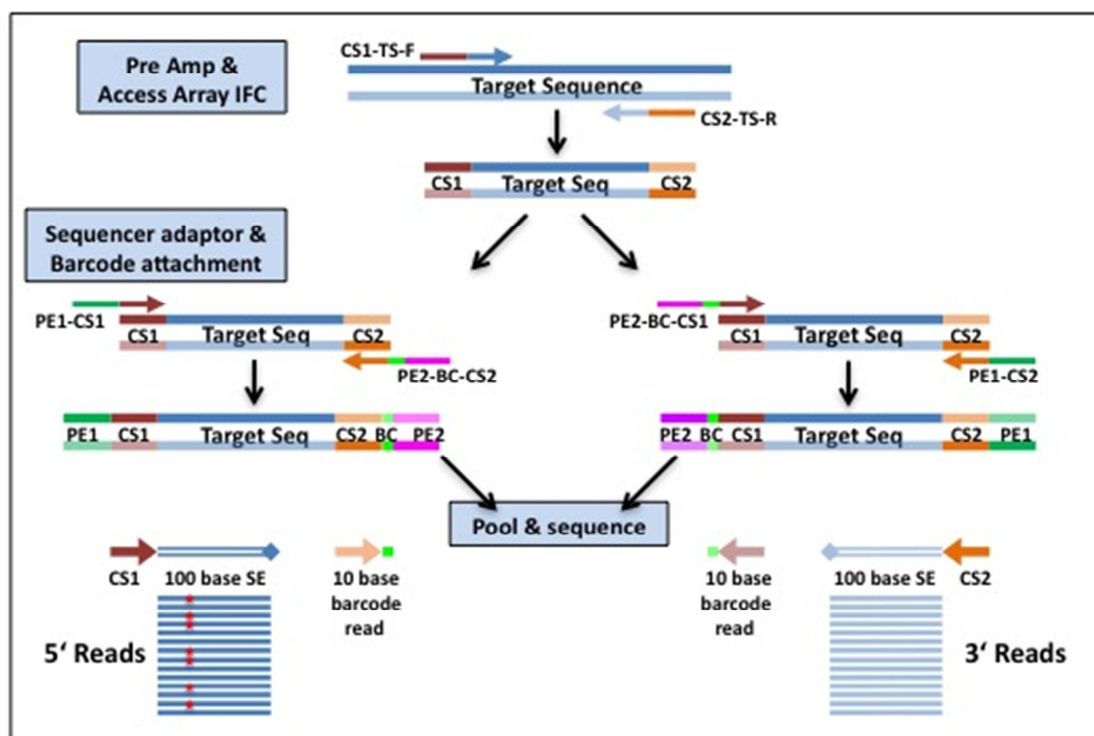


Figure S1: PCR strategy and primer design. Target-specific primers were synthesised with common adaptor sequences at their 5' ends (CS1 and CS2). These were used during the preamplification and single-plex stages to amplify the selected regions. Sequencing platform-specific adaptors and unique sample barcodes were attached during an additional round of PCR. The barcoding primers consist of Illumina sequencer adaptors (PE1 and PE2) at their 3' end, a unique 10 base barcode, and the common CS1 and CS2. Permutations of the universal tags and the sequencing adaptors enabled construction of bidirectional barcoded sequencing libraries. Using custom sequencing primers directed to the two tag sequences, sequence reads representing both strands of the amplicon pool were obtained from a single-end run. Both combinations were used in order to barcode and read amplicons in both directions. All samples were then pooled, cleaned, and then sequenced.

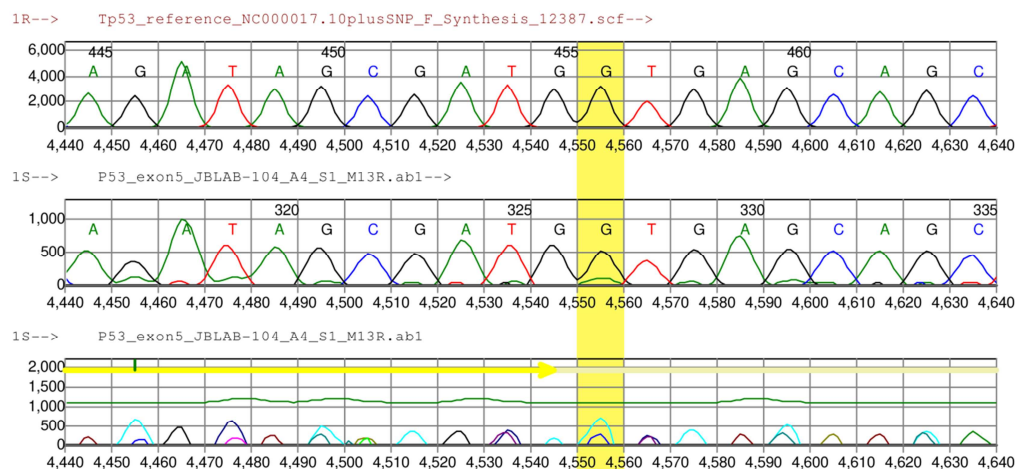
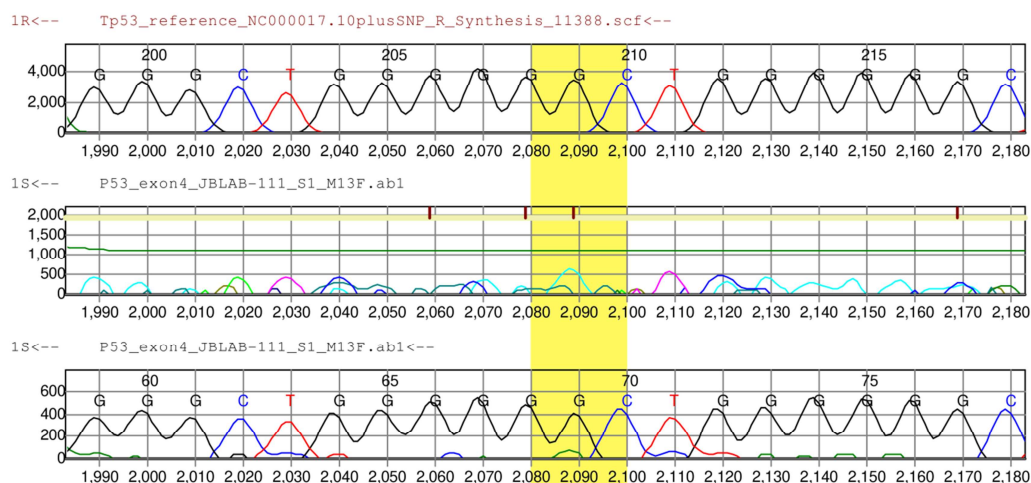
A**B**

Figure S2: Sanger traces for mutations identified by tagged-amplicon sequencing. Mutational analysis of Sanger traces was performed by Mutation Surveyor Software version 3.97 (SoftGenetics) using default settings. The *TP53* gene was on the reverse strand, therefore mutations seen in the sequencing traces were the reverse complement of the mutations as indicated by the hg19 coordinates. **(A)** Sanger sequencing of sample #104. Highlighted locus shows a mutation that was identified by TAM-Seq (17:7578370C>T), but not Sanger sequencing. The Sanger sequencing trace for the sample (middle panel) shows a weak signal above the reference (top panel), but this was at background noise levels and did not pass detection thresholds (lower panel). **(B)** Sanger sequencing of sample #111. Highlighted locus shows a mutation that was identified by TAM-Seq (17:7579642C>T), but not Sanger sequencing. The Sanger sequencing trace (lower panel) showed a weak signal above the reference (top panel), but this did not pass detection thresholds (middle panel).

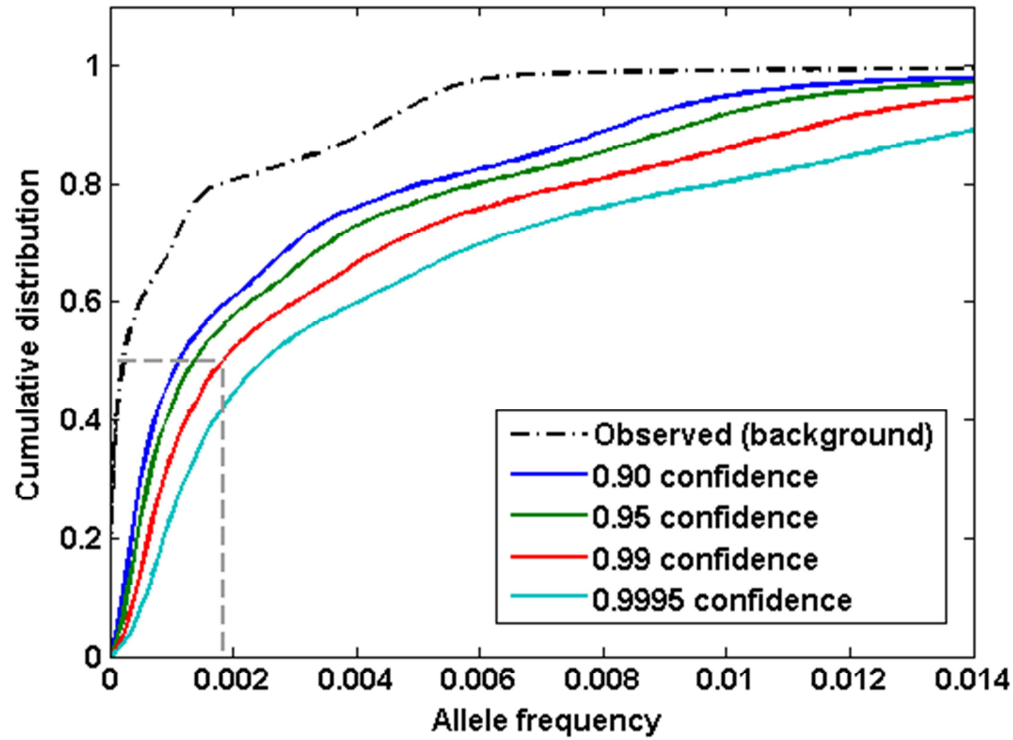


Figure S3: Background frequencies and detection limits for base substitutions. For each one of the possible base substitutions in the sequenced amplicons, we calculated the mean background read rate in the 124 barcoded libraries generated from the set of 62 plasma samples (cumulative distribution shown in black), and the standard deviation. Colored lines show the cumulative distribution of frequencies that would exceed the indicated confidence margins, using a Normal distribution model for each base substitution, given its measured mean and standard deviation. Substitutions that were covered by more than one amplicon or read direction were included multiple times, once for each amplicon/direction in which they were sequenced. Substitutions that had zero reads in all barcodes had undefined confidence limits and were omitted ($n = 589$). Dashed grey lines indicate the median value for 0.99 confidence margin.

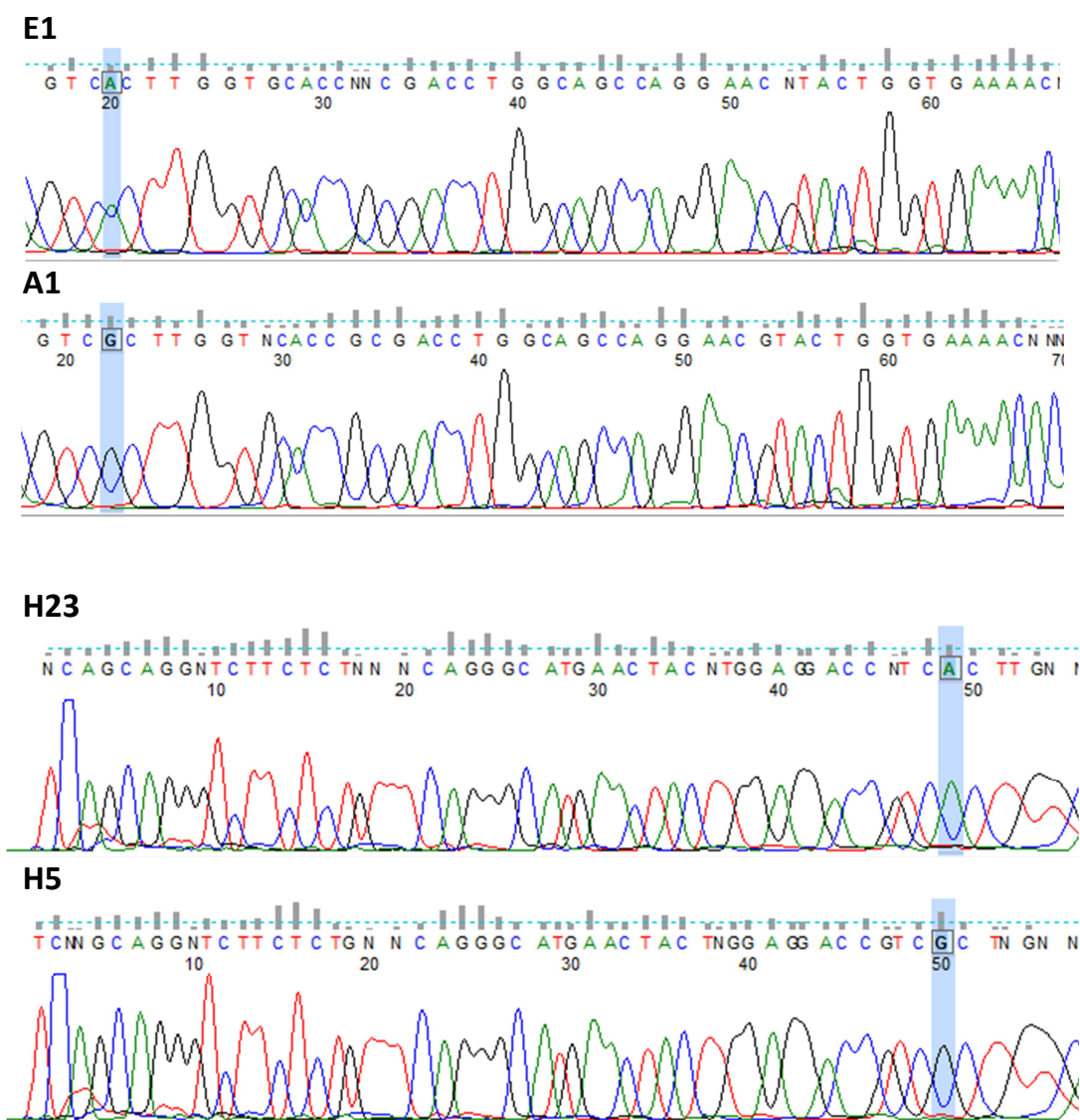


Figure S4: Replicate dilute Sanger sequencing of a mutation identified in plasma. Replicate Sanger sequencing of highly dilute template was used to validate the *EGFR* mutation that was identified *de novo* in plasma and was not found in the corresponding tumour sample from the same patient (Patient #27, Table 1). The panels show examples of mutant and wild type reads in either direction, with the mutated base (chr7:55259437G>A, genome build hg19 coordinates) highlighted in blue. E1 is an example of mutant forward read, and A1 is a matched wild-type. H23 is an example of mutant reverse read, and H5 a matched wild-type.

SUPPLEMENTARY TABLES

Table S1: Target-specific primers. Each target-specific primer consists of a universal 5' end and a target-specific 3' end as follows:

5'-ACACTGACGACATGGTTCTACA-[Target Specific-Forward]-3'.

5'-TACGGTAGCAGAGACTTGGTCT-[Target Specific-Reverse]-3'

Gene name and Amplicon ID	Target Specific Primer – Forward	Target Specific Primer- Reverse	Chr	Amplicon Start	Amplicon End
PIK3CA_E00001077674 *	CAGAGGGGAAAAATATGACAAA	AACAGAGAATCTCCATTTTAGCAC	3	178935943	178936150
PIK3CA_E00001139987	TGAGCAAGAGGCTTTGGAGT	GGTCTTTGCCTGCTGAGAGT	3	178952038	178952227
EGFR_Exon19	TCACAATTGCCAGTTAACGTCT	CCACACAGCAAAGCAGAAAC	7	55242373	55242537
EGFR_E00001601336_1	GCGTCTTCACCTGGAAGGG	CCGGACATAGTCCAGGAGG	7	55248902	55249111
EGFR_E00001601336_2	GCGTGGACAACCCCCAC	GGCTCCTTATCTCCCCTCC	7	55249005	55249213
EGFR_E00001681524_1	GGATGCAGAGCTTCTTCCCA	TTCTCTTCCGCACCCAG	7	55259352	55259542
EGFR_E00001681524_2	GGTCTTCTCTGTTTCAGGGCAT	GCTGACCTAAAGCCACCTCC	7	55259395	55259591
EGFR_E00001631695_1	GTGTCACTCGTAATTAGGTCCA	GGCCTCAGTACAACTCATTAGC	7	55260366	55260575
EGFR_E00001779947_1	TGTTCAATTCATGATCCCACTGC	CCACCAGTCACTCACACTTG	7	55266362	55266571
EGFR_E00001779947_2	TCCCTGCCAGCGAGAT	AGGGATGCAAAGGCCTCA	7	55266461	55266641
EGFR_E00001790701_1	GCCTTCTTTAAGCAATGCCATCTTTAT	CAATGGAAGCACAGACTGCAA	7	55267932	55268134
EGFR_E00001801208_1	CCCCTGCTCCTATAGCCAA	ATGAGGTACTCGTCGGCATC	7	55268806	55268987
EGFR_E00001801208_2	ACTTCTACCGTGCCCTGA	GTTCAAATGAGTAGACACAGCTT	7	55268921	55269101
EGFR_E00001773562_1	TACCCTCCATGAGGCACAC	GGAGAGCTGTAAATTCTGGCTT	7	55269336	55269516
BRAF_E00002324725	TCATAATGCTTGCTCTGATAGGA	CTGATGGGACCCACTCCAT	7	140453108	140453256
PTEN_E00001456562_1	GCAGCTTCTGCCATCTCTCT	TCCGTCTACTCCCACGTTCT	10	89624175	89624372
PTEN_E00001156351_1	TGCTGCATATTTAGATATTTCTTTCCTTA	ATGAAAACACAACATGAATATAAACATCAAT	10	89653738	89653927
PTEN_E00001156344_1	AATCTGTCTTTTGGTTTTTCTTGATAGT	AATAGTTGTTTTAGAAGATATTTGCAAGC	10	89685172	89685367
PTEN_E00001156337_4	TATATCACTTTTAACTTTTCTTTTAGTTGTGC	CTCGATAATCTGGATGACTCATTATTGTT	10	89690776	89690940
PTEN_E00001156330_1	TTCTTATTCTGAGGTTATCTTTTACCAC	TCATTACACCAGTTCGTCCCT	10	89692739	89692919

PTEN_E00001156330_3	TGACCAATGGCTAAGTGAAGATGA	TCCAGGAAGAGGAAAGGAAAAACA	10	89692840	89693048
PTEN_E00001156327_1	TCTTAAATGGCTACGACCCAG	TCCAGATGATTCTTTAACAGGTAGC	10	89711775	89711942
PTEN_E00001156327_4	CAGTCAGAGGCGCTATGTGT	TCTAGATATGGTTAAGAAAAGTGTCCA	10	89711889	89712077
PTEN_E00001156321_1	TGACAGTTTGACAGTTAAAGGCAT	CACACACAGGTAACGGCTGA	10	89717547	89717726
PTEN_E00001156321_2	TGTGGTCTGCCAGCTAAAGG	TCTCCCAATGAAAGTAAAGTACAAACC	10	89717620	89717802
PTEN_E00001156321_4m	TCCACAAACAGAACAAGATGCT	GGCCTTTTCCTTCAAACAGGATT	10	89717748	89717956
PTEN_E00001156315_1m	GCAACAGATAACTCAGATTGCCTT	GTTTCCTCTGGTCCTGGTATGA	10	89720457	89720706
PTEN_E00001156315_5 *	AGGACAAAATGTTTCACCTTTGGGTAA	ACTAGATATTCCTTGTCATTATCTGCAC	10	89720649	89720799
PTEN_E00001156315_7	CCTCAGAAAAAGTAGAAAATGGAAGTC	ACAAGTCAACAACCCCCACA	10	89720706	89720915
PTEN_E00001456541_1	AGATGAGTCATATTTGTGGGTTTTCA	TCTGGATCAGAGTCAGTGGT	10	89724997	89725180
PTEN_E00001456541_2 *	GTAGAGGAGCCGTCAAATCCA	TTCATGGTGTTTTATCCCTCTTGA	10	89725068	89725264
KRAS_E00000936617	GCCTGCTGAAAATGACTGAA	AGAATGGTCCTGCACCAGTAA	12	25398163	25398329
TP53_E00001757276_1	GACCCAAAACCCAAAATGGC	TCCCTGCTTCTGTCTCCTAC	17	7572850	7573030
TP53_E00001728015_1	GGAATCCTATGGCTTTCCAACC	CCCCCTCCTCTGTTGCTG	17	7573859	7574054
TP53_00001404886_13	TCTGTATCAGGCAAAGTCATAGAA	GCCTCAAAGACAATGGCTCC	17	7576584	7576734
TP53_E00001789298_1	AGAAAACGGCATTTTGAGTGT	AAGGGTGCAGTTATGCCTCA	17	7576786	7576983
TP53_E00001789298_2	CTGGTGTTGTTGGGCAGT	ATCTCCGCAAGAAAGGGGAG	17	7576908	7577075
TP53_E00001789298_3	TGTCCTGCTTGCTTACCTCG	GCCTCTTGCTTCTCTTTTCCT	17	7577003	7577187
TP53_E00001665758_1	GGGGTCAGAGGCAAGCAG	CTTGGGCCTGTGTTATCTCC	17	7577432	7577631
TP53_E00001255919_1	GAGAAAGCCCCCCTACTGC	AGCATCTTATCCGAGTGGAAGG	17	7578091	7578274
TP53_E00001255919_3	TCCAAATACTCCACACGCAAA	GCTGCCCCCACCATGAG	17	7578229	7578406
TP53_E00001255919_5	AGCTGCTCACCATCGCTA	CCAACTGGCCAAGACCT	17	7578361	7578525
TP53_E00001255919_6	TGTGCTGTGACTGCTTGTAG	TGCCCTGACTTTCAACTCTGT	17	7578425	7578594
TP53_E00001612188_1	ATACGGCCAGGCATTGAAGT	CCTCCTGGCCCCCTGTC	17	7579260	7579421
TP53_E00001612188_2	GGAAACCGTAGCTGCCCTG	AAGACCCAGGTCCAGATGAA	17	7579359	7579520
TP53_E00002359670	CAGCCTCTGGCATTCTGG	CCTGGTCCTCTGACTGCTCT	17	7579479	7579626
TP53_E00002419584	TCAAATCATCCATTGCTTGG	CCATGGGACTGACTTTCTGC	17	7579557	7579754
TP53_E00001596491_1	TTTCGCTTCCCACAGGTCTC	CAGCCAGACTGCCTTCCG	17	7579758	7579940

*Each of these 3 primer pairs potentially amplifies an additional non-target region, owing to large regions of homology and the short amplicon size. This was taken into account in data analysis.

Table S2: Unique sequencing barcodes. Platform-specific adaptors and barcodes are attached through PCR following the single-plex amplification step. The primers consisted of the PE1 and PE2 sequences for Illumina cluster generation, a 10-bp barcode, and the CS1 and CS2 adaptors, used in pairs: PE1-CS1 with PE2-BC-CS2, and PE1-CS2 with PE2-BC-CS1.

PE1: AATGATACGGCGACCGAGATCT.

PE2: CAAGCAGAAGACGGCATACGAGAT.

Barcode name	Barcode sequence				
FLD0001	GTATCGTCGT	FLD0060	GCACGTAGCT	FLD0121	CAGAGCTAGT
FLD0002	GTGTATGCGT	FLD0061	TCACGCTATG	FLD0122	CGCAGAGCAT
FLD0003	TGCTCGTAGT	FLD0062	CGTACTACGT	FLD0123	TGTACAGCGA
FLD0004	GTCGTCGTCT	FLD0063	CAGCTGAGTA	FLD0124	ACGTCAAGTAT
FLD0005	GTGCGTGTGT	FLD0064	GAGATCAGTC	FLD0125	TCACAGCATA
FLD0006	GCGTCGTGTA	FLD0065	TACTGAGCTG	FLD0126	ACTGCGTGTC
FLD0007	GTCGTGTA	FLD0066	TAGTAGCGCG	FLD0127	CGATCGACTG
FLD0008	GATGTAGCGT	FLD0067	GACGTCTGCT	FLD0128	GCGAGATGTA
FLD0009	GAGTGATCGT	FLD0068	GACTCGCGA	FLD0129	CTGATGCAGA
FLD0010	CGCTATCAGT	FLD0069	TCTGAGCGCA	FLD0130	GTGACGTACG
FLD0011	CGCTGTAGTC	FLD0070	TAGACGTGCT	FLD0131	CGACGCTGAT
FLD0012	GCTAGTGAGT	FLD0071	GTGACTCGTC	FLD0132	CTACGATCAG
FLD0013	GAGCTAGTGA	FLD0072	TCGAGTAGCG	FLD0133	GCTATCAGCA
FLD0014	CGTGCTGTCA	FLD0073	CGTATGATGT	FLD0134	CTAGCAGATG
FLD0015	GATCGTCTCT	FLD0074	TAGTCTGTCA	FLD0135	CATGATACGC
FLD0016	GTGCTGTCGT	FLD0075	TGCTCTATC	FLD0136	GCAGCTGTCA
FLD0017	TGAGCGTGCT	FLD0076	CTAGAGTATC	FLD0137	ACGTATCATC
FLD0018	CATGTCGTCA	FLD0077	TATCATGTGC	FLD0138	AGTATCGTAC
FLD0019	TCAGTGTCTC	FLD0078	CATGAGTGTA	FLD0139	GATACACTGA
FLD0020	GTGCTCATGT	FLD0079	TGTCGTCATA	FLD0140	GACTAGTCAG
FLD0021	CGTATCTCGA	FLD0080	TATCTCATGC	FLD0141	GATGACTACG
FLD0022	GTCATGCGTC	FLD0081	TGTGTCACTA	FLD0142	CAGAGAGTCA
FLD0023	CTATGCGATC	FLD0082	TATCGATGCT	FLD0143	TCGATCGACA
FLD0024	TGCTATGCTG	FLD0083	TAGAGTCTGT	FLD0144	ACTGATGTAG
FLD0025	TGTGTGCATG	FLD0084	CATGCATCAT	FLD0145	ACTCGATAGT
FLD0026	GAGTGTCACT	FLD0085	TGATCAGTCA	FLD0146	GACGATCGCA
FLD0027	CTAGTCTCGT	FLD0086	CGTCTATGAT	FLD0147	TCATCATGCG
FLD0028	GAGTGCATCT	FLD0087	GTGATACTGA	FLD0148	ACATGTCTGA
FLD0029	TGCGTAGTCG	FLD0088	CTAGATCTGA	FLD0149	TAGCATACAG
FLD0030	CTGTGTCGTC	FLD0089	TATCAGTCTG	FLD0150	AGAGTCGCGT
FLD0031	CTGTAGTGCG	FLD0090	TCAGATGCTA	FLD0151	TCTACGACAT
FLD0032	GTGCGCTAGT	FLD0091	TATGTACGTG	FLD0152	CTACGATGA
FLD0033	TGTGCTCGCA	FLD0092	CTATACAGTG	FLD0153	ACGCACATAT
FLD0034	GATGCGAGCT	FLD0093	TGATACTCTG	FLD0154	ACGTGCTCTG
FLD0035	CTGTACGTGA	FLD0094	TCAGCGATAT	FLD0155	ACGATCACAT
FLD0036	GCGATGATGA	FLD0095	CTACTGATGA	FLD0156	AGTGTACTCA
FLD0037	TGTGAGTCA	FLD0096	GTAGTACACA	FLD0157	TGATGTATGT
FLD0038	GTCTACTGTC	FLD0097	TGCTACATCA	FLD0158	GATATATGTC
FLD0039	CAGTCAGAGT	FLD0098	AGTGTGTCTA	FLD0159	TAGTACTAGA
FLD0040	CGCAGTCTAT	FLD0099	TCATATCGCG	FLD0160	TATAGAGATC
FLD0041	GTATGAGCAC	FLD0100	TACGTATAGC	FLD0161	TCGATATCTA
FLD0042	CGAGTGCTGT	FLD0101	CAGCTATAGC	FLD0162	TACATGATAG
FLD0043	TATAGCACGC	FLD0102	TCGATGCGCT	FLD0163	TGAGATCATA
FLD0044	TCATGCGCGA	FLD0103	GCACGCGTAT	FLD0164	CTACATACTA
FLD0045	TATGCGCTGC	FLD0104	GCAGTATGCG	FLD0165	ATCAGTGTAT
FLD0046	TCTCTGTGCA	FLD0105	TGATAGAGAG	FLD0166	ATCATATCTC
FLD0047	CTATCGCGTG	FLD0106	GCTACTAGCG	FLD0167	AGTAGATCAT
FLD0048	TACGCTGCTG	FLD0107	TGCGAGACGT	FLD0168	ACATAGTATC
FLD0049	CTGCATGATC	FLD0108	CGATGACAGA	FLD0169	ATGTATAGTC
FLD0050	CGCGTATCAT	FLD0109	GACTCATGCT	FLD0170	ACAGTCATAT
FLD0051	GTATCTCTCG	FLD0110	GTCTGATACG	FLD0171	ACATATACGT
FLD0052	GCTCATATGC	FLD0111	ACTAGCTGTC	FLD0172	AGCATCTATA
FLD0053	CACTATGTGCG	FLD0112	GCGTAGACGA	FLD0173	AGACTATATC
FLD0054	TAGCGGTAG	FLD0113	CTCAGCAGTG	FLD0174	CAGCATCTAG
FLD0055	CGTCACAGTA	FLD0114	CAGTCTACAT	FLD0175	CGAGACGACA
FLD0056	TCGCGTGAGA	FLD0115	TACTGCAGCG	FLD0176	ATCACTCATA
FLD0057	TACATCGCTG	FLD0116	TACACAGTAG	FLD0177	AGCTCTGTGA
FLD0058	GTGAGAGACA	FLD0117	CACATACAGT	FLD0178	ATGTCATGCT
FLD0059	GACTGTACGT	FLD0118	CACAGTGATG	FLD0179	GTCGACGAG
		FLD0119	CGAGCTAGCA	FLD0180	ATACAGTCTC
		FLD0120	GAGACTATGC	FLD0181	

FLD0182	CATAGACGTG	FLD0250	GAAGGAGATA	FLD0318	GAGAGGACAT
FLD0183	AGAGATATCA	FLD0251	CGAATGTATG	FLD0319	GAGCACGGAA
FLD0184	ATGCTGCGCT	FLD0252	TCGTGAATGA	FLD0320	GCTCTAACAT
FLD0185	AGTCAGACGC	FLD0253	GAATAGCTGA	FLD0321	TGCTGGCTTG
FLD0186	ACGATACACT	FLD0254	TTGTACATC	FLD0322	TGCATGGAGC
FLD0187	AGCGAGTATG	FLD0255	CTGGAGGCTA	FLD0323	GTACTAAGAG
FLD0188	ATCGCTACAT	FLD0256	TGTCAGCTTA	FLD0324	GAAGTCAAGC
FLD0189	ATGCTAGAGA	FLD0257	GTTCTTCGTA	FLD0325	GCGCATTATG
FLD0190	AGCAGTACTC	FLD0258	TTACACGTTT	FLD0326	GTCCAGACAT
FLD0191	ATCTAGATCA	FLD0259	GTAGCCAGTA	FLD0327	GAGACCTCTA
FLD0192	ATCGCATAGA	FLD0260	TGAGAAGGTA	FLD0328	TTGCACTCAG
FLD0193	TTGTTGCTGT	FLD0261	CCATATGATC	FLD0329	TGCGGCGGATA
FLD0194	GTGTGGTTGT	FLD0262	CGATCCTATA	FLD0330	AGTTGCTAGT
FLD0195	TAGGTGGAAT	FLD0263	TGACTAGCTT	FLD0331	AGGATTAGAG
FLD0196	TGTAGGTGGA	FLD0264	TAACCTCGCT	FLD0332	CCAGAACAGA
FLD0197	TTAGTGGTGA	FLD0265	TCGAATGTGC	FLD0333	CGTCAAGCAT
FLD0198	GTGAAGGTAA	FLD0266	TGCGTGAACA	FLD0334	TTGTCGAGAC
FLD0199	TGTTGTGGTA	FLD0267	GCGTTATTGC	FLD0335	GACAGGTGAC
FLD0200	GTTGATGAGT	FLD0268	GAACATCAC	FLD0336	CTGACAAGTG
FLD0201	GGTCAGTGTA	FLD0269	TCGAGGTACT	FLD0337	CACGAAGAGC
FLD0202	GTAATGGAGT	FLD0270	TGCGGATGGT	FLD0338	CATACCTGAT
FLD0203	CTCGTTATTC	FLD0271	TTCGAGCTAT	FLD0339	GACGTGCTTC
FLD0204	GGAAGTAAGG	FLD0272	GGTCTGGTGT	FLD0340	ATTGTGGAGT
FLD0205	CGGTGTGTGT	FLD0273	CTAAGTCATG	FLD0341	TCTGGTCTCA
FLD0206	CGTCTTCTTA	FLD0274	TTCAGATCA	FLD0342	AGGTAAGAGG
FLD0207	TGTGAATCTC	FLD0275	CTGCGAATGT	FLD0343	TCCTGACAGA
FLD0208	CTAATCGTGT	FLD0276	CTGTTCTAGC	FLD0344	GCACTGTTGC
FLD0209	CTCTTAGTTC	FLD0277	CACCTGTGTG	FLD0345	ACCATGAGTC
FLD0210	GGATAGGATC	FLD0278	TGGATGACAT	FLD0346	AATGCAGTGT
FLD0211	GGTGTCTTGT	FLD0279	GATCCTGAGC	FLD0347	ATATGGTGGG
FLD0212	GATGGTTGTA	FLD0280	GTGGTCTGTA	FLD0348	ACTCAGTTAC
FLD0213	CCTCGTTGTT	FLD0281	TGTTACGATC	FLD0349	AAGTGCGATG
FLD0214	GGTTGGAGTT	FLD0282	GTCTTGGCTC	FLD0350	CCACAGAGTG
FLD0215	TGGTGTCCGT	FLD0283	GGTCGTGCAT	FLD0351	AGTGGTGATC
FLD0216	CGTTAGCGTA	FLD0284	CAGGCTCAGT	FLD0352	ACTTCTTAGC
FLD0217	TACTAGGATC	FLD0285	TAGCTTCACT	FLD0353	GCCACATATA
FLD0218	GTCTCAATGT	FLD0286	CAGATGTCCT	FLD0354	ACGCAGGAGT
FLD0219	GATGAGGTAT	FLD0287	TTACGCAGTG	FLD0355	AATATGCTGC
FLD0220	GGTGTTAGTG	FLD0288	TTCGTTCCTG	FLD0356	AAGCGTAGAA
FLD0221	CATTCTCTGA	FLD0289	CACCTGCTTGA	FLD0357	GACAGCAAGC
FLD0222	CATCTGGAGT	FLD0290	TCTAGCGTGG	FLD0358	TGACCCGAGA
FLD0223	GAATGGAAGA	FLD0291	GCATAATCGC	FLD0359	CGCGACTTGT
FLD0224	GGCTGTGATC	FLD0292	GTGTAACAC	FLD0360	CATCAACATG
FLD0225	TGGTGCTGGA	FLD0293	GAGATTGCTA	FLD0361	TGGCTACGCT
FLD0226	TATGGTAAGG	FLD0294	GGACAGATGG	FLD0362	ACGCGGACTA
FLD0227	GTTTCGATTGT	FLD0295	CTTACGTTGC	FLD0363	AGAGGTCGGA
FLD0228	GGTAGAATGA	FLD0296	GTGTTCCGGT	FLD0364	AATCGAGCGT
FLD0229	TTCTCATCGT	FLD0297	CTCAAGAAGC	FLD0365	AAGTACACTC
FLD0230	CTCAATCGTA	FLD0298	TCTCGGATAG	FLD0366	AGCTGAATGA
FLD0231	CGCTAATGTA	FLD0299	CTCTGGACGA	FLD0367	ATGCCTATCA
FLD0232	GCGTCTGAAT	FLD0300	CGAGCATTGT	FLD0368	ACTGTAGGAC
FLD0233	TTCTGTTGCC	FLD0301	CCAAGAAGAA	FLD0369	ATAGCCGTGT
FLD0234	TTGTCCTTGC	FLD0302	TCCTTGTCT	FLD0370	TCACGACGAA
FLD0235	CCTGTGTAGA	FLD0303	GTAACGATGT	FLD0371	ATCTGTCCAT
FLD0236	GATAAGAAGG	FLD0304	TGGACTCAGA	FLD0372	ACTTAGAGAG
FLD0237	CAGGTCACAT	FLD0305	GGCATCATGC	FLD0373	AGTGCCAGGT
FLD0238	GCCATGTCAT	FLD0306	GTATAACGCT	FLD0374	ATTAGGTCGT
FLD0239	TCTGCCTATA	FLD0307	GCAGATAAGT	FLD0375	AGGAGAAGGA
FLD0240	CTTAGTTCGC	FLD0308	GTGCGCTCTA	FLD0376	ACAACGCAAG
FLD0241	CGTAATGAGC	FLD0309	TTGATAGCA	FLD0377	ATTAGCGAGT
FLD0242	TTGCTTAGTC	FLD0310	GTCTAGCAGG	FLD0378	ACAACGAACA
FLD0243	TCTTGTTTCA	FLD0311	GGAAACACAG	FLD0379	AGAGCGCCAA
FLD0244	GTGGCTTCGT	FLD0312	TGGTTCGCTG	FLD0380	AGGTAGCTCA
FLD0245	TGTTTCGATAG	FLD0313	CACATTAGCG	FLD0381	AACGCCAAGA
FLD0246	TCATTCAAGT	FLD0314	GAAGCGCACT	FLD0382	AAGGTATGAG
FLD0247	GTGGAGAGCT	FLD0315	GCAATGCCAGT	FLD0383	ATGGAGCACT
FLD0248	GTAGAAGTGG	FLD0316	GGAGACTGTA	FLD0384	ACGGTGCTAG
FLD0249	TGGAGCATGT	FLD0317	TCGAAGTCA		

Table S3: Mutations identified in FFPE samples. Diagnosis: type (FT, fallopian tube; O, ovarian; PP, primary peritoneal), histiotype (END, endometrioid, grade III; HGSOC, high-grade serous ovarian carcinoma; S/CC, mixed serous and clear cell; S/END, mixed serous papillary and endometrioid, high grade), and stage. FFPE source: sample type (B, biopsy; IDS, interval-debulking surgery; PS, primary surgery), sample tissue (FT, fallopian tube; O, ovary; OM, omentum; P, peritoneum; SC, sigmoid colon; U, uterus). Cellularity: estimated from H&E-stained slide of the FFPE block. Identified by Sanger sequencing: I, identified; FAIL, samples where one duplicate had a technical fault; Indel, indel identified (indels were not analyzed by the calling algorithm); M, missed; U, unsequenced region or sample. S-135 and S-165 were from the same patient, but collected at different timepoints. S-140 and S-106 were from the same block, cut in different batches. S-111, S-143, and S-122 had two mutations called each. S-133, S-169, and S-147 had known indels, and did not have any point mutations called. S-119 had no mutations called. S-176, S-177, and S-178 failed harvesting in one of the repeats and calling algorithm was not applied.

