国際調査報告　　国際出願番号　PCT／JP2014／006421

C（続き）．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
	2006/0193865 A1 & US 2004/0185053 A1 & US 2011/0305631 A1 & US 2012/0082617 A1 & US 2013/0090458 A1 & US 2013/0177526 A1 & US 2014/0058067 A1 & US 2014/0286860 A1 & US 2014/0328752 A1 & US 2011/0070156 A1 & US 2013/0209356 A1 & US 2014/0044640 A1 & US 2010/0015046 A1 & US 2012/0328564 A1 & US 2014/0099258 A1 & US 2009/0291093 A1 & US 2012/0328634 A1 & US 2013/0216561 A1 & US 2014/0004078 A1 & US 2006/0228300 A1 & US 2006/0228357 A1 & US 2009/0060862 A1 & US 2009/0191225 A1 & US 2009/0202487 A1 & US 2010/0189689 A1 & US 2010/0221210 A1 & US 2010/0233779 A1 & US 2010/0261885 A1 & US 2010/0266496 A1 & US 2011/0008251 A1 & US 2011/0020273 A1 & US 2011/0076233 A1 & US 2011/0123436 A1 & US 2011/0143417 A1 & US 2011/0158905 A1 & US 2011/0256053 A1 & US 2011/0274704 A1 & US 2011/0300066 A1 & US 2012/0009149 A1 & US 2012/0082685 A1 & US 2012/0040431 A1 & US 2012/0093769 A1 & US 2011/0300105 A1 & US 2012/0196346 A1 & US 2011/0236352 A1 & US 2011/0318306 A1 & US 2012/0225032 A1 & US 2012/0237442 A1 & US 2012/0276100 A1 & US 2013/0039861 A1 & US 2013/0072659 A1 & US 2013/0078183 A1 & US 2013/0109073 A1 & US 2013/0143296 A1 & US 2013/0171064 A1 & US 2013/0177532 A1 & US 2013/0217091 A1 & US 2013/0280250 A1 & US 2013/0289244 A1 & US 2013/0295005 A1 & US 2014/0038261 A1 & US 2014/0086832 A1 & US 2014/0161766 A1 & US 2007/0086942 A1 & US 2007/0087001 A1 & US 2007/0140966 A1 & US 2009/0202433 A1 & US 2009/0269277 A1 & US 2009/0304580 A1 & US 2010/0068137 A1 & US 2010/0189641 A1 & US 2010/0216662 A1 & US 2011/0110851 A1 & US 2011/0189083 A1 & US 2011/0189268 A1 & US 2011/0195020 A1 & US 2012/0276608 A1 & US 2013/0095033 A1 & US 2013/0164816 A1 & US 2014/0037543 A1 & US 2014/0045242 A1 & US 2014/0294722 A & US 2013/0136718 A1 & US 2011/0165072 A1 & US 2012/0141375 A1 & US 2014/0112864 A1 & US 2014/0227179 A1 & US 2011/0064754 A1 & EP 2506881 A1 & EP 2271359 A1 & EP 1994057 A1 & EP 2396036 A1 & EP 1874358 A1 & EP 1874824 A1 & EP 1937724 A1 & EP 1937851 A1 & EP 1959993 A1 & EP 2674440 A2 & EP 1572242 A1 & EP 2197468 A1 & EP 2271364 A1 & EP 2331134 A1 & EP 2473186 A1 & EP 2473187 A1 & EP 2509629 A1 & EP 2509630 1A & EP 2605788 A1 & EP 2635300 A1 & EP 2806887 A1 & EP 2764362 A1 & EP 2416807 A1 & EP 2496256 A1 & EP 2785382 A1 & WO 2011/068845 A1 & WO 2009/100194 A2 & WO 2007/112193 A2 & WO 2010/093395 A1 & WO 2006/107617 A2 & WO 2006/107786 A2 & WO 2007/046893 A2 & WO 2007/047609 A2 & WO 2007/075270 A2 & WO 2004/054622 A1 & WO 2009/055653 A1 & WO 2009/126558 A1 & WO 2010/017500 A2 & WO 2011/025904 A1 & WO 2011/026026 A1 & WO 2011/072114 A1 & WO	

国際調査報告　　国際出願番号　PCT／JP2014／006421

C（続き）．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
	2011/072124 A1 & WO 2012/024223 A2 & WO 2012/061548 A1 & WO 2013/112801 A1 & WO 2013/052484 A1 & WO 2010/117984 A1 & WO 2011/056721 A2 & WO 2013/082254 A1 & WO 2014/165506 A1	
Y	Ikuo MITSUI et al., A New Water-soluble Camptothecin Derivative, DX-8951f, Exhibits Potent Antitumor Activity against Human Tumors in vitro and in vivo, Jpn. J. Cancer Res., 1995, 86, p.776-782	1-26
Y	JP 2009-538629 A（ジェネンテック・インコーポレーテッド）2009.11.12, ［０２６７］－［０２８１］ & JP 2013-56896 A & US 2008/0050310 A1 & US 2011/0142859 A1 & US 2012/0329094 A1 & US 2013/0171170 A1 & US 2014/0127197 A1 & EP 2032606 A1 & EP 2446904 A2 & EP 2447282 A2 & WO 2007/140371 A2	1-26
Y	JP 2008-521828 A（シアトル　ジェネティックス，インコーポレイテッド）2008.06.26, 請求項１９－２１、［０１３７］－［０１４５］ & US 2008/0305044 A1 & EP 1817341 A1 & WO 2006/065533 A2	1-26
Y	JP 2005-511627 A（シアトル　ジェネティクス，インコーポレーテッド）2005.04.28, ［０１５９］－［０２０６］ & US 2005/0123536 A1 & EP 1482972 A1 & WO 2003/043583 A2	1-26
Y	WO 2000/025825 A1（第一製薬株式会社）2000.05.11, 表１、第２３頁 & US 2004/0192644 A1 & US 6811996 B1 & EP 1155702 A1 & EP 1619210 A1	1-26
Y	JP 2012-100671 A（アムジエン・インコーポレーテツド）2012.05.31, ［０１８７］、［０１８８］ & JP 2008-538506 A & JP 5087536 B2 & US 2007/0071764 A1 & US 2011/0045587 A1 & EP 1896080 A1 & WO 2006/116156 A2	1-26
Y	JP 2013-534906 A（インディアナ　ユニバーシティー　リサーチ　アンド　テクノロジー　コーポレーション）2013.09.09, ［０２２６］ & US 2013/0123178 A1 & EP 2569000 A1 & WO 2011/143209 A1	1-26
P, A	WO 2014/061277 A1（第一三共株式会社）2014.04.24, 全文 & TW 201420118 A	1-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/006069

A. CLASSIFICATION OF SUBJECT MATTER
C07K16/28(2006.01)i, *A61K39/395*(2006.01)i, *A61P35/00*(2006.01)i, *A61K31/4745*(2006.01)n, *A61K31/48*(2006.01)n

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K16/28, A61K39/395, A61P35/00, A61K31/4745, A61K31/48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2013
Kokai Jitsuyo Shinan Koho 1971-2013 Toroku Jitsuyo Shinan Koho 1994-2013

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAplus/REGISTRY(STN), PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	JP 2009-538629 A (Genentech, Inc.), 12 November 2009 (12.11.2009), entire text & US 2008/0050310 A1 & EP 2032606 A2 & WO 2007/140371 A2 & CN 101490087 A	49 1-33,35-48
X Y	JP 2008-521828 A (Seattle Genetics, Inc.), 26 June 2008 (26.06.2008), entire text & US 2008/0305044 A1 & EP 1817341 A2 & WO 2006/065533 A2	49 1-33,35-48
X Y	JP 2005-511627 A (Seattle Genetics, Inc.), 28 April 2005 (28.04.2005), entire text & US 2005/0123536 A1 & EP 1482972 A2 & WO 2003/043583 A2	49 1-33,35-48

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 December, 2013 (04.12.13)	17 December, 2013 (17.12.13)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/006069

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2002/000734 A1 (Daiichi Pharmaceutical Co., Ltd.), 03 January 2002 (03.01.2002), entire text & US 2003/0166513 A1 & EP 1298145 A1 & CN 1449412 A	1-33,35-48
Y	JP 11-092405 A (Daiichi Pharmaceutical Co., Ltd.), 06 April 1999 (06.04.1999), entire text (Family: none)	1-33,35-48
Y	JP 10-095802 A (Tanabe Seiyaku Co., Ltd.), 14 April 1998 (14.04.1998), entire text & US 5892043 A & EP 781781 A2 & CN 1158334 A	1-33,35-48
A	DOSIO, F. et al., "Antibody-targeted leucinostatin A.", JOURNAL OF CONTROLLED RELEASE, 1994.11, Vol.32, No.1, P.37-44	49

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/006069

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 34
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 34 pertains to a therapeutic method for a human being and thus relates to a subject matter on which this International Searching Authority is not required to carry out an international search under the provisions of PCT Article 17(2)(a)(i) and [PCT Rule 39.1(iv)].

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

特 許 協 力 条 約

ＰＣＴ

国際調査報告

（法８条、法施行規則第40、41条）
〔ＰＣＴ18条、ＰＣＴ規則43、44〕

出願人又は代理人 の書類記号　PD20-9003WO	今後の手続きについては、様式ＰＣＴ／ＩＳＡ／２２０ 及び下記５を参照すること。	
国際出願番号 ＰＣＴ／ＪＰ２０１３／００６０６９	国際出願日 (日.月.年)　１０．１０．２０１３	優先日 (日.月.年)　１１．１０．２０１２
出願人（氏名又は名称） 第一三共株式会社		

国際調査機関が作成したこの国際調査報告を法施行規則第41条（ＰＣＴ18条）の規定に従い出願人に送付する。
この写しは国際事務局にも送付される。

この国際調査報告は、全部で　5　ページである。

☐ この調査報告に引用された先行技術文献の写しも添付されている。

１．国際調査報告の基礎
ａ．言語に関し、この国際調査は以下のものに基づき行った。
☑ 出願時の言語による国際出願
☐ 出願時の言語から国際調査のための言語である＿＿＿＿＿＿＿＿ 語に翻訳された、
この国際出願の翻訳文（ＰＣＴ規則12.3(a)及び23.1(b)）

ｂ．☐ この国際調査報告は、ＰＣＴ規則91の規定により国際調査機関が認めた又は国際調査機関に通知された明らかな誤りの訂正を考慮して作成した（ＰＣＴ規則43.6の2(a)）。

ｃ．☑ この国際出願は、ヌクレオチド又はアミノ酸配列を含んでいる（第Ｉ欄参照）。

２．☑ 請求の範囲の一部の調査ができない（第Ⅱ欄参照）。

３．☐ 発明の単一性が欠如している（第Ⅲ欄参照）。

４．発明の名称は
☑ 出願人が提出したものを承認する。
☐ 次に示すように国際調査機関が作成した。

＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

５．要約は
☑ 出願人が提出したものを承認する。
☐ 第Ⅳ欄に示されているように、法施行規則第47条第１項（ＰＣＴ規則38.2）の規定により国際調査機関が作成した。出願人は、この国際調査報告の発送の日から１月以内にこの国際調査機関に意見を提出することができる。

６．図面に関して
ａ．要約書とともに公表される図は、
第 ＿＿＿ 図とする。
☐ 出願人が示したとおりである。
☐ 出願人は図を示さなかったので、国際調査機関が選択した。
☐ 本図は発明の特徴を一層よく表しているので、国際調査機関が選択した。

ｂ．☑ 要約とともに公表される図はない。

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１３／００６０６９

第Ⅰ欄　ヌクレオチド又はアミノ酸配列（第１ページの１．ｃの続き）

１．この国際出願で開示されたヌクレオチド又はアミノ酸配列に関して、提出された以下の配列表に基づき国際調査を行った。

ａ．提出手段　　☐ 紙形式

☑ 電子形式

ｂ．提出時期　　☑ 出願時の国際出願に含まれていたもの

☐ この国際出願と共に電子形式により提出されたもの

☐ 出願後に、調査のために、この国際調査機関に提出されたもの

２．☐ さらに、複数の版の配列表又は配列表の写しを提出した場合、出願後に提出した配列の写し若しくは追加して提出した配列の写しが、出願時に提出した配列と同一である旨又は出願時の開示を超える事項を含まない旨の陳述書の提出があった。

３．補足意見：

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１３／００６０６９

第Ⅱ欄　請求の範囲の一部の調査ができないときの意見（第１ページの２の続き）

法第8条第3項（ＰＣＴ17条(2)(a)）の規定により、この国際調査報告は次の理由により請求の範囲の一部について作成しなかった。

１．☑ 請求項　３４　は、この国際調査機関が調査をすることを要しない対象に係るものである。
つまり、
請求項３４はヒトに対する治療方法に関するものであるから、PCT17条(2)(a)(i)及び［PCT規則39.1(iv)］の規定により、この国際調査機関が国際調査をすることを要しない対象に係るものである。

２．☐ 請求項　　は、有意義な国際調査をすることができる程度まで所定の要件を満たしていない国際出願の部分に係るものである。つまり、

３．☐ 請求項　　は、従属請求の範囲であってＰＣＴ規則6.4(a)の第２文及び第3文の規定に従って記載されていない。

第Ⅲ欄　発明の単一性が欠如しているときの意見（第１ページの３の続き）

次に述べるようにこの国際出願に二以上の発明があるとこの国際調査機関は認めた。

１．☐ 出願人が必要な追加調査手数料をすべて期間内に納付したので、この国際調査報告は、すべての調査可能な請求項について作成した。

２．☐ 追加調査手数料を要求するまでもなく、すべての調査可能な請求項について調査することができたので、追加調査手数料の納付を求めなかった。

３．☐ 出願人が必要な追加調査手数料を一部のみしか期間内に納付しなかったので、この国際調査報告は、手数料の納付のあった次の請求項のみについて作成した。

４．☐ 出願人が必要な追加調査手数料を期間内に納付しなかったので、この国際調査報告は、請求の範囲の最初に記載されている発明に係る次の請求項について作成した。

追加調査手数料の異議の申立てに関する注意

☐ 追加調査手数料及び、該当する場合には、異議申立手数料の納付と共に、出願人から異議申立てがあった。

☐ 追加調査手数料の納付と共に出願人から異議申立てがあったが、異議申立手数料が納付命令書に示した期間内に支払われなかった。

☐ 追加調査手数料の納付はあったが、異議申立てはなかった。

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１３／００６０６９

Ａ．　発明の属する分野の分類（国際特許分類（ＩＰＣ））

Int.Cl.　C07K16/28(2006.01)i, A61K39/395(2006.01)i, A61P35/00(2006.01)i, A61K31/4745(2006.01)n, A61K31/48(2006.01)n

Ｂ．　調査を行った分野

調査を行った最小限資料（国際特許分類（ＩＰＣ））

Int.Cl.　C07K16/28, A61K39/395, A61P35/00, A61K31/4745, A61K31/48

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報	１９２２－１９９６年
日本国公開実用新案公報	１９７１－２０１３年
日本国実用新案登録公報	１９９６－２０１３年
日本国登録実用新案公報	１９９４－２０１３年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

CAplus/REGISTRY(STN), PubMed

Ｃ．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
X Y	JP 2009-538629 A（ジェネンテック・インコーポレーテッド）2009.11.12, 全文 & US 2008/0050310 A1 & EP 2032606 A2 & WO 2007/140371 A2 & CN 101490087 A	49 1-33, 35-48
X Y	JP 2008-521828 A（シアトル　ジェネティックス，インコーポレイテッド）2008.06.26, 全文 & US 2008/0305044 A1 & EP 1817341 A2 & WO 2006/065533 A2	49 1-33, 35-48

☑ Ｃ欄の続きにも文献が列挙されている。　☐ パテントファミリーに関する別紙を参照。

＊　引用文献のカテゴリー
「Ａ」特に関連のある文献ではなく、一般的技術水準を示すもの
「Ｅ」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「Ｌ」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「Ｏ」口頭による開示、使用、展示等に言及する文献
「Ｐ」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「Ｔ」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「Ｘ」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Ｙ」特に関連のある文献であって、当該文献と他の１以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「＆」同一パテントファミリー文献

国際調査を完了した日	国際調査報告の発送日
０４．１２．２０１３	１７．１２．２０１３

国際調査機関の名称及びあて先	特許庁審査官（権限のある職員）	４Ｂ	４１５５
日本国特許庁（ＩＳＡ／ＪＰ） 郵便番号１００－８９１５ 東京都千代田区霞が関三丁目４番３号	野村　英雄 電話番号　０３－３５８１－１１０１　内線　３４４８		

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１３／００６０６９

C（続き）．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
X Y	JP 2005-511627 A（シアトル　ジェネティクス，インコーポレーテッド）2005.04.28，全文 & US 2005/0123536 A1 & EP 1482972 A2 & WO 2003/043583 A2	49 1-33,35-48
Y	WO 2002/000734 A1（第一製薬株式会社）2002.01.03，全文 & US 2003/0166513 A1 & EP 1298145 A1 & CN 1449412 A	1-33,35-48
Y	JP 11-092405 A（第一製薬株式会社）1999.04.06，全文 （ファミリーなし）	1-33,35-48
Y	JP 10-095802 A（田辺製薬株式会社）1998.04.14，全文 & US 5892043 A & EP 781781 A2 & CN 1158334 A	1-33,35-48
A	DOSIO, F. et al., "Antibody-targeted leucinostatin A.", JOURNAL OF CONTROLLED RELEASE, 1994.11, Vol.32, No.1, P.37-44	49

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/006178

A. CLASSIFICATION OF SUBJECT MATTER
C07K16/28(2006.01)i, *A61K39/395*(2006.01)i, *A61P35/00*(2006.01)i, *A61K31/4745*(2006.01)n, *A61K31/48*(2006.01)n

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K16/28, A61K39/395, A61P35/00, A61K31/4745, A61K31/48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2013
Kokai Jitsuyo Shinan Koho 1971-2013 Toroku Jitsuyo Shinan Koho 1994-2013

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAplus/REGISTRY(STN), PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	JP 2009-538629 A (Genentech, Inc.), 12 November 2009 (12.11.2009), entire text & US 2008/0050310 A1 & EP 2032606 A2 & WO 2007/140371 A2 & CN 101490087 A	1-30,33-52 31-32
Y A	JP 2008-521828 A (Seattle Genetics, Inc.), 26 June 2008 (26.06.2008), entire text & US 2008/0305044 A1 & EP 1817341 A2 & WO 2006/065533 A2	1-30,33-52 31-32
Y A	JP 2005-511627 A (Seattle Genetics, Inc.), 28 April 2005 (28.04.2005), entire text & US 2005/0123536 A1 & EP 1482972 A2 & WO 2003/043583 A2	1-30,33-52 31-32

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 December, 2013 (04.12.13)	17 December, 2013 (17.12.13)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/006178

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	WO 2002/000734 A1 (Daiichi Pharmaceutical Co., Ltd.), 03 January 2002 (03.01.2002), entire text & US 2003/0166513 A1 & EP 1298145 A1 & CN 1449412 A	1-30,33-52 31-32
Y A	JP 11-092405 A (Daiichi Pharmaceutical Co., Ltd.), 06 April 1999 (06.04.1999), entire text (Family: none)	1-30,33-52 31-32
Y A	JP 10-095802 A (Tanabe Seiyaku Co., Ltd.), 14 April 1998 (14.04.1998), entire text & US 5892043 A & EP 781781 A2 & CN 1158334 A	1-30,33-52 31-32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2013/006178

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 53-54
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 53-54 pertain to therapeutic methods for a human being and thus relate to a subject matter on which this International Searching Authority is not required to carry out an international search under the provisions of PCT Article 17(2)(a)(i) and [PCT Rule 39.1(iv)].

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

特 許 協 力 条 約

ＰＣＴ

国際調査報告

（法８条、法施行規則第40、41条）
〔ＰＣＴ18条、ＰＣＴ規則43、44〕

出願人又は代理人 の書類記号　PD20-9004WO	今後の手続きについては、様式ＰＣＴ／ＩＳＡ／２２０ 及び下記５を参照すること。	
国際出願番号 ＰＣＴ／ＪＰ２０１３／００６１７８	国際出願日 (日.月.年)　１７．１０．２０１３	優先日 (日.月.年)　１９．１０．２０１２
出願人（氏名又は名称） 第一三共株式会社		

国際調査機関が作成したこの国際調査報告を法施行規則第41条（ＰＣＴ18条）の規定に従い出願人に送付する。
この写しは国際事務局にも送付される。

この国際調査報告は、全部で　5　ページである。

☐ この調査報告に引用された先行技術文献の写しも添付されている。

１．国際調査報告の基礎
ａ．言語に関し、この国際調査は以下のものに基づき行った。
☑ 出願時の言語による国際出願
☐ 出願時の言語から国際調査のための言語である＿＿＿＿＿＿＿＿ 語に翻訳された、
この国際出願の翻訳文（ＰＣＴ規則12.3(a)及び23.1(b)）

ｂ．☐ この国際調査報告は、ＰＣＴ規則91の規定により国際調査機関が認めた又は国際調査機関に通知された明らかな誤りの訂正を考慮して作成した（ＰＣＴ規則43.6の2(a)）。

ｃ．☑ この国際出願は、ヌクレオチド又はアミノ酸配列を含んでいる（第Ｉ欄参照）。

２．☑ 請求の範囲の一部の調査ができない（第Ⅱ欄参照）。

３．☐ 発明の単一性が欠如している（第Ⅲ欄参照）。

４．発明の名称は
☑ 出願人が提出したものを承認する。
☐ 次に示すように国際調査機関が作成した。

＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

５．要約は
☑ 出願人が提出したものを承認する。
☐ 第Ⅳ欄に示されているように、法施行規則第47条第１項（ＰＣＴ規則38.2）の規定により国際調査機関が作成した。出願人は、この国際調査報告の発送の日から１月以内にこの国際調査機関に意見を提出することができる。

６．図面に関して
ａ．要約書とともに公表される図は、
第 ＿＿＿ 図とする。
☐ 出願人が示したとおりである。
☐ 出願人は図を示さなかったので、国際調査機関が選択した。
☐ 本図は発明の特徴を一層よく表しているので、国際調査機関が選択した。

ｂ．☑ 要約とともに公表される図はない。

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１３／００６１７８

第Ⅰ欄　ヌクレオチド又はアミノ酸配列（第１ページの１．ｃの続き）

１．この国際出願で開示されたヌクレオチド又はアミノ酸配列に関して、提出された以下の配列表に基づき国際調査を行った。

ａ．提出手段　　☐ 紙形式

☑ 電子形式

ｂ．提出時期　　☑ 出願時の国際出願に含まれていたもの

☐ この国際出願と共に電子形式により提出されたもの

☐ 出願後に、調査のために、この国際調査機関に提出されたもの

２．☐ さらに、複数の版の配列表又は配列表の写しを提出した場合、出願後に提出した配列の写し若しくは追加して提出した配列の写しが、出願時に提出した配列と同一である旨又は出願時の開示を超える事項を含まない旨の陳述書の提出があった。