Patient ID	Age at diagnosis	Diagnosis: type, histiotype, and stage.	Sample ID	FFPE source	Cellularity	Gene	Mutation and base change (genome build hg19)	cDNA change	Protein change	TAm-Seq Allele frequency (repeat 1)	TAm-Seq Allele frequency (repeat 2)	Identified by Sanger sequencing
1	60	O HGSOC III	S-131	PS O	60%	TP53	17:7577570 C>T	c.711G>A	p.M237I	0.72	0.70	I
2	63	O HGSOC III	S-142	IDS O	40%	TP53	17:7578203 C>A	c.646G>T	p.V216L	0.42	0.43	I
3	54	O HGSOC III	S-150	IDS O	70%	TP53	17:7577538 C>T	c.743G>A	p.R248Q	0.87	0.91	I
4	46	O HGSOC I	S-167	PS O	70%	TP53	17:7578290 C>T	c.560-1G>A	p.?	0.73	0.73	I
5	38	O HGSOC III	S-174	PS O	90%	TP53	17:7578268 A>G	c.581T>C	p.L194P	0.69	0.72	I
6	63	O HGSOC III	S-102	IDS FT	60%	TP53	17:7578271 T>C	c.578A>G	p.H193R	0.39	0.34	I
7	68	O HGSOC III	S-112	IDS O	60%	TP53	17:7578263 G>A	c.586C>T	p.R196*	0.30	0.28	I
8	60	O HGSOC III	S-132	PS O	90%	TP53	17:7577120 C>T	c.818G>A	p.R273H	0.74	0.75	I
9	62	PP HGSOC IV	S-151	PS SC	70%	TP53	17:7578479 G>A	c.451C>T	p.P151S	0.39	0.38	I
10	58	PP HGSOC IV	S-168	IDS O	90%	TP53	17:7578508 C>T	c.422G>A	p.C141Y	0.82	0.86	I
11	55	O HGSOC III	S-104	IDS O	15%	TP53	17:7578370 C>T	c.559+1G>A	p.?	0.12	0.12	M
12	62	O HGSOC IV	S-115	IDS O	70%	TP53	17:7577579 G>T	c.702C>A	p.Y234*	0.57	0.59	I
13	45	O HGSOC IV	S-123	IDS O	80%	TP53	17:7577574 T>C	c.707A>G	p.Y236C	0.53	0.55	I
14	58	O HGSOC III	S-153	IDS O	95%	TP53	17:7578212 G>A	c.637C>T	p.R213*	0.56	0.56	I
15	61	O HGSOC IV	S-116	IDS O	98%	TP53	17:7577121 G>A	c.817C>T	p.R273C	0.76	0.75	I
16	65	O HGSOC III	S-127	IDS O	90%	TP53	17:7577094 G>A	c.844C>T	p.R282W	0.76	0.74	I
17	64	O HGSOC III	S-145	PS FT	60%	TP53	17:7578176 C>T	c.672+1G>A	p.?	0.52	0.55	I
18	63	FT HGSOC III	S-161	PS O	95%	TP53	17:7578534 C>G	c.396G>C	p.K132N	0.86	0.88	I
19	68	PP HGSOC III	S-170	IDS U	50%	TP53	17:7579312 C>A	c.375G>T	p.T125T	0.22	0.20	I

20	67	PP	HGSOC	III	S-108	IDS	OM	20%	TP53	17:7578431	G>A	c.499C>T	p.Q167*	0.74	0.93	I
21	64	O	HGSOC	I	S-117	PS	O	95%	TP53	17:7574012	C>A	c.1015G>T	p.E339*	0.70	0.70	I
22	65	O	HGSOC	III	S-128	IDS	O	95%	TP53	17:7579521	C>A	c.166G>T	p.E56*	0.68	0.67	I
23	54	O	HGSOC	III	S-138	IDS	OM	95%	TP53	17:7577569	A>C	c.712T>G	p.C238G	0.79	0.79	I
24	63	FT	HGSOC	III	S-146	PS	O	60%	TP53	17:7577559	G>T	c.722C>A	p.S241Y	0.62	0.62	I
25	61	PP	HGSOC	III	S-162	IDS	OM	40%	TP53	17:7578404	A>T	c.526T>A	p.C176S	0.11	0.19	I
26	72	PP	HGSOC	III	S-171	IDS	OM	50%	TP53	17:7578265	A>T	c.584T>A	p.I195N	0.71	0.70	I
27	68	O	HGSOC	III	S-179	IDS	O	90%	TP53	17:7578262	C>G	c.587G>C	p.R196P	0.83	0.86	I
28	46	O	HGSOC	III	S-109	IDS	O	50%	TP53	17:7577548	C>T	c.733G>A	p.G245S	0.40	0.39	I
29	82	O	HGSOC	III	S-118	IDS	OM	80%	TP53	17:7577568	C>G	c.713G>C	p.C238S	0.74	0.75	I
30	69	FT	HGSOC	III	S-130	PS	O	80%	TP53	17:7579389	G>A	c.298C>T	p.Q100*	0.75	0.79	I
31	64	O	HGSOC	III	S-172	IDS	OM	70%	TP53	17:7578406	C>T	c.524G>A	p.R175H	0.48	0.42	I
32	71	O	HGSOC	III	S-101	IDS	P	90%	TP53	17:7578370	C>T	c.559+1G>A	p.?	0.95	0.94	I
33	73	O	HGSOC	III	S-144	IDS	OM	70%	TP53	17:7577557	A>C	c.724T>G	p.C242G	0.44	0.47	I
34	54	O	HGSOC	IV	S-165	IDS	O	95%	TP53	17:7578527	A>G	c.403T>C	p.C135R	0.47	0.46	I
35	55	PP	S/END	III	S-135	B	OM	80%	TP53	17:7578527	A>G	c.403T>C	p.C135R	0.57	0.60	I
					S-106	PS	O	90%	TP53	17:7574021	C>A	c.1006G>T	p.E336*	0.96	0.96	I
					S-140			90%	TP53	17:7574021	C>A	c.1006G>T	p.E336*	0.96	0.96	I
36	52	O	HGSOC	III	S-111	PS	O	95%	TP53	17:7578502	A>T	c.428T>A	p.V143E	0.45	0.44	I
37	45	O	END	II	S-143	PS	O	95%	TP53	17:7579642	C>T	Intron	Intron	0.22	0.28	M
								95%	TP53	17:7577538	C>T	c.743G>A	p.R248Q	0.75	0.77	I
								95%	PTEN	10:89720778	A>G	c.929A>G	p.D310G	0.59	0.52	I
38	55	O	END	IV	S-122	IDS	O	80%	PTEN	10:89692791	A>G	c.275A>G	p.D92G	0.19	0.18	M
39	54	O	HGSOC	III	S-119	PS	OM	80%	PTEN	10:89685287	A>G	c.182A>G	p.H61R	0.17	0.17	I
								80%								U
								80%								Indel
40	71	O	HGSOC	III	S-133	IDS	OM	80%								Indel
41	66	O	HGSOC	III	S-169	IDS	OM	50%								Indel
42	68	FT	S/CC	III	S-147	IDS	O	90%								Indel
43	49	FT	HGSOC	III	S-176	PS	O	95%							FAIL	
44	73	PP	HGSOC	IV	S-177	PS	OM	50%							FAIL	
45	44	O	HGSOC	III	S-178	IDS	OM	30%							FAIL	

Table S4: SNPs identified in circulating DNA from two plasma control samples.

SNP ID	Genomic location (genome build hg19) and genotypes	Normal plasma DNA 1 ("plasmaDna1")	Normal plasma DNA 2 ("plasmaDna2")
rs1625895	chr17:7578115 (T/C)	TT	TC
rs1800899	chr17:7576841 (A/G)	AG	AA
rs17337360	chr7:55260440 (C/T)	CC	TT
rs1050171	chr7:55249063 (G/A)	GA	GG
rs10241451	chr7:55248926 (T/C)	TC	TT

Table S5: Frequency of SNP alleles in dilution series of DNA from control plasma.

Total amplifiable copies	Minor SNP percentage						
	200.00	80.00	32.00	12.80	5.12	2.05	0.82
2.05						40.00	
5.12					40.00	16.00	
12.80				40.00	16.00	6.40	
32.00			40.00	16.00	6.40	2.56	
80.00		40.00	16.00	6.40	2.56	1.02	
200.00	40.00	16.00	6.40	2.56	1.02	0.41	
500.00	40.00	16.00	6.40	2.56	1.02	0.41	0.16
	200.00	80.00	32.00	12.80	5.12	2.05	0.82
Minor SNP amplifiable copies							

Table S6: Additional data for Table 2 for mutations identified in plasma samples. Patient ID links to patient ID in table S3 (for patients 46 to 56, sequencing of tumour FFPE DNA was not included in table S3). Age: at diagnosis. Time: elapsed since surgery (months); chemotherapy lines: number of previous lines of chemotherapy. Vol: Plasma per amplification reaction (μL). DNA: DNA amount per amplification (in ng) was calculated from the number of total amplifiable copies (both mutant and germline allele) determined by digital PCR. Mean depth: number of sequencing reads covering this locus. AF, TAM-Seq: mean allele frequency using TAM-Seq, with the freq in each replicate given in parenthesis. Mutation call: FP, called using TAM-Seq, but not detected in FFPE DNA, and not called using more stringent calling criteria; M, mutation found in FFPE DNA and detected at allele frequency >2% in the plasma sample by digital PCR, but only found by TAM-Seq in one of the two duplicate amplicon sets; V, validated in FFPE tumour sample from the same patient (or, in the case of *EGFR* mutation in patient 27, using replicate Sanger sequencing of dilute template) and by digital PCR in plasma DNA; V*, sample failed amplification in the first run owing to a technical fault, but was reanalyzed and the mutation validated in FFPE tumour sample from the same patient and by digital PCR in plasma DNA.

Sample number	Patient ID	Age	Time (months); chemotherapy lines	Vol	DNA (ng)	Gene	Location (build hg19) and base change		Protein change	Mean depth	AF, TAM-Seq	Mean AF, digital PCR	Mutation call
1	15	61	35; 4	70	0.9	<i>TP53</i>	17:7577121	G>A	p.R273C	640	0.26 (0.227, 0.292)	0.167	V
2	3	54	44; 3	160	4.2	<i>TP53</i>	17:7577538	C>T	p.R248Q	340	0.244 (0.245, 0.242)	0.150	V
3	3	54	59; 6	160	5.7	<i>TP53</i>	17:7577538	C>T	p.R248Q	640	0.507 (0.49, 0.524)	0.410	V
4	14	58	62; 5	120	9.9	<i>TP53</i>	17:7578212	G>A	p.R213X	810	0.059 (0.056, 0.062)	0.035	V
5	10	58	48; 3	120	1.4	<i>TP53</i>	17:7578508	C>T	p.C141Y	680	0.021 (0.017, 0.024)	0.013	V
6	10	58	54; 4	120	2.1	<i>TP53</i>	17:7578508	C>T	p.C141Y	720	0.044 (0.044, 0.044)	0.038	V
7	26	72	13; 1	190	17.9	<i>TP53</i>	17:7578265	A>T	p.I195N	800	0.091 (0.081, 0.101)	0.081	V
8	31	64	21; 2	160	14.8	<i>TP53</i>	17:7578406	C>T	p.R175H	510	0.608 (0.614, 0.602)	0.627	V
9	31	64	21; 2	160	10.7	<i>TP53</i>	17:7578406	C>T	p.R175H	550	0.526 (0.529, 0.523)	0.604	V
10	31	64	21; 3	160	6.1	<i>TP53</i>	17:7578406	C>T	p.R175H	530	0.651 (0.705, 0.597)	0.682	V
11	31	64	22; 3	160	4.9	<i>TP53</i>	17:7578406	C>T	p.R175H	490	0.526 (0.548, 0.504)	0.581	V
13	47	54	12; 2	160	2.8	<i>TP53</i>	17:7578527	A>G	p.C135R	480	0.039 (0.034, 0.045)	0.045	V
14	47	54	18; 2	160	2.5	<i>TP53</i>	17:7578527	A>G	p.C135R	610	0.046 (0.047, 0.045)	0.120	V
15	47	54	20; 3	160	3	<i>TP53</i>	17:7578527	A>G	p.C135R	470	0.091 (0.133, 0.05)	0.068	V
16	27	68	25; 3	130	3.7	<i>TP53</i>	17:7578262	C>G	p.R196P	1070	0.088 (0.093, 0.084)	0.135	V
16	27	68	25; 3	130	3.7	<i>EGFR</i>	7:55259437	G>A	p.R832H	614	0.048 (0.055, 0.068, 0.008, 0.062) [†]	0.050	V

17	25	61	18; 2	160	4.2	TP53	17:7578404	A>T	p.C176S	580	0.113 (0.106, 0.12)	0.432	V
18	25	61	24; 3	160	4.4	TP53	17:7578404	A>T	p.C176S	620	0.029 (0.024, 0.033)	0.108	V
20	46	56	48; 6	140	5.2	TP53	17:7578406	C>T	p.R175H	650	0.201 (0.205, 0.197)	0.226	V
21	46	56	49; 6	140	3.6	TP53	17:7578406	C>T	p.R175H	650	0.085 (0.083, 0.087)	0.074	V
22	46	56	50; 6	140	4.1	TP53	17:7578406	C>T	p.R175H	630	0.081 (0.053, 0.109)	0.125	V
23	46	56	51; 6	140	3.7	TP53	17:7578406	C>T	p.R175H	710	0.074 (0.045, 0.103)	0.106	V
24	46	56	52; 6	140	7.1	TP53	17:7578406	C>T	p.R175H	760	0.269 (0.276, 0.261)	0.286	V
25	8	60	12; 1	130	3.9	TP53	17:7577120	C>T	p.R273H	750	0.094 (0.082, 0.106)	0.099	V
26	48	47	20; 2	160	5.7	TP53	17:7577094	G>A	p.R282W	640	0.048 (0.059, 0.036)	0.061	V
27	55	68	11; 2	150	3.6	TP53	17:7578508	C>T	p.C141Y	480	0.321 (0.342, 0.3)	0.364	V
29	50	71	0; 1	150	9.5	TP53	17:7577509	C>T	p.E258K	190	0.548 (0.534, 0.563)	0.253	V
31	51	62	0; 1	160	3.6	TP53	17:7578526	C>T	p.C135Y	620	0.04 (0.058, 0.021)	0.034	V
32	22	65	12; 1	140	2.4	TP53	17:7579521	C>A	p.E56X	1480	0.137 (0.147, 0.127)	0.122	V
33	18	63	48; 3	160	13.2	TP53	17:7578534	C>G	p.K132N	740	0.216 (0.238, 0.194)	0.206	V
34	18	63	49; 4	60	5.3	TP53	17:7578534	C>G	p.K132N	570	0.151 (0.173, 0.13)	0.201	V
36	18	63	56; 4	160	5.8	TP53	17:7578534	C>G	p.K132N	620	0.191 (0.18, 0.202)	0.275	V
37	18	63	57; 5	160	9.4	TP53	17:7578534	C>G	p.K132N	530	0.287 (0.245, 0.328)	0.362	V
38	18	63	58; 5	160	10.1	TP53	17:7578534	C>G	p.K132N	590	0.275 (0.267, 0.283)	0.331	V
39	18	63	59; 5	160	16.4	TP53	17:7578534	C>G	p.K132N	700	0.315 (0.302, 0.328)	0.323	V
40	18	63	60; 5	160	19.7	TP53	17:7578534	C>G	p.K132N	830	0.435 (0.439, 0.43)	0.482	V
41	18	63	61; 6	160	15	TP53	17:7578534	C>G	p.K132N	730	0.452 (0.461, 0.442)	0.445	V
42	18	63	62; 6	160	8.5	TP53	17:7578534	C>G	p.K132N	560	0.185 (0.2, 0.17)	0.245	V
43	56	53	15; 2	150	3.6	TP53	17:7578290	C>T	Splicing	680	0.143 (0.166, 0.12)	0.121	V
30	52	55	49; 4	170	5.2	TP53	17:7577569	A>G	p.C238R	1543	0.071(0.064, 0.077)	0.073	V*
35	18	63	50; 4	60	2.7	TP53	17:7578534	C>G	p.K132N	620	0.015 (0.002, 0.029)	0.073	M
12	53	51	23; 3	160	17	PTEN	10:89720799	T>G	Intergenic	1200	0.013 (0.014, 0.012)	-	FP
37	18	63	57; 5	160	9.4	TP53	17:7579790	G>A	Intronic	380	0.031 (0.031, 0.03)	-	FP

[†]The mutation locus in the *EGFR* gene was measured four separate times, in two overlapping amplicons in each of the two duplicates

Table S7: Mutations and amplicons studied in one breast cancer patient. To identify multiple mutations, genomic libraries from tumour and matched normal tissues were prepared using the standard Illumina paired-end sample preparation kit according to the manufacturer's instructions. DNA fragments of 300 bp were sequenced using paired-end 100-bp reads on an Illumina HiSeq 2000 sequencer to 30× genomic coverage. Sequencing reads were aligned to the hg19 reference genome using ELAND (Illumina) and subsequent data analysis was performed using the CASAVA 1.8 analysis pipeline (Illumina) to identify somatic mutations. We selected 10 mutations and designed an amplicon of <120 bp to cover each mutation. We validated these mutations by TAM-Seq in the tumour material and then followed them in serial plasma samples collected from the same patient.

Gene	Genomic location of amplicon (genome build hg19)	Genomic location of mutation (genome build hg19)	Reference allele	Mutant allele
<i>CD1A</i>	chr1:158226710-158226810	chr1:158226762	C	T
<i>BUB1</i>	chr2:111430288-111430374	chr2:111430343	G	A
<i>IQCA1</i>	chr2:237402413-237402499	chr2:237402439	G	A
<i>PIK3CA</i>	chr3:178936048-178936130	chr3:178936091	G	A
<i>ARAP3</i>	chr5:141041694-141041773	chr5:141041739	G	A
<i>MET</i>	chr7:116398593-116398675	chr7:116398635	C	T
<i>GIMAP5</i>	chr7:150439702-150439805	chr7:150439730	C	T
<i>ZFYVE21</i>	chr14:104195437-104195539	chr14:104195486	G	A
<i>KIAA0406</i>	chr20:36641162-36641252	chr20:36641201	C	T
<i>ADAMTS1</i>	chr21:28214239-28214357	chr21:28214274	C	G

ATTACHMENT 1A

LDR	01903pas a2200553 a 4500
001	9915050863406676
005	20250227140630.0
007	cr
008	090126c20099999dcumr p 0eng c
010	__ a 2009202156
022	0_ a1946-6234l1946-62349PY7(Print)
022	__ a1946-6242y1946-62349EN7(Electronic)
035	__ 9101505086
040	__ aNSDcNSDdNSD
041	0_ aeng
042	__ apccansdpanlmcopyc
044	__ 9United States
060	00 aW1bSC781Q
210	10 aSci Transl Med2DNLM
245	00 aScience translational medicine.
246	13 aSTM
260	__ aWashington, DC :bAmerican Association for the Advancement of Science
310	__ aMonthly
336	__ atextbtxt2rdacontent
337	__ aunmediatedbn2rdamedia
338	__ avolumebnc2rdacarrier
362	1_ aBegan with vol. 1, nos. 1-4 (Oct. 2009).

500	__ a Description based on: Vol. 1, nos. 1-4 (Oct. 2009); title from cover.
500	__ a Latest issue consulted: Vol. 1, nos. 9-12 (Dec. 2009).
500	__ a Print issues ceased with v. 5 (2013).
510	1_ 91a MEDLINE b v1n1, Oct 7 2009-
510	0_ 91a PubMed b v1n1, Oct 7 2009-
515	__ a Online version published on a weekly schedule.
530	__ a Also issued online.
650	12 a Translational Research, Biomedical.
655	_2 a Periodical
710	2_ a American Association for the Advancement of Science.
856	4_ u https://www.science.org/journal/stm
911	__ a Electronic only, print ceased 2013. Confirmed 20141104
914	__ a Portico b Current to 2025 as of 20250227 c ISSN_19466234
935	__ a (DNLM)1505086-nlmdb
992	__ p P2 e F a 20100218
993	__ a SDB b 20100315
994	__ a CDN b 20100315
995	__ a AUTH b 20100315 c REV d 20250227
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998	__ a ERMSLC 117972 b 20180329
999	__ a AUTH
035	__ a (OCoLC)299570656

ATTACHMENT 1B

Translational Research, Biomedical MeSH Descriptor

Data 2025

Details

Qualifiers

MeSH Tree Structures

Concepts

MeSH Heading

Translational Research, Biomedical

Tree Number(s)

H01.770.644.145.675

Unique ID

D057170

RDF Unique Identifier

<http://id.nlm.nih.gov/mesh/D057170>

Annotation

TRANSLATIONAL SCIENCE, BIOMEDICAL is available for translational research as a field or the progress or status of translational research in a particular field; do not add routinely for specific studies involving "translational research"

Scope Note

The endeavour to traverse a specific step of the translation process of turning an observation in the laboratory, clinic, or community into an intervention that will improve the health of individuals and the public. (from Nat Rev Drug Discov. 2018 July ; 17(7): 455–456.)

Entry Term(s)

Translational Medical Research

Translational Research

Translational Research, Medical

Previous Indexing

Biomedical Research (2003-2009)

Research (1996-2002)

See Also

National Center for Advancing Translational Sciences (U.S.)

Public MeSH Note

2022; see TRANSLATIONAL MEDICAL RESEARCH 2012-2021; see TRANSLATIONAL RESEARCH 2010-2011

History Note

2022 (2010)

Date Established

2010/01/01

Date of Entry

2009/07/06

Revision Date

2021/07/07

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A review on the molecular diagnostics of Lynch syndrome: a central role for the pathology laboratory

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Received: September 17, 2009; Accepted: November 10, 2009

- Lynch syndrome
- Identification of patients at risk for Lynch syndrome
- Molecular basis of Lynch syndrome and sporadic MMR-deficient tumours
- Molecular diagnostics of Lynch syndrome
- Description of molecular analyses
- MSI analysis
- Immunohistochemistry
- *MLH1* promoter hypermethylation assay
- *BRAF* mutation analysis
- Limitations of molecular analyses
- Conclusions

Abstract

Lynch syndrome (LS) is caused by mutations in mismatch repair genes and is characterized by a high cumulative risk for the development of mainly colorectal carcinoma and endometrial carcinoma. Early detection of LS is important since surveillance can reduce morbidity and mortality. However, the diagnosis of LS is complicated by the absence of a pre-morbid phenotype and germline mutation analysis is expensive and time consuming. Therefore it is standard practice to precede germline mutation analysis by a molecular diagnostic work-up of tumours, guided by clinical and pathological criteria, to select patients for germline mutation analysis. In this review we address these molecular analyses, the central role for the pathologist in the selection of patients for germline diagnostics of LS, as well as the molecular basis of LS.

Keywords: Lynch syndrome • mismatch repair genes • MSI analysis • immunohistochemistry • *MLH1* promoter methylation assay • *BRAF* mutation analysis • molecular pathology • review

Lynch syndrome

Colorectal cancer (CRC) is the most common malignancy within the European Union and ranks second to lung cancer as a cause of cancer-related mortality [1]. CRC results from both genetic and environmental factors. The most common genetic susceptibility for CRC is Lynch syndrome (LS), formerly known as hereditary non-polyposis colorectal cancer (HNPCC). LS accounts for approximately 3% of all CRCs [2, 3], and also for 2% of all endometrial cancers [4]. The burden of LS is considerably greater than these percentages imply, as the cancers are diagnosed at a young age and synchronous or metachronous malignancies occur in 30% of the patients [5, 6].

LS is characterized by a high lifetime risk for the development of CRC (20–70%), endometrial cancer (15–70%) and other extra-colonic cancers (<15%) [7–14]. These extra-colonic malignancies include carcinomas of the small intestine, stomach, pancreas and biliary tract, ovary, brain, upper urinary tract and skin. LS is caused by germline mutations in mismatch repair (MMR) genes [15], and the definitive diagnosis is currently made by identification of an inactivating germline mutation in one of the MMR genes *MLH1*, *MSH2*, *MSH6* or *PMS2* [16]. Early detection of LS is of great importance, particularly in pre-symptomatic mutation carriers,

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Table 1 Amsterdam criteria I [21]**Families must fulfil all criteria:**

1. There should be at least three relatives with a CRC.
2. One should be a first-degree relative of the other two.
3. At least two successive generations should be affected.
4. At least one should be diagnosed before the age of 50 years.
5. Familial adenomatous polyposis (FAP) should be excluded.
6. Tumours should be verified by pathological examination.

Table 2 Amsterdam criteria II [23]**Families must fulfil all criteria:**

1. There should be at least three relatives with a LS-associated cancer*.
2. One should be a first-degree relative of the other two.
3. At least two successive generations should be affected.
4. At least one should be diagnosed before the age of 50 years.
5. Familial adenomatous polyposis (FAP) should be excluded in the CRC case(s), if any.
6. Tumours should be verified by pathological examination.

*CRC, cancer of the endometrium, small bowel, ureter or renal pelvis.

Table 3 Original Bethesda Guidelines [28]**Individuals meeting any one of the following should undergo MSI testing:**

1. Individuals with cancer in families that meet the Amsterdam criteria.
2. Individuals with two LS-related cancers, including synchronous and metachronous CRCs or associated extracolonic cancers*.
3. Individuals with CRC and a first-degree relative with CRC and/or LS-related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age <45 years, and the adenoma diagnosed at age <40 years.
4. Individuals with CRC or endometrial cancer diagnosed at age <45 years.
5. Individuals with right-sided CRC with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at age <45 years ‡.
6. Individuals with signet-ring-cell-type CRC (more than 50% signet ring cells) diagnosed at age <45 years.
7. Individuals with adenomas diagnosed at age <40 years.

*Endometrial, ovarian, gastric, hepatobiliary or small-bowel cancer or transitional cell carcinoma of the renal pelvis or ureter.

‡Solid/cribriform defined as poorly differentiated or undifferentiated carcinoma composed of irregular, solid sheets of large eosinophilic cells and containing small gland-like spaces.

since colonoscopic surveillance has proven to reduce CRC morbidity and mortality by 65–70% [17–19] and prophylactic surgery may prevent endometrial and ovarium carcinoma effectively [20]. Individuals with a predisposing mutation are candidates for participation in surveillance programs.

The diagnosis of LS is hampered by the absence of specific diagnostic features and the first manifestation in many patients is the presence of an advanced cancer. Furthermore, DNA mutation analysis is time consuming and expensive. For these reasons, DNA analysis is generally preceded by a molecular diagnostic work-up to select patients as candidates for genetic tests. This molecular diagnostic work-up may be guided by several clinical and pathological criteria such as the presence of LS associated malignancies, number of malignancies and age at cancer diagnosis, family history as well as histological tumour features such as mucinous or signet-ring differentiation. In this review, we address the central role for the pathologist in the selection of patients for germline diagnostics of LS, the molecular analyses to identify LS as well as the molecular basis of LS.

Identification of patients at risk for Lynch syndrome

Different models and strategies have been developed to identify patients with LS. In 1990, the Amsterdam Criteria I were developed to provide a basis for uniformity in collaborative studies to find the disease-causing gene (Table 1) [21]. These criteria were designed to be highly specific at the expense of the sensitivity [3, 22]. They were criticized because extra-colonic tumours were not taken into account, thereby excluding classical LS families. Therefore, the Amsterdam Criteria II were established in 1999 (Table 2) [23]. However, many families with the syndrome (*i.e.* mutation carriers) do not meet these criteria [24], usually because these families are too small or there is a late onset of the disease. In addition, obtaining a thorough family history is difficult in clinical practice [25] and patients may have limited knowledge of their family history [26, 27].

In 1997, the Bethesda Guidelines were published to select patients whose tumours should be analysed for molecular features associated with LS, *i.e.* microsatellite instability (MSI), to identify potential mutation carriers (Table 3) [28]. The Bethesda Guidelines have been revised in 2004 to make them more suitable for use in clinical practice, and are not only based on family history, but also on age at cancer diagnosis, number of LS-associated carcinomas and certain histological tumour features (Table 4) [29]. These histological tumour features, associated with LS, include the presence of tumour-infiltrating lymphocytes, a Crohn's-like lymphocytic reaction, mucinous or signet-ring cell differentiation and a medullary or undifferentiated and solid growth pattern. The additional value of these pathology characteristics in the selection of tumours for further testing for LS has been described previously [30, 31]. However, these histological

Table 4 Revised Bethesda Guidelines [29]**Individuals meeting any one of the following should undergo MSI testing:**

1. CRC diagnosed in an individual under age 50 years.
2. Presence of synchronous, metachronous colorectal, or other LS-associated tumours*, regardless of age.
3. CRC with the MSI-H histology[‡], in a patient <60 years of age.
4. CRC in 1 or more first-degree relatives with a LS-related tumour*, with 1 of the cancers being diagnosed under age 50 years.
5. CRC diagnosed in 2 or more first- or second-degree relatives with LS-related tumours*, regardless of age.

*Endometrial, ovarian, gastric, small bowel, pancreas, hepatobiliary tract, renal pelvis or ureter, and brain tumours, sebaceous gland adenomas and keratoacanthomas.

[‡]Presence of tumour-infiltrating lymphocytes, Crohn's like lymphocytic reaction, mucinous or signet-ring differentiation, or medullary growth pattern.

Table 5 Dutch guideline for MSI testing (www.oncoline.nl)**The pathologist is advised to requests MSI testing (and immunohistochemistry of the MMR proteins) in the following patients:**

1. CRC or endometrial carcinoma before the age of 50 years.
2. A second CRC before the age of 70 years.
3. CRC before the age of 70 years AND another synchronous or previous LS-associated tumour*.

*CRC, endometrial, ovarian, gastric, small bowel, pancreas, hepatobiliary tract, renal pelvis or ureter, and brain tumours, sebaceous gland adenomas and keratoacanthomas.

features are related to both microsatellite unstable sporadic tumours as well as LS tumours. Therefore the ability to identify LS patients alone on the basis of these tumour features is limited [32]. In addition, the assessment of these histological tumour features indicating MSI is poorly implemented in daily clinical practice [32].

At present, the most widely accepted recommendation for the identification of patients with LS is based on the combination of these revised Bethesda Guidelines and MSI testing. This combination has proven to be an effective and efficient strategy for LS identification, with a sensitivity for detection of mutation carriers reported from 72% [3] up to 100% [33–36], and a specificity ranging from 77% to 98% [33, 35, 36]. However, these criteria have been criticized because of the use of broad and complex variables, and families with MSH6 and possibly also PMS2 mutations remain undetected [37]. It has also been shown in several studies that these criteria are poorly implemented in clinical practice [32, 38–40].

In 2005, a Dutch group therefore developed a new strategy for the detection of LS [41]. In this strategy the pathologist selects newly diagnosed patients fulfilling one of the following criteria for MSI analysis; (1) CRC before the age of 50 years, (2) Two LS-

associated tumours, including synchronous or metachronous CRCs or LS-associated tumours or (3) adenoma before the age of 40 years. These criteria, known as MIPA criteria, simplify the Bethesda guidelines in such a way that pathologists, without knowledge of family history, can easily apply them. These criteria were found to be effective, efficient and feasible in daily practice [41, 42].

In The Netherlands, the diagnosis of LS is currently based on a nationwide guideline for MSI analysis (Table 5), that was introduced in January 2008 (www.oncoline.nl). This guideline resembles the MIPA criteria. MSI analysis (and immunohistochemistry of the MMR proteins) is requested by the pathologist in patients newly diagnosed with CRC or endometrial carcinoma before the age of 50 years, or patients with two LS-associated tumours (including synchronous and metachronous CRCs or LS-associated tumours) before the age of 70 years. Presence of multiple LS-associated cancers is registered in PALGA, the nationwide network and registry of histopathology and cytopathology in The Netherlands (www.palga.nl). For MSI analysis based on a positive family history, referral to a clinical geneticist is indicated. In those cases MSI analysis will generally be performed when the (revised) Bethesda or Amsterdam Criteria are met and if archival paraffin-embedded tumour tissue can be obtained.

Since clinical criteria do not quantify the likelihood of being a mutation carrier, refined algorithms and multivariable models have been developed to make a quantitative estimation of the risk of carrying a germline MMR-gene mutation, without the requirement of tissue [36]. Several models that combine personal and familial data have been developed, such as the Leiden model, the Edinburgh Model, Premm1,2 and the MMR-pro model [43–46]. One of the advantages of the quantitative models is that the threshold for sensitivity or specificity of the model can be adjusted based upon the clinical situation. However, the role for these models in daily clinical practice remains to be determined.

At present a study (called LIMO and coordinated by the Erasmus MC, Rotterdam, The Netherlands) is performed to determine whether further improvement of LS diagnostics can be obtained by the performance of MSI analysis in CRC patients up to the age of 70 years. MSI analysis is performed in a prospective consecutive series of 1000 newly diagnosed CRC patients ≤70 years, and the results are expected in 2010.

Molecular basis of Lynch syndrome and sporadic MMR-deficient tumours

LS is caused by a germline mutation in one of the MMR genes, most commonly *MLH1* and *MSH2* (±90%) [47, 48], but also *MSH6* and *PMS2* [37, 49, 50]. LS patients are born with a germline mutation in one of these MMR genes, and acquire inactivation of the second wild-type allele in their tumours, fulfilling Knudson's two hit hypothesis for inactivation of tumour suppressor genes [51]. Because of the high chance of inactivation of the

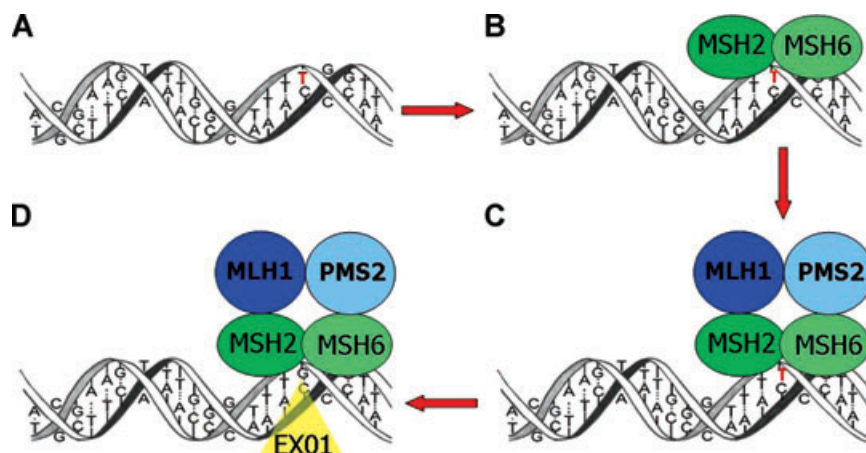


Fig. 1 The MMR system. During DNA replication, insertions or deletions of one or more nucleotides and single nucleotide mismatches may occur. For example: (A) A single nucleotide mismatch occurs (G>T in red). (B) MSH2 and MSH6 form a heterodimer and recognize the mismatch. (C) MLH1 and PMS2 dimerize and bind to the MSH2-MSH6 complex. (D) The complex of four proteins activates an exonuclease to perform the DNA repair.

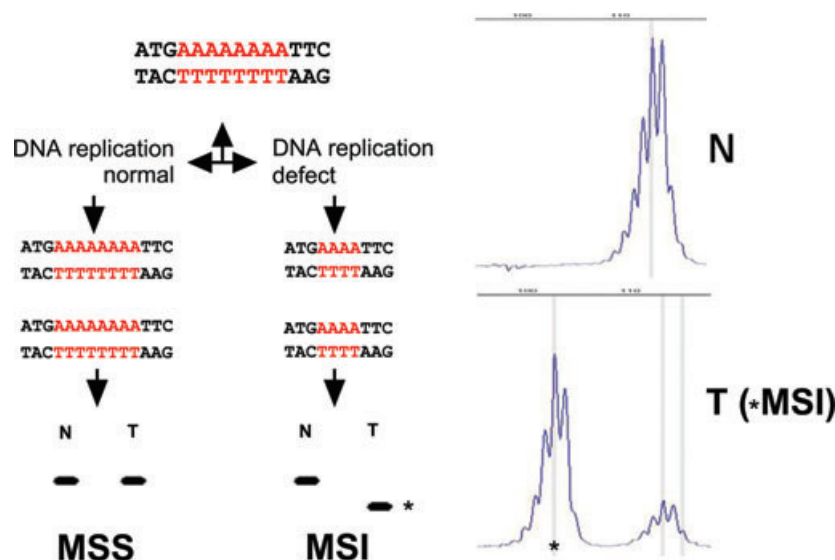


Fig. 2 MSI. A schematic microsatellite is indicated (poly A track). When the tumour cells have an intact MMR system the size of the microsatellite will be the same in DNA isolated from normal (N) and from tumour (T) cells: microsatellite stable (MSS) tumour. In case of a defect in MMR the size of the microsatellite (number of repeat units) can change (in most cases becomes shorter) when comparing N with T DNA: microsatellite unstable (MSI) tumour. Asterisks indicate the microsatellite unstable tumour DNA fragment.

homologous wild-type allele during life, LS transmits phenotypically in an autosomal dominant fashion. The somatic inactivation of the corresponding wild-type allele occurs almost exclusively by small mutations or (partial) gene loss, and bi-allelic inactivation then leads to complete abolition of the protein function. This results in a defective DNA MMR system, since the protein products of the MMR genes are involved in correction of nucleotide base mismatches and small insertions or deletions that arise during DNA replication [52–54].

The mechanism of MMR has been largely elucidated (Fig. 1). MSH2 (mutS homologue 2) forms a heterodimer with MSH6 (mutS homologue 6), sliding along the DNA as a clamp to identify single nucleotide mispairs and small insertions and deletions [55, 56]. MLH1 (mutL homologue 1) dimerizes with PMS2 (post-meiotic segregation 2) and binds to the MSH2-MSH6 complex. Together this group of four proteins recruits an exonuclease to

perform the DNA repair [57, 58]. If any of the four major proteins (MSH2, MLH1, MSH6, or PMS2) is functionally inactive, mismatches are not repaired. A defective DNA MMR system increases the mutation rate and makes the cell vulnerable to mutations in genes controlling cell growth (including tumour suppressor genes and oncogenes), resulting in an elevated cancer risk.

In case of a defective MMR system, mutations occur frequently in small (usually mononucleotide or dinucleotide) repetitive DNA sequences, known as microsatellites [59, 60]. In MMR deficient tumour cells the number of nucleotide repeat units of microsatellites can deviate from the corresponding normal DNA; the number of repeats is usually decreased, but occasionally increased (Fig. 2). This variation in repeat units and thus length or size of microsatellites is called MSI. MSI (formerly referred to as MIN, another abbreviation for MSI, or replication error abbreviated as RER) is the molecular hallmark of LS since approximately 95% of all

LS-associated cancers show MSI [61–63]. MSI thereby serves as a reliable phenotypic marker of MMR deficiency which is easy to evaluate in order to pre-select patients for germline mutation analysis of the MMR genes.

Despite the fact that tumour MSI is a reliable marker for MMR deficiency, it is a marker for LS with limited specificity since 15% of sporadic CRCs also demonstrate a MSI phenotype. This is mainly caused by somatic hypermethylation of the *MLH1*-gene promoter [64, 65]. DNA methylation is an epigenetic DNA modification that specifically targets cytosine residue at CpG dinucleotides. Genomic regions that contain a high frequency of CpG dinucleotides are called CpG islands, present in the promoters of about 40% of all human genes, including the *MLH1*-gene [66]. Hypermethylation of CpG islands in the *MLH1* promoter causes severe inhibition of gene transcription thereby functionally mimicking an inactivating gene mutation. If both copies of the gene are inactivated (mainly by bi-allelic hypermethylation), the DNA MMR function of *MLH1* is lost. This leads to microsatellite unstable cancers, especially in older patients [65]. *MLH1* deficient microsatellite unstable tumours can be assessed for *MLH1* hypermethylation to distinguish sporadic CRCs from LS-related cancers. Theoretically, sporadic hypermethylation of the other MMR genes is possible but has not yet been demonstrated.

Specific activating mutations in the *BRAF* oncogene, usually V600E missense mutations (formerly reported as V599E), can be detected in 40–87% of all sporadic microsatellite unstable tumours. An oncogenic *BRAF* mutation has been described only once [67] in numerous investigated LS tumours [68–75, 76]. These results indicate that *BRAF* mutations are closely correlated with *MLH1* methylation in sporadic CRCs [69–72, 76, 77]. Therefore, *BRAF* mutation status can be used to identify sporadic microsatellite unstable tumours, although it has been demonstrated that determination of hypermethylation of the *MLH1* gene promoter is more sensitive to detect sporadic MSI tumours [69].

In addition to sporadic forms of *MLH1* promoter hypermethylation, germline epimutations of *MLH1* (soma-wide mono-allelic hypermethylation of the gene promoter) have also been reported [78–85]. Germline *MLH1* hypermethylation, often showing some degree of mosaicism, is functionally equivalent to an inactivating mutation and produces a clinical phenotype that resembles LS. Inheritance of epimutations is weak as the methylation can be cleared on passage through the germline (germline *MLH1* promoter epimutations are reversible during meiosis) and so can display non-Mendelian inheritance. Heritability of epimutations might also be explained by the inheritance of an unknown predisposition to epimutations, rather than the inheritance of the epimutation itself [86]. Although very rare, germline *MLH1* promoter methylation should be considered in younger individuals or individuals with multiple LS-associated tumours without a family history who present with an MSI tumour showing loss of *MLH1* expression [81, 82].

Besides germline *MLH1* hypermethylation, a new mechanism of germline *MSH2* hypermethylation has recently been discovered [87]. Ligtenberg *et al.* showed that a germline deletion of the last two exons of *TACSTD1*, the gene just upstream of *MSH2* encod-

ing epithelial cell adhesion molecule (EpCAM), leads to inactivation of the *MSH2* gene by promoter hypermethylation exclusively in tissues expressing EpCAM (mosaic pattern). This mechanism may cause LS in patients with *MSH2*-deficient microsatellite unstable tumours with an undetectable *MSH2* germline mutation. Identification of these cases is possible by the determination of the methylation status of the *MSH2* gene promoter in the tumour and in EpCAM expressing normal tissues (*e.g.* normal colorectal mucosa). In addition, evidence for the presence of *MSH2* methylation can be obtained by detection of deletions in the 3' end of the *TACSTD1* gene.

Molecular diagnostics of Lynch syndrome

The molecular diagnostics of LS usually starts with MSI analysis. MSI analysis is traditionally performed with a panel of five microsatellite markers proposed by a NCI (National Cancer Institute) sponsored consensus conference, also known as the Bethesda panel [29]. With these markers, microsatellites in tumour DNA are compared to microsatellites in corresponding DNA from normal tissue. Tumours with more than one unstable marker (or $\geq 40\%$ of markers) are categorized as having a high degree of MSI (MSI-H), which is suspect for LS or epigenetic *MLH1* silencing [88–91]. Those with one unstable marker (20–40% of markers) are categorized as having a low degree of MSI (MSI-L) and tumours with no instability ($\leq 20\%$) are categorized as being microsatellite stable (MSS), seen in sporadic carcinomas [92]. Although there are no clear differences in clinical or pathological features between MSI-L and MSS tumours, it has been speculated that MSI-L tumours comprise an independent phenotype [93]. However, there is nowadays no role for separating MSI-L from MSS tumours in the diagnostic work-up. Furthermore, MSI testing seems not only important for recognition of LS, but may in the future also improve the clinical management of CRC patients. This is because patients with microsatellite unstable CRCs appear to have a better prognosis than patients with MSS tumours [94–97] and they do not seem to benefit from adjuvant chemotherapy with 5-fluorouracil [98–100].

The Bethesda panel, comprising two mononucleotide repeats (BAT-25 and BAT-26) and three dinucleotide repeats (D2S123, D5S346 and D17S250) [62], does have some limitations, mainly caused by the dinucleotide repeats. These repeats are highly polymorphic and less sensitive and specific in the identification of MSI-H tumours than mononucleotide repeats. Their use in MSI screening requires analysis of corresponding germline DNA [101] and the interpretation of size alterations in dinucleotide repeats is more difficult due to stutter, a PCR artefact. Their use can result in misclassification of MSI-L tumours as MSI-H [102, 103]. Furthermore, *MSH6* mutation carriers may develop tumours (predominantly endometrial cancer) without alteration in these dinucleotide repeats leading to false MSI-L or MSS results [104, 105].

Table 6 Immunohistochemical expression patterns associated with MMR-gene mutations

		MMR-gene mutation			
		<i>MLH1</i>	<i>MSH2</i>	<i>MSH6</i>	<i>PMS2</i>
Protein expression	MLH1	— *	+	+	+
	MSH2	+	—	+	+
	MSH6	+	—	—	+
	PMS2	—	+	+	—

* Absent MLH1 protein expression can be associated with either *MLH1* germline mutations as well as epigenetic *MLH1* silencing by promoter methylation.

+ = present nuclear protein expression in tumour cells (as well as in normal cells).

— = absent nuclear protein expression in tumour cells (and present staining in normal cells, thus serving as internal positive control).

The limitations of the Bethesda panel have led to the development of a pentaplex panel, which comprises five quasi-monomorphic mononucleotide repeats (see below). This panel shows less variation in size among different ethnic populations and has been shown to be superior to the Bethesda panel for the detection of MSI-H tumours [102, 106]. Because the pentaplex analysis is carried out in a single multiplex PCR, this method is simple to use and is free of errors due to mixing samples.

To gain insight into what gene might be affected in patients with MSI-H tumours, MLH1, MSH2, MSH6 and PMS2 protein expression can be assessed by immunohistochemistry. The combination of MSI analysis and MMR protein immunostaining is generally considered as the superior strategy for the identification of suspected LS patients [107]. Absence of MMR protein nuclear staining within the tumour cells can be compared to nuclear staining in the normal cells within the same tumour specimen (and same histological section). The latter then serve as internal positive control.

Due to their heterodimeric nature, different immunohistochemical staining patterns of the MMR proteins can be observed (Table 6). Loss of MLH1 protein due to *MLH1* gene mutation or promoter hypermethylation is usually accompanied with absence of PMS2 in the tumour (Fig. 3). Similarly, absence of MSH2 due to *MSH2* mutations results in absence of MSH6 (Fig. 3), since MSH6 and PMS2 will disintegrate without their obligatory partners MSH2 and MLH1, respectively. A mutation in either *PMS2* or *MSH6* does not lead to loss of MLH1 and MSH2 protein, respectively (Fig. 3), because of the formation of other heterodimers than MLH1-PMS2 and MSH2-MSH6. MLH1 can for instance dimerize with either MLH3 or PMS1 [108, 109] and MSH2 can also bind to MSH3 [110]. Due to the binding of MLH1 and MSH2 to other MMR proteins in the absence of PMS2 or MSH6, there is no concurrent loss of MLH1 and MSH2 [111]. To date no bona fide involvement of

PMS1, *MLH3* or *MSH3* (inactivating mutations) has been demonstrated in LS.

In general, absent MSH2, MSH6 or PMS2 expression in tumour cells with present staining in normal cells is suspect for underlying LS and calls for germline testing. Absent MLH1 (and PMS2) expression can indicate either LS or a sporadic tumour with epigenetically silenced *MLH1* [111]. If epigenetic *MLH1* silencing has been excluded by the analysis of *MLH1* hypermethylation and/or *BRAF* mutation analysis, *MLH1* germline mutation testing is indicated. Furthermore, it might theoretically be possible that immunohistochemical absence of PMS2 or MSH6 without concomitant absence of MLH1 or MSH2, respectively, is due to mutations in *MLH1* or *MSH2*. These mutations then will not lead to decreased MLH1 and MSH2 immunostaining, while binding to and expression of PMS2 and MSH6, respectively, is abrogated. Therefore, absent PMS2 or MSH6 immunostaining without detectable mutations in the *PMS2* or *MSH2* gene, asks for mutation analysis of *MLH1* or *MSH2* respectively.

At our institution, MSI analysis and immunohistochemistry are requested either by the pathologist when patients fulfil the criteria as depicted in Table 5, or by the clinical geneticist (or clinician) when individuals meet the Bethesda Guidelines. The flowchart of the molecular diagnostics of LS in The Netherlands is depicted in Fig. 4. All these different molecular diagnostic procedures will be described in more detail in the next paragraphs.

Description of molecular analyses

MSI analysis

From routine formalin fixed and paraffin embedded (FFPE) tumour tissue specimens 10 to 20 consecutive sections of 4 µm are cut and routinely glued on microscope glass slides. The number of sections is determined by the size of the tissue fragments that need to be isolated for DNA analysis. All sections are deparaffinized and the first and the last section of the series are routine Mayer haematoxylin and eosin stained. These sections are used as reference for the isolated tissue parts. The intermediate sections are stained in haematoxylin and rinsed in distilled water. The indicated tumour and normal tissue fragments are then manually scraped in distilled water from the glass slide and transferred to Eppendorf vials. From the remaining tissue fragments on the glass slides, routine microscopic preparations are made after additional staining with eosin. With these preparations the isolated tissue fragments can be verified (Fig. 5). Occasionally, when large and easy recognizable tissue fragments can be isolated, scraping is performed from paraffin sections on glass slides without deparaffinization. Furthermore, when the tissue fragments to be isolated are too small for manual isolation, laser microdissection is used on haematoxylin and eosin stained sections glued on membrane containing glass slides (PALM Membrane Slides, P.A.L.M. Microlaser Technologies AG, Bernried, Germany) (Fig. 5).

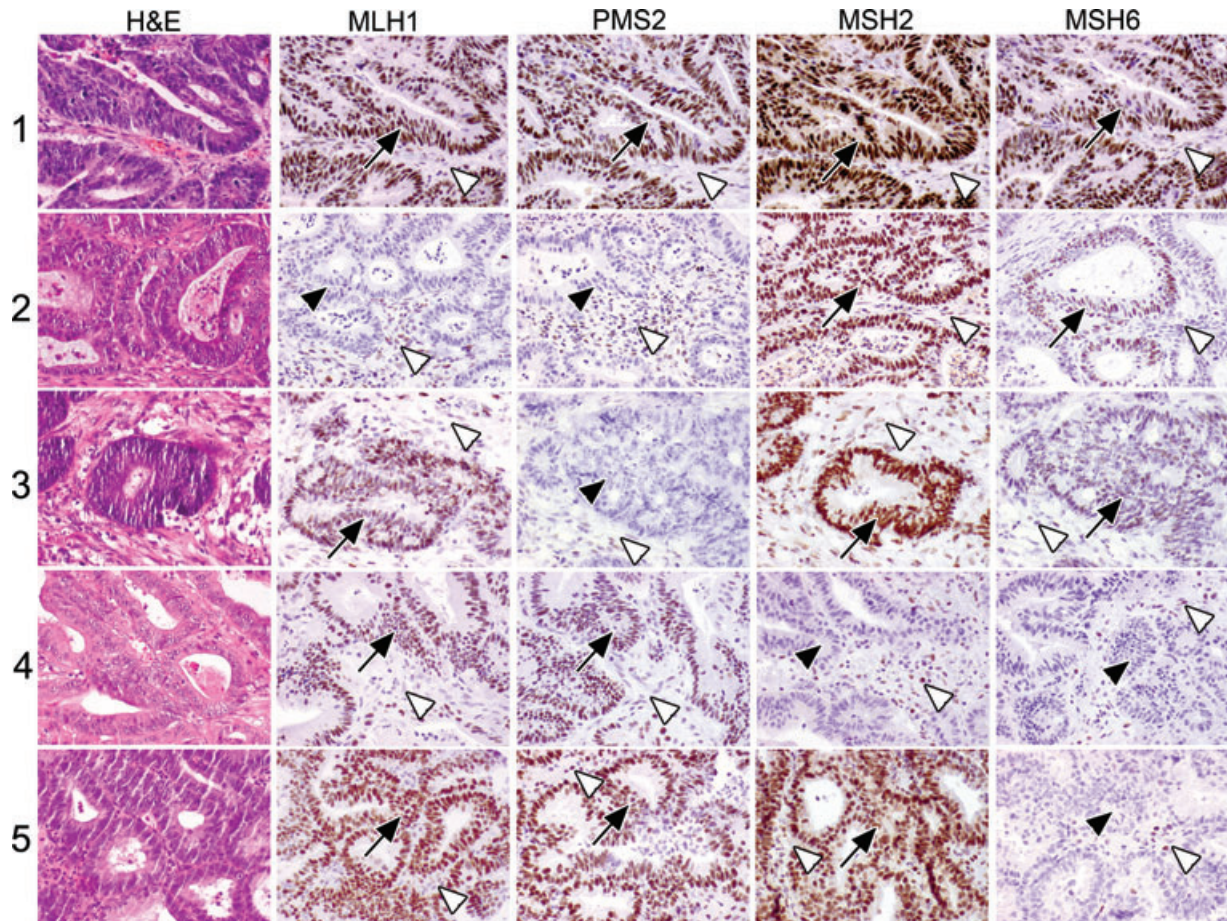


Fig. 3 Five CRC cases; haematoxylin and eosin staining and MLH1, PMS2, MSH2 and MSH6 immunohistochemistry (IHC) results. Case 1: normal IHC in the tumour cells. Case 2: absence of MLH1 and PMS2 in the tumour cells. Case 3: absence of PMS2 in the tumour cells. Case 4: absence of MSH2 and MSH6 in the tumour cells. Case 5: absence of MSH6 in the tumour cells. Arrows point to IHC⁺ tumour cells, filled arrow heads point to IHC⁻ tumour cells and open arrow heads point to IHC⁺ stromal cells.

Although MSI can be reliably detected even when DNA is isolated from a tissue fragment composed of only 10% neoplastic cells (unpublished data), tumour DNA is isolated preferably from a tissue fragment with a high percentage (>70%) of tumour cells. DNA isolated from tissue with a high percentage of tumour cells can also be used for reliable additional investigations (*BRAF* mutation and *MLH1* hypermethylation). In the case of an adenoma the fragment with the highest grade of dysplasia should be used for DNA isolation. For isolation of normal DNA a tissue fragment composed of normal cells, preferably from the normal epithelial counterpart of the tumour (e.g. normal colorectal or normal endometrial mucosa), is used to circumvent heterogeneity problems that can be caused by mosaicism (e.g. mosaic *MLH1* promoter germline hypermethylation or *MSH2* promoter hypermethylation only in Epcam expressing cells). However, since these mosaic phenomena are very rare, other normal tissue fragments (e.g. a tumour-negative lymph node) can be used for normal DNA isolation,

in cases where there is no, or not easy to isolate, normal mucosa available.

From the microdissected FFPE tissue fragments DNA is extracted by addition of 100 to 200 μ l (when very small tissue fragments are used digestion is performed in a volume down to 25 μ l) lysis buffer (10 mM Tris/HCL pH 8.0, 1 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0, 0.01% Tween 20) containing 2 mg/ml proteinase K and 5% Chelex 100 resin. Following overnight incubation at 56°C, proteinase K is inactivated at 100°C for 10 min. Next, dissolved DNA is separated from cell debris by centrifugation at maximum speed in a microcentrifuge for 5 min. The DNA-containing supernatant is carefully pipetted from the Chelex resin-containing pellet (Chelex resin inhibits polymerase activity) and transferred to another Eppendorf vial. In case undeparaffinized sections were used for DNA isolation, the DNA-containing supernatant is collected by carefully poking the pipette tip through the solidified paraffin layer on top of the supernatant.

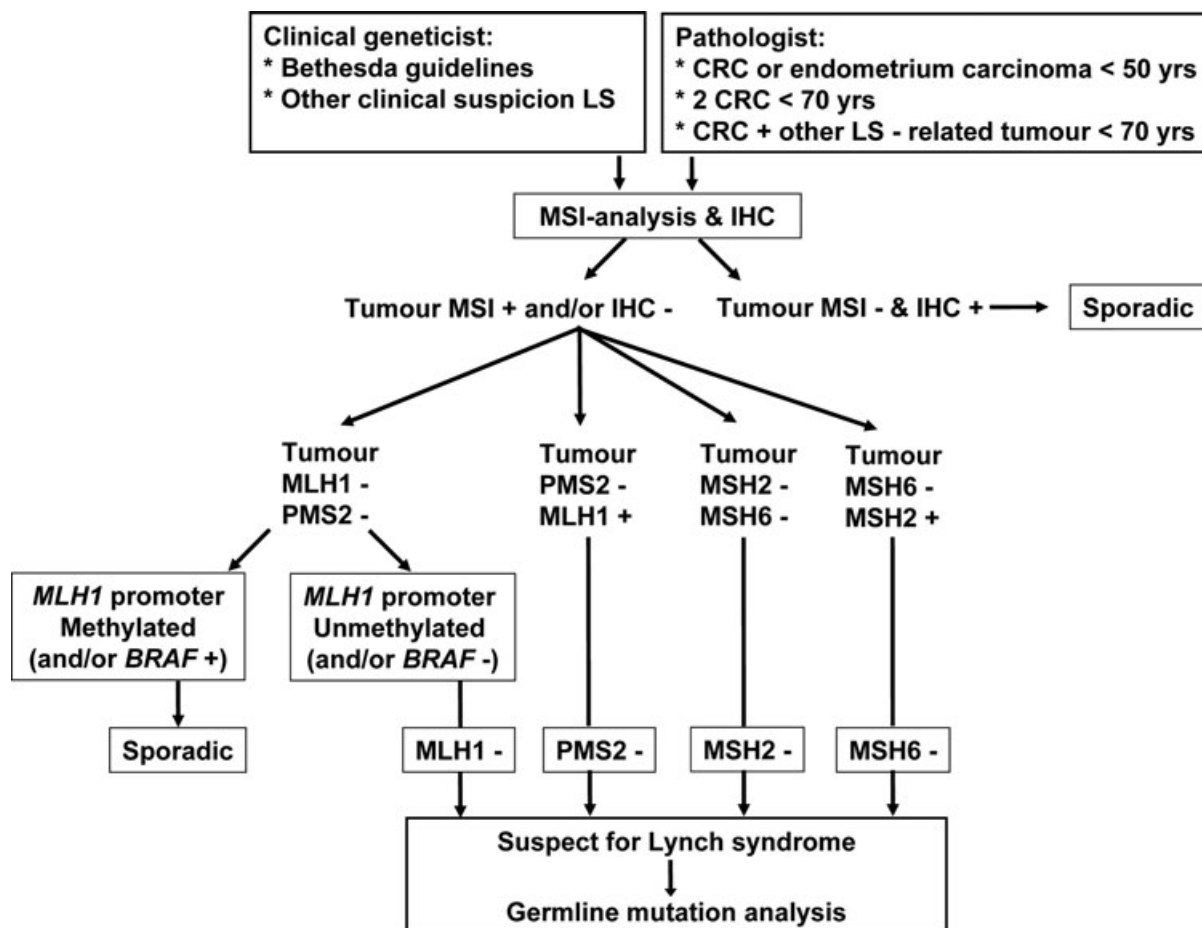


Fig. 4 Flowchart for molecular diagnostics of LS in The Netherlands.

Different methods for MSI analysis are currently available. In our laboratory we use the MSI analysis system of Promega (Promega, Madison, WI, USA) [103]; a fluorescent multiplex PCR-based assay in which the PCR products are separated by capillary electrophoresis using an ABI PRISM 3130xl genetic analyser (Applied Biosystems, Foster City, CA, USA). PCR is performed according to the kit instructions in a total volume of 10 μ l including 2 μ l of an about 80-fold dilution of the isolated DNA solution. The output data are analysed with GeneMarker software (SoftGenetics, State College, PA, USA) to determine MSI status of tumour samples. This system includes fluorescently labelled primers for co-amplification of five quasi-monomorphic mononucleotide repeat markers BAT-25, BAT-26, NR-21, NR-24 and MONO-27. In addition, 2 pentanucleotide markers (Penta C and Penta D) characterized by a high level of polymorphism have been added to provide information on possible sample mix-up or contamination. Because of the low size variation in the population of the selected mononucleotide markers this analysis allows, in most cases, that only tumour DNA is investigated for MSI. DNA from a

MSS cell line suffices as normal DNA reference. If inconclusive results are obtained, for example due to the infrequent occurrence of bi-allelic variation or borderline shifts of the marker peaks, the assay is repeated with both tumour and patient matched normal DNA. Furthermore, additional mononucleotide MSI markers such as BAT-40 can be used in the case of a MSS tumour with a strong clinical suspicion for underlying LS. Results of MSS and microsatellite unstable tumours are shown in Fig. 6.

Immunohistochemistry

Our method of immunohistochemistry was described in detail previously [112]. Briefly, FFPE tissue sections (4 μ m) are dewaxed, and antigen retrieval is performed in 10 mM Tris-EDTA buffer, (pH 9.0) in a microwave oven for 45 min. at 100°C. Primary antibodies anti-MLH1 (Pharmingen BD, Alphen aan den Rijn, The Netherlands; clone G168-728; dilution, 1:20), anti-MSH2 (Pharmingen BD; clone G219-1129; dilution, 1:300), anti-MSH6

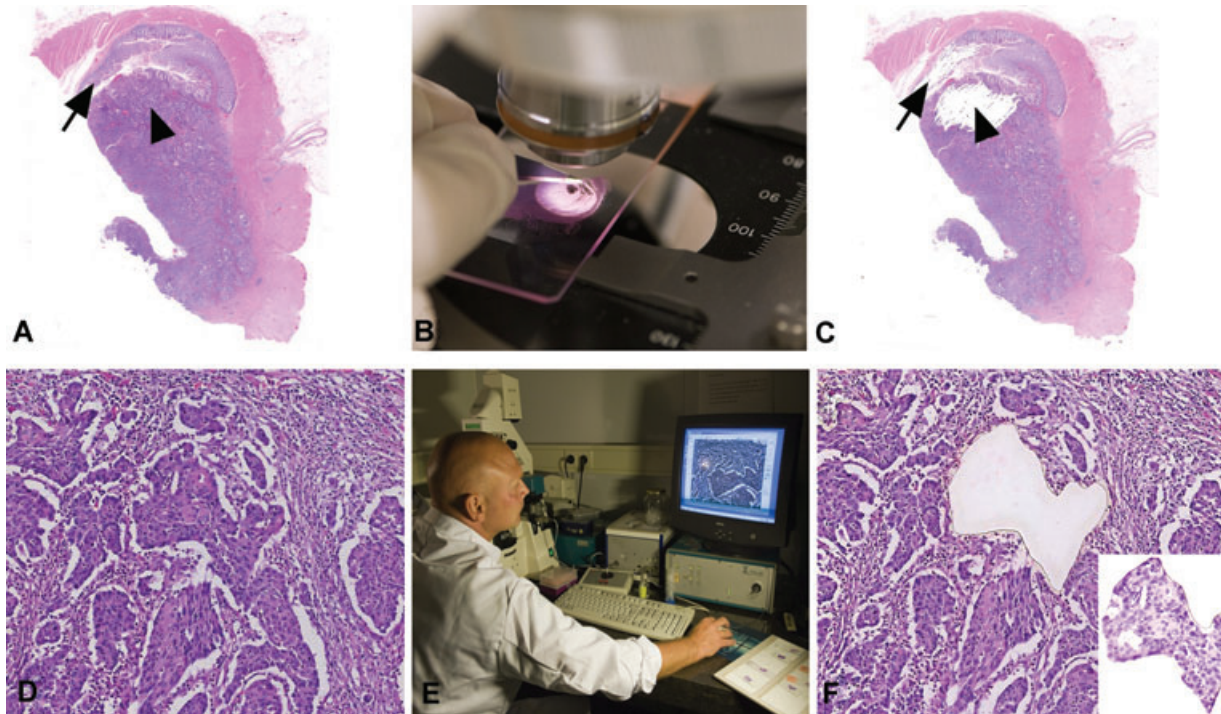


Fig. 5 (A, B, C) Manual macrodissection of a normal tissue fragment (arrow) and a region of the tumour composed of a high percentage of tumour cells (arrow head), before, during and after macrodissection, respectively. (D, E, F) Laser capture microdissection of a small tumour tissue fragment surrounded by abundant stromal cells, before, during and after laser capture microdissection, respectively. Insert in (F) shows the microdissected fragment.

(Pharmingen BD; clone 44; dilution, 1:100) and anti-PMS2 (Pharmingen BD; clone A16-4; dilution, 1:50) are applied for 1 hr at room temperature. After washing, immunoreactivity is visualized with the Envision kit (Dako, Glostrup, Denmark). Subsequently, the sections are counterstained with Mayer haematoxylin and evaluated under a light microscope (Fig. 3).

***MLH1* promoter hypermethylation assay**

In case of absent *MLH1* expression in tumour cells, the methylation status of the *MLH1* promoter can be determined by different methods such as methylation-specific PCR [113] and methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) [114]. MS-MLPA is performed with the SALSA MS-MLPA Kit ME011-A1 for MMR genes (MRC-Holland, Amsterdam, The Netherlands). The analysis is performed according to the kit instructions with 3 μ l undiluted DNA solution as input. The assay takes advantage of methylation-sensitive endonuclease *HhaI*, which only cleaves unmethylated DNA fragments. The MS-MLPA kit contains 8 control probe sequences and 21 methylation-sensitive probes of which 5 recognize CpG dinucleotides within the *MLH1* promoter. The methylation-sensitive probes contain a restriction site for *HhaI*. Comparison of a *HhaI*-digested DNA sample (yielding only signal of methylated DNA) to its undigested

counterpart (yielding signal of both methylated and unmethylated DNA) provides insight into the degree of methylation. Details of the MS-MLPA protocol are freely available on the website of the manufacturer (<http://www.mrc-holland.com>).

Basically, tumour DNA is hybridized to the probe mix. After hybridization, half of the sample is subjected to a ligation step joining both adjacently hybridized fragments of a probe set, whereas the other half of the sample is subjected to both ligation and *HhaI* digestion, leaving only methylated sequences intact. Subsequent PCR amplification exponentially amplifies all ligated, but undigested, probes. The signal generated with the part of the sample that has undergone both ligation and digestion represents the amount of methylated DNA present in the tumour. For fragment analysis, PCR products are separated by capillary gel electrophoresis using an ABI PRISM 3130xl genetic analyser (Applied Biosystems) and quantified with GeneMarker software version 1.7 (SoftGenetics). The MS-MLPA results are normalized by dividing the peak height of each *MLH1* probe signal by the mean peak height of the eight control fragments obtained with the same sample (Fig. 7). The degree of methylation for individual *MLH1* probes can be assessed by dividing normalized values of each *MLH1* probe within digested DNA samples by normalized values of the probe in corresponding undigested samples. The MS-MLPA assay is performed with both tumour and normal mucosal DNA to detect possible germline *MLH1* promoter hypermethylation.

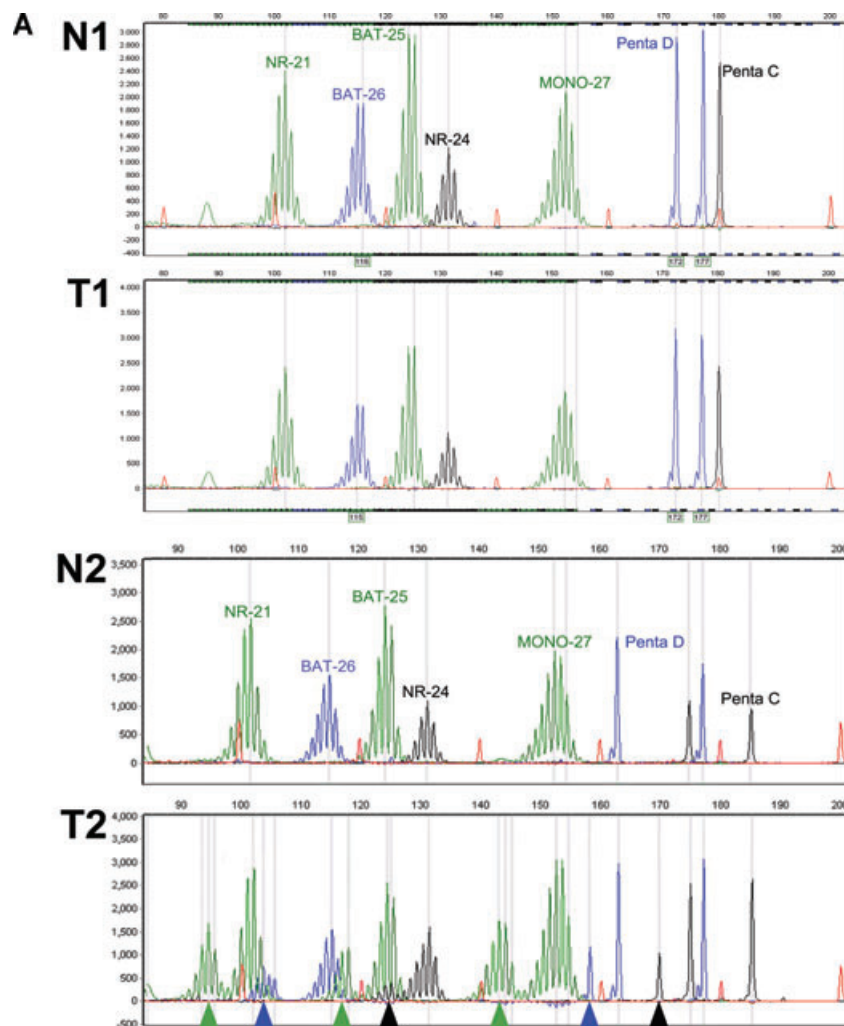


Fig. 6 MSI analysis of four cases of paired normal (N) and tumour (T) DNA. **(A)** Case 1: N1 and T1, CRC no MSI: MSS. Case 2: N2 and T2, CRC demonstrates clear MSI. **(B)** Case 3: N3 and T3, endometrial carcinoma with MSI (subtle microsatellite shifts). Case 4: N4 and T4, CRC without MSI but with heterozygous NR-21 microsatellite alleles (present in normal and tumour DNA). Arrow heads indicate the MSI shifts, arrows indicate the variant NR-21 allele.

BRAF mutation analysis

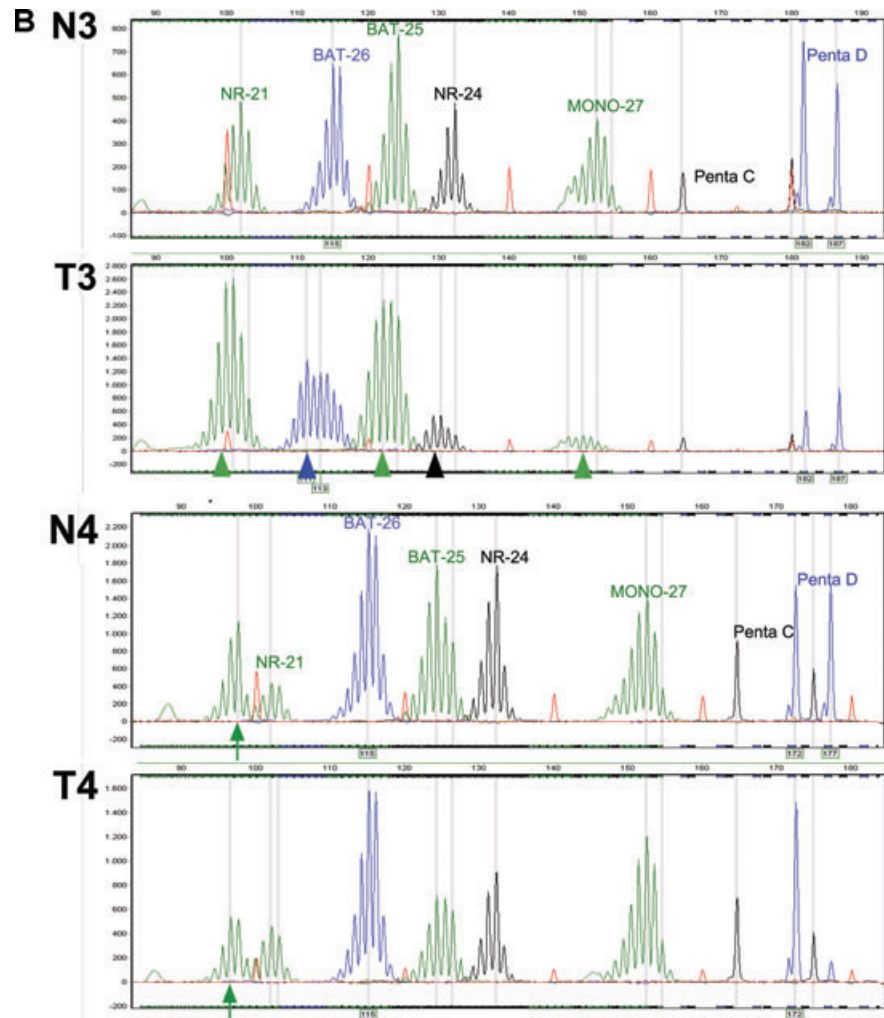
BRAF alterations of mutational hotspot codon V600 are determined by bi-directional cycle sequencing of PCR-amplified fragments. PCR amplification is performed by M13-tailed forward primer 5'-TGT AAA ACG ACG GCC AGT AAA CTC TTC ATA ATG CTT GCT CTG -3' and M13-tailed reverse primer 5'-CAG GAA ACA GCT ATG ACC GGC CAA AAA TTT AAT CAG TGG AA-3'. PCR products are generated in a 15 µl reaction mixture including 1.0 µl undiluted DNA solution, 10 µmol of each primer, 25 mM MgCl₂, 10 mM dNTPs and 1 U Taq polymerase (Promega). The PCR reaction is performed with a thermocycler (Biometra, Göttingen, Germany) with an initial denaturing step (95°C) for 3 min., followed by 35 cycles consisting of denaturation (95°C) for 30 sec., annealing (60°C) for 45 sec. and extension (72°C) for 45 sec. After the final cycle, an extension period of 10 min. at 72°C is performed. The PCR products are sequenced with M13 forward

primer 5'-TGT AAA ACG ACG GCC AGT-3' and M13 reverse primer 5'-CAG GAA ACA GCT ATG ACC-3' using the ABI PRISM BigDye Terminator v3.1 kit (Applied Biosystems). Sequence analyses are performed on an ABI PRISM 3130xl genetic analyser (Applied Biosystems). Samples are analysed using Mutation Surveyor software (SoftGenetics) and are compared with the public sequence of GenBank (NT_007914). Examples of *BRAF* mutation analysis results are shown in Fig. 8.

Limitations of molecular analyses

Over the last decade, the diagnostics of LS have improved considerably. Nevertheless, there still remain some limitations that need to be addressed. It has to be taken into account that the described procedures provide information on the chance that a

Fig. 6 Continued



certain tumour arose in the context of LS and are not diagnostic for LS in an absolute sense. The false negative rate of MSI analysis is very low (<5%) but cannot be completely ruled out. MSI can be very subtle or escape detection particularly in low grade lesions as adenomas, in endometrial carcinomas (Fig. 6B, panel N3/T3) and in samples with a low percentage of neoplastic cells [115]. These false-negative results may lead to the exclusion of LS patients (and affected family members) from necessary surveillance programs and subsequent failure to detect (secondary) cancers in an early stage. In addition, although rare, sporadic MSS tumours can occur in LS patients and MSI analysis then fails to indicate LS. To exclude false-negative MSI results as much as possible it is necessary to isolate DNA from a tissue fragment with a high percentage of tumour cells. For this, laser microdissection might be preferable instead of manual microdissection. However, laser microdissection is a time consuming and labour-intensive procedure to obtain sufficient

tissue fragments for DNA isolation [116]. In general, it is recommended to refer patients with a high clinical or familial suspicion of LS to a clinical genetics department, irrespective of the MSI status. In addition, other hereditary CRC syndromes such as attenuated familial adenomatous polyposis, MYH-associated polyposis, Cowden syndrome or Peutz-Jeghers syndrome might need to be excluded.

Although the assessment of MMR-protein expression by immunohistochemistry is a fast and simple procedure, the interpretation of the results can be difficult. Interpretation may be impeded by absence or low intensity of the nuclear staining in tumour and normal tissue due to fixation artefacts, especially in old archival specimens [117]. In case of missense mutations, the inactive protein may be (partly) expressed and detectable by immunohistochemistry. The interpretation is also hampered by some degree of observer-variation and the value of immunohistochemistry partially depends on the experience of the pathologist

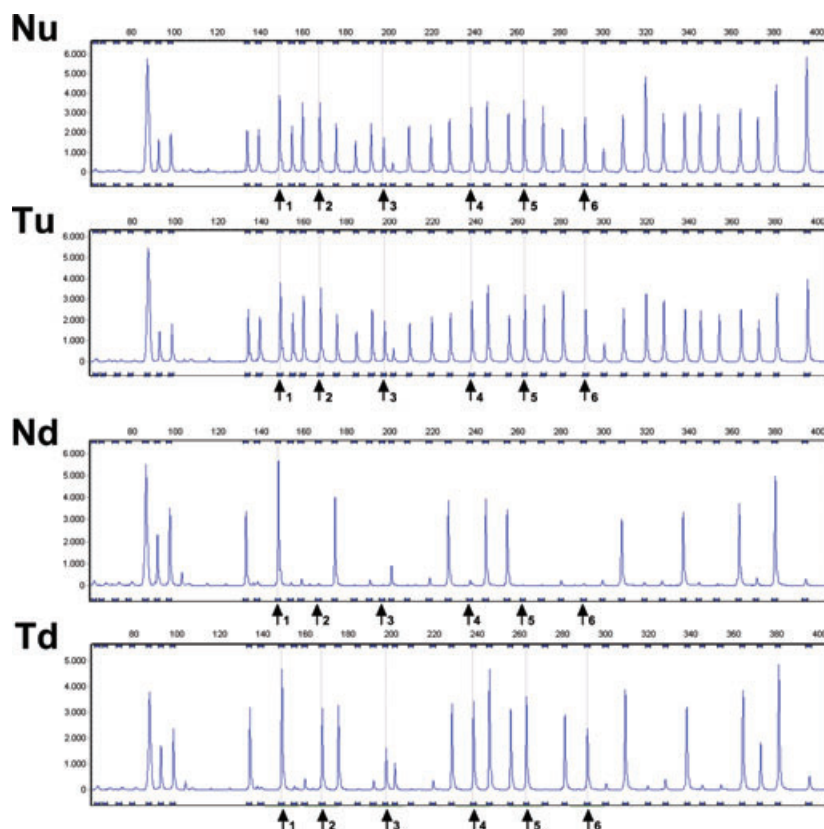


Fig. 7 *MLH1* promoter hypermethylation assay; MS-MLPA analysis of a MSI CRC with absence of *MLH1* and *PMS2* expression. Results are shown of paired undigested normal (Nu) and undigested tumour (Tu) DNA and of the methylation-sensitive endonuclease *HhaI* digested normal (Nd) and tumour (Td) DNA. Arrows indicate six *MLH1* promoter probes, nr 1 representative for a fragment without and nrs 2–6 representative for fragments with a *HhaI* restriction site. In the *HhaI* digested DNA, probes 2–6 are clearly present in the tumour DNA (Td) and not in the paired normal DNA (Nd) indicating tumour-specific methylation of the *MLH1* promoter fragments 2–6.

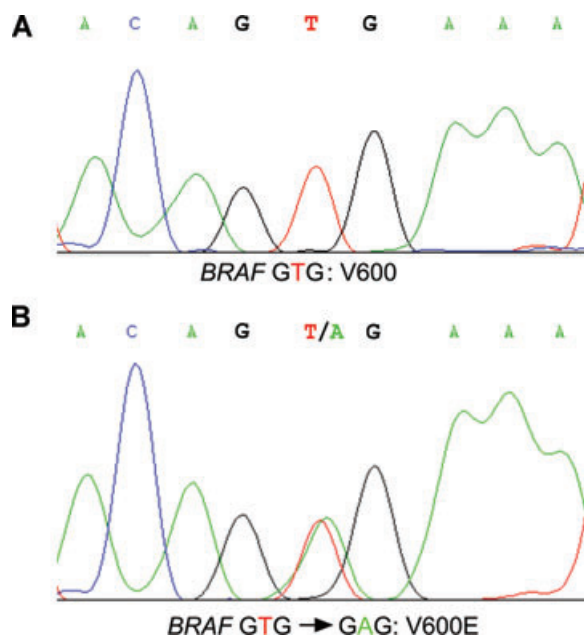


Fig. 8 Mutation analysis of codon 600 of the *BRAF* gene. (A) Tumour DNA without *BRAF* codon 600 mutation. (B) Tumour DNA with a heterozygous oncogenic mutation GTG-GAG, leading to a V600E amino acid substitution.

[118, 119]. For these reasons immunohistochemistry cannot replace MSI testing to detect LS, and this underlines the importance of the combined application of MSI analysis and MMR protein immunostaining to detect LS.

In the evaluation of *MLH1* promoter methylation, it is important to study the correct promoter regions since *MLH1* expression only correlates with methylation of the proximal promoter regions (mainly region C, but also region D) [113, 120, 121]. Nevertheless, there are still studies published in which the distal *MLH1* promoter regions were analysed, which are not or only poorly associated with gene silencing. Moreover, epigenetic inactivation of the second normal *MLH1* allele by promoter methylation (second hit), may also play a role in individuals with LS [122, 123], and it should be realized that the detection of *MLH1* promoter methylation can not completely rule out LS. In the case of a strong clinical suspicion, referral to a clinical geneticist is indicated. The exact frequencies of *MLH1* promoter methylation in LS patients (either as a second inactivating event, or as a heritable germline epimutation), are unknown. It has been reported that in tumours from *MLH1* mutation carriers, the wild-type allele is hypermethylated in 0–46% of the tumours [65, 76, 80, 122, 124–129]. However, only one study evaluated the proximal promoter region (region D) associated with gene silencing in 55 CRCs and endometrial cancers of *MLH1* germline mutation carriers [129]. Hypermethylation was seen in 7.3% of all tumours (16% of CRCs). In the other

studies, promoter regions not associated with *MLH1* silencing were investigated (*i.e.* the distal promoter regions) [65, 76, 80, 122, 124–128].

There are some other points of concern in the molecular diagnostics of LS. First, the value of MSI testing and immunohistochemistry in other LS-related tumours than CRC is largely unknown [130]. In endometrial tumours, the second most common malignancy in LS, MSI can escape detection by the occurrence of only subtle shifts in the size of the markers [131]. Therefore, MSI analysis in endometrial cancers is performed with patient matched normal DNA as the reference, and molecular pre-screening has been found feasible [4, 132, 133] (Fig. 6B, panel N3/T3). Furthermore, the quality of DNA extracted from FFPE tumours can occasionally be poor and therefore not suitable for MSI analysis [130]. And last but not least, some individuals might have ethical objections against MSI testing or immunohistochemistry, since the diagnosis of LS can be very likely after the described molecular examinations, which might have negative social consequences and raise concerns *e.g.* about insurance risks. Therefore, we believe that the clinician should inform the patient about the fact that the pathological examinations may not only give information about the nature of the tumour, but may also indicate an elevated risk of an underlying hereditary disorder.

Conclusions

Different diagnostic strategies have been developed for LS as discussed in this review and the optimal method for the identification of LS patients is still debated and in flux. In the previous paragraphs the molecular diagnostic approach of LS in The Netherlands (Erasmus MC, University Medical Center, Rotterdam) has been described (Fig. 4). This approach combines MSI analysis and MMR protein immunostaining and is in our opinion a productive way of pre-selecting patients for germline mutation analysis, with a central role for the pathologist. Nevertheless, if the clinical suspicion for LS is very high, *e.g.* because of a positive family history for LS-associated cancers or a LS-associated malignancy diagnosed at a very young age, referral to a clinical geneticist is strongly recommended, even in the case of tumours without MMR deficiency (*i.e.* MSS).