３．補足意見：

OnCusp
Ex. 1002

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１３／００６１７８

第Ⅱ欄　請求の範囲の一部の調査ができないときの意見（第１ページの２の続き）

法第8条第3項（ＰＣＴ17条(2)(a)）の規定により、この国際調査報告は次の理由により請求の範囲の一部について作成しなかった。

１．☑ 請求項　５３－５４　は、この国際調査機関が調査をすることを要しない対象に係るものである。
つまり、
請求項５３－５４はヒトに対する治療方法に関するものであるから、PCT17条(2)(a)(i)及び［PCT規則39.1(iv)］の規定により、この国際調査機関が国際調査をすることを要しない対象に係るものである。

２．☐ 請求項　　は、有意義な国際調査をすることができる程度まで所定の要件を満たしていない国際出願の部分に係るものである。つまり、

３．☐ 請求項　　は、従属請求の範囲であってＰＣＴ規則6.4(a)の第２文及び第3文の規定に従って記載されていない。

第Ⅲ欄　発明の単一性が欠如しているときの意見（第１ページの３の続き）

次に述べるようにこの国際出願に二以上の発明があるとこの国際調査機関は認めた。

１．☐ 出願人が必要な追加調査手数料をすべて期間内に納付したので、この国際調査報告は、すべての調査可能な請求項について作成した。

２．☐ 追加調査手数料を要求するまでもなく、すべての調査可能な請求項について調査することができたので、追加調査手数料の納付を求めなかった。

３．☐ 出願人が必要な追加調査手数料を一部のみしか期間内に納付しなかったので、この国際調査報告は、手数料の納付のあった次の請求項のみについて作成した。

４．☐ 出願人が必要な追加調査手数料を期間内に納付しなかったので、この国際調査報告は、請求の範囲の最初に記載されている発明に係る次の請求項について作成した。

追加調査手数料の異議の申立てに関する注意

☐ 追加調査手数料及び、該当する場合には、異議申立手数料の納付と共に、出願人から異議申立てがあった。

☐ 追加調査手数料の納付と共に出願人から異議申立てがあったが、異議申立手数料が納付命令書に示した期間内に支払われなかった。

☐ 追加調査手数料の納付はあったが、異議申立てはなかった。

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１３／００６１７８

Ａ．　発明の属する分野の分類（国際特許分類（ＩＰＣ））

Int.Cl.　C07K16/28(2006.01)i, A61K39/395(2006.01)i, A61P35/00(2006.01)i, A61K31/4745(2006.01)n, A61K31/48(2006.01)n

Ｂ．　調査を行った分野

調査を行った最小限資料（国際特許分類（ＩＰＣ））

Int.Cl.　C07K16/28, A61K39/395, A61P35/00, A61K31/4745, A61K31/48

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報	１９２２－１９９６年
日本国公開実用新案公報	１９７１－２０１３年
日本国実用新案登録公報	１９９６－２０１３年
日本国登録実用新案公報	１９９４－２０１３年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

CAplus/REGISTRY(STN), PubMed

Ｃ．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
Y A	JP 2009-538629 A（ジェネンテック・インコーポレーテッド）2009.11.12, 全文 & US 2008/0050310 A1 & EP 2032606 A2 & WO 2007/140371 A2 & CN 101490087 A	1-30,33-52 31-32
Y A	JP 2008-521828 A（シアトル　ジェネティックス，インコーポレイテッド）2008.06.26, 全文 & US 2008/0305044 A1 & EP 1817341 A2 & WO 2006/065533 A2	1-30,33-52 31-32

☑ Ｃ欄の続きにも文献が列挙されている。　☐ パテントファミリーに関する別紙を参照。

＊　引用文献のカテゴリー
「Ａ」特に関連のある文献ではなく、一般的技術水準を示すもの
「Ｅ」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「Ｌ」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「Ｏ」口頭による開示、使用、展示等に言及する文献
「Ｐ」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「Ｔ」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「Ｘ」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Ｙ」特に関連のある文献であって、当該文献と他の１以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「＆」同一パテントファミリー文献

国際調査を完了した日	国際調査報告の発送日
０４．１２．２０１３	１７．１２．２０１３

国際調査機関の名称及びあて先	特許庁審査官（権限のある職員）	４Ｂ	４１５５
日本国特許庁（ＩＳＡ／ＪＰ） 郵便番号１００－８９１５ 東京都千代田区霞が関三丁目４番３号	野村　英雄 電話番号　０３－３５８１－１１０１　内線　３４４８		

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１３／００６１７８

C（続き）．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
Y A	JP 2005-511627 A（シアトル　ジェネティクス，インコーポレーテッド）2005.04.28，全文 & US 2005/0123536 A1 & EP 1482972 A2 & WO 2003/043583 A2	1-30,33-52 31-32
Y A	WO 2002/000734 A1（第一製薬株式会社）2002.01.03，全文 & US 2003/0166513 A1 & EP 1298145 A1 & CN 1449412 A	1-30,33-52 31-32
Y A	JP 11-092405 A（第一製薬株式会社）1999.04.06，全文 （ファミリーなし）	1-30,33-52 31-32
Y A	JP 10-095802 A（田辺製薬株式会社）1998.04.14，全文 & US 5892043 A & EP 781781 A2 & CN 1158334 A	1-30,33-52 31-32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2015/000355

A. CLASSIFICATION OF SUBJECT MATTER
C07K16/28(2006.01)i, *A61K31/4745*(2006.01)i, *A61K39/395*(2006.01)i, *A61P35/00*(2006.01)i, *C07D491/052*(2006.01)n

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K, A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho	1922-1996	Jitsuyo Shinan Toroku Koho	1996-2015
Kokai Jitsuyo Shinan Koho	1971-2015	Toroku Jitsuyo Shinan Koho	1994-2015

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAplus/REGISTRY/MEDLINE/EMBASE/BIOSIS(STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP 2006-511526 A (Immunomedics, Inc.), 06 April 2006 (06.04.2006), claims; paragraphs [0027] to [0032] & WO 2004/054622 A1 & EP 1572242 A & US 2004/0185053 A1 & US 2009/0291093 A1 & US 2012/0328634 A1 & US 2013/0216561 A1 & US 2014/0004078 A1	1-26
A	JP 2013-500253 A (Enzon Pharmaceuticals, Inc.), 07 January 2013 (07.01.2013), claims; paragraphs [0008] to [0023] & WO 2011/011474 A1 & US 2012/0171201 A1 & EP 2456464 A & CN 102481364 A & KR 10-2012-0104158 A	1-26

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 April 2015 (14.04.15)	21 April 2015 (21.04.15)
Name and mailing address of the ISA/ Japan Patent Office 3-4-3,Kasumigaseki,Chiyoda-ku, Tokyo 100-8915,Japan	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2015/000355

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP 2013-534535 A (Covx Technologies Ireland, Ltd.), 05 September 2013 (05.09.2013), claims & US 2012/0201809 A1 & WO 2012/007896 A1 & EP 2593142 A & CN 103096933 A & KR 10-2013-0050966 A	1-26
P,A	WO 2014/107024 A1 (CELLTRION), 10 July 2014 (10.07.2014), claims; abstract & KR 10-2014-0088828 A & KR 10-2014-0088841 A & KR 10-2014-0088842 A & KR 10-2014-0088843 A	1-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2015/000355

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [x] Claims Nos.: 27
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 27 pertains to a method for treatment of the human body by therapy and thus relates to a subject matter on which this International Searching Authority is not required to carry out an international search under the provisions of PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv).

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

特 許 協 力 条 約

ＰＣＴ

国際調査報告

（法８条、法施行規則第40、41条）
〔ＰＣＴ18条、ＰＣＴ規則43、44〕

出願人又は代理人 の書類記号　PD20-9008WO	今後の手続きについては、様式ＰＣＴ／ＩＳＡ／２２０ 及び下記５を参照すること。	
国際出願番号 ＰＣＴ／ＪＰ２０１５／０００３５５	国際出願日 (日.月.年)　２８．０１．２０１５	優先日 (日.月.年)　３１．０１．２０１４
出願人（氏名又は名称） 第一三共株式会社		

国際調査機関が作成したこの国際調査報告を法施行規則第41条（ＰＣＴ18条）の規定に従い出願人に送付する。
この写しは国際事務局にも送付される。

この国際調査報告は、全部で　5　ページである。

☐ この調査報告に引用された先行技術文献の写しも添付されている。

１．国際調査報告の基礎
ａ．言語に関し、この国際調査は以下のものに基づき行った。
☑ 出願時の言語による国際出願
☐ 出願時の言語から国際調査のための言語である＿＿＿＿＿＿＿＿ 語に翻訳された、この国際出願の翻訳文（ＰＣＴ規則12.3(a)及び23.1(b)）

ｂ．☐ この国際調査報告は、ＰＣＴ規則91の規定により国際調査機関が認めた又は国際調査機関に通知された明らかな誤りの訂正を考慮して作成した（ＰＣＴ規則43.6の2(a)）。

ｃ．☑ この国際出願は、ヌクレオチド又はアミノ酸配列を含んでいる（第Ｉ欄参照）。

２．☑ 請求の範囲の一部の調査ができない（第Ⅱ欄参照）。

３．☐ 発明の単一性が欠如している（第Ⅲ欄参照）。

４．発明の名称は
☑ 出願人が提出したものを承認する。
☐ 次に示すように国際調査機関が作成した。

＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

５．要約は
☑ 出願人が提出したものを承認する。
☐ 第Ⅳ欄に示されているように、法施行規則第47条第１項（ＰＣＴ規則38.2）の規定により国際調査機関が作成した。出願人は、この国際調査報告の発送の日から１月以内にこの国際調査機関に意見を提出することができる。

６．図面に関して
ａ．要約書とともに公表される図は、
第＿＿＿＿図とする。
☐ 出願人が示したとおりである。
☐ 出願人は図を示さなかったので、国際調査機関が選択した。
☐ 本図は発明の特徴を一層よく表しているので、国際調査機関が選択した。

ｂ．☑ 要約とともに公表される図はない。

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１５／０００３５５

第Ⅰ欄　ヌクレオチド又はアミノ酸配列（第１ページの１．ｃの続き）

１．この国際出願で開示されたヌクレオチド又はアミノ酸配列に関して、提出された以下の配列表に基づき国際調査を行った。

ａ．提出手段

☐ 紙形式

☑ 電子形式

ｂ．提出時期

☑ 出願時の国際出願に含まれていたもの

☐ この国際出願と共に電子形式により提出されたもの

☐ 出願後に、調査のために、この国際調査機関に提出されたもの

２．☐ さらに、複数の版の配列表又は配列表の写しを提出した場合、出願後に提出した配列の写し若しくは追加して提出した配列の写しが、出願時に提出した配列と同一である旨又は出願時の開示を超える事項を含まない旨の陳述書の提出があった。

３．補足意見：

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１５／０００３５５

第Ⅱ欄　請求の範囲の一部の調査ができないときの意見（第１ページの２の続き）

法第8条第3項（ＰＣＴ17条(2)(a)）の規定により、この国際調査報告は次の理由により請求の範囲の一部について作成しなかった。

1．☑ 請求項　27　は、この国際調査機関が調査をすることを要しない対象に係るものである。
つまり、
請求項27は、治療による人体の処置方法に関するものであって、PCT 第17 条(2)(a)(i)及びPCT 規則39.1(iv)の規定により、この国際調査機関が国際調査を行うことを要しない対象に係るものである。