Acknowledgements

The currently ongoing LIMO study is supported by an Erasmus MC-grant. We thank Frank van der Panne for photographic work.

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ATTACHMENT 2A

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999	___ a AUTH
035	___ a (OCoLC)46362607

ATTACHMENT 2B

Cell Biology MeSH Descriptor Data 2025

[Details](#)[Qualifiers](#)[MeSH Tree Structures](#)[Concepts](#)**MeSH Heading**

Cell Biology

Tree Number(s)

H01.158.100.433

H01.158.273.160

Unique ID

D003585

RDF Unique Identifier

<http://id.nlm.nih.gov/mesh/D003585>

Annotation

the discipline only (education, history, etc); entry term CYTOLOGY: for cytological structures use the qualifier /cytol with specific anatomical term or organism

Scope Note

The study of the structure, behavior, growth, reproduction, and pathology of cells; and the function and chemistry of cellular components.

Entry Term(s)

Cellular Biology

NLM Classification #

QU 21

See Also

Cells

Public MeSH Note

2010; see CYTOLOGY 1984-2009

History Note

2010 (1984)

Entry Combination

methods:Cytological Techniques

Date Established

2010/01/01

Date of Entry

1999/01/01

Revision Date

2022/07/14

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ATTACHMENT 2C

Medicine MeSH Descriptor Data 2025

[Details](#)[Qualifiers](#)[MeSH Tree Structures](#)[Concepts](#)**MeSH Heading**

Medicine

Tree Number(s)

H02.403

Unique ID

D008511

RDF Unique Identifier

<http://id.nlm.nih.gov/mesh/D008511>

Annotation

medicine only as a field, profession or discipline: differentiate from DELIVERY OF HEALTH CARE where the patient is emphasized; very general; it is divided broadly into experimental medicine (= BIOMEDICAL RESEARCH) & CLINICAL MEDICINE, a specialty devoted to the diagnosis & management of human patients; / legislation & jurisprudence = LEGISLATION, MEDICAL or JURISPRUDENCE or FORENSIC MEDICINE; LEGAL MEDICINE see FORENSIC MEDICINE is also available

Scope Note

The art and science of studying, performing research on, preventing, diagnosing, and treating disease, as well as the maintenance of health.

Entry Term(s)

Insurance Medicine
Medical Specialities
Medical Specialties
Medical Specialty
Specialities, Medical
Specialties, Medical
Specialty, Medical

See Also

Economics, Medical
Education, Medical
History of Medicine
Insurance, Health
Legislation, Medical

Public MeSH Note

1963; for SPECIALTIES, MEDICAL see SPECIALTIES, MEDICAL 1968-2009

History Note

1963

Entry Combination

economics:Economics, Medical
education:Education, Medical
ethics:Ethics, Medical
history:History of Medicine
legislation & jurisprudence:Legislation, Medical

Date Established

1963/01/01

Date of Entry

1999/01/01

Revision Date

2021/07/06

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ATTACHMENT 2D

Molecular Biology MeSH Descriptor Data 2025

[Details](#)[Qualifiers](#)[MeSH Tree Structures](#)[Concepts](#)**MeSH Heading**

Molecular Biology

Tree Number(s)

H01.158.201.636

H01.158.273.343.595

H01.181.122.650

Unique ID

D008967

RDF Unique Identifier

<http://id.nlm.nih.gov/mesh/D008967>

Annotation

use for the discipline only (education, history, etc)

Scope Note

A discipline concerned with studying biological phenomena in terms of the chemical and physical interactions of molecules.

Entry Version

MOL BIOL

Entry Term(s)

Biochemical Genetics

Biology, Molecular

Genetics, Biochemical

Genetics, Molecular

Molecular Genetics

Previous Indexing

Biology (1966-1967)

See Also

Biochemical Phenomena

Genetic Phenomena

Genetic Structures

Public MeSH Note

70

History Note

70(68)

Date Established

1970/01/01

Date of Entry

1999/01/01

Revision Date

2016/06/17

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Microsatellite Instability in Colorectal Cancer



C. Richard Boland



Ajay Goel

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Microsatellite instability (MSI) is a hypermutable phenotype caused by the loss of DNA mismatch repair activity. MSI is detected in about 15% of all colorectal cancers; 3% are of these are associated with Lynch syndrome and the other 12% are caused by sporadic, acquired hypermethylation of the promoter of the *MLH1* gene, which occurs in tumors with the CpG island methylator phenotype. Colorectal tumors with MSI have distinctive features, including a tendency to arise in the proximal colon, lymphocytic infiltrate, and a poorly differentiated, mucinous or signet ring appearance. They have a slightly better prognosis than colorectal tumors without MSI and do not have the same response to chemotherapeutics. Discovery of MSI in colorectal tumors has increased awareness of the diversity of colorectal cancers and implications for specialized management of patients.

Keywords: Lynch Syndrome; DNA Mismatch Repair; Hereditary Nonpolyposis Colorectal Cancer.

Cancers can be characterized by patterns of changes in gene expression. Genes that mediate tumorigenesis can be broadly characterized as oncogenes, which are activated by alterations; and tumor suppressor genes, which are inactivated during tumorigenesis. Oncogenes can encode growth factors or their receptors, signaling molecules, regulators of the cell cycle, and other factors that regulate cell proliferation and survival. Their oncogenicity can be induced by mutations that lead to overactive gene products, amplifications that alter copy number, alterations or rearrangements that affect promoter function, or modified interactions with regulators of transcription or epigenetic modification. Tumor suppressors restrain growth and proliferation, passage through the cell cycle, motility, invasion, or other functions related to stable differentiation. Genes that encode tumor suppressors are commonly inactivated by deletion, mutations, promoter methylation, or other changes in reg-

ulation. Colorectal cancers (CRCs) develop gradually over a long period of time through the sequential accumulation of genetic alterations that overcome the redundant control mechanisms built into each cell.¹ Only a few mutations are common to most colorectal tumors, but each tumor has a unique combination of genetic alterations.² It is possible that no 2 CRCs are alike, a daunting consideration for the rational planning of treatment.

Models of CRC Pathogenesis

It was not immediately obvious that CRCs would be so diverse genetically. The initial attempt to characterize multistep carcinogenesis resulted in a novel conceptual model in which specific genetic alterations were associated with the sequential evolution of the neoplastic phenotype in the colon. In the model proposed by Fearon and Vogelstein, inactivation of the *adenomatous polyposis coli* (*APC*) gene led to the initial appearance of the adenoma from normal colonic mucosa, mutations in *KRAS* were associated with growth of the adenoma, genetic alterations (mainly deletions) to genes on chromosome 18q resulted in adenoma growth and progression, and biallelic loss or inactivation of *p53* mediated the adenoma-to-carcinoma transition.³ Chromosomal instability and loss of heterozygosity (LOH) are important steps in this process, and there is a relationship between the degree of LOH and tumor progression.^{4,5} Although this model,

Abbreviations used in this paper: *APC*, *adenomatous polyposis coli* gene; CIMP, CpG island methylator phenotype; CRC, colorectal cancer; EMAST, elevated microsatellite alterations at selected tetranucleotide repeats; 5-FU, 5-fluorouracil; HNPCC, hereditary nonpolyposis colorectal cancer; IDL, insertion-deletion loop; LOH, loss of heterozygosity; *MLH*, Mut L homologue; MMR, mismatch repair; *MSH*, Mut S homologue; MSI, microsatellite instability; PCR, polymerase chain reaction; PMS1, post-meiotic segregation-1; TGF- β , transforming growth factor- β ; TGF β R2, TGF- β type II receptor.

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0016-5085/10/\$36.00
doi:10.1053/j.gastro.2009.12.064

proposed in 1990, has been useful, only a few CRCs actually evolve along this pathway.^{2,6}

Fearon and Vogelstein³ were careful to characterize the genetic changes as “alterations” and did not presume to have determined the mechanisms involved. Some of the alterations were point mutations (as occurs in *KRAS*), whereas many were deletions of relatively large pieces of chromosome (such as on 18q, in which an arm or whole chromosome can be deleted). Point mutations can occur through a number of mechanisms, such as chemical carcinogenesis or unrepaired “natural” degradation of DNA. A diet-induced increase in proliferation can create replicative errors in DNA that exceed the ability of the cell to repair them. The origin of genetic deletions and LOH is complex and is addressed in the accompanying review of chromosomal instability.

A controversy emerged between investigators who thought that some type of genomic instability was required to produce all the mutations that are present in colorectal tumors vs investigators who reasoned that cells continuously generate a small number genetic alterations, and that mutations that provide proliferative or survival advantages are selected for during clonal expansion. It is not clear which model occurs during CRC pathogenesis, but at least 1 type of colorectal tumor is caused by the hypermutable phenotype known as microsatellite instability (MSI).

MSI as a Unique Mechanism in Tumor Development

Shortly after publication of the multistep genetic model of colorectal carcinogenesis, many investigators began searching the genome for novel tumor suppressor genes using powerful techniques. In 1992, Manuel Perucho used an arbitrarily primed polymerase chain reaction (PCR), extracting DNA from colonic tissues and amplifying thousands of sequences using a small number of randomly chosen PCR primers. This technique yielded unique but reproducible electrophoretic signatures from each sample.⁷ Perucho's group used arbitrarily primed PCR to amplify matched samples of DNA from colorectal tumors and adjacent normal colonic tissue, separated the PCR products electrophoretically, and compared the results to identify differences in PCR amplicons between normal and tumor tissues—DNA missing from the tumor would presumably represent somatic cell deletions that were candidate regions for new tumor suppressor genes.

Deleted DNA bands were found in most colorectal tumor samples.⁸ However, upon careful analysis of the comparative autoradiograms, the authors noted that 12% of ~130 tumors had bands that were not actually deleted, but were shorter in length and migrated slightly further down the gel. They analyzed the sequences in these bands and discovered that they contained simple repetitive sequences (ie, microsatellites), principally in polyadenine (A_n) tracts associated with *Alu* sequences.

The DNA obtained from this group of tumors had undergone somatic deletion of ≥ 1 of the adenine residues. The method was unbiased and quantitative, permitting the estimate that each tumor contained $\sim 10^5$ such mutations. The tumors with these “ubiquitous somatic mutations at simple repeated sequences” had unique clinical and pathological characteristics. First, the tumors with these deletion mutations were significantly more likely to arise in the proximal colon, were less likely to be invasive, were less likely to have mutations in *KRAS* or *p53*, were more likely to appear poorly differentiated, and came from younger patients. Based upon these findings, the authors concluded that these deletions represented a unique pathway to tumor development and predicted that the “catastrophic loss of fidelity in the replication machinery of normal cells” that caused them might be hereditary, although they had no evidence for this.⁸

Simultaneously, Stephen Thibodeau's laboratory was studying dinucleotide repeat sequences (a type of microsatellite) that were useful as tools for genetic mapping and analysis of LOH, and looking for novel tumor suppressor genes on chromosomes 5q, 15q, 17p, and 18q in colorectal tumors. They observed deletion mutations in the $[CA]_n$ sequences (called “C A repeats”) in these regions and coined the term *microsatellite instability* (which they termed *MIN*). They detected MSI in 25 of 90 CRCs (28%), and noted that these aberrations were heterogeneous in different tumors. They referred to a large deletion or expansion within the $[CA]_n$ tract as a “type I mutation,” and called a single 2-base-pair repeat change a minor or “type II mutation.” The implications of type I vs II mutations have never been elucidated, but they might reflect the cumulative nature of mutations in dinucleotide repeat sequences as they occur over time.⁹ Importantly, they found that 89% of tumors with MSI were in the proximal colon and that the patients with MSI CRCs had a better prognosis; like Perucho, Thibodeau et al recognized that this represented a unique pathway for tumor development that “does not involve loss of heterozygosity.”¹⁰

Studies from an international consortium led by Bert Vogelstein in the United States and Albert de la Chapelle in Finland helped elucidate the clinical implications of MSI, using microsatellites (mainly, but not exclusively dinucleotide repeats) as tools for genome-wide linkage analysis of hereditary CRC. Aaltonen et al found significant linkage at chromosome 2p using the marker D2S123 in 2 large kindreds with Lynch syndrome (then called hereditary nonpolyposis colorectal cancer [HNPCC]).¹¹ The authors used D2S123 to look for LOH at that locus in the CRC DNA, testing the hypothesis that this was the site of the tumor suppressor gene that caused hereditary CRC and that the second hit to the gene would be LOH. Instead, they observed MSI with the marker and then noted that deletion mutations in microsatellite sequences were widespread in hereditary CRCs.

They referred to this as the replicative error phenotype. More importantly, they found MSI in 13% of sporadic CRC cases and correctly reasoned that the hereditary tumors and a subset of sporadic tumors shared a unique but common pathway of tumor development.¹²

It became apparent that a subset of colorectal tumors (at least 12% and perhaps as much as 28%) were characterized by a large number of mutations at microsatellite sequences, formed in the proximal colon, had unique clinical features (such as a better prognosis than other types of CRC), and were hereditary. MSI was the first DNA marker available to identify hereditary CRC, although none of the investigators who discovered MSI in CRC understood mechanisms by which it developed.

A series of investigations led to the realization that MSI arises from defects in the DNA mismatch repair (MMR) system and the identification of the 4 genes that cause Lynch syndrome. Interestingly, microbial geneticists predicted that defects in the MMR system were probably responsible for MSI. The timeframe of these discoveries are illustrated in Figure 1.

DNA MMR System

The “mutator phenotype” was discovered in bacteria in the 1970s and 1980s and was well-characterized by the beginning of the 1990s.¹³ It is caused by mutational inactivation of genes involved in DNA repair. The first human disease that was clearly associated with defects in DNA repair was xeroderma pigmentosa, a rare autosomal recessive disease caused by biallelic inactivating mutations in genes involved in nucleotide excision repair. Several other DNA repair systems had been characterized that included MMR, base excision repair, and a variety of nucleases and other DNA excision repair enzymes, yet no human diseases were associated with these.

In prokaryotes, the MMR system consists of a family of enzymes that detect S-phase DNA replication errors (ie,

those that result in mismatches between the 2 strands of DNA) in which the newly synthesized strand has incorporated the wrong nucleotide. DNA polymerase sometimes makes errors incorporating the correct number of bases during replication of long repetitive DNA sequences, such as microsatellites. Slippage during replication of a repetitive sequence creates a temporary insertion-deletion loop (IDL) that can be recognized and repaired by the MMR system, along with single base-pair mismatches. If these are not repaired, during the second round of replication the original parental strand is copied correctly, but the erroneously synthesized daughter strand (with the mismatch or IDL) contains a mutation. Single base-pair mismatches result in point mutations, whereas IDLs result in frame-shift mutations that usually lead to a downstream nonsense mutation; this results in production of a truncated, nonfunctional protein. This is the basis of MSI.

MMR is more complex in yeast and mammalian systems. The yeast homologues of the bacterial *mutS* and *mutL* genes were cloned and given the names *Mut S homologue* (*MSH*) and *Mut L homologue* (*MLH*). Then, additional homologous copies of these genes were cloned from yeast, giving rise to the terms *MSH1* through *MSH6*, and *MLH1* through *MLH3*. Another *MutL* homologue, called *post-meiotic segregation-1* (*PMS1*), was also identified in yeast. Each of these genes had diverged from the initial *mutS* and *mutL* sequences and has been associated with specific repair functions in nuclear DNA, during meiosis, or in mitochondrial DNA. Phylogenetic analysis indicates that *MSH1* was the founding member of the *Mut S* family and that the homologues diverged from this progenitor gene, but no highly homologous *MSH1* gene is present in higher organisms.¹⁴

More importantly, in the yeast and other eukaryotes, *MutS* and *MutL* proteins no longer function as homodimers. Instead, *MSH2* forms a heterodimer with

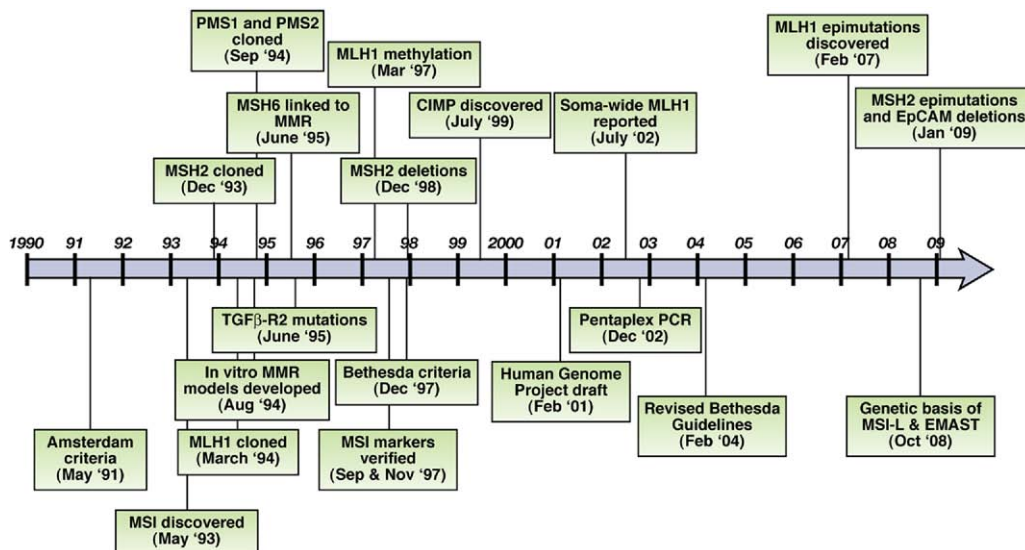


Figure 1. MSI research associated with colorectal cancer from 1990 to 2010. EpCAM, epithelial cell adhesion molecule.

MSH6 or MSH3, giving rise to MutS α or MutS β , respectively.¹⁵ These heterodimers have different relative abilities to bind to DNA mismatches and, as a result, yeast and other eukaryotes have a broader ability to recognize and repair different types of DNA misincorporation. For example, MutS α has a higher affinity for recognizing single base-pair mismatches. In fact, MSH6 was initially called the "GT-binding protein."^{16,17} It was later discovered that the *MSH3* gene encoded another MutS homologue that dimerized with MSH2 to create a complex with increased ability to bind to larger IDLs.¹⁸ The evolution of diverse homologues of *mutS* increased the cell's ability to recognize and repair synthetic errors in DNA and increased replication fidelity in higher organisms. The phylogeny and biochemistry of factors involved in MMR have been reviewed.^{19–22}

Mammals have 4 homologues of the prokaryotic *MutL* gene: *MLH1*, *MLH3*, *PMS1*, and *PMS2*. *PMS1* was the first *mutL* homologue cloned in yeast; it was given a unique name because of its functional role in meiosis. Tracing the genes phylogenetically is complicated because the yeast *PMS1*, *MLH1*, *MLH2*, and *MLH3* genes correspond to human *PMS2*, *MLH1*, *PMS1*, and *MLH3* genes, respectively. The function of the products of the *MutL* homologues are not as clear as those of the *MutS* homologues, but the encoded proteins function as heterodimers. *MLH1* and *PMS2* form MutL α , which mediates the interaction between the MutS proteins and enzymes involved in long-patch excision in postreplication mismatch repair. However, *MLH1* (the major *MutL* homologue in humans) encodes a product that can dimerize with *PMS1* to form MutL β , which suppresses mutagenesis in yeast but has an uncertain function in humans. Moreover, the dimer of *MLH1* and *MLH3* (MutL γ) helps suppress IDL mutations and functions during meiosis in yeast, but its function in humans is unknown. Loss of *MLH1* results in total loss of MMR activity, but loss of *PMS2* can be partially compensated by *MLH3*. It is not known how *PMS1* fits into this model, but the *MLH1*–*PMS1* heterodimer is not part of the canonical human MMR system. This system is illustrated in the model in Figure 2.

MSI is Caused by Deficiencies in MMR

By 1993, it was recognized that about 15% of colorectal tumors have a unique mechanism of pathogenesis; they might have been first solid tumors with subclasses that had features so distinct they could be considered as separate diseases. To identify the different pathogenic mechanisms, researchers analyzed the PCR products of colorectal tumors with MSI.

Microsatellite sequences are abundant throughout the genome; they are polymorphic among individuals but are unique and uniform in length in every tissue in each person. The heterogeneity of dinucleotide repeats made them valuable for forensic, gene mapping, and allele

discrimination analyses. Figure 3 illustrates amplification of dinucleotide and mononucleotide repeats in colonic tissues using autoradiography and gel electrophoresis.

The initial published examples of MSI were autoradiograms of radiolabeled PCR products; findings in yeast indicated that defects in genes that encode MMR factors might be responsible for the MSI observed, although no human MMR genes had been cloned.

MMR Genes, HNPCC, and Lynch Syndrome

MSH2 and HNPCC

Because much of the attention was focused on MSI in hereditary CRC, several groups set out to determine whether germline mutations in *MMR* genes were responsible for Lynch syndrome. Fishel et al cloned the human *MSH2* gene based on its homology to the yeast sequence, mapped it to human chromosome 2p22–21 (close to the locus implicated earlier in the year¹²), and found a sequence variation in patients with familial CRC²³ at the –6 position of intron 13 of *MSH2*. It was proposed that this variant altered splicing, inactivated the gene product, and was a germline mutation that mediated CRC pathogenesis. In fact, this is a common intronic polymorphism, but it was linked (in *cis*) to a true inactivating mutation that was found shortly thereafter. The association between *MSH2* and hereditary CRC explained the MSI phenotype observed in colorectal tumor samples; *MSH2* is located near the locus described in the first reported linkage study.

Leach et al confirmed the role of *MSH2* mutations in hereditary CRC, identifying germline mutations in 3 kindreds with familial CRC: 1 leucine to proline missense mutation, 1 large in-frame deletion, and 1 nonsense mutation.²⁴ These mutations were found in multiple affected members of the kindreds but not in individuals without cancer. Parsons et al showed that the CRC cell line, HCT116, which had MSI at [CA]_n sequences, was deficient in MMR activity.²⁵ Within 7 months of the discovery of MSI in CRC, it was linked to a class of genes that had not previously been of interest to cancer biologists. It was recognized that colorectal tumors might develop through loss of a DNA repair mechanism and that germline mutations in at least 1 of these genes mediated HNPCC.²⁶

MLH1, PMS2, PMS1, and MSH6

Shortly after the 2p locus was linked with familial CRC, CRC in 3 Swedish families was linked to chromosome 3p21–23.²⁷ The association between *MSH2* and Lynch syndrome indicated that other genes that encode MMR factors might be located on 3p, which would represent a second familial CRC locus. Two groups found *MLH1* on 3p21—Bronner et al identified the human homologue of yeast *MLH1* and found germline mutations

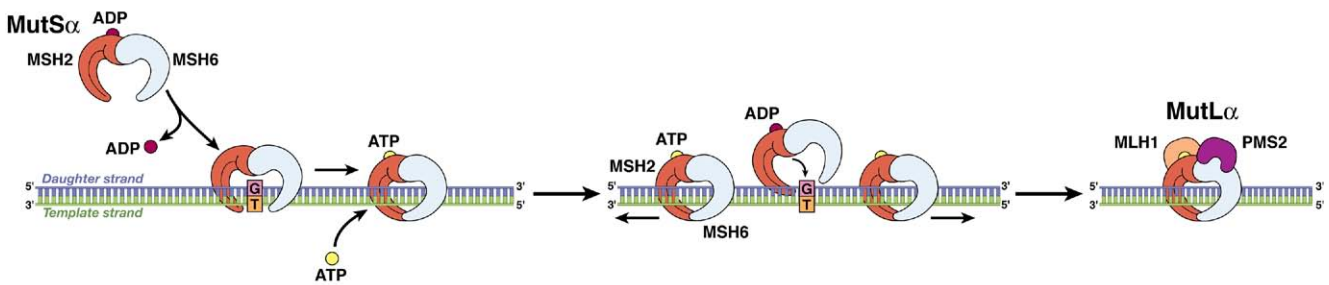
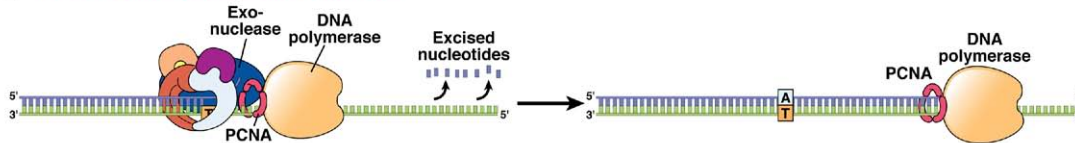
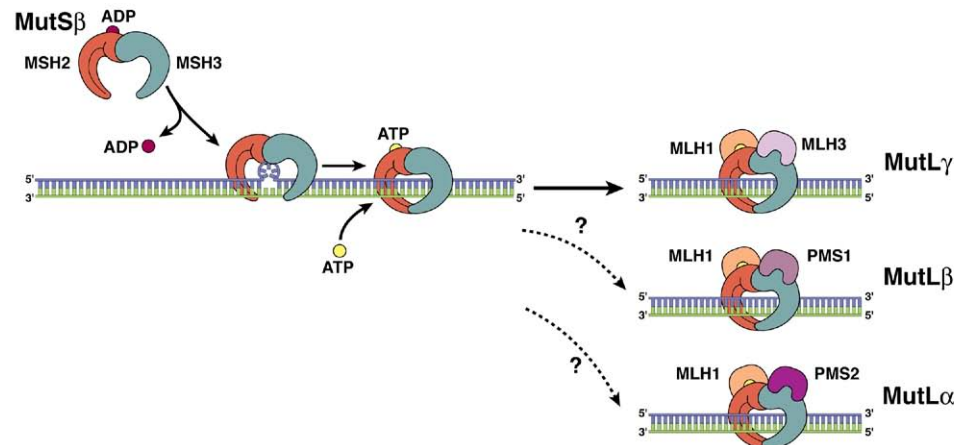
A Single mismatch**B** Exonuclease complex and resynthesis**C** Insertion/deletion loop and variations in MutL complexes

Figure 2. The DNA MMR system functions through a series of steps. (A) MSH2–MSH6 (MutS α) recognizes single base-pair mismatches, in which the DNA polymerase has matched the wrong base (G) with the T on the template (shown on *left*), and creates a sliding clamp around the DNA. This step that requires the exchange of adenosine triphosphate (ATP) for adenosine diphosphate (ADP) (by MSH2, but not MSH6 or MSH3). The complex diffuses away from the mismatch site, which is then bound by the MLH1–PMS2 (MutL α) complex (*right*). This “matchmaker” complex moves along the new DNA chain until it encounters the DNA polymerase complex. (B) The DNA MMR protein sliding clamp interacts with exonuclease-1, proliferating cell nuclear antigen (PCNA), and DNA polymerase. This complex excises the daughter strand back to the site of the mismatch (shown on *left*). Eventually, the complex falls off the DNA and resynthesis occurs, correcting the error. (C) Variations on the DNA MMR theme. Whereas MSH2–MSH6 recognizes single pair mismatches and small IDLs, MSH2–MSH3 (MutS β) complements this by also recognizing larger IDLs (shown on *left*). The *right side* shows the possible interactions with different MutL dimers, as MLH1 can dimerize with PMS2, PMS1, or MLH3. The preferred interaction with MSH2–MSH3 is MLH1–MLH3 (MutL γ), but the precise roles of the other MutL heterodimers in this reaction are not entirely understood.

in multiple members of a CRC family with significant linkage to the 3p locus.²⁸

At virtually the same time, the groups of Vogelstein and de la Chapelle scanned a DNA database for sequences homologous to the yeast *MLH1*, mapped one to chromosome 3p, and cloned the gene. Papadopoulos et al identified germline mutations in 7 members of 1 large Finnish CRC kindred that had been linked to 3p, found mutations in 3 more CRC kindreds, and reported a mutation in this gene in a CRC cell line.²⁹ Using the same strategy of searching databases for homologous sequences, the human *PMS1* and

PMS2 genes were implicated in familial CRC shortly thereafter.³⁰ The association between *PMS2* and CRC has been established, but the role of *PMS1* in sporadic or familial CRC is not clear. In the period from December 1993 to September 1994, 3 more genes were linked to familial CRC; it took another several years to identify the relationship between the *MSH6* gene¹⁷ and Lynch syndrome.³¹ *MSH6* is associated with somewhat atypical CRC kindreds with delayed onset of the cancer, which obscured the initial efforts at discovery. Once it was realized that *MSH5* families did not fit the classical expectations of the

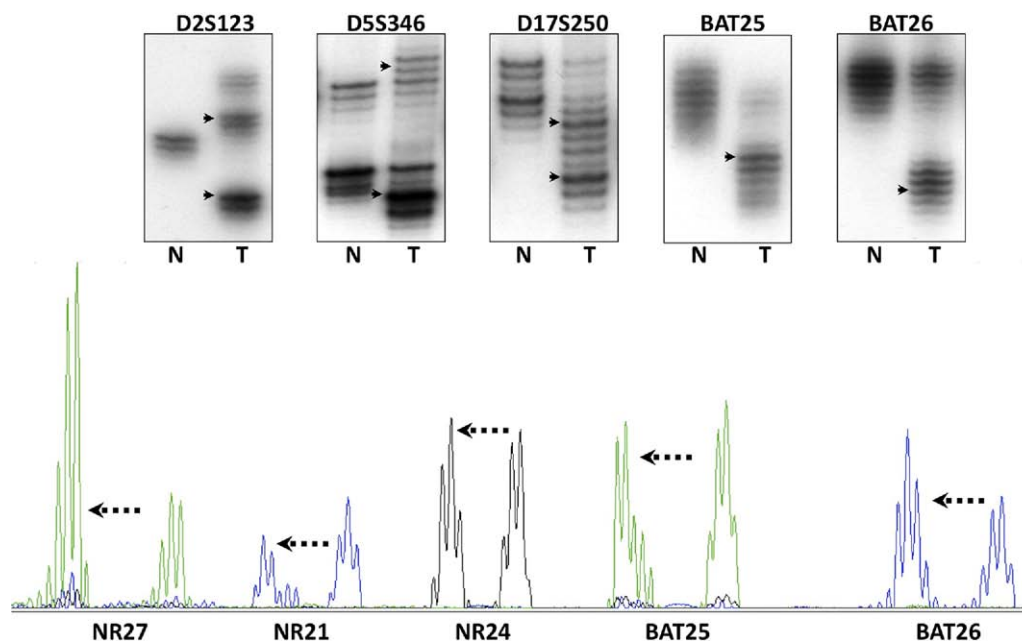


Figure 3. MSI was initially discovered by autoradiography analyses of the PCR products after separation by gel electrophoresis (*upper panel*). N refers to DNA from the normal colon, and T from the tumor. The DNA polymerase used in PCR also has difficulty with the accurate amplification of templates, which is thought to be the explanation for the “ladder” of DNA bands that can be seen in the lanes for normal and tumor DNA. The upper panel illustrates the use of the 5 markers recommended by the National Cancer Institute consensus group; these consist of 3 dinucleotide repeats and 2 mononucleotide repeats (BAT25 and BAT26). In each instance, the DNA in the tumor has undergone somatic mutations (frequently, but not always, deletions), and the PCR product migrates to a different position on the gel, as indicated by the *arrowheads*. The lower panel shows the PCR products as they are analyzed by most laboratories using automated DNA sequencing with fluorescent primers. In this instance, 5 mononucleotide repeats have been analyzed, and in each instance, the mutations consist of deletions with different electrophoretic mobility (mutant alleles indicated by the *arrows*).

Lynch Syndrome phenotype, the case for its involvement was secured.³²

Lynch Syndrome

The focus of MSI in CRC was immediately aimed at Lynch syndrome because of its inherited and unique features.³³ These patients develop tumors at early ages, often between 20 and 30 years old. They frequently have multiple tumors, including those of the colon, rectum, endometrium, stomach, ovary, urinary tract, small intestine, and other sites, but no increase in the frequency of cancers of the breast, lung, or prostate.³⁴ Before 1993, there was no agreement on whether this syndrome actually represented a distinct disease.²⁶

Families with clusters of CRC were therefore evaluated in linkage studies to identify loci of putative hereditary CRC genes. The Amsterdam Criteria were developed in 1991 so that research groups would have a uniform collection of families to study.³⁵ These criteria called for 3 CRC cases in a family in which 1 individual was a first-degree relative of the other 2, CRC occurred in at least 2 generations (in which familial polyposis was excluded), and 1 affected family member was younger than 50 years of age. The Amsterdam II Criteria were developed in 1999 to acknowledge the involvement of noncolonic tumors in the phenotype.³⁶

Studies of these kindreds facilitated the identification of genes responsible for this disease. Lynch syndrome is the hereditary disease caused by inactivating germline mutations in genes that encode MMR factors.³⁷ There are 4 definite Lynch syndrome genes: *MSH2*, *MLH1*, *MSH6*, and *PMS2*.³⁸ There are 2 genes that are candidates for Lynch syndrome (*MLH3*³⁹ and *Exo1*⁴⁰), but have been only rarely associated with familial CRC or the reported associations are believed to be coincidental. There has been no independent confirmation of a role for *MLH3* in CRC and there has been a contradictory report on the role of *Exo1* in this disease.⁴¹ However, both genes are an integral part of the MMR system, so there is rationale for considering these genes.

The Amsterdam Criteria are rigorous; it was expected that these would exclude many actual Lynch syndrome families because of the small sizes of families, missing information, and other practical issues. Although this was the case, it was surprising that 40%–60% of families that met the Amsterdam Criteria did not have a germline mutation in an MMR gene and the tumors did not have MSI.^{42,43} Families with non-Lynch syndrome CRC have been termed *familial colorectal cancer, type X* by 1 large collaboration.⁴² These families are characterized by microsatellite-stable tumors, a lower relative risk for CRC, the absence of excess

tumors outside of the colon, and later onset of CRC. The genetic basis of this group is not known.

MMR Gene Mutations in Lynch Syndrome

To diagnose Lynch syndrome, MMR genes are analyzed, preferably using exon-by-exon sequencing with PCR primers that include relevant portions of the intron–exon boundaries; these detect point mutations and small insertion-deletion mutations that involve DNA sequences between the primers but not larger deletions or rearrangements, such as DNA sequence that are rejoined after a break, because the breakpoints are usually located deep within introns or completely outside of the coding regions. When DNA sequencing is performed, the tracing is examined for a double signal, ie, the simultaneous presence of a wild-type and mutant nucleotide in the same sequence position. When there is a deletion of an entire exon in which the breakpoints are outside of the primers for that exon, the analysis gives normal results because only 1 strand is sequenced, and conventional techniques cannot determine whether 1 or 2 alleles are detected. Large deletions can be detected by multiplex ligation-dependent probe amplification, which quantifies the number of alleles at each exon.⁴⁴ Large deletions account for >33% of all *MSH2* mutations associated with HNPCC⁴⁵; *MSH2* is located within a dense cluster of *Alu* sequences, which are prone to internal recombination followed by excision of the DNA loop. Large rearrangements also occur with *MLH1* and *MSH6*.⁴⁶

Analysis for mutations in DNA MMR genes does not end when a sequence variation is found; interpretation is another issue. Most deletions and nonsense mutations have pathologic consequences, but missense mutations are not always interpretable. It is important to determine how well the sequence has been preserved (indicating its functional significance) and if it is located in a region where the MMR proteins interact. Disruption of regions of protein–protein interaction by a nonconservative amino acid change could disrupt complex formation and function (see Figure 4). The mutant allele might need to be cloned and its product tested in a functional assay.^{22,47} A proportion of mutations identified in genetic testing are reported as “variants of uncertain significance,” and will remain so until we are better able to predict protein function based on its structure. It is interesting that there is a missense mutation in *MLH1* (c415G→C; D132H) that affects adenosine triphosphate binding of the mutant gene product, increases risk for CRC, but does not always cause MSI in CRC cells.⁴⁸

MSI in Sporadic CRC

Two of the 3 initial descriptions of MSI were made in samples from sporadic colorectal tumors, rather than tumors from patients with familial CRC.^{8,10} Much of the subsequent attention to MSI was directed to familial CRC, but only about 3% of all CRCs come from

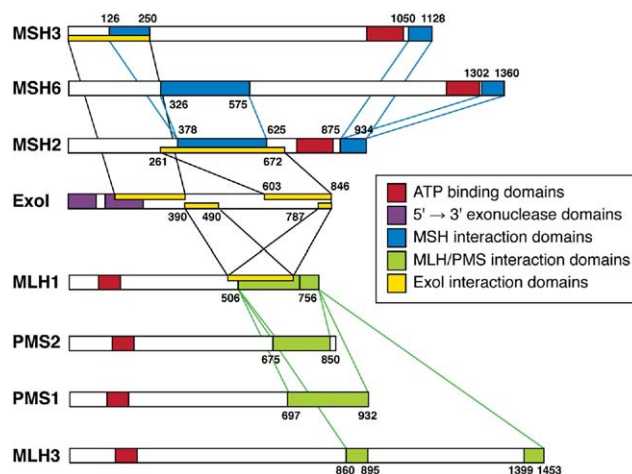


Figure 4. The regions of protein–protein interactions among the members of the MMR system. Mutations in the regions of protein interactions are particularly likely to disrupt MMR function. Key interactions are illustrated among the MutS proteins, among the MutL proteins, and between these proteins and Exo1. ATP, adenosine triphosphate; PMS, post-meiotic segregation.

Lynch syndrome families.^{49,50} Approximately 12%–17% of all colorectal tumors have MSI, depending upon the methods used to detect it,^{51,52} (references 51–144 can be found in the Supplementary Material) most CRCs with MSI are sporadic.

The majority of colorectal tumors with MSI have lost expression of MLH1 and PMS2 protein. In 1997, Kane et al showed that *MLH1* was silenced by methylation in most of these tumors.⁵³ The characteristic features of sporadic CRC with MSI include the absence of significant familial clustering, biallelic methylation of the *MLH1* promoter⁵⁴; absence of MLH1 and PMS2 proteins; and frequent mutation (usually V600E) in *BRAF*.⁵⁵ The tumors are frequently diploid (74%) and patients with sporadic colorectal tumors with MSI have a better prognosis than those with non-MSI tumors.⁵⁶ Whereas patients with Lynch syndrome patients are younger than those with sporadic CRC, those with sporadic CRCs with MSI are older. Loss of *MLH1* expression increases with age; the gene is lost in ~50% of CRC patients older than 90 years of age.⁵⁷

Sporadic MSI and CpG Island Methylator Phenotype

MSI-associated sporadic CRCs arise through a process that involves the CpG island methylator phenotype (CIMP).⁵⁸ About half of the genes in the human genome have promoters that are embedded in clusters of cytosine-guanosine residues called CpG islands.⁵⁹ Cytosines in these regions can be methylated by DNA methyltransferases. Methylation is a means by which a cell permanently silences genes; it remains in the progeny of each cell. In vitro, it is possible to demethylate at least some promoters using the DNA methyltransferase inhib-

itor 5-azacytidine; however, upon removal of this agent, remethylation occurs. Histone modifications indicate which CpG sites are marked for permanent methylation.⁶⁰

Methylation increases with advancing age, is accelerated in the colon in response to chronic inflammation, and might be an adaptive response to injury.^{61,62} A subset of CRCs has been identified that is characterized by CIMP.^{63,64} These tumors progress by methylating tumor suppressor genes and have distinctive clinical features compared to non-CIMP colorectal tumors.⁶⁴ Some of the commonly methylated promoters have no apparent role in tumor development and have been labeled “methylated in tumor” or MINT genes, but methylation also occurs in promoters of known tumor suppressor genes, such as *p16* and *insulin-like growth factor 2*, and DNA repair genes such as *methylguanine methyltransferase* and *MLH1*.

The region of the *MLH1* promoter in which methylation mediates gene silencing is the 3' end, close to the start codon. The 5' end of the promoter is also prone to methylation, but this is not of functional importance unless the methylation extends to the critical 3' region.⁶⁵ Therefore, specific CpG residues are more important than others in mediating gene silencing. Importantly, most of the CRCs with sporadic MSI come from a CIMP background, which creates an important distinction from Lynch syndrome tumors.^{66,67}

Consequently, it is essential to know more about a colorectal tumor than whether there is MSI or not. Lynch syndrome tumors are associated with germline mutations in DNA MMR genes,³⁷ occur in younger individuals, can have *KRAS* mutations (but never *BRAF* mutations), and are associated with a better prognosis than non-MSI tumors.⁶⁸ Most sporadic colorectal tumors with MSI occur in older individuals, have *BRAF* mutations in about half of the cases (V600E), are associated with a background of CIMP, and are associated with a reduced mortality. Curiously, *BRAF* mutations, which are detected in sporadic but not familial CRCs with MSI are associated with reduced mortality.⁶⁴

Germline Epimutations

To aide in the diagnosis of Lynch syndrome, researchers have searched for germline mutations in a coding regions, splice sites, or promoter regions of MMR genes. However, instead of a genetic mutation, some cases of Lynch syndrome are caused by epigenetic inactivation of genes by promoter methylation. These events, originally described as germline epimutations, are better referred to as constitutional epimutations, to reflect the aberrant silencing of a gene that is normally active in somatic tissues in the absence of a sequence mutation. Constitutional epimutations of *MLH1*^{69–71} and *MSH2*^{72,73} have been identified in families with Lynch syndrome who have no apparent MMR gene sequence mutations. *MSH2* methylation can arise from a deletion in the 3' end

of the gene just upstream of *MSH2* (*TACSTD1*)—the loss of the stop codon somehow results in methylation of the CpG island downstream, which leads to constitutional silencing of *MSH2*. Similar to germline mutations, constitutional epimutations in *MSH2* demonstrate classic autosomal dominant inheritance and are associated with a 50% risk of transmission to the offspring.^{73,74}

Pathophysiology of Colorectal Carcinogenesis with MSI

CRCs with MSI were discovered, in part, because they were different from most other CRCs; the tumor cells had a tendency to be diploid and had less LOH. The mutational patterns were of interest because of the unique clinical features of these tumors. In 1995, Markowitz et al provided an important insight into the carcinogenetic pathway.⁷⁵ Transforming growth factor- β (TGF- β) signaling inhibits proliferation in the colonic epithelium; Markowitz et al showed that the TGF- β type II receptor (TGF β R2) was not expressed in cell lines with MSI or in xenografts of these cells grown in nude mice, but was expressed by cell lines without MSI. TGF β R2 includes 2 microsatellite sequences. From nucleotides 1931 to 1936, there is a 6-base pair [GT]₃ sequence (the inverse of a C-A repeat) and from nucleotides 709 to 718 there are 10 consecutive adenines (A₁₀). Deletion mutations in these microsatellite sequences create frame shifts that inactivate the gene product. These were found predominantly in the MSI cell lines; cells with mutant forms of TGF β R2 did not slow proliferation in response to TGF- β . The most frequent mutation was a single base-pair deletion in the A₁₀ sequence; Markowitz et al demonstrated that this mutation made the protein nonfunctional.⁷⁶ Furthermore, the authors identified mutations in the A₁₀ tract of 90% of 111 colorectal tumor samples found to have MSI, proving that this was a relevant site of this form of genomic instability.⁷⁷ In a new paradigm, repetitive DNA sequences were proposed to be particularly sensitive to the loss of DNA MMR activity, resulting in frame-shift mutations that led to premature stop codons and gene inactivation.

Several other genes affected by MSI were then identified that encoded regulators of cell proliferation (*GRB1*, *TCF-4*, *WISP3*, *activin receptor-2*, *insulin-like growth factor-2 receptor*, *axin-2*, and *CDX*), the cell cycle or apoptosis (*BAX*, *caspase-5*, *RIZ*, *BCL-10*, *PTEN*, *hG4-1*, and *FAS*), and DNA repair (*MBD-4*, *BLM*, *CHK1*, *MLH3*, *RAD50*, *MSH3*, and *MSH6*)⁷⁸ (see Table 1). Remarkably, every human MMR gene except *MLH1* includes a mononucleotide repeat of at least A₇,⁷⁹ so the process of MMR could become increasingly defective with cumulative losses of components on the system.⁸⁰ However, it is not clear how many of the mutations at these loci are of functional significance (which has been determined for TGF β R2), or whether some are simply markers of MSI, because bial-

Table 1. The Genetic Targets of Microsatellite Instability in Colorectal Cancer

Microsatellite length	Gene
A10	<i>AIM2</i>
	<i>CASPASE-5</i>
	<i>MBD-4</i>
	<i>OGT</i>
	<i>SEC63 (also, A9)</i>
	<i>TGFβ₁R2</i>
A9	<i>BLM</i>
	<i>CHK1</i>
	<i>GRB-14</i>
	<i>MLH3</i>
	<i>RAD50</i>
	<i>RHMM</i>
	<i>RIZ (also, A8)</i>
	<i>TCF-4</i>
	<i>WISP3</i>
	<i>ACVR11</i>
A8	<i>APAF</i>
	<i>BCL-10</i>
	<i>hG4-1</i>
	<i>MSH3</i>
	<i>PTEN (2 A6's)</i>
A6	<i>OGT</i>
T10	<i>KIAA0971</i>
T9	<i>NADH-UOB</i>
G8	<i>BAX</i>
	<i>IGF2R</i>
C9	<i>SLC23A1</i>
C8	<i>MSH6</i>
G7	<i>AXIN-2 (A6, A6, C6)</i>
	<i>CDX2</i>
T7	<i>FAS</i>

Data from Duval and Hamelin.⁷⁸

lelic inactivation of these genes has not been documented in all the tumors.

The identification of the proximate targets of carcinogenesis in the setting of defective MMR activity indicates that MSI-associated colorectal tumorigenesis occurs through a different biological pathway compared to sporadic tumors. CRC cells with MSI can activate or inactivate the same signaling pathways as those without MSI, but different proteins within these pathways are involved. Genes are altered by different mechanisms in CRC cells with MSI compared to those without MSI. For example, sporadic colorectal tumors arise from a combination of mutations and LOH, resulting in biallelic inactivation of *APC*. Colorectal tumors with MSI have an increase in the number of point mutations compared to cancer cells without MSI, are more likely to be diploid, and do not have experienced widespread allelic imbalance or LOH. A substantial portion of colorectal tumors with MSI have normal expression of *APC*, but have mutations in β -catenin that make it unable to interact with APC protein and undergo degradation,^{81,82} the biological equivalent of having no APC protein. Moreover, some colorectal tumors with MSI that have neither inactivated *APC* nor mutated β -catenin could have frame-shift mutations in

factors further downstream in the WNT pathway, such as in the A₉ sequence of *TCF-4*.

Of equal importance are the differences in the pathogenesis of colorectal tumors with MSI that come from patients with Lynch syndrome compared with sporadic CRC cells with CIMP, as illustrated in Figure 5. For reasons that are not clear, *BRAF* mutations are found in the majority of sporadic tumors with MSI but not in tumors that arise in patients with Lynch syndrome, which often have *KRAS* mutations.^{64,66} Activating mutations in *BRAF* and *KRAS* are functionally equivalent because their products function in series, in the mitogen-activated kinase signaling pathway, which regulates epithelial cell proliferation.⁸³

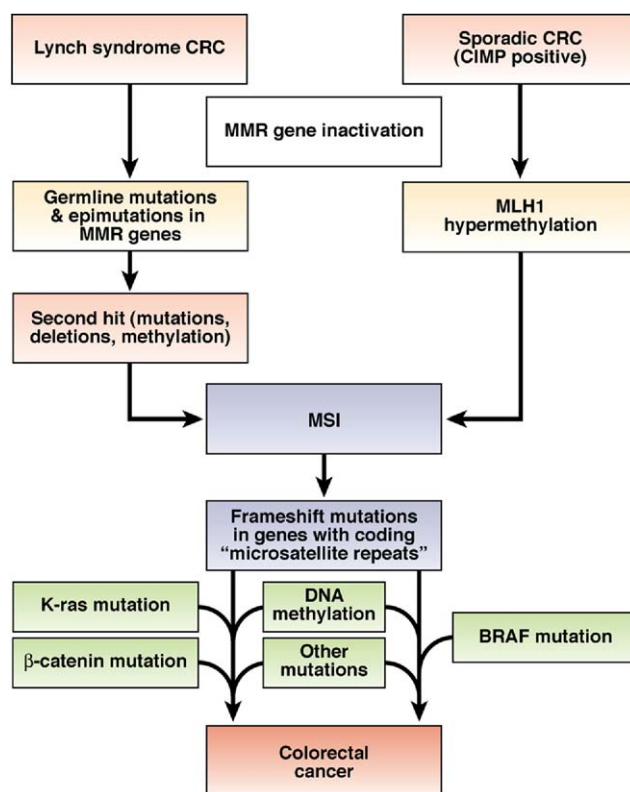


Figure 5. There are 2 molecular pathways to the development of CRC with MSI. Approximately 20%–25% of colorectal tumors with MSI arise in individuals with Lynch syndrome. These tumors contain a germline mutation in one of the MMR genes, followed by a second hit to the wild-type copy (inherited from the unaffected parent); this could occur via LOH, methylation, or point mutation. Defects in MMR result in MSI and rapid accumulation of somatic mutations. It has been proposed that tumors arise via mutations in a few critical genes, but that large numbers of microsatellite mutations also occur—most of which are simply passengers that provide the mutational signature used to identify tumors with MSI. Colorectal tumors that arise in patients with Lynch syndrome often have mutations in *KRAS*. Most cases of CRC associated with MSI are not inherited (familial), but arise through sporadic methylation-induced silencing of *MLH1*. These sporadic tumors have the CIMP signature, resulting in methylation of many gene promoters. When the *MLH1* promoter is methylated, MMR activity fails and MSI ensues. Thus, the mutational signature of sporadic tumors includes CIMP and MSI. *BRAF* mutations are also observed in most sporadic colorectal tumors, but do not occur in tumors of patients with Lynch syndrome.

MSI and Inflammatory Bowel Disease

The risk of CRC is increased in patients with inflammatory bowel disease, but it is not completely clear how chronic inflammation mediates carcinogenesis. Inflammation potentially increases mutagenesis via the generation of oxidative stress and formation of free radicals; mucosal repair involves the stimulation of proliferation, which could expand populations of colon cells with mutations that provide proliferative or survival advantages. However, studies in 2 different in vitro models have shown that oxidative stress can “relax” DNA MMR activity,^{84,85} and another study has demonstrated an increase in mutation rates in cells exposed to oxidative stress.⁸⁶ So, at the time of greatest mutational load, the DNA MMR system is underactive—a biological paradox. The same has been reported for base excision repair, which might also lead to MSI.⁸⁷

There is evidence for MSI in colon cells of patients with chronic ulcerative colitis. Using a panel of 5 dinucleotide repeat markers, Suzuki et al found that 21% of 63 colitis-associated tumors had at least 1 mutated microsatellite marker; a similar finding was reported for dysplastic lesions.⁸⁸ Perhaps more surprisingly, Brentnall et al reported instability in at least 1 of 7 dinucleotide repeat markers in 50% of non-neoplastic mucosa samples from individuals with chronic ulcerative colitis, but not in samples from patients with acute infectious colitis⁸⁹; this finding was independently confirmed.⁹⁰ These studies were done before the MSI marker panels were standardized, but an unpublished review of the primary data indicated that only 1 dinucleotide repeat was mutated in each instance, indicating low level MSI (MSI-low) rather than high MSI level (MSI-high).

Models of MSI

Studies of MMR expanded from *Escherichia coli* and *Saccharomyces cerevisiae* to human cells and diseases in the mid-1990s. Parsons et al discovered that HCT116 cells had defects in MMR activity and MSI²⁵ that were associated with biallelic mutations in *MLH1*. Stable transfer of human chromosome 3 into HCT116 (HCT116 + ch3 cells) restored *MLH1* and created a model to study the role of MMR in the response to DNA damage and regulation of the cell cycle.⁹¹ MMR activity was restored in HCT116 + ch3 cells, which had reduced tolerance to DNA alkylation by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.⁹¹ The model also demonstrated that MMR regulates passage through the G₂/M cell cycle checkpoint and the response to 6-thioguanine, which is incorporated into DNA as guanine but acts like a mismatch in newly synthesized DNA.⁹² MMR activity is required to restrain clonal expansion of cells exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and prevent proliferation of cells with damaged DNA.⁹³ Cultured CRC cells with intact MMR activity were significantly more sensitive to therapeutic concentrations of 5-fluorouracil (5-FU; 5–10 μ M)

than DNA MMR-deficient cells,⁹⁴ as well as other chemotherapeutics.^{95,96} This provided the theoretical basis for later observations that patients with MSI CRCs might not respond to traditional 5-FU-based chemotherapy.

Cell models were subsequently developed to study other genes in the DNA MMR family. Although stable transfer of human chromosome 3 corrected some DNA MMR activity, HCT116 + ch3 cells were still devoid of *MSH3* activity. Therefore, the HCT116 + 3 + 5 cell line was created by stable transfer of human chromosome 5 into HCT116 + ch3 cells, permitting an exploration of the effects of *MSH3* on MMR.⁹⁷ The CRC cell line HCT15 does not express *MSH6* and the endometrial cancer cell line HEC59 does not express *MSH2* or *MSH6*. Stable transfer of human chromosome 2 (which contains *MSH2* and *MSH6*) into these cells created cell lines that permitted analysis of the role of the MutS system in human cells.⁹⁸ In the modified HCT15 and HEC59 cell lines, *MSH2* and *MSH6* were re-expressed, MSI and the hypermutable phenotype were corrected (reducing mutations at the *HPRT* locus by 96% in HCT15), and the cells became more sensitive to cell death after exposure to mutagens. Similarly, in LoVo cells, which have defects at the *MSH2* locus, MMR activity and sensitivity to mutagenic agents were restored following transfer of a fragment of chromosome 2.⁹⁹

Lynch syndrome is an autosomal dominant disease, associated with the inheritance of a single mutated gene. However, in cultured cells, defects in MMR are recessive; a single copy of the gene restores DNA MMR activity. MMR activity is lost in cells that lack *MSH2* or *MLH1*, but only partially disrupted in cells without *MSH6* or *MSH3*. These findings are important for understanding the pathogenesis of Lynch syndrome; patients with inactivating mutations in *MSH6* develop an attenuated form of the disease and Lynch syndrome has not been associated with mutations in *MSH3*.¹⁰⁰

Stoichiometry of MMR Proteins

HCT116 (deficient in *MLH1*), LoVo (deficient in *MSH2*), HCT15 and DLD1 (deficient in *MSH6*), and SW48 (hypermethylated at *MLH1*) were used together with the single chromosome transfer-mediated restoration models and control lines (SW480, HEL, WI38) to study the stoichiometry of the members of the human DNA MMR system.¹⁰¹ *MSH2* was the most abundantly expressed MMR protein; cells expressed 3–5-fold more molecules than of its partner *MLH1*, depending upon the cell line studied. *MSH6* protein was 4–12-fold more abundant than *MSH3* protein and the combined amount of *MSH6* and *MSH3* expressed equaled that of *MSH2* in cells. *MSH6* might therefore have a more important role in DNA homeostasis than *MSH3*, and there are probably no other binding partners in the cell for *MSH2*. The relationship between expression levels of *MSH2* and *MLH1* have implications for MMR; a substantially larger

number of MutS complexes are involved in identifying DNA mismatches than MutL complexes are required for interaction with the exonuclease complex. PMS2 was expressed in the lowest concentrations; the molar ratios of MLH1:PMS2 ranged from 1.4 to 2.75. Unfortunately, antibodies were not available to measure levels of PMS1 or MLH3 when these studies were performed. However, these studies helped provide a biochemical framework for the relative roles of *MSH2* and *MLH1* in MMR and their importance in human disease. Perhaps more importantly, these findings provided insight into the clinical differences between Lynch syndrome caused by mutations in *MSH2* and *MLH1*, which have the most highly penetrant phenotypes, and the more attenuated phenotypes associated with germline mutations in *MSH6* and *PMS2*.¹⁰⁰

Methylation of *MLH1*

CIMP and the acquired form of MSI induced by hypermethylation of the *MLH1* locus were studied in the CRC cell lines SW48 and RKO. SW48 cells do not express MLH1 or PMS2 protein,¹⁰¹ they lack *MLH1* messenger RNA but do express stable *PMS2* messenger RNA. Exposure of SW48 cells to the demethylating agent 5-azacytidine led to expression of the *MLH1* messenger RNA and stable expression of the MLH1 and PMS2 proteins, which had been rapidly degraded in the absence of the stabilizing MLH1 protein. Demethylation-induced re-expression of *MLH1* and *PMS2* restored sensitivity to 5-FU,¹⁰² suggesting that demethylation might be used in conjunction with other therapeutic agents to overcome the drug resistance associated with acquired MSI in patients with CRC.

Measuring MMR Deficiency

Another model has been developed to measure mutation rates in cells with varying degrees of MMR deficiency. A green fluorescent protein reporter gene can be used to identify sites of microsatellite mutation as a surrogate for a direct measurement of MMR activity (which is labor intensive)⁹¹ to quantify the effects of physiological manipulations. This technology has shown that oxidative stress induces relaxation of MMR activity and increases frame-shift mutations. Furthermore, it has been shown that mesalamine improves replication fidelity in cultured cells whereas aspirin does not.^{86,103}

There is no naturally occurring animal model of MMR deficiency, but knockout strains of mice have been created for each of the Lynch syndrome genes: *MSH2*,^{104,105} *MLH1*,^{106,107} *PMS2*,¹⁰⁷ and *MSH6*.¹⁰⁸ In spite of contrary claims, none of the heterozygous knockout models produces a phenotype that is similar to the human disease. The principal tumors that develop in these mice are lymphomas. Homozygous disruption of MMR genes produces animals with a constitutional deficiency of MMR activity, a variety of interesting genetic lesions, and some intestinal tumors, but are not useful in screens for agents

that have therapeutic or preventive effects in humans. Animals with compound knockouts of *Apc* and a DNA MMR gene have accelerated polyp progression, but these tumors predominantly form in the small intestine.¹⁰⁹

The study of MMR in mouse models is limited because human genes associated with colorectal carcinogenesis include coding microsatellites, in places where the mouse genes do not. Furthermore, genes that are associated with colorectal tumorigenesis encode factors that are required for cell proliferation and survival, such as TGF β 2, BAX, and caspase-5, so it is a challenge to create mice with defects in these genes for cancer studies.

MSI-High and MSI-Low

At an international consensus meeting in 1997, the definition of MSI was standardized.¹¹⁰ The term *MSI*, when not otherwise modified, refers to MSI-high, in which >30% of the microsatellite marker panel is mutated, as defined in the Bethesda guidelines.¹¹⁰ The definition of MSI-high permits the characterization of a group of CRCs with similar clinical and pathological characteristics. However, if enough different microsatellite markers are used, a larger proportion of CRCs will be found to have at least 1 mutated microsatellite. Those CRCs in which at least 1 but <30% of the markers are mutated are called MSI-low and have clinical features of microsatellite-stable tumors. MSI-low has been observed in many tumor types.¹¹¹⁻¹¹³

Another type of MSI has been recognized that does not fit into the definition of MSI-high. This signature has been called "elevated microsatellite alterations at selected tetranucleotide repeats" (EMAST). EMAST is most frequently found in noncolonic tumors and is associated with *p53* mutations. There is no evidence that it is caused by mutational inactivation of the MMR system.¹¹⁴

Haugen et al have resolved the mechanistic basis of MSI-L and EMAST.⁹⁷ They found evidence for EMAST in ~60% of sporadic CRCs and in all of the tumors that were MSI-high or MSI-low. EMAST was also found in 35 of 82 (42.6%) of microsatellite-stable CRCs. Based on immunohistochemical analyses, 2%–50% of tumor cells were deficient in MSH3; its expression was heterogeneous throughout the tumor samples. Those parts of the tumor that did not express MSH3 were microdissected and found to be significantly more likely to have EMAST. Using in vitro models in which deficiencies in MMR can be studied in isolation, MSH3-deficient cells (including HCT116 + ch3, which has constitutional MSH3 deficiency, or cells in which MSH3 was conditionally knocked down), were found to have instability at di- and tetra-nucleotide repeats (ie, they had the EMAST and MSI-low phenotypes). This was important because MSI-low was detected through instability at dinucleotide repeat microsatellites.¹¹⁵ MSI-L and EMAST can therefore be induced by downregulation of *MSH3*, which occur

heterogeneously (ie, non-clonally) throughout a tumor. It is possible that a similar mechanism is responsible for the appearance of MSI-low in the colonic mucosa of patients with chronic ulcerative colitis.⁸⁹

Diagnosis of MSI

Patients with colorectal tumors with MSI have longer survival times than patients with tumors without MSI.¹⁰ A study of 175 patients with Lynch syndrome (120 of whom had Lynch syndrome-MLH1 type), compared to more than 14,000 population-based patients with CRC, showed that the 5-year cumulative relative survival for patients with Lynch syndrome was 65%, compared to 44% of patients with sporadic CRC who were older than 65 years of age.¹¹⁶ A pooled analysis of MSI that included 32 studies and 7642 CRC cases found an overall hazard ratio of 0.65 for patients with tumors that have MSI.⁵¹ Patients with Lynch syndrome have lower-stage disease when they are identified compared to patients with other types of CRC, and it is less common for patients with Lynch syndrome to present with metastatic disease.^{117–119} Gryfe et al reported that 17% of 607 CRC patients who were younger than 50 years old had MSI; the hazard ratio for patients with MSI-associated tumors was 0.42, compared with patients in the same cohort with non-MSI tumors. Patients with tumors with MSI had lower mortality rates when patients were stratified by tumor stage, including patients with stage IV cancer.¹²⁰

Tumors with MSI have greater numbers of tumor-infiltrating lymphocytes that are activated and cytotoxic;¹²¹ the lymphocytic reaction is independently associated with longer survival.¹²² Studies report that colorectal tumors with lymphocytic infiltrates are associated with longer survival times; in fact, lymphocyte infiltration may be a better prognostic factor than routine pathology staging.¹²³ Abnormal results from immunohistochemical analyses of MSH2 and MLH1 independently predict better outcomes in patients with stage II–T3 colorectal tumors.¹²⁴ Therefore, detection of MSI in a patient with CRC is a positive prognostic factor, particularly among young patients.

However, not all studies have confirmed the value of MSI detection in prognosis.^{125,126} This might be because there are technical challenges in identifying tumors with MSI. Some studies could have included CRC cases that were falsely reported to have MSI-positive tumors, which might have altered the modest differences in predicted patient outcomes.^{126,127} Second, MSI analyses include 2 groups of patients: those with Lynch syndrome (who tend to be younger) and those with acquired methylation of the *MLH1* gene (who are older). If Lynch syndrome patients are excluded from a cohort, the average age of patients with MSI is older—older patients have shorter overall survival times regardless of whether or not they have CRC. Most evidence, however, indicates that MSI predicts positive outcomes in patients with CRC.

The principal use of MSI testing in the clinic is to identify patients with Lynch syndrome. Approximately 15% of all colorectal tumors have MSI, and 75%–80% of this group have acquired methylation of *MLH1*; only ~2%–3% of all CRCs have germline mutations in one of the MMR genes.⁴⁹ Although MSI analysis is the first approach to identifying patients with Lynch syndrome, immunohistochemical analysis is equivalent in levels of sensitivity and specificity and is accessible to most pathology laboratories. Identification of abnormally expressed proteins can be used to identify genes that should be sequenced. MSI identifies MMR-deficient colorectal tumors with approximately 93% sensitivity; most insensitivity is caused by mutations in *MSH6*.^{128,129} Initial reports estimated that immunohistochemical analysis of MLH1 and MSH2 identified tumor samples with MMR defects with 92.3% sensitivity and 100% specificity.¹²² A comprehensive review of 16 studies that compared MSI with immunohistochemical analysis of MLH1 and MSH2 proteins suggested that immunohistochemistry was somewhat less sensitive overall, mainly because of its poor performance with MLH1. However, when expression of MSH6 and PMS2 were included in the analyses, the performance of immunohistochemistry improved, resolving the problems encountered with samples from patients with Lynch syndrome caused by MSH6 and PMS2.¹²⁹ The limitation of immunohistochemistry is that staining can be heterogeneous throughout the tumor, which affects the sensitivity of the test.¹²⁹

Several panels of microsatellites have been used to diagnose MSI. The first consensus meeting recommended a panel of 3 dinucleotide repeats and 2 mononucleotide repeats that had been validated by a German consortium.^{110,111,130,131} This panel required that normal tissue be compared with tumor tissue, so many groups use a panel of 5 mononucleotide repeats that can be amplified and analyzed in a single assay. The location and nature of each of the microsatellite targets used for diagnostic purposes are listed in Table 2. The Pentaplex panel is as sensitive and specific as the initial panel proposed by the National Cancer Institute, but has some advantages that improve specificity. The T₂₅ repeat in the 3' untranslated region of *caspase-2* is useful in detecting loss of *MSH6*.

MSI as a Predictor of Response to Chemotherapy

Tests for MSI can be used to predict patient response to adjuvant chemotherapy, although this practice is controversial. MMR genes were identified in bacteria; inactivating mutations created a mutator phenotype and allowed tolerance to DNA-damaging agents.¹³² A similar phenotype was found in mammalian cell lines.^{16,133} The stable correction of MMR activity in cell lines restored the cytotoxic response to alkylating agents,^{91,93} 6-thioguanine,⁹² 5-FU,⁹⁴ platinum compounds, and other

Table 2. Microsatellite Markers Used in Diagnosis of Microsatellite Instability in Colorectal Cancer

Marker	Gene	MS repeat	Chromosomal location	Location of MS repeat
D2S123	Linked to MSH2	CA (n)	2p16	
D5S346	Linked to APC	CA (n)	5q22-23	
D17S250	Linked to p53	CA (n)	17q12	
BAT-25	c-kit	A (25)	4q12	Intron 16
BAT-26	MSH2	A (26)	2p21	Intron 5
Pentaplex panel of mononucleotide repeat markers				
BAT-25	c-kit	A (25)	4q12	Intron 16
BAT-26	MSH2	A (26)	2p21	Intron 5
NR-21	SLC7A8	T (21)	14q11	5' UTR
NR-24	Zinc finger 2 (ZNF-2)	T (24)	2q11	3' UTR
NR-27	Inhibitor of apoptosis protein-1	A (27)	11q22	5' UTR
Other relevant microsatellite markers				
CAT25	Caspase-2 (CASP2)	T (25)	7q34	3' UTR

MS, microsatellite; NCI, National Cancer Institute.

agents.^{96,134} This indicated that tumors with MSI might be resistant to some chemotherapeutic regimens.

However, the first study of the responsiveness of colorectal tumors with MSI reported just the opposite—tumors with MSI were more responsive to adjuvant chemotherapy than tumors without MSI.¹³⁵ In this study, the assignment of patients to the treatment groups was flawed; instead of random assignment, oncologists selected patients for chemotherapy based on those with the fewest comorbidities and best performance status. Only 23 patients with MSI received chemotherapy, and the median age of these patients was 13 years younger than those who were not treated. Overall survival was significantly better in the MSI group, regardless of treatment. This created the impression that such patients were ideal candidates for adjuvant chemotherapy.

However, 11 subsequent studies have shown no benefit for chemotherapy among patients with colorectal tumors with MSI (see Table 3^{51,126,136–143}). In the largest of these,

a prospective, multicenter study in which patients were prospectively randomly assigned to treatment groups, the investigators found a nonsignificant, 2-fold excess in mortality among stage III CRC patients with MSI and a 3-fold increase in mortality among the stage II CRC patients with MSI.¹⁴¹ The MSI-associated increases in mortality might be related to the immunosuppressive effects of the treatment regimens, which would counter the increased anti-tumor immunity observed in patients with MSI.

A study by Fallik et al associated inclusion of the topoisomerase-I inhibitor, irinotecan, in the chemotherapeutic regimen with increased survival times of in patients with MSI-associated CRC,¹⁴⁴ a finding that has been supported by Bertagnolli et al.¹⁴⁰ It is possible that a combination of drugs without excessive immunotoxicity is best for patients with MSI-associated CRC. There is no evidence for the efficacy of traditional 5-FU-based adjuvant chemotherapy in patients with MSI-associated

Table 3. Chemotherapy in Colorectal Cancer with Microsatellite Instability

First author	Year	Study design	Adjuvant chemotherapy regimen	No. of patients (MSI/MSS)	Benefit of chemotherapy in patients with MSI
Elsaleh ¹³⁵	2000	Consecutive patients	5-FU	63/669	Yes
Ribic ¹⁴¹	2003	Randomized controlled study	5-FU	95/475	No
Carethers ⁹⁴	2004	Consecutive patients	5-FU	36/168	No
de Vos tot Nederveen Cappel ¹⁴³	2004	Lynch syndrome patients	5-FU	28/0	No
Storojeva ¹³⁶	2005	Randomized controlled study	5-FU/mitomycin	21/139	No
Benatti ¹⁴²	2005	Consecutive patients	5-FU	256/1007	No
Popat ⁵¹	2005	Pooled data from multiple studies	5-FU	1277/6365	No
Lanza ¹³⁷	2006	Consecutive patients	5-FU	75/288	No
Jover ¹³⁸	2006	Consecutive patients	5-FU	66/688	No
Kim ¹²⁶	2007	Prospective study	5-FU/leucovorin	98/444	No
Des Guetz ¹³⁹	2009	Meta-analysis	—	454/2871	No
Bertagnolli ¹⁴⁰	2009	Randomized controlled study	5-FU/irinotecan/leucovorin	106/677	No

5-FU, 5-fluorouracil; MSS, microsatellite stable.

CRC, but there might be regimens that would benefit these patients. Patients with MSI-associated CRC should be included in clinical trials until the appropriate treatment regimen is identified. Also, these studies did not distinguish whether the colorectal tumors with MSI were sporadic or inherited. Because most tumors with MSI are sporadic, it is likely that these observations are more relevant to those tumors; additional studies focused on patients with Lynch syndrome will be needed to confirm this concept.

Supplementary Material

Note: The first 50 references associated with this article are available below in print. The remaining references accompanying this article are available online only with the electronic version of the article. Visit the online version of *Gastroenterology* at www.gastrojournal.org, and at [doi:10.1053/j.gastro.2009.12.064](https://doi.org/10.1053/j.gastro.2009.12.064).

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Received November 5, 2009. Accepted December 30, 2009.

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Conflicts of interest

The authors disclose no conflicts.

Funding

Supported by grants from the National Cancer Institute and National Institutes of Health R01 CA72851 (to Dr Boland) and R01 CA129286 (to Drs Goel and Boland).

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ATTACHMENT 3A

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530	__ a Also issued online.
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ATTACHMENT 3B

Gastroenterology MeSH Descriptor Data 2025

[Details](#)[Qualifiers](#)[MeSH Tree Structures](#)[Concepts](#)**MeSH Heading**

Gastroenterology

Tree Number(s)

H02.403.429.405

Unique ID

D005762

RDF Unique Identifier

<http://id.nlm.nih.gov/mesh/D005762>

Scope Note

A subspecialty of internal medicine concerned with the study of the physiology and diseases of the digestive system and related structures (esophagus, liver, gallbladder, and pancreas).

Entry Version

GASTROENTEROL

Entry Term(s)

Hepatology

NLM Classification #

WI 21

See Also

Gastrointestinal Diseases

Date Established

1966/01/01

Date of Entry

1999/01/01

Revision Date

2009/07/06

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Meeting Report

A National Cancer Institute Workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition: Development of International Criteria for the Determination of Microsatellite Instability in Colorectal Cancer

C. Richard Boland,¹ Stephen N. Thibodeau, Stanley R. Hamilton, David Sidransky, James R. Eshleman, Randall W. Burt, Stephen J. Meltzer, Miguel A. Rodriguez-Bigas, Riccardo Fodde, G. Nadia Ranzani, and Sudhir Srivastava²

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Abstract

In December 1997, the National Cancer Institute sponsored "The International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition," to review and unify the field. The following recommendations were endorsed at the workshop. (a) The form of genomic instability associated with defective DNA mismatch repair in tumors is to be called microsatellite instability (MSI). (b) A panel of five microsatellites has been validated and is recommended as a reference panel for future research in the field. Tumors may be characterized on the basis of: high-frequency MSI (MSI-H), if two or more of the five markers show instability (i.e., have insertion/deletion mutations), and low-frequency MSI (MSI-L), if only one of the five markers shows instability. The distinction between microsatellite stable (MSS) and low frequency MSI (MSI-L) can only be accomplished if a greater number of markers is utilized. (c) A unique clinical and pathological phenotype is identified for the MSI-H tumors, which comprise ~15% of colorectal cancers, whereas MSI-L and MSS tumors appear to be phenotypically similar. MSI-H colorectal tumors are found predominantly in the proximal colon, have unique histopathological features, and are associated with a less aggressive clinical course than are stage-matched MSI-L or MSS tumors. Preclinical models suggest the possibility that these tumors may be resistant to the cytotoxicity induced by certain chemotherapeutic agents. The implications for MSI-L are not yet clear. (d) MSI can be measured in fresh or fixed tumor specimens equally well; microdissection of pathological specimens is recommended to enrich for neoplastic tissue; and normal tissue is required to document the presence of MSI. (e) The "Bethesda guidelines," which were developed in 1996 to assist in the selection of tumors for microsatellite analysis, are endorsed. (f) The spectrum of microsatellite alterations in noncolonic tumors was reviewed, and it was concluded that the above recommendations apply only to colorectal neoplasms. (g) A research agenda was recommended.

Introduction

The discovery of MSI³ in colorectal cancers and its linkage to HNPCC in 1993 opened new chapters in tumor biology and in the

clinical management of patients with heightened cancer susceptibility. Over the past 5 years, the scope of MSI has been expanded to encompass a unique form of genomic instability broadly involved in the genesis of cancer and limited to neither HNPCC nor colorectal cancer. MSI is caused by a failure of the DNA MMR system to repair errors that occur during the replication of DNA and is characterized by the accelerated accumulation of single nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences that occur ubiquitously throughout the genome. MSI is seen in most HNPCC tumors and in a proportion of nonhereditary colorectal tumors. The presence of MSI in tumor tissue is associated with certain unique clinical and pathological characteristics. The literature on MSI is growing rapidly, and the need for development of uniform criteria for its detection and definition is recognized.

A series of prior workshops led to the progressive evolution of criteria and guidelines to deal with the emerging information on the subject. The "Amsterdam Criteria" were developed in 1991 to permit the uniform identification of familial clusters of colorectal cancer so that HNPCC families might be recognized and studied (1). In 1995, the Early Detection Branch of the National Cancer Institute convened a workshop to discuss the role of genetic testing for HNPCC. In 1996, a second NCI sponsored workshop entitled "The Intersection of Pathology and Genetics in the Hereditary Nonpolyposis Colorectal Cancer (HNPCC) Syndrome" was held, which led to the development of the "Bethesda guidelines" for testing of colorectal tumors for MSI (2). To facilitate communication among investigators, the Early Detection Branch sponsored a third workshop entitled the "International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition" on December 8-9, 1997, in Bethesda, MD, to define uniform criteria for MSI; to propose technical guidelines for its detection; to review the literature pertaining to the implications of this phenotype; and to develop a research agenda for future research. In particular, the identification of potential areas of clinical application to cancer detection, prognosis, and therapeutic response was a high priority.

MSI was initially described in independent publications from three groups that appeared nearly simultaneously in 1993, and each used unique descriptors for their findings. Thibodeau *et al.* (3) referred to the process as MSI (which they abbreviated MIN), and recognized that tumors with MSI predominately occurred in the proximal colon, were associated with an enhanced survival, and were notable for the absence of "LOH." Peltomaki *et al.* (4) used the term RER phenotype, and the same international collaborating group, Aaltonen *et al.* (5), linked this to a locus that would later yield the first of several DNA MMR genes responsible for HNPCC. Ionov *et al.* (6) referred to the

Received 2/26/98; accepted 9/9/98.

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³ The abbreviations used are: MSI, microsatellite instability; HNPCC, hereditary nonpolyposis colorectal cancer; MMR, mismatch repair; NCI, National Cancer Institute; LOH, loss of heterozygosity; RER, replicative error; MSI-H, high-frequency MSI; MSI-L, low-frequency MSI; MSS, microsatellite stable; FAP, familial adenomatous polyposis; SEER, Surveillance, Epidemiology, and End Results; RER, replicative error.

process as ubiquitous somatic mutations at simple repetitive sequences, linked the process to familial cancer, and made other observations on this novel tumor progression pathway (7). Over the past 5 years, a variety of laboratories have developed their own tools for measuring MSI, and the profusion of approaches has made it difficult to compare results from different groups and has confused investigators attempting to enter the field.

At the 1997 Workshop, ~120 investigators and other interested parties from North America, Europe, Asia, and Australia convened to discuss MSI. The NCI intramural and extramural programs, academia, and biotechnology industry were represented. After a general session that reviewed the state of the art, three "breakout" groups were formed. The first of these was charged with developing a consensus definition of MSI, establishing the criteria for the measurement of MSI, and recommending markers that would facilitate uniformity among those doing research in the field. The second group reviewed the existing published and unpublished data on the natural history of colorectal tumors with MSI, discussed animal models relevant to the study of MSI, and explored whether the presence of MSI carries implications for therapy at this time. The third group was asked to address the issue of MSI in noncolonic tumors and in colorectal (and other) tumors outside of the context of HNPCC. After meeting individually, each of the breakout groups presented their findings to the entire workshop for the development of consensus.

Workshop Summary

Definitions and Technologies

Since its initial description, numerous studies have reported MSI in colorectal cancer and other extracolonic malignancies. Despite the large number of studies, there remains some confusion over how to define this phenomenon, specifically, how many markers should be used, which markers should be used, and how many must display instability before a tumor is defined as having a particular tumor phenotype? Because of the absence of uniform criteria defining this phenomenon, there has been considerable variability in the frequency of MSI reported within a given tumor type. Clearly, if we are to determine the underlying molecular basis of microsatellite instability, as well as potential clinical and pathological associations, it is critical that uniform definitions and criteria be established.

The workshop was established in part to define uniform criteria for the identification and characterization of tumors with MSI. The breakout session "Definition, Correlations, and Technologies," chaired by S. N. T. was charged specifically with this task, as well as discussing other methodological and quality control issues. Overall, there were seven major areas of discussion: Nomenclature, Definition, Methodology, Quality Control, Alternative Strategies, Criteria for Colon *versus* other Malignancies, and Indications for Testing. It is important to note that the discussion and recommendations reported here pertain only to colorectal cancer. As will be discussed later, the applicability of the recommendations to other tumor systems and premalignant states remains uncertain.

Nomenclature. Considerable variability has arisen in the literature as to how to best describe alterations in the length of microsatellites discovered in certain tumors when compared to normal tissue. As a result, there are several different acronyms currently in use. Although there are advantages and disadvantages for each of the different terms, there was general agreement that a single term would be preferred. Proposed terms included: MSI, MIN, or MI (for MSI); RERs, USM (for ubiquitous somatic mutations), and MMP (for the microsatellite mutator phenotype). The majority of participants agreed that MSI was the most appropriate term to describe this phenomenon. It is important to recognize that MSI refers solely to novel length alleles and is

distinct from the observation of LOH or allelic imbalance, in which one of the pre-existing alleles has been lost in the tumor.

Definitions. MSI is defined as a change of any length due to either insertion or deletion of repeating units, in a microsatellite within a tumor when compared to normal tissue. It is important to stress that MSI, as defined, does not describe a particular tumor phenotype but refers only to the observation of instability at a given marker. The definitions and criteria used to define a particular tumor phenotype are discussed below.

Several studies (8–12) have now shown that a subset of colorectal cancer demonstrates the phenomenon of MSI and that such tumors can be divided into several groups. The first is characterized by MSI-H (that is, the majority of markers exhibit MSI); a second group with MSI-L (i.e., only a minority of markers exhibit MSI); and a third group lacking apparent instability (none of the markers exhibit MSI; MSS). Overall, ~60–70% of tumors fall into the group lacking MSI, and the remaining tumors are nearly evenly split between the MSI-H and MSI-L groups. The distinction between tumors demonstrating MSI-H and those demonstrating MSI-L was generally accepted. However, there was considerable debate and controversy regarding the separation of tumors with low frequency *versus* those lacking MSI. Arguments in favor of combining the MSI-L and MSS groups included the fact that the baseline mutation rate for microsatellites in apparently stable colorectal cancers is not precisely known. Second, the distinction between these two groups is highly dependent on both the type and the number of microsatellites analyzed. Given the use of enough markers, it may be that all colorectal cancers will exhibit some level of MSI. With the "reference" panel of five microsatellites suggested below, there may be an insufficient number of markers to conclude with certainty whether or not an apparently stable tumor would demonstrate MSI if an expanded panel were analyzed. Finally, as discussed in another section, differences between MSI-H and MSI-L/MSS but not MSI-L and MSS have been noted with many clinical and pathological parameters, such as tumor site, stage, sex, ploidy, histology, and clinical outcome (8–16). Arguments in favor of the distinction between MSI-L and MSS included the likelihood that a baseline of instability would eventually be established for MSS tumors. Furthermore, given that the cohort of human genes responsible for MSI is not yet fully known and that some of these appear to be associated with more attenuated phenotypes (e.g. *hMSH6*), it may be important to separate those lacking apparent instability from MSI-L, at least in the investigational setting, at this time. Those arguing in favor of the distinction between MSI-L and MSS groups recognized that it is conceivable, in the future, the additional clinical correlation may prove the distinction between MSI-L and MSS to be superfluous.