2．☐ 請求項　　は、有意義な国際調査をすることができる程度まで所定の要件を満たしていない国際出願の部分に係るものである。つまり、

3．☐ 請求項　　は、従属請求の範囲であってＰＣＴ規則6.4(a)の第２文及び第3文の規定に従って記載されていない。

第Ⅲ欄　発明の単一性が欠如しているときの意見（第１ページの３の続き）

次に述べるようにこの国際出願に二以上の発明があるとこの国際調査機関は認めた。

1．☐ 出願人が必要な追加調査手数料をすべて期間内に納付したので、この国際調査報告は、すべての調査可能な請求項について作成した。

2．☐ 追加調査手数料を要求するまでもなく、すべての調査可能な請求項について調査することができたので、追加調査手数料の納付を求めなかった。

3．☐ 出願人が必要な追加調査手数料を一部のみしか期間内に納付しなかったので、この国際調査報告は、手数料の納付のあった次の請求項のみについて作成した。

4．☐ 出願人が必要な追加調査手数料を期間内に納付しなかったので、この国際調査報告は、請求の範囲の最初に記載されている発明に係る次の請求項について作成した。

追加調査手数料の異議の申立てに関する注意

☐ 追加調査手数料及び、該当する場合には、異議申立手数料の納付と共に、出願人から異議申立てがあった。

☐ 追加調査手数料の納付と共に出願人から異議申立てがあったが、異議申立手数料が納付命令書に示した期間内に支払われなかった。

☐ 追加調査手数料の納付はあったが、異議申立てはなかった。

国際調査報告

国際出願番号　ＰＣＴ／ＪＰ２０１５／０００３５５

Ａ．　発明の属する分野の分類（国際特許分類（ＩＰＣ））

Int.Cl.　C07K16/28(2006.01)i, A61K31/4745(2006.01)i, A61K39/395(2006.01)i, A61P35/00(2006.01)i, C07D491/052(2006.01)n

Ｂ．　調査を行った分野

調査を行った最小限資料（国際特許分類（ＩＰＣ））

Int.Cl.　C07K, A61K, A61P

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報	１９２２－１９９６年
日本国公開実用新案公報	１９７１－２０１５年
日本国実用新案登録公報	１９９６－２０１５年
日本国登録実用新案公報	１９９４－２０１５年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

CAplus/REGISTRY/MEDLINE/EMBASE/BIOSIS(STN)

Ｃ．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
A	JP 2006-511526 A (イミューノメディクス、インコーポレイテッド) 2006.04.06, 特許請求の範囲、【００２７】－【００３２】 & WO 2004/054622 A1 & EP 1572242 A & US 2004/0185053 A1 & US 2009/0291093 A1 & US 2012/0328634 A1 & US 2013/0216561 A1 & US 2014/0004078 A1	1 - 26

☑　Ｃ欄の続きにも文献が列挙されている。　　☐　パテントファミリーに関する別紙を参照。

＊　引用文献のカテゴリー
「Ａ」特に関連のある文献ではなく、一般的技術水準を示すもの
「Ｅ」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「Ｌ」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「Ｏ」口頭による開示、使用、展示等に言及する文献
「Ｐ」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「Ｔ」国際出願日又は優先日後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「Ｘ」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Ｙ」特に関連のある文献であって、当該文献と他の１以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「＆」同一パテントファミリー文献

国際調査を完了した日 １４．０４．２０１５	国際調査報告の発送日 ２１．０４．２０１５
国際調査機関の名称及びあて先 日本国特許庁（ＩＳＡ／ＪＰ） 郵便番号１００－８９１５ 東京都千代田区霞が関三丁目４番３号	特許庁審査官（権限のある職員）　４Ｎ　３３４１ 山本　晋也 電話番号　０３－３５８１－１１０１　内線　３４８８

国際調査報告　　国際出願番号　ＰＣＴ／ＪＰ２０１５／０００３５５

Ｃ（続き）．　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求項の番号
A	JP 2013-500253 A (エンゾン　ファーマシューティカルズ，インコーポレーテッド) 2013.01.07, 特許請求の範囲、【０００８】－【００２３】 & WO 2011/011474 A1 & US 2012/0171201 A1 & EP 2456464 A & CN 102481364 A & KR 10-2012-0104158 A	1 - 26
A	JP 2013-534535 A (コヴェックス・テクノロジーズ・アイルランド・リミテッド) 2013.09.05, 特許請求の範囲 & US 2012/0201809 A1 & WO 2012/007896 A1 & EP 2593142 A & CN 103096933 A & KR 10-2013-0050966 A	1 - 26
P, A	WO 2014/107024 A1 (CELLTRION) 2014.07.10, Claims, Abstract & KR 10-2014-0088828 A & KR 10-2014-0088841 A & KR 10-2014-0088842 A & KR 10-2014-0088843 A	1 - 26

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Ex. 1002

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Notes:
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Translated: 15:45:58 JST 09/27/2018
Dictionary: Last updated 06/28/2018 / Priority:

Decision to Grant a Patent

Application number: Patent Application No. 2016-166850
Date of Drafting: Heisei 28(2016) October 7
Patent examiner: Atsuta 1** 5575 4N00
Title of the invention: Anti-HER2 antibody-medicine conjugate
The number of claims: 3
Applicant: DAIICHI SANKYO Co., Ltd.
Representative: OHNO, Seiji (and 4 others)

This application is to be granted a patent, since no reason for refusal has been found.

Director General(p.p.) Director(p.p.) Examiner Assistant examiner Manager for Determination of Classification UEHARA, Katsunori Atsuta 1 ** OTA, Yuzo 98405575 3959
1. Distinction of Patent: Division

2. Reference: see below

3. Application of Patent Act Article 30: not applicable

4. Change of the Title of the Invention: No

5. International Patent Classification (IPC)
C07K 16/32 ZNA ,A61K 39/395 L,A61K 39/395 C,A61K 31/4745 =A61P 35/00 ,C07K 5/103

6. Deposit of microorganisms

7. Indication of unacceptance of retroactivity to the filling date

Reference information

Application number: Patent Application No. 2016-166850

1. Technical fields searched (IPC, DB name)
C07K 1/00 - 19/00A61K. 6/00 - 51/12A61P 1/00 -
43/00JSTPlus/JMEDPlus/JST7580(JDreamIII)CAplus/REGISTRY/MEDLINE/EMBASE/BIOSIS(STN)

2. Reference patent documents
JP 2006-511526 A (JP, A) JP 2013-500253 A (JP, A) JP 2013-534535 A (JP, A) JP
2011-524001 A (JP, A) JP 2011-519864 A (JP, A) International Publication the 2013/188740
(WO, A1)

3. Reference books and magazines

[Translation done.]

整理番号:PD20-0014D　発送番号:468490　発送日:平成28年10月18日　1

特許査定

（分割）

特許出願の番号	特願２０１６－１６６８５０
起案日	平成２８年１０月　７日
特許庁審査官	厚田　一拓　５５７５　４Ｎ００
発明の名称	抗ＨＥＲ２抗体－薬物コンジュゲート
請求項の数	３
特許出願人	第一三共株式会社
代理人	大野　聖二（外　４名）

この出願については、拒絶の理由を発見しないから、特許査定をします。

上記はファイルに記録されている事項と相違ないことを認証する。
認証日　平成28年10月13日　経済産業事務官　高地　伸幸

注意：この書面を受け取った日から３０日以内に特許料の納付が必要です。

整理番号:PD20-0014D　発送番号:468490　発送日:平成28年10月18日　2

1．出願種別　分割
2．参考文献　有
3．特許法第３０条適用　無
4．発明の名称の変更　無

5．国際特許分類（ＩＰＣ）

Ｃ０７Ｋ　１６／３２　ＺＮＡ，
Ａ６１Ｋ　３９／３９５　Ｌ，
Ａ６１Ｋ　３９／３９５　Ｃ，
Ａ６１Ｋ　３１／４７４５　＝
Ａ６１Ｐ　３５／００　，
Ｃ０７Ｋ　５／１０３

6．菌寄託

7．出願日の遡及を認めない旨の表示

整理番号:PD20-0014D　発送番号:468490　発送日:平成28年10月18日　3/E

参考情報

特許出願の番号　特願２０１６－１６６８５０

１．調査した分野（ＩＰＣ，ＤＢ名）

Ｃ０７Ｋ　１／００　－　１９／００
Ａ６１Ｋ　６／００　－　５１／１２
Ａ６１Ｐ　１／００　－　４３／００
ＪＳＴＰｌｕｓ／ＪＭＥＤＰｌｕｓ／ＪＳＴ７５８０（ＪＤｒｅａｍＩＩＩ）
ＣＡｐｌｕｓ／ＲＥＧＩＳＴＲＹ／ＭＥＤＬＩＮＥ／ＥＭＢＡＳＥ／ＢＩＯＳＩＳ（ＳＴＮ）

２．参考特許文献

引用文献
特表２００６－５１１５２６　（ＪＰ，Ａ）
特表２０１３－５００２５３　（ＪＰ，Ａ）
特表２０１３－５３４５３５　（ＪＰ，Ａ）
特表２０１１－５２４００１　（ＪＰ，Ａ）
特表２０１１－５１９８６４　（ＪＰ，Ａ）
国際公開第２０１３／１８８７４０　（ＷＯ，Ａ１）

３．参考図書雑誌

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Notes:
1. Untranslatable words are replaced with asterisks (****).
2. Texts in the figures are not translated and shown as it is.

Translated: 15:51:04 JST 09/27/2018
Dictionary: Last updated 06/28/2018 / Priority:

Notification of Reasons for Refusal

Application number: Patent Application No. 2016-540705
Date of Drafting: Heisei 28(2016) December 2
Patent examiner: TAKAHASHI, Juri 4498 4C00
Representative/Applicant: OHNO, Seiji (and 4 others)
Applied Provisions: Article 36

This application should be refused for the reason that the following Reason. If the applicant has any argument against the reason, please submit a Written Argument within three months after the day of dispatch of this notification.
Reason
1. (Clarity) This patent application does not fulfill the requirement of the provision of Patent Law Article 36(6)(ii) in terms of the item(s) listed below regarding the statement of the scope of claims.

Note

**Reason 1

- Claim: 12
The description "SEQ ID NO 583 and 584 are contained in a heavy chain and a light chain, respectively... antibody-medicine conjugate" Becoming is ambiguous as Japanese. Please correct to the same description as Claim 11.

<Claim(s) in which no reason for refusal is found>
At present for the invention concerning Claims 1-11 and 13-15, no reason for refusal is found. If any reason for refusal is found later, it will be notified. ---------- <Record of the result of prior art search>
- Technical fields to be searched: IPC A61K47/48
DB name CAPLUS/MEDLINE/BIOSIS/EMBASE (STN) JSTPLUS/JMEDPLUS/JST7580 (JDreamIII)

- Prior art documents International Publication No. WO2014/057687 International Publication No. WO2012/019024 International Publication No. WO2012/064733 International Publication No. WO2000/25825 International Publication No. WO2013/163229
<Suggestions in amending>
(1) When making amendment to the description or the scope of claims, the amended parts should be underlined so as to be clearly identified (refer to Note 6 and 7 of Form 13, Regulations under the Patent Act). (2) The amendment(s) shall be made within the scope of the

matters stated in the description, scope of claims, or drawings of the present application as originally filed. By a Written Argument, after description parts, such as a Description of the time of application which serves as a basis in the Reason correction is legitimate about each correction matter, are shown clearly, please claim. (3) One should make sure not to perform any corrections that invoke a violation against Patent Act Article 17-2(4) when amending the scope of claims.
If you have any question or are willing to make an interview with the examiner to discuss issues on this notification of reasons for refusal, please contact us at:
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Director General(p.p.) Director(p.p.) Examiner Assistant examiner MAEDA, Hitoshi TAKAHASHI, Juri 91574498

[Translation done.]

2020-900935

整理番号:　　発送番号:557833　発送日:平成28年12月 6日　　1

拒絶理由通知書

特許出願の番号	特願２０１６－５４０７０５
起案日	平成２８年１２月　２日
特許庁審査官	高橋　樹理　　４４９８　４Ｃ００
特許出願人代理人	大野　聖二（外　４名）　様
適用条文	第３６条

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●理由１について

・請求項　１２

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ＤＢ名　CAPLUS/MEDLINE/BIOSIS/EMBASE(STN)

・先行技術文献　国際公開第２０１４／０５７６８７号
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DX-8951F, A WATER-SOLUBLE CAMPTOTHECIN ANALOG, EXHIBITS POTENT ANTITUMOR ACTIVITY AGAINST A HUMAN LUNG CANCER CELL LINE AND ITS SN-38-RESISTANT VARIANT

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We previously reported that DX-8951f, a novel water-soluble camptothecin analog, significantly inhibits the growth of various human and murine tumors *in vitro* and *in vivo*. The antitumor effects and topoisomerase I inhibitory activity of DX-8951f are stronger than those of other current camptothecin analogs. In this study, we established an SN-38-resistant cell line, PC-6/SN2-5, from the human oat cell carcinoma PC-6 cell line by a stepwise selection system, investigated the mechanism of resistance of this cell line and then compared the antitumor activity of camptothecin analogs against the cell line. PC-6/SN2-5 cells were resistant to SN-38 (32-fold) and SK&F104864 (topotecan; 14-fold), but barely resistant to CPT-11 (3-fold) and DX-8951f (2-fold). Topoisomerase I protein levels and topoisomerase I activities of parental cells were similar to those of resistant cells. Determination of the cellular drug concentration by either flow cytometric analysis or the high-performance liquid chromatography method confirmed that the cellular accumulation of SN-38 and topotecan was significantly reduced in PC-6/SN2-5 cells, whereas that of DX-8951f was only slightly reduced. Furthermore, DX-8951f stabilized the cleavable complex formations in intact PC-6/SN2-5 cells as well as in parental cells, but SN-38 and topotecan did not in the resistant cells. Our data suggest that PC-6/SN2-5 cells may have acquired resistance to camptothecin analogs by a decrease in intracellular drug accumulation and that DX-8951f may have the potency to overcome such a type of resistance mechanism induced by camptothecin compounds. *Int. J. Cancer* 72:680–686, 1997.
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Irinotecan hydrochloride, CPT-11, is a semi-synthetic analog of camptothecin and is one of the most active anticancer agents for various cancers (Ohno *et al.*, 1990; Masuda *et al.*, 1992; Fukuoka *et al.*, 1992; Shimada *et al.*, 1993; Futatsuki *et al.*, 1994; Takeuchi *et al.*, 1991). Previous studies have indicated that the antitumor activity of camptothecin analogs is mediated through interaction of the drug with its target enzyme, DNA topoisomerase I (Topo I) (Hsiang *et al.*, 1985; Hsiang and Liu, 1988; Hertzberg *et al.*, 1989). CPT-11 is a pro-drug that is converted to 7-ethyl-10-hydroxycamptothecin (SN-38) (Kanzawa *et al.*, 1990; Kawato *et al.*, 1991), which is responsible for much of the efficacy and toxicity of CPT-11 *in vivo*. Due to the polymorphism of enzymes such as carboxylesterase, which is known to convert CPT-11 to SN-38 in rat serum, interpatient variation is inevitable (Ohe *et al.*, 1992). To solve this problem, we synthesized non-pro-drug type water-soluble camptothecin derivatives and selected one of the hexacyclic camptothecin analogs, DX-8951f, which exhibits rather higher Topo I inhibitory activity and antitumor activity than the other camptothecin analogs under development including i.e., SN-38, topotecan, 9-aminocamptothecin (9-AC) (Mitsui *et al.*, 1995). Furthermore, DX-8951f was effective on a P-glycoprotein-mediated multidrug-resistant cell line (Mitsui *et al.*, 1995). Because of its high and broad antitumor activity, the clinical effects of DX-8951f have been expected, and preparations for phase I trials of DX-8951f are in progress.

Since the development of resistance to antitumor agents is the main reason for treatment failure in cancer patients, several mechanisms of drug resistance related to camptothecin analogs have been investigated. The typical mechanisms of resistance to camptothecin analogs are reduction in Topo I levels (Andoh *et al.*, 1987; Gupta *et al.*, 1988; Tan *et al.*, 1989; Sugimoto *et al.*, 1990; Kubota *et al.*, 1992; Madelaine *et al.*, 1993; Saijo *et al.*, 1994; Kijima *et al.*, 1994; Nagai *et al.*, 1995), alteration of Topo I activity (Tan *et al.*, 1989; Kubota *et al.*, 1992; Tanizawa *et al.*, 1993; Madelaine *et al.*, 1993; Kapoor *et al.*, 1995), mutations in Topo I (Andoh *et al.*, 1987; Tan *et al.*, 1989; Kubota *et al.*, 1992; Tanizawa *et al.*, 1993) and decreased metabolic activation of CPT-11 (Saijo *et al.*, 1994; Ogasawara *et al.*, 1995). Decrease in drug accumulation in resistant cells has been shown to be one of the mechanisms in the camptothecin-induced resistance (Chang *et al.*, 1992).

To evaluate the antitumor effect of DX-8951f on camptothecin-resistant cell lines, we established an SN-38-resistant cell line from human oat cell carcinoma, PC-6, by stepwise selection. We investigated the mechanism of resistance of this cell line and compared the antitumor effects and other related effects of several camptothecin analogs on the cell line.

MATERIAL AND METHODS

Material

DX-8951f ((1*S*, 9*S*)-1-amino-9-ethyl-5-fluoro-2, 3-dihydro-9-hydroxy-4-methyl-1*H*, 12*H*-benzo [*de*] pyrano [3′,4′:6,7] indolizino [1,2-*b*] quinoline-10, 13 (9*H*, 15*H*)-dione monomethanesulfonate dihydrate), camptothecin, topotecan and 9-aminocamptothecin were synthesized in our laboratory, and SN-38 and CPT-11 were supplied by Yakult Honsya (Tokyo, Japan). All compounds were dissolved in DMSO to obtain stock solutions of 1–10 mM.

Anticancer agents

Adriamycin (ADM), 5-fluorouracil (5-FU) and mitomycin C (MMC) were purchased from Kyowa Hakko Kogyo (Tokyo, Japan), cis-diaminedichloroplatinum (CDDP) and VP-16 were from Nippon Kayaku (Tokyo, Japan), amsacrine (AMSA) was from Warner-Lambert (Scarborough, Canada), vincristine (VCR) was from Shionogi (Osaka, Japan), taxol was from Sigma (St. Louis, MO) and mitoxantrone (MIT) was from Takeda (Osaka, Japan). Actinomycin D (Act-D) was purchased from Banyu (Tokyo, Japan), and cytarabine (Ara-C), nimustine (ACNU) and thiotepa (TESPA) were from Nippon Shinyaku, (Kyoto, Japan), Sankyo (Tokyo, Japan) and Sumitomo (Osaka, Japan), respectively. All compounds were dissolved according to the manufacturer's instructions.

Cell culture and isolation of SN-38-resistant cell line

Human oat cell carcinoma cell line PC-6 was obtained from Immuno-Biological Laboratories (Takasaki, Japan). PC-6/SN2-5, an SN-38-resistant PC-6 mutant, was obtained as a result of stepwise, continuous exposure to SN-38. The cells were at first treated with SN-38 at 0.1 nM and finally maintained with 4.8 nM of

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TABLE I – CYTOTOXICITY OF VARIOUS ANTICANCER AGENTS AGAINST PARENTAL AND RESISTANT PC-6[1]

Compound	Number of experiments	PC-6 GI_{50}[2] Mean ± SE	PC-6/SN2-5 GI_{50}[2] Mean ± SE	Extent of resistance[3] (mean ± SE)
Camptothecin derivatives				
SN-38	11	0.706 ± 0.405	19.1 ± 1.77	32.0 ± 4.53
CPT-11	9	821 ± 81.5	2448 ± 112	3.18 ± 0.294
Topotecan	7	3.57 ± 0.674	41.1 ± 3.52	13.9 ± 2.44
9-AC	7	1.55 ± 0.238	8.55 ± 0.805	6.21 ± 1.12
DX-8951f	9	0.186 ± 0.019	0.395 ± 0.039	2.24 ± 0.325
Alkylating agents				
TESPA	6	731 ± 102	689 ± 200	0.923 ± 0.183
ACNU	1	1630	1240	0.761
Antibiotics				
ADM	7	6.33 ± 0.855	7.96 ± 0.942	1.31 ± 0.103
MMC	5	48.7 ± 4.97	54.2 ± 13.9	0.977 ± 0.203
Act-D	1	0.223	0.181	0.812
Antimetabolites				
5-FU	9	463 ± 46.6	426 ± 44.5	0.947 ± 0.093
Ara-C	1	12.2	12.9	1.057
Others				
VP-16	7	216 ± 14.2	230 ± 25.7	1.15 ± 0.107
MIT	8	0.851 ± 0.180	15.9 ± 3.86	21.6 ± 4.01
AMSA	3	45.1 ± 15.1	37.9 ± 6.64	0.935 ± 0.144
CDDP	11	287 ± 37.8	287 ± 33.5	1.25 ± 0.278
VCR	6	0.304 ± 0.047	0.383 ± 0.070	1.34 ± 0.189
Taxol	1	0.712	0.653	0.917

[1]Cytotoxicity was determined by MTT assay (3-day exposure).–[2]GI_{50}, drug concentration required to 50% growth inhibition, ng/ml. Dose levels of camptothecin derivatives are expressed as free base. The mean and standard errors were obtained from the data of independent experiments.–[3]Extant of resistance: (GI_{50} for PC-6/SN2-5)/(GI_{50} for PC-6).

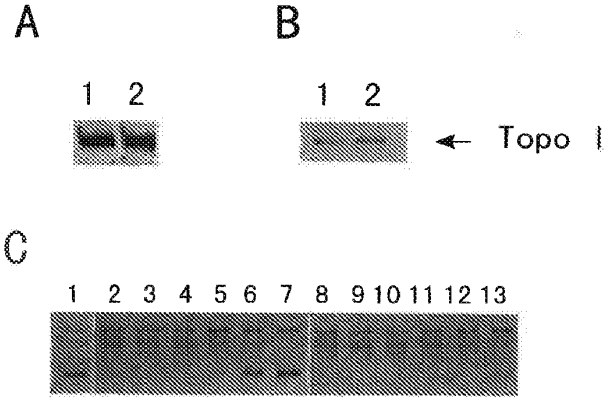

FIGURE 1 – DNA topoisomerase levels and activities in PC-6 and PC-6/SN2-5 cells. *(a* and *b)* Immunoblotting of topoisomerase I in PC-6 (lane 1) and PC-6/SN2-5 (lane 2) cells. Whole cell lysates (20 μg protein) *(a)* and nuclear extract (88 ng protein) *(b)* were each added to a lane and separated by SDS-PAGE. Topo I protein was then detected with topoisomerase I antibody. *(c)* SV40 supercoiled DNA relaxation by different amounts of nuclear extract protein from PC-6 and PC-6/SN2-5 cells. Lane 1, DNA only; lanes 2–7, DNA and nuclear protein from PC-6 cells; lanes 8–13, DNA and nuclear protein from PC-6/SN2-5 cells. The amounts of nuclear protein applied were 250 ng (lanes 2 and 8), 125 ng (lanes 3 and 9), 62.5 ng (lanes 4 and 10), 31.3 ng (lanes 5 and 11), 15.6 ng (lanes 6 and 12) and 7.81 ng (lanes 7 and 13).

SN-38. We selected one well-growing clone, PC-6/SN2-5, from the final 5 clones.

In vitro *cytotoxicity*

Growth inhibition experiments were carried out as previously described (Mitsui *et al.*, 1995). Briefly, PC-6 or PC6/SN2-5 cells were plated in 96-well microplates, and on the next day, drugs were added at various concentrations. The solvents of each drug were added to control wells. After 3 days of cultivation, the amount of viable cells was determined by MTT assay (Mosmann, 1983). Growth inhibition of 50% (GI_{50}) was calculated from $100 \times [(T - T_0)/(C - T_0)] = 50$, which is the drug concentration causing a 50% reduction in optical density in control cells during the drug incubation (where C is control optical density, T is test optical density and T_0 is optical density at time zero).