The next discussion addressed the minimum criteria required to define and distinguish the MSI-H and MSI-L tumor groups. How many and which markers should be used, and how many markers need to demonstrate instability to be placed in the MSI-H group? Critical to the discussion was whether or not markers had been tested for their usefulness in identifying the various tumor phenotypes. Ideally, a given microsatellite sequence should demonstrate MSI frequently (e.g. >80% of the time) for a tumor defined as MSI-H but infrequently (e.g. <20% of the time) for a tumor in the MSI-L group. It was clear that some, but not many, studies examining these specific issues have been performed. Although dinucleotide repeats have been used most frequently to study MSI, instability has also been observed with mono-, tri-, and tetranucleotide repeats (11, 17). The likelihood that a microsatellite will be susceptible to instability may relate to the inherent mutation rate at that locus. For example, some markers that demonstrate very high rates of instability may be desirable markers for clonality (18) but might overestimate the presence of MSI resulting from certain causes such as defective MMR. It was generally

agreed, therefore, that the proper selection of markers was critical for the identification of MSI-L and MSI-H tumor phenotypes and that certain markers should not be used. Several mononucleotide repeats, such as the ones defined by *BAT25*, *BAT26*, and *BAT40* (11, 17, 19), have proven to be very useful for the identification of the MSI-H group of tumors. Instability at these loci appears in the majority of tumors with widespread MSI (*i.e.*, MSI-H) but rarely in tumors defined as MSI-L. Another potential advantage for some of these markers (*e.g.*, *BAT26*) is the presence of a quasimonomorphic profile in populations studied to date (17, 19). The separation of shortened unstable alleles can be clearly distinguished from the normal allele in the majority of cases. Although the availability of matching normal DNA is not an absolute requirement when the germ-line allele size is considered constant in the population, it was recommended that all studies assessing MSI using these markers should continue using matched normal/tumor pairs. This concern was based on a lack of information regarding possible wild-type length variability among different racial and ethnic groups.

On the basis of those studies performed to date, it was clear that many markers could be used to define and distinguish MSI-H from MSI-L/MSS tumors. For the purposes of providing some uniformity in this area, however, the workshop participants agreed to identify a working "reference" panel of markers with guidelines for defining the MSI-H tumor groups (Table 1). The recommended panel validated by Ruschhoff and Fishel (11, 20) is composed of two mononucleotide repeats (*BAT26* and *BAT25*) and three dinucleotide repeats (*D5S346*, *D2S123*, and *D17S250*). Using this reference panel, MSI-H tumors are defined as having instability in two or more markers, whereas MSI-L tumors are defined as having instability in one marker. Tumors showing no apparent instability may either be included in the MSI-L group or the MSS group, with the limitations discussed above. Importantly, historical data suggests that these reference markers will identify those tumors characterized by defective mismatch repair, primarily in the two major mismatch repair genes, *hMSH2* and *hMLH1* (11). If it is important to distinguish MSI-L from MSS tumors, then the evaluation of additional markers may be necessary. If more than five markers are used to identify these particular tumor phenotypes, then the participants recommended that the criteria be modified to assess the percentage of markers demonstrating instability

rather than the absolute number. In this case, the MSI-H group of tumors would be defined as having MSI in ≥ 30 –40% of the markers tested, whereas the MSI-L group would exhibit MSI in < 30 –40% of the markers. Additional microsatellites that have proven to be of use for such studies are shown in Table 1. However, unless problems are encountered or additional information is desired to address specific issues, the use of the "reference marker panel" should be more than adequate to distinguish the MSI-L/MSS group from the MSI-H group of colorectal cancers.

Although these loci are presented as a "working reference panel," the group recognizes that other loci and panels may prove to be of equal utility. Specifically, an argument can be made for choosing markers from the alternate panel, which are in regions displaying high levels of allelic loss (*e.g.*, 17p and 18q), because, if the tumor proves to be MSS, the same assay may provide prognostic information about LOH. This reference panel, therefore is not intended to replace existing markers or panels that have been validated, or to be the only panel available for such studies. Rather, this reference panel provides an opportunity to compare existing markers in the field against which to test the utility of new markers.

As the MSI assay is applied to clinical testing in the future, further analysis of those tumors demonstrating instability in only one or two shifted loci should be considered. This may allow the determination of the validity of studying a limited (*e.g.*, five or fewer) number of markers. Furthermore, such studies may provide more detailed information to determine whether the MSI-L and MSS groups should or should not be separate. Additional data may also help in the identification of the genes responsible for the presence of low-level instability in the MSI-L group.

When results involving MSI are published, the group recommends that as much detail as is feasible should be provided in regard to each tumor and the specific loci shifted. Ideally, it should include a two-dimensional matrix comparing tumors on one axis with the loci tested on the other axis. Such information will be critical for future comparison of new loci and clinical correlation, as well as providing the potential for merging data sets. It may also help eliminate redundant markers and replace them with more informative ones.

Overall, these recommendations are consistent with those being formulated by the International Collaborating Group on HNPCC (ICG-HNPCC), represented by Dr. Hans Vasen, and R. F., and G. N. R. at the workshop. Given the similarities between the two, a single international set of criteria is recommended.

Methodology. In general, a variety of techniques to characterize MSI have been found to be equally effective. A number of studies have shown good correlation of results across multiple sites using different technologies for assessing MSI (20). Instability can be detected in both fresh and paraffin-embedded material, but in either case, it is essential to compare normal to tumor DNA in adjacent lanes.

It is generally recognized that tissue processing should include microdissection to enrich for tumor cell populations. Enriching for tumor cells will enhance the likelihood of detecting novel shifted microsatellites in inherently heterogeneous cell populations. Microdissection should be done in collaboration with an anatomic pathologist for purposes of quality control.

Quality Control. Although they have presented a reference panel here, the group recognizes the need for and encourages the replacement of loci in the current panel with others that have been quality controlled to work on formalin-fixed/paraffin-embedded material and shown to be more sensitive at detecting underlying gene defects resulting in MSI.

When these analyses are performed, a variety of technical and interpretative problems may result at a given locus. One potential

Table 1 International guidelines for evaluation of MSI in colorectal cancer

Reference panel			
Marker	Repeating unit	GenBank accession no.	
<i>BAT25</i>	Mononucleotide	9834508	
<i>BAT26</i>	Mononucleotide	9834505	
<i>D5S346</i>	Dinucleotide	181171	
<i>D2S123</i>	Dinucleotide	187953	
<i>D17S250</i>	Dinucleotide	177030	
Criteria for interpretation			
	5 loci analyzed	>5 loci analyzed	Interpretation
No. of markers	≥2	≥30–40%	MSI-H
Exhibiting instability	1	<30–40%	MSI-L
Length changes	0	0	MSS or MSI-L
Alternative loci ^a			
<i>BAT40</i>	<i>D18S55</i>	<i>D3S1029</i>	<i>D5S107</i>
<i>BAT34C4</i>	<i>D18S58</i>	<i>D10S197</i>	<i>D8S87</i>
<i>TGF-β-RII</i>	<i>D18S61</i>	<i>D13S175</i>	<i>D18S69</i>
<i>ACTC (635/636)</i>	<i>D18S64</i>	<i>D17S588</i>	<i>D13S153</i>
			<i>D17S787</i>
			<i>D7S519</i>
			<i>D20S100</i>

^a Marker information was contributed by J. Ruschhoff, R. Fishel, S. Hamilton, R. Fodde, J. Gilbert, R. Hamelin, I. Kirsch, S. Markowitz, B. Bapat, and S. N. Thibodeau.

problem is PCR failure, in which a locus simply does not amplify. In situations in which one or more PCR failures are present, the need to reanalyze is dependent upon the information obtained from the remaining markers that successfully amplified. For example, a case having one PCR failure, one marker not demonstrating MSI, and three markers demonstrating MSI could be scored as MSI-H, regardless of whether the PCR failure would have been positive or negative for instability (*i.e.*, three or more of five). In the instance in which there is one PCR failure and only one marker positive for MSI, then reamplification would be required because the results of the one missing marker could alter the final interpretation (*i.e.*, one of five for MSI-L or two of five for MSI-H). Although it is not feasible to outline all of the possible combinations here, the participants agreed that scoring should be done in the context of what would be informative for these five markers.

LOH or MSI? Another potential scoring problem may arise when one of the normal alleles for a given marker is missing and no other novel fragments are present for that marker. One cannot easily discern whether this represents true LOH or MSI in which the shifted allele has comigrated with the remaining wild-type allele. In this situation, it is recommended that such a finding for that locus be scored as negative for MSI. Although scoring results in this fashion would appear to bias the data in favor of the MSI-L group of tumors, this does not appear to be a serious problem because most of the tumors in the MSI-H group tend to show MSI with the majority of markers. Furthermore, it was felt that the frequency that a shifted allele would comigrate with the remaining wild-type allele in the absence of other novel fragments would be relatively low. This issue, however, may require further clarification.

LOH and MSI? Conversely, when a specific marker shows both allelic imbalance (apparent LOH) and the presence of novel fragments (MSI), it will be difficult to distinguish whether the apparent allelic loss occurred as a result of the MSI or whether it was truly present in addition to the MSI. In this situation, the participants suggested that those markers showing MSI (novel fragments) should be scored as noninformative for LOH in those tumors exhibiting the MSI-L phenotype, whereas all markers should be scored as noninformative for LOH in those tumors exhibiting the MSI-H phenotype. As before, these recommendations may require further study. In general, markers should be scored as having either LOH and MSI, but not both.

As this assay becomes more widely applied clinically, it will be important to establish a bank of paired genomic DNAs (with tumors representing each of the categories) that can be distributed to laboratories for the purposes of quality control. In some cases, additional markers may be required to properly distinguish MSI-L from MSI-H tumors. Those markers shown in Table 1 are alternative markers that can be used for such purposes.

Alternative Strategies. Immunohistochemistry represents a useful alternative strategy for identifying tumors with defective MMR activity. Several studies reported on the close correlation between tumors displaying MSI-H and the absence of protein expression for either hMSH2 or hMLH1 (11, 12, 21). This technique should be evaluated in more detail to explore the potential utility of this approach. Current data suggest that the combined approach of concurrent testing for MSI and hMSH2/hMLH1 immunohistochemistry may provide the highest detection rate for the MSI-H group of tumors. Additionally, immunohistochemistry provides information on the specific defective gene involved and may, therefore, be cost-effective by limiting the numbers of genes to be sequenced to identify the at-risk individuals in a given kindred.

Criteria for Colorectal versus Other Malignancies. The reference panel is recommended for the characterization of MSI in colorectal cancer only. The utility of these markers in other malignancies,

such as endometrial or gastric cancers, has not yet been fully evaluated. The committee encourages evaluation of this panel in other tumors but fully recognizes that the optimal set of loci to diagnose MSI in cancers other than colorectal cancer is likely to be different. Additionally, the role of defective MMR in other malignancies is still not well understood.

Indications for Testing. The group briefly discussed indications for testing and generally felt that the Bethesda guidelines (2) were reasonable. These criteria suggest that tumors should be tested for MSI in the following situations: (a) individuals with cancer in families that meet the Amsterdam Criteria; (b) individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, small bowel adenocarcinoma, or transitional cell carcinoma of the renal pelvis or ureter); (c) individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma, in which one of the cancers was diagnosed at age <45 years, and the adenoma was diagnosed at age <40 years; (d) individuals with colorectal cancer or endometrial cancer diagnosed at age <45 years; (e) individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cirriiform, defined as poorly differentiated or undifferentiated carcinoma composed of irregular, solid sheets of large eosinophilic cells and containing small gland-like spaces; medullary carcinoma) on histopathology diagnosed at age <45 years; (f) individuals with signet ring cell-type colorectal cancer (composed of >50% signet ring cells) diagnosed at age <45 years; and (g) individuals with adenomas diagnosed at age <40 years.

Clinical Associations and Preclinical Models

The second breakout session, chaired by S. R. H., reviewed the current state of knowledge of clinical associations with MSI and preclinical models of MMR. The participants discussed the clinical importance of MSI and tumor pathology, the natural history and prognosis of patients with MSI+ tumors, and the potential impact of MSI on therapeutic decisions. Additional issues mentioned included interactions of environmental factors with deficient MMR, animal models, and *in vitro* systems that may be useful in research. These presentations were made prior to the development of uniform terminology for MSI, and a variety of criteria and definitions were used by individual groups. The data are presented in the terms used by the investigative group.

Pathology and Clinical Correlates of Tumors with MSI. Dr. Jeremy Jass of the University of Queensland presented a study, performed in collaboration with Dr. Barbara Leggett and S. J. M., that examined the clinical tumor traits of 303 sequential frozen colorectal cancers, according to the MSI status of the tumors. MSI status was based on examination of two mononucleotide markers, three dinucleotide markers, and one tetranucleotide microsatellite marker. Eighty % of tumors showed no MSI. Eleven % showed weak positivity, and 9% showed strong positivity. The weak and negative groups were combined for comparison with the strongly reacting group because the clinical features were similar in the first two groups, except for possibly the Crohn's-like/lymphoid reaction in some tumors. The strongly reacting MSI+ group was significantly different from the MSI- group in terms of proximal colonic location, mucinous and undifferentiated histology, and the presence of tumor-infiltrating lymphocytes. There was a trend toward disease stage without liver metastases and expansile growth pattern. Decision trees were developed to use pathological characteristics to predict MSI status. The combined pathological characteristics had a 93% accuracy for predicting

MSI status. These indicative pathological results deserve prospective investigation.

During the discussion, it was noted that the pathological findings of sporadic and HNPCC tumors with MSI appear to be similar, although there may be a difference in the types of alterations that occur in the microsatellite markers, particularly in sporadic tumors showing infrequent MSI. The issue of MSI+ status in appendiceal tumors was also raised, and it was noted that this has not been studied. The significance of the weak MSI+ tumors remains unclear. This pattern is observed in ulcerative colitis and in serrated adenomas and may reflect the early phases of clonal expansion or minor instability in hyperplasia. The implications may thus be different in cancer and precursor lesions. The pathological characteristics of MSI+ extracolonic tumors as well as the immunopathology are not well defined and require further examination.

Several conclusions came from the presentation and discussion. The pathological observations may be used as a surrogate for MSI+ testing with a high degree of accuracy, although the clinical utility of this approach in identifying HNPCC families and managing MSI+ sporadic tumors needs clarification.

Natural History and Prognosis of Tumors with MSI. Dr. Shozo Baba of Hamamatsu University, presented details of the HNPCC registry in Japan. They found 4104 high-risk families, including 394 colorectal cancer cases that met the Amsterdam criteria. In his personal series, Dr. Baba found 137 extracolonic malignancies and 98 colorectal cancers. Among the extracolonic tumors in the series, gastric cancer was most common in men, and gynecological tumors were the most common in women. Other malignancies were similar in occurrence to series in other countries. Only one-third of non-Amsterdam criteria pedigrees were found to have MMR germ-line mutations. One HNPCC family was found to have an hMSH6 mutation. Most families with HNPCC were not identified by Amsterdam Criteria. MSI+ status was found in 95% of HNPCC cancers, 47% of cancers in families with high risk but not meeting Amsterdam criteria, 36% of patients with multiple cancers, and 13% of sporadic tumors. MSI status was positive in 3% of sporadic adenomas and in all HNPCC adenomas. One uterine tumor was MSI+ and exhibited tumor-infiltrating lymphocytes. The crude 5-year survival of patients with colorectal cancer tumors quoted from a study by Jarvinen was 85% in HNPCC, 53% in FAP, and 43% in sporadic cancers. The corresponding survival figures from Japan were 80, 78, and 64%, respectively. There was no clear consensus over whether a positive germ-line mutation in a DNA MMR gene should lead to a recommendation for prophylactic colectomy. Mutations in the transforming growth factor- β type II receptor gene, the *BAX* gene, and the insulin-like growth factor gene were frequent in HNPCC tumors but not in sporadic MSI cases. In contrast, *K-ras*, *p53*, and *APC* gene mutations were unusual in HNPCC but common in sporadic cases.

Dr. Donald Henson from the NCI presented SEER data on colon cancer. Of note, the SEER registry does not include family history data. The purpose of this study was to ascertain if HNPCC could be identified in the SEER database by determining whether features of HNPCC occurred more frequently than would be expected by chance in sporadic tumors. There were 171,229 cases of colorectal cancer from 1973 to 1994, excluding nonadenocarcinoma cases. Thirty-six % of the cases were proximal colonic (cecum and ascending colon only). Age of diagnosis was <50 years in 5.9%. HNPCC-type histology was present in 10.8%. Multiple primary colon cancers were found in 26.4%. The combination of HNPCC features revealed that 89 cases met the criteria, whereas 106 were expected assuming that all HNPCC cancers would have such features. Thus, SEER cases with features of HNPCC were not overrepresented in the sample. The speakers concluded that the occurrence of these tumor features in the SEER

database did not exceed that expected by chance. It was commented from the audience that the frequency of HNPCC may actually be as low as 1% of colorectal cancer cases.

Dr. Anneka Lindblom from the Karolinska Institute briefly presented data on 190 cases from her institution, indicating that MSI status did not correlate with survival even when diagnostic stage was taken into account.

Group participants concluded that population-based studies to determine the frequency and natural history of MSI+ colonic adenomas, cancers, and extracolonic tumors are needed. Similarly, such studies should be done with an emphasis on precursor lesions in persons with known germ-line mutations of MMR genes. The issue of MSI status as an independent predictor of prognosis for any MSI+ lesions is not resolved.

Therapy. Dr. Stephen Howell at the University of California, San Diego, presented data concerning the effects of chemotherapeutic agents in MMR-deficient cell lines. He first discussed cisplatin sensitivity in MMR-deficient cell lines. Tumor cell lines that were deficient in MMR activity exhibited less sensitivity to cisplatin than matched lines made MMR-proficient by stable chromosomal transfer. This difference was observed for cisplatin and carboplatin but not other platinum-containing drugs. These observations suggest that MMR serves to signal the presence of adducts in tumor DNA and is a functional part of the response to chemotherapeutic agents.

Another set of experiments involving a mixture of MSI+ and MSI- tumor cells, both in cell culture and xenograft animal models, demonstrated that treatment with cisplatin enriched the population for MMR-deficient cells. MMR deficiency appears to account for some of the resistance to cisplatin in patients with ovarian cancer, as evidenced by an increased fraction of MMR-deficient cells in tumor biopsies after treatment. These same chemotherapeutic agents also appear to be capable of causing mutations in MMR-deficient cells at a higher rate than in MMR-proficient tumor cells. Loss of MMR also causes resistance to doxorubicin and etoposide but not 5-fluorouracil in certain MMR-proficient and -deficient cell lines.

During the discussion, it was noted that MMR deficiency may be related to a failure to activate proapoptotic signal transduction pathways. There are some data indicating that radiation sensitivity is decreased in MMR-deficient cells, but this is not yet certain. Tumors with defective MMR remain an important target for chemotherapy; it may be possible to identify drugs that selectively target MMR-deficient cells. The potential of increased toxicity of such agents in patients with germline mutations remains to be clarified. To address these issues, it would be helpful to have truly isogenic MMR proficient and deficient tumor cell lines available.

Environmental and Genetic Interactions with MMR Deficiency. Dr. Russell Jacoby of the University of Wisconsin discussed the factors influencing adenomas in the MIN mouse model of *APC*. He reported that nonsteroidal anti-inflammatory drugs result in a 10-fold decrease in adenomatous polyps in MIN mice and cause a similar regression in FAP patients. He speculated on the role of genetic background (such as *mom1/pla2s* alleles from AKR versus B6 mice), which are associated with a 4-fold difference in polyp number in these rodents. He also reported that a 2-fold difference in adenomas is observed in germ-free animals compared with conventional control animals. The role of these factors in HNPCC and MSI+ tumors has not been examined.

The role of MSI in adenomas was discussed as a predictor of the presence of a germ-line MMR mutation. There is evidence that adenomas with MSI progress more quickly to carcinoma and more often contain foci of cancer. The familial standardized incidence ratio, as a way of measuring familiarity, was correlated with the severity of MSI in colonic neoplasms as a predictor of HNPCC. The familial

standardized incidence ratio ranks the family history relative to the general population mean and incorporates information about the number of cancers, age at diagnosis, and degree of relatedness to the individual tested. This correlation showed that when MSI is more common, family history is stronger. The finding of MSI in adenomas is more specific but less sensitive than finding it in cancers for predicting HNPCC, particularly in smaller adenomas.

In the discussion period, the speculation was raised that HNPCC adenomas may start sporadically, with MMR deficiency being activated after an adenoma is formed.

A number of issues were raised for future research: To what degree are environmental factors involved in the tumor spectrum of HNPCC? How is the declining rate of gastric cancer in HNPCC in the United States related to environmental factors? Why do MSI tumors occur predominantly in the proximal colon? Are MMR-deficient cells more sensitive to mutations induced by environmental mutagenic agents?

In Vitro Models. C. R. B. (workshop chairman) addressed the need for different approaches to *in vitro* studies of MSI. Because gene transfer techniques have been unsuccessful in this setting, human chromosomal transfer has been used. Whole chromosome transfer permits the stable introduction of a physiologically regulated copy of the missing DNA MMR genes, but in addition to the gene of interest, numerous other genes are simultaneously transferred. Families of cell lines have been developed and characterized with the reconstitution of *hMLH1* and *hMSH2*. Studies of these cell lines have revealed that MMR-deficient cells do not respond to DNA damage with the expected G₂-M cell cycle checkpoint arrest. Cells that have had DNA MMR activity restored continue to manifest malignant behavior because they can grow in monolayer culture and nude mice, but they respond to certain types of DNA damage with an appropriate G₂-M cell cycle arrest and cell death. DNA MMR-deficient cells are tolerant of 6-thioguanine, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and 5-fluorouracil when compared with their MMR-proficient counterparts.

MSI in Non-HNPCC, Noncolonic Tumors

The third breakout session, chaired by D. S., addressed the phenomenon of MSI in other settings. MSI has been described across a spectrum of microsatellites and at varying frequencies in other tumor types. Some of these tumors with MSI (*e.g.*, endometrial cancer) occur sporadically in non-HNPCC kindreds and other tumors with MSI are not part of the usual HNPCC spectrum. The classification of these tumors is difficult because the absolute background frequency of a given microsatellite alteration in somatic tissue is largely unknown. Background rates of microsatellite alterations in germ-line tissues have been obtained from germ-line linkage studies and analysis of single sperm (22, 23). In these cells, approximately one new allele is generated at a dinucleotide repeat every 1000 divisions (0.1%) and at tetranucleotide repeats every 30–100 divisions (1–3%). Analysis in peripheral lymphocytes suggests some relaxation of microsatellite replication fidelity occurs in somatic compared to the germline tissues. Analysis of these repeats in primary tumors suggests elevated frequencies of these alterations for a given repeat unit (24, 25). As a group, ~1 in 150 dinucleotides and 1 in 20–30 tri- and tetranucleotides demonstrate an alteration across tumor types. However, reliable studies suggest there is a great difference in these observed frequencies from one marker to another (*i.e.* one dinucleotide compared with a similar dinucleotide) and from one type of cancer to another (*e.g.*, bladder cancer *versus* lung cancer). In addition, investigator variability for microsatellite analysis and potential artifacts (*e.g.*, dilution of malignant cell DNA in heterogeneous tissue specimens containing large numbers of normal cells) and other PCR artifacts may contribute to these differences.

There are two general types of noncolonic non-HNPCC tumors that display elevated frequencies of MSI. The first group, commonly found in gastric (26–46) and endometrial (23, 27, 47, 48) neoplasms, has a similar phenotype to MSI in colorectal cancer and displays instability at dinucleotide and mononucleotide markers and, to a lesser degree, at larger repeats. Like colon cancer, these tumors with a high frequency of MSI (similar to MSI-H) are almost always seen in sporadic cases, and the majority of these cases are not from familial clusters or HNPCC kindreds. Like sporadic cases of colon cancer with MSI-H, they have not been commonly found to have mutations in the known MMR genes (49–55). The reference panel of markers described above (or a similar panel) should be established for the identification and characterization of this group of tumors. The presence of and molecular basis for a non-colonic MSI-L and MSI-H group of tumors remains to be established. The mechanism(s) underlying these phenotypes, such as diminished MMR gene expression or more subtle alterations in the MMR or other pathways, thus remain to be elucidated for these cancers (Refs. 56–66; Table 2).

A second group of noncolonic non-HNPCC tumors displays elevated frequencies of instability only at highly selected tri- and tetranucleotide repeats. These markers have been empirically tested in certain primary tumors to identify shifts that may be useful as clonal markers for cancer detection (25). In certain tumors, larger repeats are more commonly altered than smaller repeats; this finding stands in stark contrast to what is found in HNPCC and most sporadic gastric and endometrial tumors, which have alterations in mono- and dinucleotide repeats at higher frequency. One group of tetranucleotide alterations, (AAAG)_n, seems to be particularly susceptible to these alterations in non-HNPCC tumors of different types, including lung, bladder, and head and neck cancer.⁴ However, the frequency of alterations at any given locus varies substantially from one tumor type to another. Evidence also suggests a binomial distribution of instability at tri- and tetranucleotide markers in primary lung cancer in which a small peak of tumors demonstrates an overall shift rate of >10% at the most susceptible repeats, and there is a large peak at 0–2% of susceptible repeats for most lung cancers. This observation is reminiscent of the binomial distribution for mono- and dinucleotide alterations reported for MSI-H and MSI-L tumors above. This different type of MSI has been termed elevated microsatellite alterations at selected tetranucleotide repeats, to achieve uniformity of classification in future research studies. The mechanism underlying these phenotypes also remains to be elucidated, but the relative absence of overlap between traditional MSI+ and EMAST+ tumors already suggests that a non-MMR pathway may be involved.

In addition to underlying deficiencies in repair mechanisms, it is possible that environmental factors may play a role in generating some microsatellite alterations. The issue of microenvironment has been raised, with a special focus on DNA damage from oxygen radicals or lipid adducts leading to MSI at a microsatellite locus. General environmental exposures, including smoking and diet, were also raised as potential generators of increased microsatellite alterations in tumors, regardless of the endogenous proficiency of repair pathways. These exposures could act alone or in concert with DNA repair pathways to generate the specific phenotypes described in the different tumor types.

Tumors with MSI have been found to potentially inactivate certain target genes by permitting an increased frequency of mutations in short repeat tracts in the DNA encoding the expressed portions of

⁴ D. Sidransky and J. Jen, unpublished observations.

Table 2 Frequency of MSI in noncolonic, non-HNPCC tumors

Tumor	Loci	MSI ^a	Frequency	Ref
Endometrial	Dinucleotide	≥2/8 loci	10/109 (9%)	67
Endometrial	D1S126 D2S393 D3S1067 D5S644 TP53	≥2/5 loci	18/77 (22%)	68
Ovarian		≥2/5 loci	2/68 (3%)	52
Endometrial	Dinucleotide	≥2/8 loci	12/68 (18.5%)	69
Gastric	D2S123 D2S136 D3S1067 D11S922 TP53	≥2/5 loci	15/24 (62.5%)	70
Gastric	AP(delta)3 (A) ₁₈ hMSH2 (A) ₂₆ D1S158 D5S421 D8S199 BAX (G) ₈ hMSH3 (A) ₈ hMSH6 (G) ₈	≥2/5 loci	25/167 (15%)	70
Gastric	Dinucleotide	≥2/6 loci	16/25 ^a (64%)	32
Cervix	Dinucleotide	≥2/30 loci	16/25 ^a (64%) 13/25 ^a (52%) 5/98 (2%) 3/89 (3.3%)	71
Breast	Tetranucleotide			
Skin cancers	Dinucleotide	≥2/12 loci	2/100 (2%)	72
Basal cell	47 loci ^b	≥2 of loci tested		73
Squamous cell			1/47 (2%)	
Melanoma			1/49 (2%)	
Lymphoma			0/41 (0%)	
MALT ^c	D3S1262 D3S1265 D3S11 D3S1261 D6S262	≥2/5 loci	21/40 (52.5%)	74
HIV-positive	Nine loci	≥4/6 (66%)		75
Lung	Dinucleotide	≥2/8 loci	12/35 (34%)	76
Gliomas	D2S123 D3S1067	≥1/2 loci		77
Glioblastomas			5/24 (21%)	
Astrocytomas			2/16 (12.5%)	
Glioblastomas	TGFβRII(A) ₁₀		4/24 (17%)	
Astrocytomas			2/16 (12.5%)	
Glioblastomas	TGFβRII(GT) ₃		0	
Astrocytomas			0	
Prostate	23 loci	>2/23 loci	4/47 (8.5%)	78

^a These 25 tumors had MSI in ≥2/5 loci.^b Not all tumors investigated at all loci.^c MALT, mucosa-associated lymphoid tissue.

these genes. Most of these microsatellite alterations result in frame-shifts that truncate proteins, presumably leading to inactivation of the affected allele. Because MSI affecting repeat tracts within some, but not all, coding regions may represent chance events rather than specific gene targeting, certain criteria are hereby set forth by which to gauge whether an affected gene is a true target of inactivation involved in tumorigenesis. These include: (a) a high frequency of inactivation; (b) biallelic inactivation by simultaneous alteration of the other allele's repeat tract, point mutation, or loss; (c) involvement of the candidate MSI target gene in a bona fide growth suppressor

pathway; (d) inactivation of the same growth suppression pathway in MSI-negative tumors through inactivation of the same gene, or of another gene within the same pathway; and (e) functional suppressor studies in *in vitro* or *in vivo* models, such as cell lines or animals. Several potential target genes have already been identified as listed in Table 3.

Explorations of the genome for additional targets of MSI was thought to hold great potential for elucidating the MMR pathway in human tumorigenesis. Furthermore, the discovery of unstable sequences in the coding regions could help to identify new tumor suppressor genes, as well as to suggest new areas of research into targets of molecular-based anticancer therapy or cancer prevention.

MSI at any locus represents a potential clonal marker for the detection of cancer (25). Although these alterations at anonymous repeat tracts may not provide any growth advantage to neoplastic cells, they are still carried and propagated as genetic material is passed on to daughter cells during clonal propagation. Recent pilot studies in bladder cancer indicate the potential for use of these alterations in cancer detection and monitoring (16, 116). In combination with LOH studies, the majority of bladder cancers were identified by microsatellite analysis of the urine. In three cases, microscopic tumors were identified before direct visualization by cystoscopy (16). These markers may also be useful in the detection of other tumor types by analysis of bodily fluids and can also be found at lower frequency in the serum or plasma of cancer patients (117, 118). These markers may also provide useful prognostic information in addition to establishing the diagnosis in tested patients. Further characterization of these markers in preneoplastic lesions would help establish their role in detection of preclinical lesions for preventive approaches.

Future studies should focus on better cataloging and characterization of microsatellite alterations (including length and type of alteration) in normal tissues and cancers. Precise documentation will help define accurate phenotypes that may eventually lead to novel biological and mechanistic insights. Correlation of these phenotypes in the various MSI groups with emerging molecular events and clinical-pathological data needs to be carried out. To ensure optimal use of this knowledge, a database will need to be set up to collect, store, and disseminate information about the rate and frequencies of MSI in different tumor types. The database will be limited if we do not develop optimal high-throughput assays for running and interpreting microsatellite assays.

To better decipher complex phenotypes and establish parameters for testing different repair pathways, we need to develop new eukaryotic models for MSI and genomic instability. In addition, we need to pursue the establishment of mammalian cell lines and transgenic animals that demonstrate microsatellite or genomic instability. These systems are mandatory as resources for elucidating the underlying mechanisms of various types of instability. In addition, they will be valuable resources for testing novel therapeutic approaches in various tumor types with distinct instability phenotypes.

It may be appropriate to initiate a massive screen of known cDNAs

Table 3 Mutated genes in tumors with MSI

Putative gene target	Frequency	Biallelic inactivation	Suppressor pathway	Inactivated in MSS	Functional data	Refs.
TGF-β type II receptor	High (colon)	Yes	Yes	Yes	Yes	28, 29, 77, 79–95
IGF-IIR ^a	Moderate	Yes	Yes	Yes	Yes	96–106
Bax	High	No	Unclear	No	No	70–107
MSH3/MSH6	Moderate	Yes	Yes	No	No	70, 108–110
PTEN (MMAC1)	High	Yes	Yes	Yes	Yes	111, 112
APC	Moderate	Yes	Yes	Yes	Yes	113, 114
β2-microglobulin	Low	No	No	No	No	115

^a IGF-IIR, insulin-like growth factor type II receptor.

with repetitive DNA tracts in multiple tumor types to identify potential target genes involved in the pathogenesis of MSI tumors. This may be viewed as a gene discovery tool to identify important pathways relevant to neoplasms with instability and many other tumor types. In addition to the above criteria, more definitive assays to confirm the involvement of these new gene targets in tumorigenesis would be useful.

Recommendations for Future Research Initiatives

The identification of colorectal cancer patients with MSI-H tumors is important because: (a) some have germline mutations in a mismatch repair gene which has implications for patient management and family members; and (b) the biology of MSI-H tumors differs from that of MSI-L tumors and those without MSI. At the present time, the workshop members are of the opinion that MSI testing has one clinical purpose: to identify patients with HNPCC. The Bethesda guidelines specify which tumors should be tested for this purpose, including criteria based on age, pathological characteristics, family history and the presence of extracolonic tumors.

For research purposes, studies of MSI should address several areas, as follows: (a) Prognosis. The use MSI-H tumor status as an independent prognostic factor in tumors of the colorectum and extracolonic sites in both the sporadic and HNPCC settings requires further investigation. Several lines of evidence relevant to the area of prognosis were discussed. The natural history and response to therapy of MSI-H tumors appear to be different than the other tumors. *In vitro* studies have shown chemoresistance to some agents (e.g., cisplatin) and decreased radiosensitivity, whereas anecdotal clinical reports have suggested improved response to therapy in some settings (e.g., radiotherapy for glioblastoma multiforme in Turcot syndrome; Ref. 119). Defective MMR also represents a potential for novel therapeutic agents. Finally, the pathology of MSI-H tumors characterized by tumor-infiltrating lymphocytes and a Crohn's-like lymphoid response raised the possibility of evaluating immunomodulating agents, such as levamisole. Further examination of prognosis in patients with MSI-H tumors and design of clinical trials is needed. (b) Prevention. Identification of environmental factors which interact with defective MMR in producing MSI-H tumors and studies of chemopreventive agents are needed. (c) Biology of tumors. Further characterization of the biology of both colonic and extracolonic neoplasms is needed, including information on growth rates, progression to malignancy in premalignant lesions, and metastasis. (d) Testing strategies. The utility of immunohistochemistry for defining the expression of *hMSH2* and *hMLH1* gene products needs confirmation. It was suggested that this may be used to supplement MSI testing. Algorithms for efficient characterization of MSI-H in sporadic and HNPCC tumors should be developed and validated in prospective clinical studies. Strategies for distinguishing HNPCC from other inherited predispositions, including attenuated FAP and *APCII307K* premutation in Ashkenazi Jews are needed. (e) High-risk clinics and registries. The continued development of high-risk clinics and registries is encouraged. These can provide optimal identification, education, genetic testing, and management of those with HNPCC. They also serve as a vital resource for basic and clinical investigation of this condition. (f) Preclinical models. Further development and characterization of animal models and *in vitro* systems, including yeast and cell lines, would be of great help in addressing the above issues. In addition, the preclinical models can direct translational application of functional tests for defective MMR. (g) Nomenclature. It is suggested that the name HNPCC be changed to a more appropriate name in view of present genetic knowledge. One suggestion was the "hereditary MMR deficiency syndrome;" however, no consensus was reached on this issue.

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ATTACHMENT 4A

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530	__ aAvailable also online.
550	__ aOfficial organ of the American Association for Cancer Research.
555	__ aVols. 1-10, 1941-50. 1 v; Vols. 11-25, 1951-65. 1 v; Vols. 26-30, 1966-70. 1 v.
583	__ 3 Vol. 55, no. 7 (APR1 1995) c 19950519 f Notice not published l Printed on acid-free paper x Further review is required to determine exactly when acid-free paper was first used for this title
650	12 aNeoplasms
650	22 aNeoplasms x therapy

650	22 a Neoplasms, Experimental
655	_2 a Periodical
710	2_ a American Association for Cancer Research.
770	0_ t Cancer research. Supplement g 1953-56 x 0576-6656 w (OCOLC)02258973 w (DNLM)0373144
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998	__ a ERMSLC 117978 b 20100526
999	__ a AUTH
035	__ a (DNLM)C05900000(s)
035	__ a (OCOLC)01553285

ATTACHMENT 4B

Neoplasms MeSH Descriptor Data 2025

[Details](#)[Qualifiers](#)[MeSH Tree Structures](#)[Concepts](#)**MeSH Heading**

Neoplasms

Tree Number(s)

C04

Unique ID

D009369

RDF Unique Identifier<http://id.nlm.nih.gov/mesh/D009369>**Annotation**

general; prefer specifics; familial: consider also NEOPLASTIC SYNDROMES, HEREDITARY; metastatic cancer of unknown origin: index NEOPLASM METASTASIS

Scope Note

New abnormal growth of tissue. Malignant neoplasms show a greater degree of anaplasia and have the properties of invasion and metastasis, compared to benign neoplasms.

Entry Version

NEOPL

Entry Term(s)

Benign Neoplasm

Benign Neoplasms

Cancer

Malignancy

Malignant Neoplasm

Malignant Neoplasms

Neoplasia

Neoplasm

Neoplasms, Benign

Tumor

Tumors

Consider Also

consider also terms at CANCER, CARCINO-, ONCO-, and TUMOR

Public MeSH Note

/diagnosis was NEOPLASM DIAGNOSIS 1964-65; /etiology was NEOPLASM ETIOLOGY 1964-65; /immunology was NEOPLASM IMMUNOLOGY 1964-65; /radiotherapy was NEOPLASM RADIOTHERAPY 1964-65; /therapy was NEOPLASM THERAPY 1964-65; NEOPLASM STATISTICS was heading 1964-65; CARCINOGENESIS was heading 1977

History Note

/diagnosis was NEOPLASM DIAGNOSIS 1964-65; /etiology was NEOPLASM ETIOLOGY 1964-65; /immunology was NEOPLASM IMMUNOLOGY 1964-65; /radiotherapy was NEOPLASM RADIOTHERAPY 1964-65; /therapy was NEOPLASM THERAPY 1964-65; NEOPLASM STATISTICS was heading 1964-65; CARCINOGENESIS was heading 1977

Entry Combination

secondary:Neoplasm Metastasis

Date Established

1966/01/01

Date of Entry

1999/01/01

Revision Date

2021/06/30

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ATTACHMENT 4C

Neoplasms, Experimental MeSH Descriptor Data 2025

[Details](#)[Qualifiers](#)[MeSH Tree Structures](#)[Concepts](#)**MeSH Heading**

Neoplasms, Experimental

Tree Number(s)

C04.619

E05.598.500.496

Unique ID

D009374

RDF Unique Identifier

<http://id.nlm.nih.gov/mesh/D009374>

Annotation

IM general only; not a routine coordinate for specific neoplasms in experimental animals; policy: Manual 24.5+; /chem ind: Manual 24.5.6+; spontaneous: Manual 24.5.5

Scope Note

Experimentally induced new abnormal growth of TISSUES in animals to provide models for studying human neoplasms.

Entry Version

NEOPL EXPER

Entry Term(s)

Experimental Neoplasms

NLM Classification #

QZ 206

See Also

Neoplasm Transplantation

Tumor Virus Infections

Date Established

1966/01/01

Date of Entry

1999/01/01

Revision Date

2004/07/13

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Clinical implications of microsatellite instability in sporadic colon cancers

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Current Opinion in Oncology 2009, 21:369–373

Purpose of review

To review data demonstrating the prognostic and predictive impact of microsatellite instability (MSI) in human colon carcinomas.

Recent findings

MSI is a molecular marker of defective DNA mismatch repair that is detected in approximately 15% of sporadic colon cancers. Most, but not all retrospective studies, have shown that colon cancers with MSI have better stage-adjusted survival rates compared with non-MSI tumors. Furthermore, analyses of colon cancers from participants in randomized adjuvant therapy trials have suggested that MSI tumors do not benefit from treatment with 5-fluorouracil. Recent studies, including a pooled analysis, validate prior data demonstrating the prognostic and predictive impact of MSI status in colon cancer.

Summary

MSI is a molecular marker that can provide valuable prognostic and predictive information in colon cancer patients. In the appropriate clinical setting, MSI data can be used in clinical decision-making. Specifically, the favorable outcome of stage II colon cancers with MSI indicates that such patients should not receive adjuvant chemotherapy. Although data for stage III colon cancers with MSI suggest a lack of benefit from 5-fluorouracil alone, the benefit of the current standard treatment, 5-fluorouracil, leucovorin, and oxaliplatin, in this subgroup remains unknown and awaits further study.

Keywords

colon cancer, microsatellite instability, prediction, prognosis

Curr Opin Oncol 21:369–373
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1040-8746

Introduction

Although surgical tumor staging remains the key determinant of colorectal cancer (CRC) prognosis and treatment, there is considerable stage-independent variability in clinical outcome underscoring the need for robust prognostic and predictive markers to guide the use of adjuvant chemotherapy. Approximately 15% of sporadic colon cancers develop via an alternative pathway of tumorigenesis characterized by microsatellite instability (MSI), a molecular marker of defective function of the DNA mismatch repair (MMR) system [1•,2]. Multiple retrospective studies [3–6], as well as a population-based study [7] and meta-analysis [8], have shown that patients whose colon cancers exhibit MSI, that is, MSI-high-frequency (MSI-H) tumors [9], have a more favorable stage-adjusted prognosis compared with patients with non-MSI-H tumors. Evidence also suggests that patients with MSI-H colon cancers do not benefit from 5-fluorouracil (5-FU)-based adjuvant therapy in contrast to those with non-MSI-H tumors [8,10–13]. Despite these results, some studies have failed to show an impact of MSI status upon patient outcome [14•] or suggest a better outcome for non-MSI tumors

[15,16]. Recently, a retrospective analysis was reported that has validated the prognostic and predictive impact of MSI-H in colon cancers from participants in randomized adjuvant therapy trials [17••]. It is our objective to highlight the importance of recognizing the MSI-H phenotype in clinical practice and to discuss its implications for clinical decision-making, including the use of adjuvant chemotherapy.