Detection of topoisomerase I in whole cell lysates and nuclear extracts

Cells (5×10^6) were lysed with SDS buffer (10 mM HEPES, 2 mM orthovanadate, 10 mM NaF, 10 mM pyrophosphate, 1 mM PMSF, 10 μg/ml leupeptin, 10% 2-mercaptoethanol, 10% glycerol, 8% SDS, 42 mM Tris-HCl, 0.002% bromophenol blue, pH 7.4). Protein in the whole cell lysates was separated in 7.5% polyacrylamide gel and blotted onto nitrocellulose membrane. The membrane was treated with anti-Topo I human antibody (TopoGEN, Columbus, OH) and subsequently, with horseradish peroxidase-conjugated protein A. The Topo I-specific band was detected with ECL reagents (Amersham, Aylesbury, UK). To obtain a nuclear extract, cells (5×10^7) were washed with ice-cold buffer (2 mM K_2HPO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, 0.1 mM dithiothreitol), resuspended in buffer containing 0.35% Triton-X100 and PMSF and then incubated on ice for 10 min. The resulting lysates were centrifuged, and precipitates were then incubated with buffer containing 0.35 M NaCl for 1 hr at 4°C. After centrifugation (18,000*g*, 10 min), the protein concentration of the supernatant (nuclear extract) was determined by Bradford's method using a protein assay kit (Bio-Rad, Melville, NY). The same amount of nuclear protein was analyzed by Western blotting analysis using anti-Topo I antibody, as described above.

Determination of topoisomerase I activity

DNA Topo I activity in PC-6 and PC-6/SN2-5 cells was determined using the DNA relaxation assay (Kawato *et al.*, 1991). Nuclear extracts prepared by the method described above were incubated with supercoiled SV40 DNA (GIBCO, Grand Island, NY) at 37°C for 10 min. Relaxed DNA and supercoiled DNA were separated in 0.8% agarose gel by electrophoresis and visualized by ethidium bromide staining.

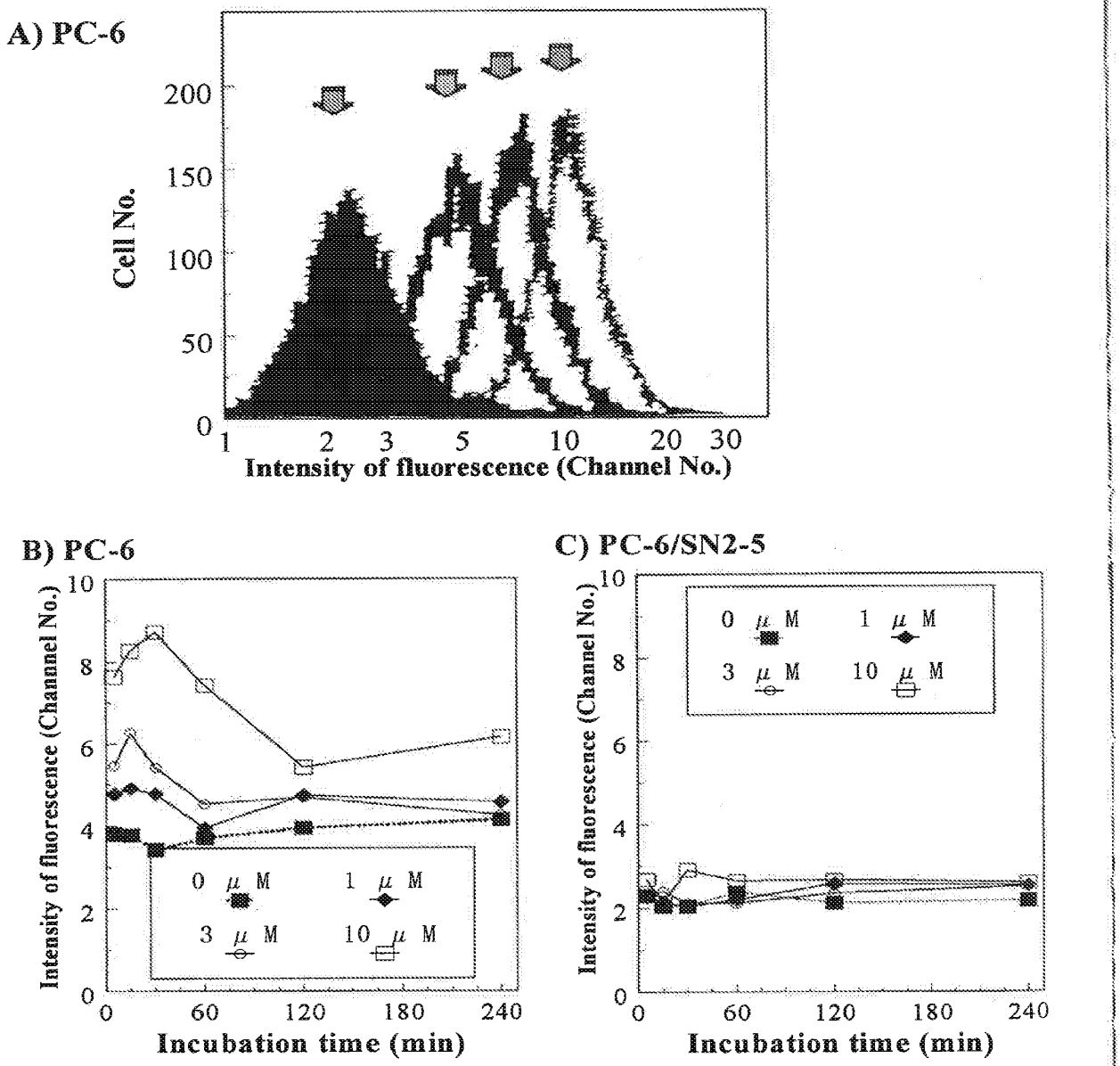

FIGURE 2.—Intracellular accumulation of topotecan in PC-6 and PC-6/SN2-5 cells. *(a)* A representative example of the actual cell sorting result with PC-6 cells treated with 0, 1, 3 and 10 μM of topotecan for 30 min. Arrows indicate positions of peak channel. PC-6 *(b)* and PC-6/SN2-5 *(c)* cells were incubated with 0 μM (■), 1 μM (◆), 3 μM (○) or 10 μM (□) of topotecan for the indicated times, and the autofluorescence intensity of topotecan in the cells was measured by flow cytometry. Intensity of fluorescence is indicated as the peak channel number of each sample.

Measurement of intracellular accumulation of topotecan by flow cytometry

The flow cytometry method described by Hendricks *et al.* (1992) was used to measure topotecan accumulation in PC-6 and PC-6/SN2-5 cells. Various concentrations of topotecan were added to the cells and incubated for 5–240 min at 37°C. Each sample was immediately subjected to flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA). A 15-mW argon laser was used to deliver a 488-nm excitation to the cells, and fluorescence in the cells was detected using a 585-nm filter with a band width of 42 nm.

Measurement of intracellular accumulation of camptothecin analogs by HPLC

Cells (1×10^5) were incubated with 10 μM of drugs for 30 min at 37°C and then washed twice with ice-cold PBS(−). After centrifugation, 100 μl of H_2O was added to the cell pellet, and the cells were disrupted by vigorous sonication. Next, the cell solution was centrifuged at 10,000*g*, and then 75 μl of the resulting supernatant were mixed with the same amount of stop-solution (0.1 N HCl/ethanol, 1:10). The insoluble protein fraction was removed by centrifugation, and then the supernatant was subjected to HPLC.

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which was performed using a C_{18} guard-pak precolumn (Waters, Milford, MA) and a 4.6 mm × 25 cm analytical column of 5 μM ODS-80TS TSK-gel (Tosoh, Tokyo, Japan). Fifty microliters of the sample were injected into the column using a Bio-Rad AS-100 autosampler. To prepare the mobile phase, 20% (for DX-8951f), 27% (for SN-38) or 15% (for topotecan) of MeCN were added to 0.01 N HCl and de-gassed with helium prior to use. The chromophore for each drug was detected using an F1000 fluorescent detector (Hitachi, Tokyo, Japan). The excitation (Ex) or emission (Em) wavelengths for the drugs were as follows: Ex = 375 nm, Em = 443 nm for DX-8951f, Ex = 380 nm, Em = 556 nm for SN-38, and Ex = 383 nm, Em = 533 nm for topotecan. Retention times and peak areas were calculated using the C-R4AX chromatographic data system (Shimadzu, Kyoto, Japan). Peak areas were fitted to the linear regression of a calibration curve produced by adding 1–100 μM of each compound in 0.01 N HCl.

Detection of topoisomerase I covalently bound to DNA in cells

Intracellular DNA-Topo I complexes were detected by using TopoGEN *in vivo* Topoisomerase Cross Linking Kit, following the manufacturer's instruction. Cells (1×10^7) were plated in 100-mm plastic dish, incubated for 18 hr and further exposed to drug for 30 min. Cells were scraped off and rapidly lysed by the addition of 1% sarkosyl buffer. Following CsCl step gradient centrifugation (100,000*g* for 18 hr at 25°C), gradients of cell lysate were divided into 23–24 fractions. The DNA content of each fraction was measured by reading the absorbance at 260 nm. Free and DNA-bound Topo I in each fraction was detected by dot blot analysis using the specific antibody to human Topo I (TopoGEN) and developed on X-ray film by the ECL system. The density of Topo I-specific dots was measured with a densitometer (Vilber Lourmat, Marne-la-Vallée, France) and analyzed by a Bio Profile ver. 6.0.

RESULTS

In vitro *cytotoxicity of various anticancer agents against parental and resistant PC-6 cell lines*

Table I shows the cytotoxicity of 5 different camptothecin analogs and other well-known anticancer agents against human oat cell carcinoma PC-6 cells and its SN-38-resistant line PC-6/SN2-5. PC-6/SN2-5 cells were about 32-fold resistant to SN-38, 14-fold to topotecan, and 6-fold to 9-AC but less resistant to other camptothecin analogs, namely, DX-8951f (2-fold) and CPT-11 (3-fold). PC-6/SN2-5 cells were not resistant to any of the other anticancer drugs tested in this study, but were resistant only to mitoxantrone (MIT, 22-fold).

Topo I protein level and Topo I activity

The decrease in the Topo I protein level has been reported as one of the mechanisms for camptothecin resistance; we thus determined the Topo I protein levels in PC-6 and PC-6/SN2-5 cells by Western blotting analysis. As Figure 1*a* shows, the Topo I levels in cell lysates of both cell line were similar. Specifically, the Topo I level in the PC-6/SN2-5 cell line appeared to be slightly higher than that in the PC-6 cell line. In some camptothecin-resistant cells, it has been shown that Topo I protein cannot enter the nucleus, although Topo I might be expressed at normal levels in the cytosol. We next determined Topo I levels in nuclear extracts of PC-6 and PC-6/SN2-5 cells. The results obtained in nuclear extracts were similar to those in whole cell lysates: Topo I levels in the nuclear extract of PC-6/SN2-5 cells were slightly higher than that in parental cells (Fig. 1*b*). Mutation in protein is often known to cause a band shift on SDS-PAGE, but Topo I-specific bands on SDS-PAGE of samples derived from both cell lines were detected at the same position (Fig. 1*b*). Reduction of Topo I activity caused by some mutations in Topo I has also been reported with regard to camptothecin-resistant cells, but the supercoiled DNA relaxation activity of nuclear extracts from PC-6/SN2-5 cells was slightly higher, rather than lower, than that in parental cells (Fig. 1*c*).

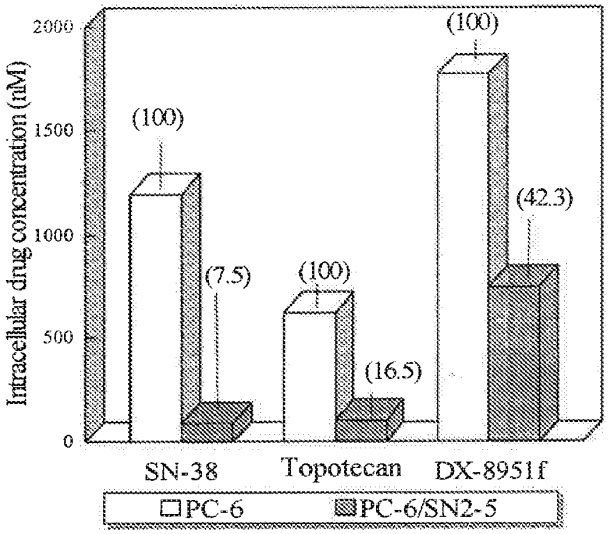


FIGURE 3 – Intracellular accumulation of SN-38, topotecan and DX-8951f in PC-6 and PC-6/SN2-5 cells. PC-6 (open column) and PC-6/SN2-5 (closed column) cells were incubated with 10 μM of SN-38, topotecan or DX-8951f for 30 min, and the autofluorescence intensity of each drug in the cells was measured by HPLC. The figures shown in parenthesis are the relative drug concentrations (%) compared with the respective parental lines. Each column represents the mean of duplicate samples, and standard error is indicated by an error bar.

Topotecan accumulation (flow cytometry method)

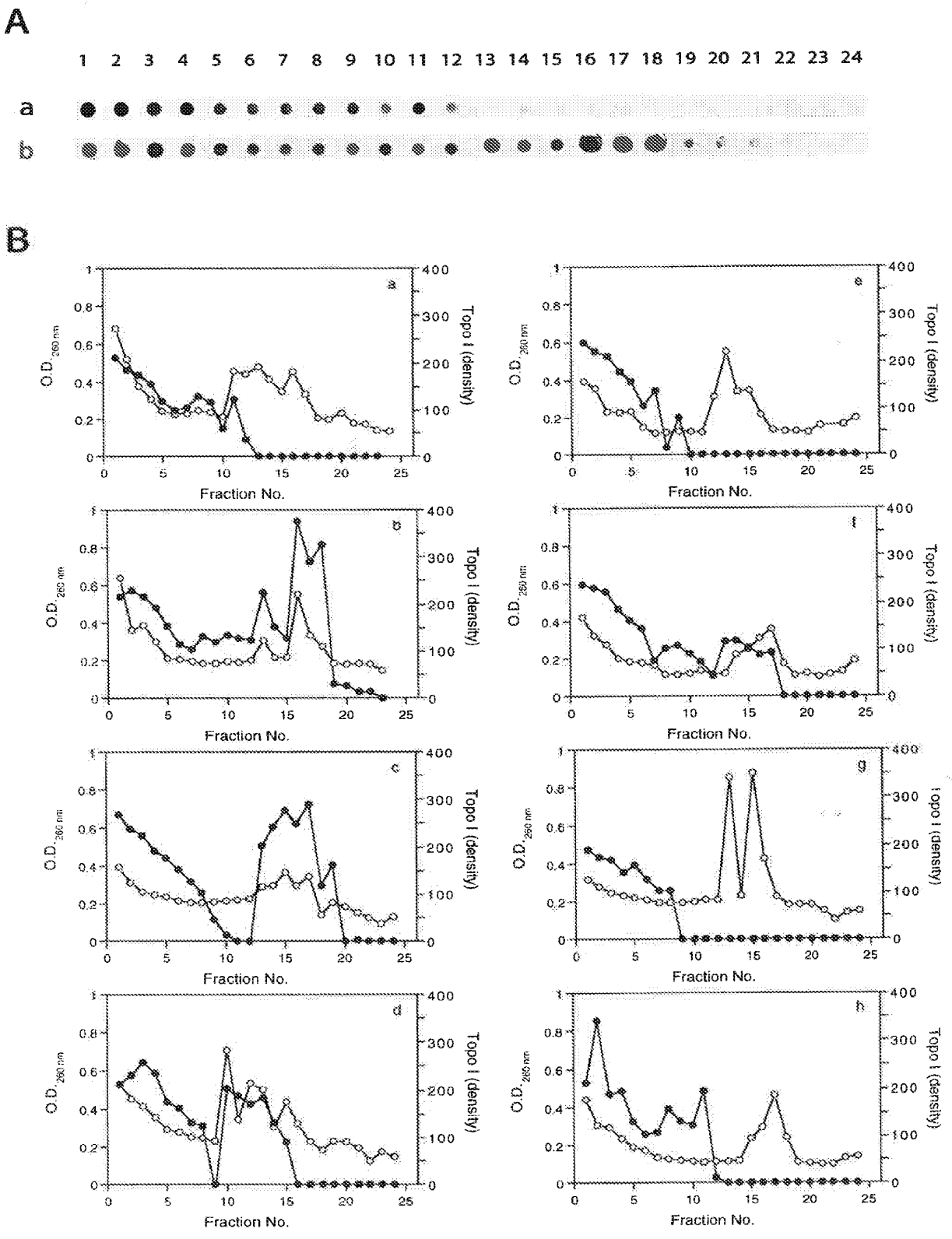
To test whether the resistance of PC-6/SN2-5 cells to camptothecin analogs is due to a defect in drug accumulation, we next measured topotecan accumulation using a FACScan by detecting its fluorescence, since the fluorescence of topotecan could be detected by the apparatus. As Figure 2*a* and *b* indicates, topotecan accumulated in PC-6 cells in a dose-dependent manner. The accumulation peaked at 30 min after adding the drug, and the intracellular concentration decreased slowly. However, in PC-6/SN2-5 cells, drug accumulation was apparently reduced and was detected only when the cells were treated with the highest concentration (10 μM) of topotecan (Fig. 2*c*).

Drug accumulation (HPLC method)

Since the autofluorescence of DX-8951f and SN-38 could not be detected by FACScan, intracellular accumulation of these drugs and topotecan was measured by the HPLC method. The intracellular concentration of these drugs in PC-6/SN2-5 cells was lower than in PC-6 cells (Fig. 3). The differences in drug accumulation between these 2 cell lines were about 10 times for SN-38 and about 5 times for topotecan, but only double for DX-8951f (Fig. 3). While P-glycoprotein expression in both cell lines was determined by Western blotting using P-glycoprotein-specific antibody (clone C219), P-glycoprotein was not detected in either cell line (data not shown).

Stabilization of DNA-Topo I complex by camptothecin analogs

Next, the formation of covalent DNA-Topo I complexes in PC-6 and PC-6/SN2-5 cells was measured, since camptothecin analogs are known to inhibit Topo I via stabilization of covalent DNA-Topo I complexes. Topo I protein was detected only in the protein-rich fraction of non-treated PC-6-cell lysates (Fig. 4*A-a, B-a*), but in the lysates of cells treated with 34 nM of DX-8951f (Fig. 4*A-b, B-b*), 120 nM of SN-38 (Fig. 4*B-c*) and 190 nM of topotecan (Fig. 4*B-d*), Topo I was also detected in DNA-rich fractions. This result suggests that DX-8951f, topotecan and SN-38 stabilize DNA-Topo I complexes in PC-6 cells at the concentrations examined. This


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
a
b
B
a
b
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O.D.260 nm
Topo I (density)
Fraction No.

FIGURE 4

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stabilizing activity of these compounds was correlated with their antitumor activity. The stabilizing activity of DX-8951f was about 2.5 times and 5 times higher than that of SN-38 and of topotecan, respectively. DX-8951f also showed stabilizing activity in PC-6/SN2-5 cells at the same concentration as in PC-6 cells (Fig. 4*B-f*), although the stabilizing activity appeared to be a little weaker than in parental cells. By contrast, SN-38 (Fig. 4*B-g*) and topotecan (Fig. 4*B-h*) showed no stabilizing activity in PC-6/SN2-5 cells at the same dose as in PC-6 cells. Two-fold (68 nM) and 10-fold (1,900 nM) concentrations were required for DX-8951f and for topotecan, respectively, to show stabilizing activity in PC-6/SN2-5 cells to the same extent as in the parental cells. SN-38 at a 5 times higher concentration (600 nM), however, did not show activity in the resistant cells (data not shown).

DISCUSSION

To evaluate our novel camptothecin-derived compounds, we have established various PC-6 subclones resistant to CPT-11 or SN-38 and selected 15 CPT-11-resistant clones and 5 SN-38-resistant clones including PC-6/SN2-5. These 20 clones exhibited some differences in resistance levels but showed similar sensitivity patterns against various anticancer drugs, the sensitivity of all clones to various anticancer agents including camptothecin derivatives being similar to that of PC-6/SN2-5. Our stepwise selection system may select a type of resistant cell line that exhibits a similar sensitivity to drugs.

Some camptothecin-resistant cell lines are known to reduce the level or the activity of Topo I, or both, and this reduction may be the dominant camptothecin-resistant mechanism (Andoh *et al.*, 1987; Gupta *et al.*, 1988; Tan *et al.*, 1989; Sugimoto *et al.*, 1990; Kubota *et al.*, 1992; Madelaine *et al.*, 1993; Tanizawa *et al.*, 1993; Kapoor *et al.*, 1995). It has been suggested that when the Topo I level or activity is clearly reduced, another topoisomerase (Topo II) may in its place perform the topoisomerase activity necessary to cell proliferation, and thus Topo I inhibitors were unable to exhibit cell killing activity (Ishida and Andoh, 1994). The overall cellular and nuclear Topo I expression levels of the PC-6/SN2-5 cell line were not lower than those of the parental PC-6 cell line (Fig. 1*a,b*). In addition, the Topo I activity of the PC-6/SN2-5 cells was not lower than that of the parental PC-6 cell line (Fig. 1*c*), and the shift of the Topo I-specific band on SDS-PAGE was not detected in the PC-6/SN2-5 cell-derived sample (Fig. 1*a,b*). The resistance of the PC-6/SN2-5 cell line is therefore not due to alteration in amount or activity of Topo I protein.

The above findings prompted us to consider that reduced intracellular drug accumulation, which is also a well-characterized mechanism of resistance to camptothecin analogs (Chang *et al.*, 1992), may be the relevant resistance mechanism of the PC-6/SN2-5 cell line. Flow cytometry analysis showed a marked reduction of topotecan-accumulation in PC-6/SN2-5 cells (Fig. 2), and HPLC analysis demonstrated similar results (Fig. 3). In addition, decreases in drug accumulation were detected after treatments not only with topotecan but also with other camptothecin analogs, and the decreased level was well correlated with the resistance ability to each compound observed in cytotoxicity tests. These data suggest that the resistance of the PC-6/SN2-5 cell line may have been caused mainly by a defect in intracellular drug accumulation. P-glycoprotein-mediated multidrug-resistant cell lines have been reported to be resistant to topotecan (Hendricks *et al.*, 1992; Chen *et al.*, 1993). It is, however, unlikely that P-glycoprotein-mediated drug efflux participates in the mechanism of this resistant cell line since Western blot analysis revealed that P-glycoprotein is not expressed in PC-6/SN2-5 cells (data not shown). Other unknown mechanisms by which intracellular drug concentrations are reduced are probably responsible for the production of the resistant cells.

As Figure 4 illustrates, stabilization of DNA-Topo I complexes by SN-38 and topotecan was not detected in PC-6/SN2-5 cells at the same concentrations as it was in parental cells. Figure 4 also shows that the stabilizing activity of DX-8951f was weakened in the resistant cells. These findings suggest 2 possibilities: the drug could not enter the nucleus in a sufficiently large amount to induce the Topo I inhibition, or it could not bind to DNA-Topo I complexes because of the mutation of the binding domain. Since topotecan at a 10 times higher concentration and DX-8951f at a 2 times higher concentration succeeded in stabilizing cleavable complexes in the resistant cells to the same extent as they did in the parental cells; furthermore, since the reduced level of drug accumulation and the stabilizing activity of cleavable complex were well correlated in PC-6/SN2-5 cells, reduced drug accumulation was considered to be a more likely explanation of the phenomenon.