Sporadic microsatellite instability-high-frequency colon cancers

MSI-H can be detected in nearly all cases of Lynch syndrome and in about 15% of sporadic colon cancers [1•]. Whereas Lynch syndrome is due to germline mutations in MMR genes, sporadic colon cancers develop MSI-H due to inactivation of the *hMLH1* gene by DNA methylation, an epigenetic and nonheritable event [1•,18,19••]. Of approximately 108 000 incident colon cancers that were diagnosed in the United States in 2008 [20], 16 000 are expected to be MSI-H tumors. Sporadic MSI-H colon cancers and those from patients

with Lynch syndrome share distinct clinical and pathological features that include proximal colon predominance, poor differentiation with or without mucinous histology, a marked peritumoral lymphocytic infiltration, wild-type *p53* status, and diploid DNA content [3–5,11,21,22]. In contrast to Lynch syndrome cases, sporadic MSI-H cancers are distinct epidemiologically in that they are associated with older age at diagnosis, female sex, and with cigarette smoking [19^{••},22,23]. MSI-H status has been shown to be very rare in rectal carcinomas [24,25].

Whereas Amsterdam and Bethesda criteria were developed to identify patients with Lynch syndrome [26,27], established criteria to identify sporadic colon cancers with MSI-H are lacking and the clinician must rely upon clinical and pathological features. MSI testing can be performed in paraffin-embedded tumor tissues using a polymerase chain reaction (PCR)-based assay for detection of instability at selected microsatellite loci [2,9]. CRCs are categorized according to the extent of MSI exhibited as high frequency (MSI-H), low frequency (MSI-L) or microsatellite stable (MSS) [9]. MSS tumors show no instability at any loci that is typical of most sporadic CRCs that show chromosomal instability [1[•]]. Nearly all studies have shown that MSI-L and MSS colon cancers have similar clinicopathological features and clinical outcome; therefore, they are generally grouped together [4,5,9,14[•],22,28]. Analysis of tumor tissue for MMR protein expression by immunohistochemistry (IHC) is a complementary or alternative test whose results are highly concordant with MSI testing [6,29]. IHC will reveal loss of the protein product of MMR genes of which only hMLH1 loss has been described in sporadic MSI-H colon cancers [18,19^{••},22]. Therefore, this test can suggest Lynch syndrome if loss of any protein besides hMLH1 is found. Importantly, neither IHC nor MSI indicates the mechanism of the MMR defect, namely germline mutation versus epigenetic inactivation. Accordingly, these data must be interpreted within the relevant clinical context, which dictates whether any additional testing and/or genetic evaluation/counseling is warranted.

Prognostic impact of microsatellite instability phenotype

When MSI was discovered in CRCs, it was noted that patients with MSI-H tumors had better survival rates compared with those with non-MSI-H (i.e., MSS, MSI-L) tumors [2]. MSI-H was also found to be associated with lower tumor stage at diagnosis [30] and is rare in metastatic CRCs [31,32]. Subsequently, several retrospective case studies as well as a population-based study [7] found that MSI-H colon cancers have a more favorable survival compared with non-MSI-H tumors that was independent

of tumor stage [4–6,11,30]. Similar results were found among participants in phase III adjuvant trials, in which patients with MSI-H stage II and III colon cancers not treated with chemotherapy showed significantly better overall survival rates compared with untreated patients with non-MSI-H tumors [3,10]. Not all studies [14[•],33], however, have shown a prognostic impact for MSI-H, including a cooperative group trial whereby no survival differences were found for patients with MSI-H versus those with non-MSI-H colon cancers [14[•]]. Several factors may account for these differences, including an insufficient sample size as MSI-H tumors represent a relatively small subset, and that the magnitude of the MSI-H prognostic effect is modest. Furthermore, selection bias is a potential confounder in retrospective case series including adjuvant clinical trials, in which tissue availability has generally been limited to a patient subset. In addition, the microsatellite markers used to detect MSI-H cases have not been uniform, which can result in false positive results that can dilute the prognostic effect [9]. Despite these limitations, the prognostic impact of MSI status is strongly supported by a meta-analysis of 32 studies including 1277 MSI-H CRCs that demonstrated a 35% reduction in the risk of death for patients with MSI-H colon cancers compared with those with non-MSI-H tumors [8]. This analysis included untreated patients as well as patients treated in phase III randomized, 5-FU-based adjuvant therapy trials. The observed survival benefit of MSI-H was maintained when restricting the analysis to clinical trial participants.

In a recent study aimed at validating the prognostic and predictive impact of MSI status, stage II and III colon cancers from participants in North American and European adjuvant therapy trials were analyzed for MSI or MMR protein expression [17^{••}]. MMR expression, determined by IHC, has been shown to be highly concordant with MSI test results [29] and confers similar prognostic information [4,6]. After pooling of studies and considering only patients not receiving chemotherapy ($n = 515$), tumors with MSI-H or loss of an MMR protein showed a 49% reduction in disease-free survival compared with non-MSI-H cases or those with intact protein expression [17^{••}]. These results from an independent dataset confirm prior studies demonstrating the prognostic impact of MSI-H. To date, the exact mechanism underlying the better outcome of MSI-H colon cancers remains unknown and awaits further study. However, the pronounced lymphocytic infiltration seen in these cancers may contribute to their better prognosis [34[•],35,36].

Predictive role of microsatellite instability

Human CRC cell lines with MSI-H display resistance to compounds that induce DNA damage, including 5-FU

[37]. Importantly, resistance to 5-FU was overcome by restoring normal DNA MMR function in CRC cells [38]. As predicted by the preclinical data, retrospective studies have suggested that patients with sporadic MSI-H colon cancers did not derive benefit from 5-FU-based chemotherapy in contrast to those with non-MSI tumors [8,10–13]. Among these reports is a study of 256 MSI-H colon cancer patients from three institutions in Italy, whereby 5-FU-based chemotherapy failed to improve survival in these patients [11]. Further support derives from adjuvant therapy trials, whereby patients with MSI-H tumors (stage II and III) did not benefit from 5-FU-based chemotherapy, whereas patients with non-MSI cancers demonstrated a significant survival advantage in favor of treatment [10]. In a study in which stage II and III CRC patients received adjuvant chemotherapy and were followed prospectively, the survival benefit of 5-FU treatment was again limited to non-MSI tumors or those with intact MMR proteins [12]. Extended follow-up of this cohort has confirmed these earlier findings [39[•]]. Despite these data, the predictive utility of MSI has generated much controversy, given that some retrospective studies have failed to demonstrate a predictive impact of MSI [14[•],33] and other reports [15,16,40] have shown that patients with MSI colon cancers receive a greater benefit from 5-FU-based adjuvant treatment than do non-MSI patients. In one of these reports, patients receiving 5-FU were on average 13 years younger than those who did not [16]. In addition to potential selection bias, another issue is the need to compare treated with untreated MSI cases given the prognostic impact of MSI. Another report evaluating the predictive value of MSI-H in colon cancers from adjuvant therapy trials failed to confirm that MSI testing can predict response to 5-FU-based chemotherapy [14[•]]. As previously stated, potential explanations include retrospective study design, limited statistical power to detect survival differences, and technical issues related to defining MSI-H status.

Importantly, a meta-analysis of retrospective data from patients with tumor node metastasis (TNM) stage II and III colon cancers enrolled in randomized clinical trials found that MSI-H tumors do not derive benefit from adjuvant 5-FU compared with non-MSI-H cases [8]. A study was recently reported at the 2008 American Society of Clinical Oncology (ASCO) meeting, in which the effect of 5-FU in relation to MSI status was determined in pooled data from stage II and III colon cancer patients treated in randomized adjuvant studies from North America and Europe. This study confirmed that patients whose tumors showed MSI-H or loss of MMR protein expression did not benefit from 5-FU compared with those patients randomized to untreated control arms [17^{••}].

Implications of microsatellite instability for adjuvant therapy

Since publication of the ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer [41], further evidence has accumulated confirming the prognostic utility of MSI status in colon cancer patients and more recently, validating its predictive impact for 5-FU-based adjuvant therapy [3,6,12,17^{••}]. Although nearly all of the data are from retrospective analyses, results from more recent, large studies are concordant and statistically robust. These studies demonstrate that MSI-H is a favorable prognostic marker and that MSI-H tumors do not benefit from adjuvant 5-FU. A prospective phase III study in stage II colon cancer patients is being conducted by the Eastern Cooperative Oncology Group (ECOG 5202), whereby MSI status and chromosome 18q allelic imbalance (an adverse prognostic marker in some colon cancer studies [42,43]) are determined in tumor specimens. Patients are categorized into low-risk (MSI-H or 18q intact) or high-risk (non-MSI-H or 18q allelic imbalance) groups. In this trial, low-risk patients receive observation, whereas high-risk patients are treated with 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX) with or without bevacizumab. Although this study aims to provide definitive information regarding the influence of these molecular markers on clinical outcome, the trial will not provide needed prospective data as to responsiveness of MSI-H tumors to FOLFOX therapy, as no MSI cases are being treated. This clinical trial is currently accruing patients and will require several years for the data to mature.

Although prospective studies remain the ‘gold standard’ for validating prognostic and predictive biomarkers, circumstances exist in which the preponderance of evidence is consistent and in which the results have been validated in an independent dataset [17^{••}]. We regard this situation to be the case for the use of MSI status to inform patient prognosis and to influence treatment decisions in stage II colon cancer patients. Similarly, the predictive utility of *K-ras* mutational status for responsiveness to antibodies against the epidermal growth factor receptor (EGFR) has been consistently shown in retrospective analyses and these data have led to changes in clinical practice [44]. We believe that MSI testing can provide useful prognostic information in CRC patients and inform decision-making with regard to adjuvant therapy. The use of adjuvant chemotherapy in patients with stage II colon cancer remains controversial [45[•],46,47] and represents a dilemma for medical oncologists with regard to the potential benefits versus risks of treatment. It is estimated that one-third of all stage II patients receive adjuvant therapy in the United States. We believe that the strength of the available evidence is sufficient at the present time to recommend that MSI-H stage II colon cancer patients not receive adjuvant chemotherapy given

that such tumors have a favorable prognosis and exhibit resistance to 5-FU. This recommendation is evidence-based and can spare such patients unnecessary treatment-related toxicities and reduced quality of life during chemotherapy. We emphasize that non-MSI-H status by itself does not indicate a high-risk stage II tumor nor does it provide a rationale for adjuvant chemotherapy. The majority (~85%) of stage II CRCs are non-MSI-H tumors and decisions regarding adjuvant therapy in these patients should be individualized. In stage III disease, however, the issue is more complex. Standard adjuvant therapy for stage III colon cancer is the FOLFOX regimen [48] and the predictive impact of MSI status upon the chemosensitivity to FOLFOX is unknown. At present, the available data do not justify the exclusion of MSI-H stage III colon cancer patients from receiving FOLFOX chemotherapy despite the fact that MSI-H tumors demonstrate resistance to 5-FU. We regard the impact of MSI status upon responsiveness to FOLFOX as a critical research question that awaits further evaluation. Clearly, MSI status needs to be taken into account in future adjuvant therapy trials in CRC.

As previously stated, sporadic MSI-H colon cancers are associated with proximal tumor site, poor differentiation, older age at diagnosis, and with female sex [3,4,19^{••},21,22]. We and others [49] are developing a model to predict the likelihood of MSI-H colon cancers using clinically available data. To identify MSI-H CRC patients in clinical practice and to utilize such information for clinical decision-making at this time, we recommend that MSI or IHC testing for defective DNA MMR testing be performed on all resected, node-negative nonmetastatic cancers of the proximal colon, wherein adjuvant therapy is a consideration. An advantage of MSI testing is that it does not identify the suspected mutated gene and is therefore not a genetic test but is a clinical test that can be utilized to influence patient management. An argument can be made that the cost of MMR testing may be offset by more efficient use of adjuvant chemotherapy.

Conclusion

Accumulated evidence demonstrates the prognostic and predictive utility of MSI status, including a recent study validating the clinical utility of defective MMR (MSI-H or loss of MMR protein expression) in colon cancers from patients treated in adjuvant therapy trials [17^{••}]. We believe that the current evidence is sufficient to utilize MSI results for prognosis and to influence clinical decision-making regarding the use of adjuvant chemotherapy in stage II colon cancer patients. At present, we restrict our recommendations to stage II patients. While patients with stage III MSI-H tumors have a better prognosis and may not benefit from 5-FU, data regarding the benefit of FOLFOX in such patients are lacking.

Therefore, all stage III patients should continue to receive standard adjuvant chemotherapy as of this writing. An analysis of MMR status in colon cancers from completed adjuvant studies that evaluated FOLFOX, for example, the Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) [45[•]], as well as ongoing adjuvant studies (NSABP C-08, NCCTG N0147) could shed further light on this issue. Going forward, prospective clinical trials are needed to evaluate non-5-FU-based regimens in patients with MSI-H stage III colon cancers. In summary, MSI is an important molecular marker of tumor biology that can provide clinically useful information and represents one further step toward individualized cancer therapy.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 391).

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ATTACHMENT 5A

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ATTACHMENT 5B

Neoplasms MeSH Descriptor Data 2025

[Details](#)[Qualifiers](#)[MeSH Tree Structures](#)[Concepts](#)**MeSH Heading**

Neoplasms

Tree Number(s)

C04

Unique ID

D009369

RDF Unique Identifier<http://id.nlm.nih.gov/mesh/D009369>**Annotation**

general; prefer specifics; familial: consider also NEOPLASTIC SYNDROMES, HEREDITARY; metastatic cancer of unknown origin: index NEOPLASM METASTASIS

Scope Note

New abnormal growth of tissue. Malignant neoplasms show a greater degree of anaplasia and have the properties of invasion and metastasis, compared to benign neoplasms.

Entry Version

NEOPL

Entry Term(s)

Benign Neoplasm

Benign Neoplasms

Cancer

Malignancy

Malignant Neoplasm

Malignant Neoplasms

Neoplasia

Neoplasm

Neoplasms, Benign

Tumor

Tumors

Consider Also

consider also terms at CANCER, CARCINO-, ONCO-, and TUMOR

Public MeSH Note

/diagnosis was NEOPLASM DIAGNOSIS 1964-65; /etiology was NEOPLASM ETIOLOGY 1964-65; /immunology was NEOPLASM IMMUNOLOGY 1964-65; /radiotherapy was NEOPLASM RADIOTHERAPY 1964-65; /therapy was NEOPLASM THERAPY 1964-65; NEOPLASM STATISTICS was heading 1964-65; CARCINOGENESIS was heading 1977

History Note

/diagnosis was NEOPLASM DIAGNOSIS 1964-65; /etiology was NEOPLASM ETIOLOGY 1964-65; /immunology was NEOPLASM IMMUNOLOGY 1964-65; /radiotherapy was NEOPLASM RADIOTHERAPY 1964-65; /therapy was NEOPLASM THERAPY 1964-65; NEOPLASM STATISTICS was heading 1964-65; CARCINOGENESIS was heading 1977

Entry Combination

secondary:Neoplasm Metastasis

Date Established

1966/01/01

Date of Entry

1999/01/01

Revision Date

2021/06/30

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Mechanisms and functions of DNA mismatch repair

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DNA mismatch repair (MMR) is a highly conserved biological pathway that plays a key role in maintaining genomic stability. The specificity of MMR is primarily for base-base mismatches and insertion/deletion mispairs generated during DNA replication and recombination. MMR also suppresses homeologous recombination and was recently shown to play a role in DNA damage signaling in eukaryotic cells. *Escherichia coli* MutS and MutL and their eukaryotic homologs, MutSa and MutLa, respectively, are key players in MMR-associated genome maintenance. Many other protein components that participate in various DNA metabolic pathways, such as PCNA and RPA, are also essential for MMR. Defects in MMR are associated with genome-wide instability, predisposition to certain types of cancer including hereditary non-polyposis colorectal cancer, resistance to certain chemotherapeutic agents, and abnormalities in meiosis and sterility in mammalian systems.

Keywords: MutS, MutL, microsatellite instability, cancer

Cell Research (2008) 18:85-98. doi: 10.1038/cr.2007.115; published online 24 December 2007

Introduction

DNA damage accumulates in cells over time as a result of exposure to exogenous chemicals and physical agents (i.e., benzo[a]pyrene, polychlorinated biphenyls, dioxin, cigarette smoke, asbestos, ultraviolet light, radon), as well as endogenous reactive metabolites including reactive oxygen and nitrogen species (ROS and NOS). Another source of DNA damage is errors that occur during normal DNA metabolism or aberrant DNA processing reactions, including DNA replication, recombination, and repair. Nucleotide misincorporation generates DNA base-base mismatches during DNA synthesis at variable rates, depending on many factors, including the specific DNA polymerases. In general, the replicative DNA polymerases have relatively high replication fidelity (see McCulloch and Kunkel, this issue), while translesion DNA polymerases, which specifically bypass sites of DNA damage, have lower replication fidelity (see Andersen *et al.* and Gan *et al.* in this issue). DNA damage, if unrepaired, has the potential to generate mutations in somatic or germline cells, which can alter cellular phenotype and cause dysfunction and disease. To prevent such deleterious effects and safeguard the integ-

egrity of the genome, cells possess multiple mechanisms to repair DNA damage and thus prevent mutations. One such system is the critical pathway known as DNA mismatch repair (MMR).

MMR corrects DNA mismatches generated during DNA replication, thereby preventing mutations from becoming permanent in dividing cells [1-3]. Because MMR reduces the number of replication-associated errors, defects in MMR increase the spontaneous mutation rate [4]. Inactivation of MMR in human cells is associated with hereditary and sporadic human cancers [1, 3, 5], and the MMR system is required for cell cycle arrest and/or programmed cell death in response to certain types of DNA damage [6, 7]. Thus, MMR plays a role in the DNA damage response pathway that eliminates severely damaged cells and prevents both mutagenesis in the short term and tumorigenesis in the long term.

The prototypical *Escherichia coli* MMR pathway has been extensively studied and is well characterized both biochemically and genetically. Thus, *E. coli* MMR is a useful and important framework for understanding eukaryotic MMR. In this review, the biochemistry and genetics of *E. coli* MMR will be described briefly by way of introduction, and the remainder of the discussion will focus on the cellular functions of MMR and their roles in cancer avoidance in mammalian cells. For areas of research on human MMR not discussed in this paper or an additional discussion of MMR in other species, readers are referred to the following

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excellent reviews: [1-3, 8-11].

Mechanism of mismatch correction

DNA MMR in E. coli

E. coli MMR requires the following protein components: MutS, MutL, MutH, DNA helicase II (MutU/UvrD), four exonucleases (ExoI, ExoVII, ExoX, and RecJ), single-stranded DNA binding protein (SSB), DNA polymerase III holoenzyme, and DNA ligase [12, 13]. MutS, MutL, and MutH initiate MMR and play specialized biological roles in MMR in *E. coli*.

MutS recognizes base-base mismatches and small nucleotide insertion/deletion (ID) mispairs, and thus MutS has been called the “mismatch recognition” protein [3]. MutS possesses intrinsic ATPase activity. High-resolution structures of MutS bound to DNA have been determined by X-ray crystallography [14, 15]. These structures revealed that MutS binds to a mismatch as a homodimer. Interestingly, the mismatch-binding site is comprised of sequence-wise identical but structurally and functionally different domains from the two subunits, indicating asymmetry in the protein-DNA complex. Hence, the MutS homodimer acts as a virtual heterodimer when bound to a DNA mismatch. This characteristic is mimicked by eukaryotic MutS homologs (MSH), which function as heterodimers instead of homodimers (see below). MMR in *E. coli* is ATP-dependent, and requires the functional MutS ATPase.

MutL interacts physically with MutS, enhances mismatch recognition, and recruits and activates MutH. Defects in MutL completely inhibit MMR in *E. coli*. Despite the fact that a functional human MutL homolog, MutL α , possesses an endonuclease activity that is essential for mammalian MMR [16], no hydrolytic activity has been detected in MutL. However, MutL may play a role as a molecular matchmaker that facilitates assembly of a functional MMR complex [3, 17], because it stimulates the loading and the processivity of helicase II (or UvrD) at the MMR initiation site [18, 19]. Like MutS, MutL functions as a homodimer and possesses ATPase activity [20]. Mutations in the ATP-binding domain lead to a dominant negative mutator phenotype [21]. MutL mutants that are defective in ATP hydrolysis but proficient in ATP binding can activate MutH but cannot stimulate MutH in response to a mismatch or MutS, suggesting that ATP hydrolysis by MutL is essential for mediating the activation of MutH by MutS [22]. Recent studies show that MutL interacts physically with the clamp loader subunits of DNA polymerase III [23, 24], suggesting that MutL may promote binding of DNA polymerase III to MMR intermediates. These observations suggest that MMR is coupled with DNA replication.

In *E. coli*, DNA is methylated at the N6 position of

adenine in dGATC sequences. In replicating DNA, the daughter strand is transiently unmethylated, and it is the presence of hemimethylated dGATC sequences that molecularly distinguishes the newly synthesized daughter strand from the parental DNA strand. In MMR, hemimethylated dGATC sites determine the strand specificity of repair. MutH, which recognizes hemimethylated dGATC sequences, functions as a monomer and belongs to a family of type-II restriction endonucleases [25, 26]. Upon its recruitment and activation by MutS and MutL in the presence of ATP, MutH specifically incises the unmethylated daughter strand of hemimethylated dGATC [3, 22], and this strand-specific nick provides the initiation site for mismatch-provoked excision.

The first step of the MMR pathway is binding of a MutS homodimer to the mismatch. Subsequently, a hemimethylated dGATC site 5' or 3' to the mismatch is located and cleaved by the concerted action of MutS, MutL, MutH, and ATP. Three models have been proposed to address how mismatch binding by MutS leads to cleavage of the hemimethylated dGATC site (see the section of Unsolved Fundamental Problems in MMR for details). The strand-specific nick generated by MutH at hemimethylated dGATC is a starting point for excision of the mispaired base. In the presence of MutL, helicase II (UvrD) loads at the nick and unwinds the duplex from the nick towards the mismatch [18], generating single-strand DNA, which is rapidly bound by single-stranded DNA-binding protein (SSB) and protected from nuclease attack [27]. Depending on the position of the strand break relative to the mismatch, ExoI or ExoX (3'→5' exonuclease), or ExoVII or RecJ (5'→3' exonuclease) excises the nicked strand from the nicked site (the dGATC site) up to and slightly past the mismatch. The resulting single-stranded gap undergoes repair DNA resynthesis and ligation by DNA polymerase III holoenzyme, SSB, and DNA ligase [3].

These early studies on *E. coli* MMR demonstrate three key features of this important pathway: first, repair is strand specific (i.e., restricted to the newly synthesized DNA strand); second, repair is bi-directional, proceeding 5'→3' or 3'→5' from the nick to the site of the mismatch; and third, MMR has broad substrate specificity including base-base mismatches and small ID mispairs. All of these properties require functional MutS, MutL, and MutH. Because the mechanism of MMR is highly conserved throughout evolution, *E. coli* MMR is an excellent model for MMR in eukaryotic cells.

MMR in human cells

MMR is a highly conserved biological pathway with strong similarities between human MMR and prototypical *E. coli* MMR [2, 3]. These similarities include substrate

specificity, bidirectionality, and nick-directed strand specificity. The role of hemi-methylated dGATC sites as a signal for strand discrimination is not conserved from *E. coli* MMR to human MMR, but because the hemi-methylated dGATC site directs MutH-dependent nicking, and because human MMR is presumed to be nick-directed *in vivo*, both systems are thought to discriminate daughter and template strands using a strand-specific nick.

Several human MMR proteins have been identified based on their homology to *E. coli* MMR proteins (Table 1). These include human homologs of MutS [28-32], MutL [33-36], EXO1 [37-39], single-strand DNA-binding protein RPA [27, 40], proliferating cellular nuclear antigen (PCNA) [41-43], DNA polymerase δ (pol δ) [44], and DNA ligase I [45]. Although *E. coli* MutS and MutL proteins are homodimers, human MutS and MutL homologues are heterodimers [32, 34, 46]. hMSH2 heterodimerizes with hMSH6 or hMSH3 to form hMutS α or hMutS β , respectively, both of which are ATPases that play a critical role in mismatch recognition and initiation of repair [2]. hMutS α preferentially recognizes base-base mismatches and ID mispairs of 1 or 2 nucleotides, while hMutS β preferentially recognizes larger ID mispairs. At least 4 human MutL homologs (hMLH1, hMLH3, hPMS1, and hPMS2) have been identified [33, 35, 36, 47]. hMLH1 heterodimerizes with hPMS2, hPMS1, or hMLH3 to form hMutL α , hMutL β , or hMutL γ , respectively [2]. hMutL α is required for MMR and hMutL γ plays a role in meiosis, but no specific biological role has been identified

for hMutL β [2]. hMutL α possesses an ATPase activity and defects in this activity inactivate MMR in human cells. In a reconstituted human MMR system, hMutL α regulates termination of mismatch-provoked excision [45]. Recent studies show that MutL α possesses a PCNA/replication factor C (RFC)-dependent endonuclease activity which plays a critical role in 3' nick-directed MMR involving EXO1 [16].

PCNA interacts with MSH2 and MLH1 and is thought to play roles in the initiation and DNA resynthesis steps of MMR [41, 43]. PCNA also interacts with MSH6 and MSH3 [48-51] via a conserved PCNA interaction motif termed the PIP box [52]. It has been proposed that PCNA may help localize MutS α and MutS β to mispairs in newly replicated DNA [53, 54]. Although PCNA is absolutely required during 3' nick-directed MMR, it is not essential during 5' nick-directed MMR [55]. This observation might be explained by the fact that EXO1, a 5'→3' exonuclease, is involved in both 5' and 3' directed MMR. Like PCNA, EXO1 also interacts with MSH2 and MLH1 [37-39, 56-58]. While EXO1 can readily carry out 5' directed mismatch excision in the presence of MutS α or MutS β and RPA [45, 59], its role in catalyzing 3' nick-directed excision requires the MutL α endonuclease, which is activated by PCNA and RFC [16, 60]. Although it has been suggested that EXO1 possesses a cryptic 3'→5' exonuclease activity [60, 61], current data do not support that hypothesis. Instead, recent studies suggest the following steps for EXO1-catalyzed 3'

Table 1 MMR components and their functions

<i>E. coli</i>	Human	Function
(MutS) ₂	hMutS α (MSH2-MSH6) ^a hMutS β (MSH2-MSH3)	DNA mismatch/damage recognition
(MutL) ₂	hMutL α (MLH1-PMS2) ^a hMutL β (MLH1-PMS1) hMutL γ (MLH1-MLH3)	Molecular matchmaker; endonuclease, termination of mismatch-provoked excision
MutH	? ^b	Strand discrimination
UvrD	? ^b	DNA helicase
ExoI, ExoVII, ExoX, RecJ	ExoI	DNA excision; mismatch excision
Pol III holoenzyme	Pol δ	DNA re-synthesis
	PCNA	Initiation of MMR, DNA re-synthesis
SSB	RPA	ssDNA binding/protection; stimulating mismatch excision; termination of DNA excision; promoting DNA resynthesis
	HMGB1	Mismatch-provoked excision
	RFC	PCNA loading; 3' nick-directed repair; activation of MutL α endonuclease
DNA Ligase	DNA ligase I	Nick ligation

^aMajor components in cells.

^bNot yet identified.

nick-directed repair: (1) after recognition of the 3' nick and the mismatch, MutL α endonuclease makes an incision 5' to the mismatch in a manner dependent on PCNA and RFC; and (2) EXO1 performs 5'→3' excision from the MutL α -incision site through and beyond the site of the mismatch [16]. However, *exo1* null mutants in mice and yeast have a weak mutator phenotype [56, 62]; thus, it is likely that additional as yet unidentified exonucleases are involved in eukaryotic MMR.

Other protein components involved in human MMR include single-strand DNA-binding protein RPA, RFC, high mobility group box 1 protein (HMGB1), and DNA pol δ . RPA seems to be involved in all stages of MMR: it binds to nicked heteroduplex DNA before MutS α and MutL α , stimulates mismatch-provoked excision, protects the ssDNA gapped region generated during excision, and facilitates DNA resynthesis [27, 45, 60, 63]. Furthermore, RPA is phosphorylated after pol δ is recruited to the gapped DNA substrate. Recent studies indicate that phosphorylation reduces the affinity of RPA for DNA, that unphosphorylated RPA stimulates mismatch-provoked DNA excision more efficiently than phosphorylated RPA, and that phosphorylated RPA facilitates MMR-associated DNA resynthesis more efficiently than unphosphorylated RPA [63]. These results are consistent with the fact that a high-affinity RPA-DNA complex might be required to protect nascent ssDNA and to displace DNA-bound MutS α /MutL α [27, 45], while a lower-affinity RPA-DNA complex might facilitate DNA resynthesis by pol δ [63]. HMGB1 is a mismatch-binding protein and has a DNA-unwinding activity [64-66]. It interacts with MSH2 and MSH6 *in vitro* [67]. Recent studies show that HMGB1 can substitute for RPA in an *in vitro* reconstituted MMR system [45]. Additional studies are needed to precisely define the function of HMGB1 in MMR.

Unsolved fundamental problems in MMR

Despite great progress in identifying MMR proteins and genes and application of state-of-the-art biochemical and genetic approaches to analyze the mechanism of MMR in prokaryotic and eukaryotic cells, several key questions about this pathway remain unanswered. One of these questions concerns the mechanism by which MMR proteins facilitate the communication between two physically distant DNA sites: the mismatch and the strand discrimination signal. It is generally agreed that the strand discrimination signal is a strand-specific nick in both prokaryotic and eukaryotic cells (see above), although the source of the nicking activity, at least for the leading strand, is not known in eukaryotic cells. Previous studies have proposed several alternative models for this process, which can be classified into “*cis*-” or “moving” and “*trans*-” or “stationary”

models (Figure 1). The “stationary” model (Figure 1, right) proposes that interactions among MMR proteins induce DNA bending or looping that brings the two distant sites together, while MutS (or the MSH heterodimers, i.e. MutS α and MutS β) remains bound at the mismatch [19, 22]. In this model, the MutS (or MSH heterodimers) ATPase activity acts in a proofreading role to verify mismatch binding and authorize the downstream excision [22]. Support for the stationary model came from the following experiments. Junop *et al.* showed that recognition of a mismatch by MutS on a DNA molecule activated MutH cleavage of a GATC site located on a separate DNA molecule without a mismatch [22]. Consistent with this observation, a second study demonstrated that mismatch-provoked excision could be initiated when a biotin-streptavidin blockade was placed between the mismatch and pre-existing nick [68]. The “*cis*” or “moving” models suggest that MutS-MutL (or MutS α / β -MutL α) complexes load at a mismatch site and then move away from the site to search for the strand break, where exonucleases can be recruited to initiate excision.

There are two moving models, one called the “translocation” model and the other called the “molecular switch” or “sliding clamp” model (Figure 1). In the translocation model [69], ATP reduces the mismatch-binding affinity of MutS or the MSH heterodimers, and ATP hydrolysis drives unidirectional translocation of MutS proteins along the DNA helix. DNA is threaded through the protein complex until the latter reaches a strand discrimination signal in either orientation, a process that forms a DNA loop (Figure 1, left). In the molecular switch model, MutS or the MSH heterodimer binds to mismatched DNA in an ADP-bound state. The mismatch binding by MutS or the MSH heterodimer triggers a conformational change that allows an ADP to ATP exchange, which promotes a second conformational change that allows MutS or the MSH heterodimers to form a sliding clamp [70-73]. In this model (Figure 1, middle), it is the binding of ATP, not ATP hydrolysis, that signals downstream events including formation of ternary complex with MutL (or MLH heterodimers) and sliding of the ternary complex from the mismatch to the strand break [70-73].

Recent studies by Pluciennik and Modrich [74] argue in favor of a moving rather than a stationary mechanism, because their data demonstrate that a dsDNA break [75] or a protein “roadblock” between the mismatch and the nick inhibits *in vitro* MMR with recombinant *E. coli* proteins. It is not clear why two “roadblock” experiments [68, 74] obtained distinct results. In a reconstituted human MMR reaction, Zhang *et al.* [45] show that multiple MutS α -MutL α complexes are essential for processing a single mismatch, providing evidence to support the molecular switch model. Additional studies are needed to address these unresolved

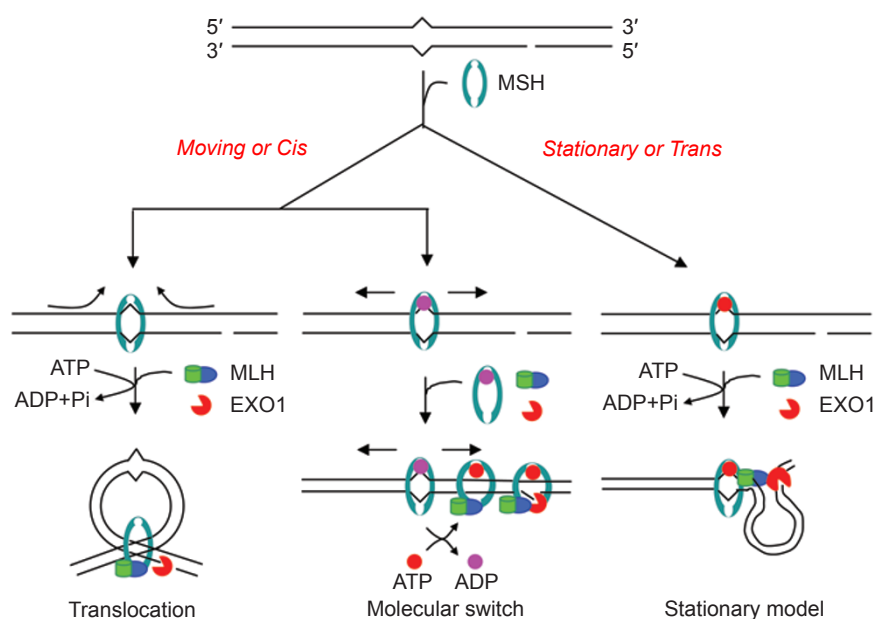


Figure 1 Models for signaling downstream MMR events following mismatch recognition. A schematic diagram for signaling between the mismatch and the strand discrimination signal is shown. Here, a 5' nick is the strand discrimination signal. Similar models apply for 3' nick-directed MMR. The “stationary” or “trans” model (right) emphasizes that MutS or its homolog (MSH) proteins remain bound at the mismatch. It is the protein-protein interactions that induce DNA bending or looping that brings the two distant sites together. The two DNA sites can cooperate in a “trans” configuration. In two “cis” or “moving” models, one called the “translocation” model (left) and the other called the “molecular switch” or “sliding clamp” model (middle), it is hypothesized that the MSH proteins bind to the mismatch and then move away from the site to search for the strand discrimination signal. The translocation model suggests that ATP hydrolysis drives unidirectional movement of the MSH proteins, resulting in the formation of an α -like loop. In the molecular switch model (center), binding of an MSH protein (in its ADP-bound state) to the mismatch triggers an ADP to ATP exchange that promotes bi-directional sliding of the protein away from the mismatch, thereby emptying the mismatch site for an incoming MSH protein. Mismatch excision begins when an MSH protein reaches the strand break.

questions about the molecular mechanism of MMR.

MMR mediates DNA damage signaling

MMR deficiency and drug resistance

DNA-damaging agents such as the alkylating agents *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), temozolomide, or procarbazine are cytotoxic agents that kill most of the replicating cells. Many cancer therapeutics are genotoxic and cytotoxic agents that induce apoptotic cell death. Interestingly, many cells that acquire resistance to such agents are deficient in MMR. For example, the human lymphoblastoid cell line MT1, which has a defect in *hMSH6*, was derived by culturing TK6 cells in the presence of a high concentration of MNNG. The resulting MNNG-resistant MT1 cells are defective in strand-specific MMR [76]. Many human colorectal cancer cell lines are also resistant to alkylating agents and have associated defects in MMR. The causal relationship between drug resistance and MMR is demonstrated by the fact that *hMLH1*-defective MNNG-resistant cells lose drug resistance when the

hMLH1 defect is genetically complemented with wild-type *hMLH1* on chromosome 3 [77]. It has also been observed that defects in *MSH2* and *PMS2* confer resistance to alkylating agents (reviewed in [78]). The mechanism by which MMR influences drug cytotoxicity is discussed further below.

Resistance to methotrexate (MTX) has also been associated with phenotypic changes in MMR in human cells. This occurs by the unusual mechanism of co-amplification of the human chromosomal region that encodes dihydrofolate reductase (DHFR, the target of MTX) and *hMSH3* [79, 80]. Amplification of *DHFR* lowers sensitivity to MTX by overexpressing the target of the drug. However, overexpression of *hMSH3* sequesters *hMSH2* in the *hMutS β* heterodimer, effectively preventing formation of the *hMutS α* (*hMSH2/hMSH6*) heterodimer, which leads to degradation of uncomplexed *hMSH6*, significant dysregulation of MMR and hypermutability [81, 82]. Overexpression of DHFR combined with genome-wide hypermutability and defective MMR are likely responsible for the MTX resistance of HL60 and other tumor cells.

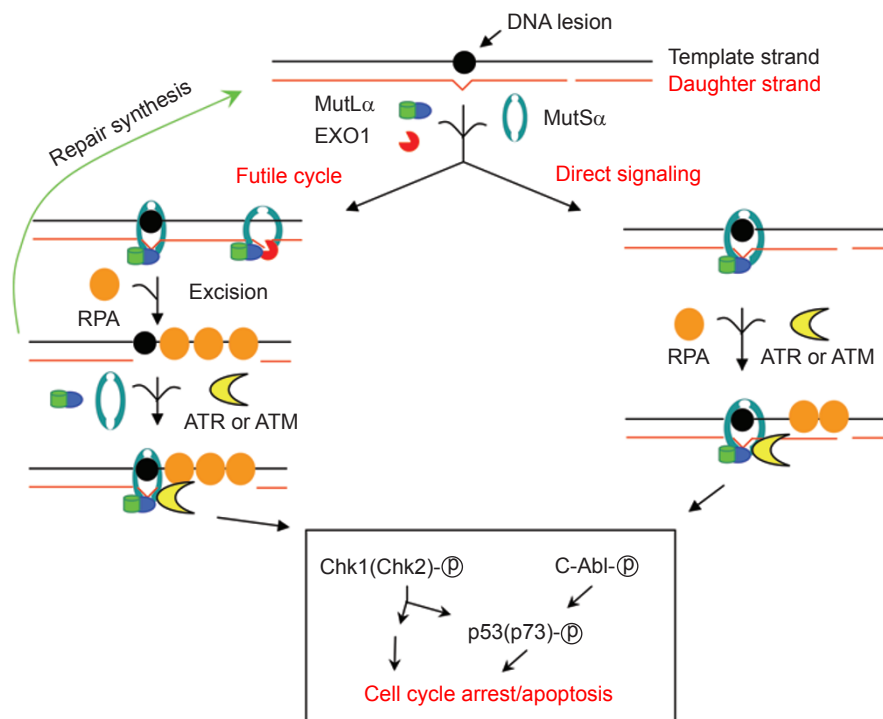


Figure 2 Models for MMR-dependent DNA damage signaling. The “futile DNA repair cycle” model (left) suggests that DNA adducts (solid black circle) induce misincorporation, which triggers the strand-specific MMR reaction. Since MMR only targets the newly synthesized strand for repair, the offending adduct in the template strand cannot be removed, and will provoke a new cycle of MMR upon repair resynthesis. Such a futile repair cycle persists and activates the ATR and/or ATM damage signaling network to promote cell cycle arrest and/or programmed cell death. The direct signaling model proposes that recognition of DNA adducts by MSH-MLH complexes allows the proteins to recruit ATR and/or ATM to the site, activating the downstream damage signaling.

MMR proteins promote DNA damage-induced cell cycle arrest and apoptosis

Cell cycle arrest is an important mechanism for preventing DNA damage-induced genomic instability. A large number of studies have characterized the so-called G2 or S phase checkpoints, and identified proteins required for cell cycle arrest, including ATM, ATR, p53, p73, Chk1, and Chk2. However, it was a somewhat unexpected finding that hMutSα- and hMutLα-deficient cells are defective in cell cycle arrest in response to multiple types of DNA damaging agents [6, 7, 83]. While the molecular basis of this effect is not precisely known, it has been reported that MMR-deficient cells fail to phosphorylate p53 and p73 in response to DNA damage [84, 85]. This implicates ATM, ATR, and/or c-Abl, because these kinases phosphorylate p53 and p73 during the response to DNA damage [85, 86]. In support of this, it has been reported that hMutSα and hMutLα interact physically with ATM, ATR-ARTIP, c-Abl, and p73 in cells treated with DNA damaging agents/drugs [83, 87–89]. These observations implicate hMutSα and hMutLα in a signaling cascade that leads from DNA damage to cell cycle arrest and/or apoptosis. They also at least in part explain the fact that drug-induced cytotoxicity is lost in MMR-deficient cells, as discussed above [6]. Very recently, EXO1 has been shown to be essential for upstream induction of DNA damage response, possibly by reducing ssDNA formation and recruiting RPA and ATR to the damage site [90]. It remains to be seen if MutSα and/or

MutLα act to recruit EXO1 in DNA damage response as they do in MMR.

Two models have been proposed to describe the role of MMR in DNA damage signaling. The “futile DNA repair cycle” model (Figure 2, left) proposes that strand-specific MMR, which targets only newly replicated DNA, engages in a futile DNA repair cycle when it encounters DNA lesions in the template strand, and this futile cycling activates DNA damage signaling pathways to induce cell cycle arrest and apoptosis [6]. Support for this model came from both *in vivo* and *in vitro* experiments. Stojic *et al.* [86] showed that exposure to MNNG induces DNA breaks/gaps, cell cycle arrest, and persistent nuclear foci at sites of DNA damage. The DNA damage-associated repair foci contain both damage signaling and DNA repair proteins, including ATR, γ-H2AX, and RPA. York and Modrich [91] showed that nicked circular heteroduplex plasmid DNA containing a single O⁶-methylguanine (O⁶-me-G)-thymine (T) mispair cannot be repaired by the MMR system when the lesion (O⁶-me-G) and the nick are on opposite strands; this suggests a futile repair process. An alternative model, referred to as the direct signaling model (Figure 2, right), argues that hMutSα/hMutLα directly trigger DNA damage signaling by recruiting ATM or ATR/ARTIP to the lesion, which activates a checkpoint response. This model is supported by an elegant study from the Hsieh laboratory showing that ATR and ARTIP form a complex with MutSα/MutLα in the presence of O⁶-me-G/T, which activates the ATR kinase

and phosphorylates Chk1 [89]. Because mammalian MMR proteins interact with a broad spectrum of DNA lesions [6], this model is consistent with the notion that MutS α /MutL α acts as a sensor for DNA damage in mammalian cells. Both models provide a reasonable explanation for decreased DNA damage-induced apoptotic signaling and increased drug resistance in MMR-deficient cells.

Role of MMR in other DNA metabolic pathways

MMR proteins have also been implicated in homeologous recombination, immunoglobulin class switching, somatic hypermutation, interstrand-crosslink repair, and trinucleotide repeat (TNR) expansion. Homeologous recombination (recombination between related but non-identical DNA sequences) generates mispairs/heteroduplexes, and induces genomic instability via chromosomal translocations, deletions, or inversions [92, 93]. The frequency of homeologous recombination is much lower than that of homologous recombination in normal cells, but the frequency of homeologous recombination is dramatically elevated in MMR-deficient cells, suggesting that MMR suppresses homeologous recombination [3, 94]. MutS and MutL inhibit DNA strand exchange between divergent sequences *in vitro*, most likely by binding to the mismatches generated during strand exchange [95]. Recent studies in yeast reveal that suppression of homeologous recombination is mediated by MutS α and a RecQ family helicase, SGS1 [96, 97]. Consistent with this notion, yeast strains defective in *sgs1* fail to suppress homeologous recombination [97, 98]. It has been postulated that MutS α recruits SGS1 to DNA mismatches, where it unwinds the heteroduplex and blocks homeologous recombination [96]. Although suppression of homeologous recombination by MMR proteins in human cells is less well understood, two human SGS1 homologous proteins, BLM and RECQ1, interact with MutS α [99, 100]. This suggests that a similar mechanism is used to suppress homeologous recombination in yeast and human cells.

Recent studies also implicate MMR in repair of inter-strand crosslinks (ICLs), in a process that involves protein components from homologous recombination, double-strand break repair, and nucleotide excision repair [101–103]. The precise nature of this involvement is not yet clear, and the specific MMR proteins that participate remain somewhat controversial. However, hMutS β appears to directly bind ICLs *in vitro* [102], and hMutL α interacts specifically with the helicase domain of Fanconi Anemia protein FANC-J to facilitate ICL repair [101].

The studies discussed above suggest that MMR promotes genomic stability. However, during immunoglobulin class switching and somatic hypermutation, it appears that MMR proteins play a highly specialized role in promoting genetic

variation. Immunoglobulin class switching and somatic hypermutation are mechanisms for increasing antibody diversity during antigen-stimulated B-cell differentiation. During this process, activation-induced cytidine deaminase (AID) deaminates cytosine residues to uracil, generating G:U mispairs, which can be recognized and processed by MMR [104, 105]. However, during repair resynthesis, the high-fidelity replicative pol δ and ϵ are thought to be replaced by the translesion polymerases, which are error-prone and crucially introduce base substitutions and frameshift mutations [106]. Additionally, MMR proteins play an important role in class switch recombination, an event where the IgM constant region is substituted by downstream constant sequences. In this capacity, MMR proteins utilize strand breaks generated by uracil DNA glycosylase to repair the AID-induced G:U mispairs in a strand-indiscriminate manner, leading to double-strand DNA breaks. It is these breaks that stimulate class switch recombination [107]. B cells from mice deficient in MMR genes (*MSH2*, *MSH6*, *MLH1*, *PMS2*, or *EXO1*) display a low level of somatic hypermutation and reduced class switch recombination [8, 62, 108, 109].

MMR proteins also promote TNR expansion, a phenomenon associated with a number of neurological disorders in humans, including Huntington's disease, myotonic dystrophy, and fragile X syndrome [110] (also see Kovtun and McMurray in this issue). TNRs such as (CAG) $_n$ form single-stranded DNA loop/hairpin structures *in vitro* [111]. Surprisingly, transgenic CAG repeats undergo expansion in wild-type, but not in knockout mice defective in MMR genes *MSH2* and *MSH3* [112, 113], suggesting that the expansion mutations of the transgenic CAG repeats require functional MMR proteins MSH2 and MSH3. *In vitro* biochemical studies indeed show that MutS β (the MSH2-MSH3 heterodimer) specifically binds to the (CAG) $_n$ hairpin structure [113]. One model proposes that MutS β inhibits repair or resolution of the (CAG) $_n$ hairpin, thus stimulating (CAG) $_n$ expansion [113]. However, many of these studies were conducted using transgenic mouse models for TNR expansion. Thus, the role of MMR in human neurological diseases involving TNR expansion is at present unclear, and additional genetic and biochemical studies are needed to define the mechanism of TNR expansion in human cells.

MMR deficiency leads to cancer development

MMR defects in hereditary non-polyposis colorectal cancer (HNPCC) and other cancers

In the early 1990s, it was shown that HNPCC and some cases of sporadic colon cancer are caused by defects in human MMR [3]. For HNPCC, Kolodner and co-workers,

and Vogelstein and co-workers independently identified germ-line mutations in *hMSH2* at chromosome 2p16-p21 in HNPCC families [29, 30]. Genetic analyses of HNPCC kindreds revealed a large increase in frequency of insertion and deletion mutations in simple repeat (microsatellite) sequences, a phenomenon known as microsatellite instability (MSI) [114]. MSI was also observed at lower incidence in sporadic colon cancers [114-116]. Additional studies showed 100- to 700-fold decreased stability of poly(GT) tracts in yeast strains with single or double knockouts in *MSH2*, *MLH1*, or *PMS1* [117]. Biochemical studies by Modrich and co-workers [118] and Kunkel and co-workers [119] also showed that extracts of MSI-positive tumor cells were severely defective in repair of base-base and ID mispairs. Thus, genetic and biochemical evidence converged to support the hypothesis that defective MMR plays a causal role in carcinogenesis leading to HNPCC, and strongly implicated such defects in some sporadic human cancers such as colorectal cancer.

The second locus linked to HNPCC was *hMLH1* at 3p21-p23 [33, 36, 120]. Furthermore, HNPCC has also been linked to mutations in two additional MutL homolog genes *hPMS1* and *hPMS2*, on 2q and 7p, respectively [35]. Defects in *hMLH1* represent the majority of all HNPCC cases [35, 36], with mutations in *hMSH2* accounting for a large fraction of all the remaining HNPCC cases for which a genetic defect has been identified. In contrast, germ-line mutations in *hMSH3* have not yet been linked to HNPCC, and mutations in *hPMS1*, *hPMS2*, and *hMSH6* are relatively rare in HNPCC patients [1-3]. These observations are consistent with the fact that *hMSH2* and *hMLH1* are essential MMR components, while *hPMS1*, *hPMS2*, *hMSH6*, and *hMSH3* play important but partially redundant and/or dispensable roles in MMR.

Genetic evidence described above shows that defects in MMR correlate with HNPCC, and biochemical studies provide compelling additional evidence for this hypothesis. In particular, cell lines from HNPCC patients and sporadic tumors with MSI are defective in strand-specific MMR, and these cells can be divided into at least two complementation groups, corresponding to hMutL α [34] and hMutS α [32]. Importantly, purified hMutL α or hMutS α specifically complements the biochemical defect in these cells. Similar evidence was obtained from chromosome or gene transfer experiments: expressions of the exogenous MMR genes complement the biochemical defect and stabilize simple repetitive sequences in the transfected cells (reviewed in [121]).

These genetic and biochemical complementation studies essentially prove the causal role of MMR defects in HNPCC. However, many questions remain, including whether or not mutations in *hMLH3* or *hEXO1* also cause

HNPCC, a possibility that remains controversial [47, 122, 123]. Mice defective in *EXO1* exhibit a mild defect in genomic stability, are partially defective in strand-specific MMR, and have increased rates of some cancers [62]. Thus, it seems possible that germ-line mutations in *hEXO1* could potentially have similar phenotypic effects in humans. Additional studies are needed to clarify this point.

Although MSI was first correlated with MMR defects in tumors from HNPCC patients, MSI is also associated with a wide variety of non-HNPCC and non-colonic tumors (reviewed in Ref. [124]). These include endometrial, ovarian, gastric, cervical, breast, skin, lung, prostate, and bladder tumors as well as glioma, leukemia, and lymphoma. Biochemical studies confirmed that MSI cell lines from sporadic leukemia, endometrial, ovarian, prostate, and bladder cancers are defective in strand-specific MMR [32, 125, 126]. Interestingly, MSI in sporadic non-colonic tumors is often associated with hypermethylation of the promoter of *hMLH1* (see below for details), and few mutations in MMR genes have been identified in these cells. These findings suggest that MMR defects are a likely cause of MSI in non-colonic sporadic cancers, although other mechanisms may also be involved in causing the MSI mutator phenotype.

Mouse models for MMR demonstrate roles in cancer and meiosis

Knockout mouse models have been developed for *MSH2*, *MSH3*, *MSH6*, *MLH1*, *MLH3*, *PMS1*, *PMS2*, and *EXO1* (reviewed in [8, 62, 127, 128]) and their phenotypes have been somewhat informative. Most of the knockout mice have a mutator phenotype, are MSI-positive, and are cancer-prone. However, the primary cancer susceptibility of *MSH2*, *MLH1*, and *PMS2* knockout mice is lymphoma, not colorectal cancer as in humans, and secondary cancer susceptibilities are to gastrointestinal tumors, skin neoplasms, and/or sarcomas (Table 2).

MSH2^{-/-} deficient mice are fertile [129, 130], are MSI-positive, develop lymphoma within 1 year of age, and have a significantly shorter lifespan than wild-type mice (i.e., 50% mortality by 6 months of age). The phenotype of *MSH6*^{-/-} deficient mice is similar to that of *MSH2*^{-/-} deficient mice, but lacking MSI [129, 131], a phenotype resembling that of atypical HNPCC with an *hMSH6* defect as the tumors in these *MSH6*-defective individuals have longer latency and low MSI [132]. Cells from *MSH3*^{-/-} mice are defective in repair of ID mispairs but can repair base-base mismatches. *MSH3*^{-/-} mice are either tumor free [133] or develop tumors at a very late age [134], essentially consistent with the fact that no *MSH3* mutations have been identified in HNPCC patients. However, in *MSH3*^{-/-} and *MSH6*^{-/-} double deficient mice, the tumor predisposition phenotype is indistinguishable from *MSH2*^{-/-} or *MLH1*^{-/-}

mice [133, 134], suggesting that *MSH3* cooperates with *MSH6* in tumor suppression.

Sterility is a characteristic feature of *MLH* mutant mice (except *PMS1*) [135-137]. These animals are also susceptible to cancer and display genomic instability, reflecting defective MMR. However, male and female *MLH1* and *MLH3* knockout mice [135-137], and male *PMS2* knockout mice are completely sterile [138]. *PMS1* knockout mice are exceptional, because they are fertile, they lack cancer susceptibility, and, apart from a very small increase in mutations in mononucleotide repeats, they appear to be MSI-negative [127]. *EXO1* defective mice are also sterile [62]. It is clear that the loss of fertility in these knockout mice is caused by abnormal meiosis [62, 128, 135-137].

The characteristics of all MMR knockout mouse models are summarized in Table 2. These data strongly support the ideas that MMR is a basic genome surveillance mechanism and that defects in MMR can promote cancer development. The effects of MMR defects on carcinogenesis appear to be tissue- and species-specific, in a manner that is poorly understood. The effects of MMR defects on meiosis in humans remain poorly characterized. However, MMR clearly plays a critical role during meiosis and/or gamete formation in mice.

Epigenetic silencing of MMR gene expression leads to cancers

Mutations in MMR genes cause genomic instability and MSI in HNPCC and in a subset of sporadic colorectal cancers. However, in a significant fraction of MSI-positive sporadic colon tumors that have an MMR defect, mutations have not been identified in MMR genes. Epigenetic silencing of *hMLH1* via promoter hypermethylation strongly down-regulates MMR in many of these cases [139, 140]. In contrast, hypermethylation of the *hMSH2* gene is rarely observed in tumors with MSI. In fact, it has been reported that more than 95% of MSI-H sporadic tumors demonstrate mutation and/or epigenetic silencing of *hMLH1* [141]. While most studies demonstrate epigenetic silencing of the

hMLH1 promoter in sporadic tumors, hypermethylation of the *hMLH1* promoter was also recently demonstrated in an HNPCC patient who does not have a germ-line mutation in any MMR gene [142]. Interestingly, recent studies suggest that this effect may be heritable [143-145]. Direct evidence that *hMLH1* promoter hypermethylation down-regulates *hMLH1* gene expression was obtained by treating cells with 5-aza-deoxycytidine, which reversed promoter hypermethylation, and restored *hMLH1* gene expression and normal MMR capacity [146, 147].

MMR deficiency and mutations in coding repeat sequences

Previous studies demonstrate that defects in MMR increase the mutation rate in genes containing a simple repeat sequence in coding regions, often referred to as target genes [148]. Thus, defects in MMR confer a mutator phenotype. It is presumed that such a mutator phenotype has genome-wide consequences and could increase the frequency of additional genome-destabilizing and cancer-promoting mutations; however, this is a difficult hypothesis to test experimentally. One approach is to selectively analyze the stability of di- and tri-nucleotide tracts within coding regions. For example, Markowitz *et al.* [149] reported two mutation “hotspots” in the *type II transforming growth factor- β receptor (TGF- β RII)* gene in MMR-deficient tumor cells from a patient with sporadic colorectal cancer. One of these mutational hotspots fell within a 6-bp GT dinucleotide repeat and the other fell within an (A)₁₀ mononucleotide repeat [149]. Both of these hotspots were sites of frequent frameshift mutations that truncated the *TGF- β RII* gene product. Similar observations have been made in other colorectal tumor cells and many other MSI-positive tumor cells including glioma, gastric, uterine, cervical and squamous head and neck tumors, as well as ulcerative colitis-associated cancer and cecum cancer. Furthermore, in some of these tumor cells, somatic frameshift mutations have been documented in many other genes including *Bax*, *insulin-like growth factor 2 receptor (IGF2-R)*, transcrip-

Table 2 Phenotypes of MMR-deficient knockout mice

Gene	MSI	Tumor	Fertility	Reference
<i>MSH2</i>	Yes	Lymphoma, GI, skin, and other tumors	Yes	[129, 130]
<i>MSH3</i>	Yes	Tumor free or GI tumors at a very late age	Yes	[133, 134]
<i>MSH6</i>	Low MSI in dinucleotide repeats	Lymphoma, GI and other tumors	Yes	[131, 133, 134]
<i>MLH1</i>	Yes	Lymphoma, GI, skin, and other tumors	No	[135, 136]
<i>PMS1</i>	Mononucleotide repeats only	None	Yes	[127]
<i>PMS2</i>	Yes	Lymphoma and sarcoma	Male infertile	[127, 138]
<i>MLH3</i>	Yes	Not available	No	[137]
<i>EXO1</i>	Yes	Lymphoma	No	[62]

tion factor *E2F-4*, *APC*, *PTEN*, *hMSH3*, *hMSH6*, *Mre11*, *MBD4/MED*, *ACTRII*, *AIM2*, *APAF-1*, *AXIN-2*, *BCL-10*, *BLM*, *Caspase-5*, *CDX-2*, *CHK-1*, *FAS*, *GRB-14*, cell cycle protein *hG4-1*, *KIAA0977*, ubiquinone oxidoreductase gene *NADH*, *OGT*, *Rad50*, *RHAMM*, *RIZ*, *SEC63*, *SLC23AT*, *TCF-4*, and *WISP-3* (reviewed in Ref. [150]). These data are consistent with the idea that similar mutations occur on a genome-wide basis and at a much higher rate in MMR-deficient cells than in wild-type cells. Because the genes noted above play critical roles in regulating cell growth or genomic stability, loss-of-function mutations in these genes may be crucial steps in the multi-step pathway of carcinogenesis.

Conclusion and perspectives

The discovery that defects in MMR play a causal role in HNPCC and many MSI-positive sporadic cancers brought immediate clinical relevance to research in the field of eukaryotic MMR. This discovery led to intensive research on and better understanding of the biological roles of MMR in eukaryotic cells, which relate to cancer prevention and therapy. Although the primary role of MMR is to improve replication fidelity by correcting replication-associated base-base mismatches or ID mispairs, important secondary roles are to modulate DNA recombination and facilitate DNA damage signaling. Thus, it is abundantly clear that defects in MMR are “permissive” for carcinogenesis.

The identification that MMR-deficient cells are resistant to certain chemotherapeutic drugs such as temozolomide, procarbazine, or cisplatin has significant impacts on cancer treatments, especially for patients with tumors defective in MMR. It is also known that MMR deficiency can be acquired during chemotherapy by selective mutations in MMR genes [6]. Therefore, the risks for chemotherapy are two-fold. First, for patients with MMR-deficient tumors, chemotherapeutic treatments could selectively kill patients’ MMR-proficient cells (e.g., blood cells) that undergo proliferation, thereby leading to rapid deaths of the cancer patients. Second, if a patient’s tumor is not caused by loss of MMR function, chemotherapeutic treatments may kill the tumor cells; at the same time, the treatment may induce or select for a mutation in MMR genes, which could lead to a secondary cancer. Therefore, novel chemotherapeutic or alternative approaches are needed for cancer patients with or without MSI-positive tumors. Such approaches might include targeted gene therapy, which could selectively restore drug sensitivity in tumor cells defective in MMR or treatment with agents that stimulate apoptosis downstream of MMR in tumor cells. Additional research on mechanisms that selectively kill MMR-deficient cells is also warranted. Such efforts should also include more basic research on the

molecular mechanisms of eukaryotic MMR. Understanding these mechanisms will support efforts for developing new therapeutic approaches for patients with HNPCC or other MSI-positive MMR-deficient tumors.

Acknowledgments

The author acknowledges research support from the National Institutes of Health (GM072756 and CA115942) and the Kentucky Lung Cancer Research Program, USA. The author regrets the lack of citations for many important observations mentioned in the text, but their omission is made necessary by restrictions in the preparation of this review. The author holds the James-Gardner Endowed Chair in Cancer Research.

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ATTACHMENT 6A

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022	__ a 1001-0602 l 1001-0602 9PY7 (Print)
022	__ a 1748-7838 9EN7 (Electronic)
030	__ a CREEB6
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040	__ a DNLM c DNLM
041	0_ a eng
044	__ 9 England
060	00 a W1 b CE128WD
210	10 a Cell Res 2 DNLM
245	00 a Cell research.
260	__ a Beijing, China : b Science Press, c 1990-
260	3_ a Basingstoke, England : b Nature Publishing Group
300	__ av.
310	__ a Monthly, b 2005-
321	__ a Bimonthly, b 2002-2004
336	__ a text b txt 2 rd a content
337	__ a unmediated b n 2 rd a media

338	__ a volume bnc2rdac carrier
362	0_ a Vol. 1, no. 1 (Mar. 1990)-
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510	1_ 91a MEDLINE bv7n1 ,June 1997-
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510	0_ a PMC
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530	__ a Available also online.
550	__ a Vols. for 2005- issued by: Institute of Biochemistry and Cell Biology.
650	_2 a Cells
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710	2_ a Zhongguo ke xue yuan. b Shanghai xi bao sheng wu xue yan jiu suo.
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787	08 i Sister journal: t Cell discovery x2056-5968w (DLC) 2015243403 w (OCoLC)915037241
856	4_ u https://pmc.ncbi.nlm.nih.gov/journals/?term=9425763
856	4_ u http://www.nature.com/cr
911	__ a Electronic only, print ceased 2016. Confirmed 20160930
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ATTACHMENT 6B

Cells MeSH Descriptor Data 2025

[Details](#)[Qualifiers](#)[MeSH Tree Structures](#)[Concepts](#)

MeSH Heading

Cells

Tree Number(s)

A11

Unique ID

D002477

RDF Unique Identifier

<http://id.nlm.nih.gov/mesh/D002477>

Annotation

general; prefer specific cell or specific anatomical term or organism with /cytol; cell stages = CELL CYCLE

Scope Note

The fundamental, structural, and functional units or subunits of living organisms. They are composed of CYTOPLASM containing various ORGANELLES and a CELL MEMBRANE boundary.

Previous Indexing

Cytology (1966-1971)

Public MeSH Note

1972; was see under CYTOLOGY 1963-71

History Note

1972; was see under CYTOLOGY 1963-71

Entry Combination

physiology:Cell Physiological Phenomena

transplantation:Cell Transplantation

Date Established

1972/01/01

Date of Entry

1999/01/01

Revision Date

2018/02/28

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Digital RNA sequencing minimizes sequence-dependent bias and amplification noise with optimized single-molecule barcodes

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Edited by Jonathan S. Weissman, University of California, San Francisco, CA, and approved December 14, 2011 (received for review November 1, 2011)

RNA sequencing (RNA-Seq) is a powerful tool for transcriptome profiling, but is hampered by sequence-dependent bias and inaccuracy at low copy numbers intrinsic to exponential PCR amplification. We developed a simple strategy for mitigating these complications, allowing truly digital RNA-Seq. Following reverse transcription, a large set of barcode sequences is added in excess, and nearly every cDNA molecule is uniquely labeled by random attachment of barcode sequences to both ends. After PCR, we applied paired-end deep sequencing to read the two barcodes and cDNA sequences. Rather than counting the number of reads, RNA abundance is measured based on the number of unique barcode sequences observed for a given cDNA sequence. We optimized the barcodes to be unambiguously identifiable, even in the presence of multiple sequencing errors. This method allows counting with single-copy resolution despite sequence-dependent bias and PCR-amplification noise, and is analogous to digital PCR but amendable to quantifying a whole transcriptome. We demonstrated transcriptome profiling of *Escherichia coli* with more accurate and reproducible quantification than conventional RNA-Seq.

systems biology | digital counting | next generation sequencing | gene expression | genomics

The central goal of transcriptome profiling is to accurately quantify the abundance of RNA transcripts in a sample. Although hybridization-based approaches like DNA microarrays can provide only a relative, analog measure of transcript abundance, sequencing-based approaches, such as RNA sequencing (RNA-Seq), have the advantage of removing hybridization bias among genes (1, 2) and offer the promise of true digital quantification.

The interpretation of conventional RNA-Seq is complicated by sequence-dependent bias and amplification noise from reverse transcription, adapter ligation, library amplification by PCR, solid-phase clonal amplification, and sequencing (3–5). NanoString technology mitigates these complications by eliminating enzymatic reactions and hybridizing color-coded probes directly to RNA for single-molecule detection (6), although it requires many specific probes. Other methods reduce bias in RNA-Seq by eliminating PCR and directly sequencing single molecules of RNA (7), or sequencing single molecules (8) or clonal populations (9) of cDNA. However, library amplification is desirable for sequencing small samples or single cells (10).

Conventional library amplification is based on PCR, but the exponential amplification afforded by PCR introduces noise, especially at low copy numbers (11). Digital PCR was introduced to circumvent this problem by distributing DNA molecules into many containers, each receiving zero or one molecules, which are amplified and detected by PCR (12). This technique has been successfully applied to RNA counting (13); however, it requires specific primers for each gene, which hinders high-throughput measurements.

Here we report a system-wide method for bias and noise reduction in RNA-Seq that allows the use of PCR to amplify a cDNA library before sequencing, providing accurate digital

quantification of the transcriptome. In our approach, each cDNA molecule is attached to a unique barcode sequence from a large pool of barcodes before amplification (Fig. 1A) (14). Deep sequencing then allows quantification of the number of cDNA molecules in the original sample by counting the number of unique barcode sequences associated with a given cDNA sequence. This concept has been applied recently for studying protein-RNA interactions (15), to improve the sensitivity of DNA mutation detection (16, 17) and accuracy of DNA copy-number measurements for individual genes by threshold detection (18), and to perform karyotyping and mRNA profiling (19). However, barcode identification in these studies was not immune to errors incurred during library preparation, amplification, and sequencing, which can convert one barcode into another. Hence, a substantial fraction of reads contained misidentified barcodes (16–19), which in some cases were discarded using an artificial threshold (17–19). To avoid this complication, we designed optimized barcodes that can be ligated and amplified with minimal bias and distinguished from one another despite the accumulation of PCR mutations and sequencing errors.

Results

Barcoding Strategy for Digital RNA-Seq. Fig. 1A depicts the general concept of digital counting by random labeling of all target nucleic acid molecules in a sample with unique barcode sequences. To achieve unique barcoding of as many target sequences as possible, the set of barcode sequences introduced to the sample must be (i) much larger than the copy number of the most abundant target sequence and (ii) sampled randomly by the target sequences. If these two criteria are satisfied, then digital quantification of the target molecules by this method is limited only by sequencing depth and accuracy. Unlike conventional sequencing-based approaches to nucleic-acid quantification, the digital counting technique is no longer limited by intrinsic amplification noise and bias in downstream sample preparation and sequencing (Fig. 1A).

Implementation of the scheme in Fig. 1A for digital RNA-Seq requires several critical considerations. As noted above, if the barcode sequences are random, then a sequencing error at one position in a barcode will cause that barcode to be misidentified. This error-induced interconversion will occur even if the barcode sequences are nonrandom (18), unless the barcodes are carefully

Author contributions: K.S., T.Z.J., P.A.S., and X.S.X. designed research; K.S. and T.Z.J. performed research; K.S., T.Z.J., and P.A.S. analyzed the data; K.S., T.Z.J., P.A.S., and X.S.X. wrote the manuscript.

Conflict of interest statement: Harvard University has filed a provisional patent application based on this work.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE34449).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1118018109/-DCSupplemental.

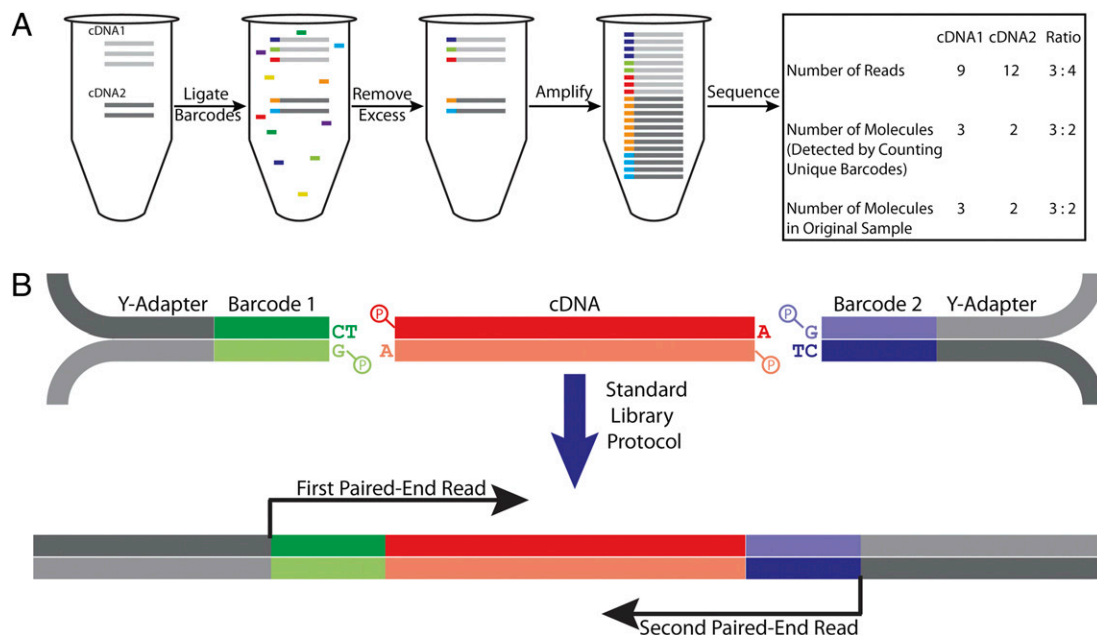


Fig. 1. Our scheme of digital RNA-Seq. (A) General principle of digital RNA-Seq. Assume the original sample contains two cDNA sequences, one with three copies and another with two copies. An overwhelming number of unique barcode sequences are added to the sample in excess, and five are randomly ligated to the cDNA molecules. Ideally, each cDNA molecule in the sample receives a unique barcode sequence. After removing the excess barcodes, the barcoded cDNA molecules are amplified by PCR. Because of intrinsic noise and sequence-dependent bias, the barcoded cDNA molecules are amplified unevenly. Consequently, after the amplicons are sequenced, it appears that there are three copies of cDNA1 for every four copies of cDNA2 based on the relative number of reads for each sequence. However, the ratio in the original sample was 3:2, which is accurately reflected in the relative number of unique barcodes associated with each cDNA sequence. (B) In our implementation of A, we found it advantageous to randomly ligate both ends of each phosphorylated cDNA fragment to a barcoded phosphorylated Illumina Y-shaped adapter. Note that the single T and A overhangs present on the barcodes and cDNA, respectively, are to enhance ligation efficiency. After this step, the sample is amplified by PCR and prepared for sequencing using the standard Illumina library protocol. For each amplicon, both barcode sequences and both strands of the cDNA sequence are read using paired-end deep sequencing.

designed so that multiple substitution errors and indels do not obscure their identities (20). Because DNA secondary structure can reduce amplification efficiency, the barcodes should not have significant sequence overlap or complementarity with each other, the adapter and primer sequences used in library preparation and sequencing, or the transcriptome-of-interest. Ideally, the barcode set will not contain sequence motifs that are known to be problematic for sequencing chemistries, such as long homopolymers and regions with high or low GC-content.

We used a computer program to generate a set of 145 barcode sequences 20-bp in length (Dataset S1) that satisfies the above criteria (*Materials and Methods* and *SI Materials and Methods*). The barcode sequences can sustain up to four substitution errors and remain unambiguously identifiable. In addition, a barcode that incurs up to nine substitution errors or the combination of one indel and five substitution errors will not take on the sequence of another barcode.

Instead of using a single barcode sequence to identify each target molecule in our sample (15–19), we attached a barcode sequence to both ends of each target molecule (Fig. 1B and *SI Materials and Methods*). If both ends of a target sequence sample all of the barcodes randomly, the target sequence will have access to $145 \times 145 = 21,025$ unique labels. The two barcode sequences along with the target molecule sequence were then read out by paired-end sequencing (Fig. 1B). This paired-end strategy dramatically reduces the number of barcodes that must be designed and synthesized, is compatible with conventional paired-end library protocols, and provides long-range sequence information that improves mapping accuracy (21, 22). In addition, attaching barcodes to both ends increases the overall randomness of barcode sampling because the two ends of a target molecule are unlikely to have a similar degree of bias. We tested and characterized this

method on a set of quantified DNA spike-in sequences and a cDNA library derived from the transcriptome of *Escherichia coli*.

Quantification of Spike-in Sequences and Barcode Sampling Bias. To calibrate our digital RNA-Seq system, we measured the concentrations of five synthetic DNA spike-in sequences using the Fluidigm digital PCR platform and used them as internal standards. The spike-in samples were barcoded, added to the barcoded *E. coli* cDNA library, and quantified using the sequencing-based digital counting strategy described above. Fig. 2A shows that the number of digital counts (i.e., unique barcodes) observed in deep sequencing is well-correlated with the digital PCR calibration of the spike-in sequences.

To evaluate the difference between using random barcode sequences and our optimized barcode sequences, we conducted two experiments. In one experiment, we labeled the spike-in molecules with random barcode sequences (*SI Materials and Methods*), and in the second experiment we used our optimized, predetermined barcode set. We constructed the histograms of the number of reads for all barcodes observed from the most abundant spike-in sequence (Fig. 2B). When using random barcodes (red histogram in Fig. 2B and *SI Materials and Methods*), the left-most bin exhibits a large peak because a substantial fraction of barcodes are infrequently read because of sequencing errors; this causes barcodes to interconvert, generating quantification artifacts that were also evident in previous reports (16–19). In stark contrast, the left-most bin, when using optimized barcodes (green histogram in Fig. 2B), has no such peak because our optimized barcode sequences avoid misidentification because of sequencing errors. The effect of sequencing error on both random and optimized barcode counting is clearly shown by simulation (Fig. S1 and *SI Materials and Methods*).

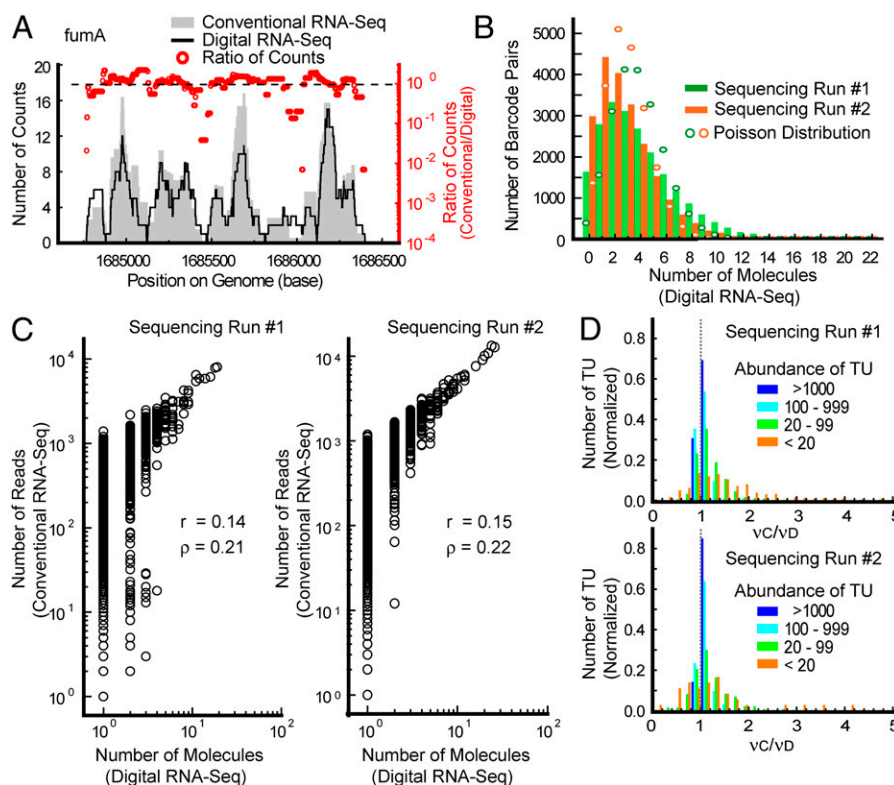


Fig. 3. Digital quantification of the *E. coli* transcriptome. (A) Conventional and digital counting results for the *fumA* transcription unit (TU) as a function of genome position. The conventional counts were calculated by using a conventional calibration curve that allows regression of the number of reads against the number of input molecules for all spike-in molecules (Fig. 2A). The digital counts were obtained by counting the number of unique barcodes associated with each fragment. The red dots are the ratios of these two numbers for each base. (B) Histograms of the number of times a barcode pair was observed with the *E. coli* cDNA sequences (i.e., the number of cDNA molecules attached to a given barcode pair) in the two replicates. Barcode sampling is more biased on average for *E. coli* cDNA fragments, but is still in reasonably good agreement with Poisson statistics. (C) Correlation between the number of reads (conventional counting) and the number of molecules obtained from digital counting of unique barcodes for every mapped fragment in the two replicates. For low copy molecules, the conventional counts are distributed over three orders-of-magnitude; this is because the conventional method counts amplicons, which are subject to intrinsic noise (11), rather than directly counting molecules in the original samples like the digital counting method. We note that higher copy fragments are less affected by intrinsic noise (11), as the number of molecules sequenced is greater; this effectively allows averaging over the read counts of many molecules in conventional RNA-Seq, decreasing the variance of counting in the process. (D) Uniformity of conventional vs. digital counting along the length of each transcription unit as a function of transcription unit abundance across the whole *E. coli* transcriptome for both replicates. We calculated the variation $\nu_D = s_D/\mu_D$ (where μ_D and s_D are the mean and sample SD of the digital counts among 99-base bins in a transcription unit, respectively) associated with digital counting and the variation $\nu_C = s_C/\mu_C$ associated with conventional counting within each transcription unit for which at least three bins contained on average at least one read. We then created the histogram of the ratio between conventional and digital counting variation (ν_C/ν_D) for transcription units in different abundance ranges for each replicate. Transcription unit abundance is the sum of all digital counts for each fragment in the transcription unit.

the ratio of counts between the two replicates for both conventional and digital counting along the *fumA* transcript. This ratio is consistently close to one for digital counting, but fluctuates over three orders-of-magnitude for conventional counting. We analyzed the global reproducibility of the whole transcriptome for quantification of transcription units and genes for both conventional and digital counting in Fig. 4B and C, respectively. In both cases, the correlation between replicates is noticeably better for digital counting than conventional counting, particularly for low copy transcripts.

Discussion

Unlike previously reported methods of eliminating bias and noise from RNA-Seq (7–9), our strategy allows amplification by PCR and uses standard commercial protocols for sample preparation. However, the implementation described above also leaves considerable room for improvement. For example, one could ligate barcoded adapters directly to RNA (23, 24), reducing the bias that occurs during reverse transcription. Alternatively, a recently described protocol for processing mature mRNA from single mammalian cells could be modified to include barcoded primers

for reverse transcription and second-strand synthesis before amplification (10), obviating the need for ligation.

One disadvantage of our technique is that it requires higher sequencing coverage than conventional RNA-Seq. This requirement is because both the transcriptome and the barcode set must be evenly sampled for accurate counting. However, the cost-per-base of deep sequencing continues to decrease rapidly. In our experiment, the mean number of reads per fragment was ~400. However, the spike-in sequencing reads can be randomly down-sampled 10-fold (*SI Materials and Methods*) without perturbing the correlation between abundance measured by digital PCR and digital barcode counting (Fig. S3), which implies that significantly lower coverage will suffice in many cases.

For applications where many cycles of PCR are required for sensitive detection, bias and noise reduction are crucial for accurate quantification. Although we demonstrated our technique on the *E. coli* transcriptome, we note that the maximum copy number for polyadenylated mRNA in a single mouse blastomere was found to be ~2,400 (10). With 155 optimized barcode sequences (10 more than were used in this study), one could uniquely label nearly every identical molecule in this system

ACKNOWLEDGMENTS. Illumina sequencing was performed at the Harvard Faculty of Arts and Sciences Center for Systems Biology, with the help of Christian Daly, and at the Tufts University School of Medicine Genomics Core Facility, with the help of Kip Bodi and James Schiemer; digital PCR was performed by the Fluidigm Genetic Analysis Facility at the Molecular Genetics Core Facility of the Children's Hospital Boston Intellectual and Developmental Disabilities Research Center, with the help of Hal Schneider and Ta-Wei Lin; and base-calling analysis was performed at the Harvard

Faculty of Arts and Sciences Research Computing Group by Jiangwen Zhang. This work was supported by National Institutes of Health (NIH) National Human Genome Research Institute Grant HG005097-01 (to X.S.X.) and NIH National Human Genome Research Institute Recovery Act Grand Opportunities Grant 1RC2HG005613-01 (to X.S.X.); and K.S. was supported by a Postdoctoral Fellowship for Research Abroad from the Japanese Society for the Promotion of Science and a fellowship from The Uehara Memorial Foundation.

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ATTACHMENT 7A

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ATTACHMENT 7B

Science MeSH Descriptor Data 2025

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MeSH Heading

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Tree Number(s)

H01.770

Unique ID

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RDF Unique Identifier

<http://id.nlm.nih.gov/mesh/D012586>

Annotation

use for the discipline (education, history, etc) only

Scope Note

The study of natural phenomena by observation, measurement, and experimentation.

See Also

Religion and Science

Date Established

1966/01/01

Date of Entry

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Revision Date

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