Yang *et al.* (1995) reported cross-resistance to camptothecin derivatives in a mitoxantrone-resistant human cell line. They confirmed that the resistance was associated with defects in accumulation of mitoxantrone and topotecan in the absence of P-glycoprotein overexpression and found a reduced stabilization of cleavable complexes in their resistant cells without decrease in Topo I level or activity. Interestingly, in the PC-6/SN2-5 cell line, high resistance to mitoxantrone was observed, although no resistance was observed against other non-camptothecin-type anticancer drugs (Table I), suggesting a common mechanism of resistance in their and our resistant cell lines. The reason for the cross-resistance is not understood, but some physico-chemical or electrical similarities may exist between mitoxantrone and camptothecin analogs.

Intracellular accumulation of DX-8951f was highest among the camptothecin analogs tested in this study, not only in parental PC-6 cells but also in PC-6/SN2-5 cells. DX-8951f was previously reported to exhibit good tissue permeability, on the basis of data from calculated log P values of various hexacyclic camptothecin derivatives synthesized in our laboratory (Mitsui *et al.*, 1994). This characteristic of DX-8951f may improve cytotoxicity, overcoming the defect in drug accumulation. All the results described above suggest that DX-8951f possesses more potent anticancer activity than SN-38 and topotecan against various tumors including various types of camptothecin-resistant cells.

FIGURE 4 – DNA-topoisomerase I complexes in drug-treated or untreated PC-6 and PC-6/SN2-5 cells. Cell lysates were separated to 23 or 24 fractions by CsCl gradients. *(a)* Representative dot blots carried out using anti-Topo I antibody. Fraction 1 came from the top of the tube, and fraction 24 came from the bottom of the tube. *(a)* PC-6 cells untreated. *(b)* PC-6 cells treated with 34 nM DX-8951f. *(b)* Closed symbols refer to the density of Topo I-specific dots and open symbols to absorbance at 260 nm of each sample. PC-6 *(a–d)* and PC-6/SN2-5 *(e–h)* cells were incubated with 34 nM of DX-8951f *(b, f)*, 120 nM of SN-38 *(c, g)*, 190 nM of topotecan *(d, h)* or without additive *(a, e)*. The data are representative of 2 similar results.

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REFERENCES

ANDOH, T., ISHII, K., SUZUKI, Y., IKEGAMI, Y., KUSUNOKI, Y., TAKEMOTO, Y. and OKADA, K., Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. *Proc. nat. Acad. Sci. (Wash.)*, **84**, 5565–5569 (1987).

CHANG, J.Y., DETHLEFSEN, L.A., BARLEY, L.R., ZHOU, B.S. and HENG, Y.C., Characterization of camptothecin-resistant Chinese hamster lung cells. *Biochem. Pharmacol.*, **43**, 2443–2452 (1992).

CHEN, A.Y., YU, C., POTMESIL, M., WALL, M.E., WANI, M.C. and LIU, L.F.,

Camptothecin overcomes MDR1-mediated resistance in human KB carcinoma cells. *Cancer Res.*, **51**, 6039–6044 (1991).

Fukuoka, M., Nitani, H., Suzuki, A., Motomiya, M., Hasegawa, K., Nishiwaki, Y., Kuriyama, T., Abiyoshi, Y., Negoro, S., Masuda, N., Nakahara, S. and Taguchi, T., A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer. *J. clin. Oncol.*, **10**, 16–20 (1992).

Futatsuki, K., Wakui, A., Nakao, I., Sakata, Y., Kambe, M., Shimada, Y., Yoshino, M., Taguchi, T. and Ogawa, N., Late phase II study of irinotecan hydrochloride (CPT-11) in advanced gastric cancer. *Jpn. J. Cancer Chemother.*, **21**, 1033–1038 (1994) (in Japanese).

Gupta, R.S., Gupta, R., Eng, B., Lock, R.B., Ross, W.E., Hertzberg, R.P., Caranfa, M.J. and Johnson, R.K., Camptothecin-resistant mutants of Chinese hamster ovary cells containing a resistant form of topoisomerase I. *Cancer Res.*, **48**, 6404–6410 (1988).

Hendricks, C.B., Rowinsky, E.K., Grochow, L.B. and Kaufmann, S.H., Effect of P-glycoprotein expression on the accumulation and cytotoxicity of topotecan (SK&F 104864), a new camptothecin analogue. *Cancer Res.*, **52**, 2268–2278 (1992).

Hertzberg, R.P., Caranfa, M.J. and Hecht, S.M., On the mechanism of topoisomerase I inhibition by camptothecin: evidence for binding to an enzyme-DNA complex. *Biochemistry*, **28**, 4629–4638 (1989).

Hsiang, Y.H., Hertzberg, R., Hecht, S. and Liu, L.F., Camptothecin induces protein-linked DNA break via mammalian DNA topoisomerase I. *J. biol. Chem.*, **260**, 14873–14878 (1985).

Hsiang, Y.H. and Liu, L.F., Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. *Cancer Res.*, **48**, 1722–1726 (1988).

Ishida, Y. and Andoh, T., Resistance mechanisms related to topoisomerase inhibitors. *Jpn. J. Cancer Chemother.*, **21**, 952–961 (1994) (in Japanese).

Kanzawa, F., Sugimoto, M., Minato, K., Kasahara, K., Bungo, M., Nakagawa, K., Fujiwara, Y., Liu, L.F. and Saijo, N., Establishment of camptothecin analogue (CPT-11)-resistant cell line of human non-small cell lung cancer: characterization and mechanism of resistance. *Cancer Res.*, **50**, 5919–5924 (1990).

Kapoor, R., Slade, D.L., Fujimori, A., Pommier, Y. and Harker, W.G., Altered topoisomerase I expression in two subclones of human CEM leukemia selected for resistance to camptothecin. *Oncology Res.*, **7**, 83–95 (1995).

Kawato, Y., Furuta, T., Aonuma, M., Yasuoka, M., Yokokura, T. and Matsumoto, K., Antitumor activity of a camptothecin derivative, CPT-11, against human tumor xenografts in nude mice. *Cancer Chemother. Pharmacol.*, **28**, 192–198 (1991).

Kibma, T., Kubota, N. and Nishio, K., Establishment of a CPT-11-resistant human ovarian cancer cell line. *Anticancer Res.*, **14**, 799–804 (1994).

Kubota, N., Kanzawa, F., Nishio, K., Takeda, Y., Ohmori, T., Fujiwara, Y., Terashima, Y. and Saijo, N., Detection of topoisomerase I gene point mutation in CPT-11 resistant lung cancer cell line. *Biochem. biophys. Res. Comm.*, **188**, 571–577 (1992).

Madelaine, I., Prost, S., Naudin, A., Riou, G., Lavelle, F. and Riou, J.F., Sequential modifications of topoisomerase I activity in a camptothecin-resistant cell line established by progressive adaptation. *Biochem. Pharmacol.*, **45**, 339–348 (1993).

Masuda, N., Fukuoka, M., Kusunoki, Y., Matsui, K., Takifuji, N., Kudoh, S., Negoro, S., Nishioka, M., Nakagawa, K. and Tanaka, M., CPT-11: a new derivative of camptothecin for treatment of refractory or relapsed small-cell lung cancer. *J. clin. Oncol.*, **10**, 1225–1229 (1992).

Mitsui, I., Kumazawa, E., Hirota, Y., Aonuma, M., Sugimori, M., Ohsuki, S., Uoto, K., Ejima, A., Terasawa, H. and Saito, K., A new water-soluble camptothecin derivative, DX-8951f, exhibits potent antitumor activity against human tumors *in vitro* and *in vivo*. *Jpn. J. Cancer Res.*, **86**, 776–782 (1995).

Mitsui, I., Ohsuki, S., Hirota, Y., Sugimori, M., Terasawa, H. and Saito, K., High potency of a camptothecin derivative DX-8951f might be attributed to good membrane permeability. *Proc. Amer. Assoc. Cancer Res.*, **35**, 455 (1994).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65**, 55–63 (1983).

Nagai, S., Yamauchi, M., Andoh, T., Nishizawa, M., Saita, T., Kubota, Y., Kondou, K., Akiyama, S., Ito, K. and Takagi, H., Establishment and characterization of human gastric and colonic xenograft lines resistant to CPT-11 (a new derivative of camptothecin). *J. Surg. Oncol.*, **59**, 116–124 (1995).

Ogasawara, H., Nishio, K., Kanzawa, F., Lee, Y.S., Funayama, Y., Ohira, T., Kohsaishi, Y., Isogai, Y. and Saijo, N., Intracellular carboxyl esterase activity is a determinant of cellular sensitivity to the antineoplastic agent KW-2189 in cell lines resistant to cisplatin and CPT-11. *Jpn. J. Cancer Res.*, **86**, 124–129 (1995).

Ohe, Y., Sasaki, Y., Shinkai, T., Eguchi, K., Tamura, T., Kohna, A., Kunikane, H., Okamoto, H., Kakato, A., Ohmatsu, H., Kanzawa, F. and Saijo, N., Phase I study and pharmacokinetics of CPT-11 with 5-day continuous infusion. *J. nat. Cancer Inst.*, **84**, 972–974 (1992).

Ohno, R. and 13 others, An early phase II study of CPT-11: a new derivative of camptothecin, for treatment of leukemia and lymphoma. *J. clin. Oncol.*, **8**, 1907–1912 (1990).

Saijo, N., Nishio, K., Kanzawa, F., Shinkai, T., Kakato, A., Sasaki, Y., Eguchi, K., Tamura, T., Ohe, Y., Oshita, F. and Nishio, M., 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy camptothecin: mechanism of resistance and clinical trials. *Cancer Chemother. Pharmacol.*, **34**, 112–117 (1994).

Shimada, Y., Yoshino, M., Wakui, A., Nakao, I., Futatsuki, K., Sakata, Y., Kambe, M., Taguchi, T. and Ogawa, N., Phase II study of CPT-11, a new camptothecin derivative, in metastatic colorectal cancer. *J. clin. Oncol.*, **11**, 909–913 (1993).

Sugimoto, Y., Tsukahara, S., Oh-hara, T., Isoe, T. and Tsuruo, T., Decreased expression of DNA topoisomerase I in camptothecin-resistant tumor cell lines as determined by a monoclonal antibody. *Cancer Res.*, **50**, 6925–6930 (1990).

Takeuchi, S., Takamizawa, H., Takeda, Y., Okawa, T., Tamaya, T., Noda, K., Sugiura, T., Sekimia, K., Yakushiji, M. and Taguchi, T., Clinical study of CPT-11, camptothecin derivative, on gynecological malignancy. *Proc. Amer. Soc. clin. Oncol.*, **10**, 189 (1991).

Tan, K.B., Mattern, M.R., Eng, W.K., McCabe, F.L. and Johnson, R.K., Nonproductive rearrangement of DNA topoisomerase I and II genes: correlation with resistance to topoisomerase inhibitors. *J. nat. Cancer Inst.*, **81**, 1732–1735 (1989).

Tanizawa, A., Bertrand, R., Kohlhagen, G., Taguchi, A., Jenkins, J. and Pommier, Y., Cloning of Chinese hamster DNA topoisomerase I cDNA and identification of a single point mutation responsible for camptothecin resistance. *J. biol. Chem.*, **268**, 25463–25468 (1993).

Yang, C.H.J., Horton, J.K., Cowan, K.H. and Schneider, E., Cross-resistance to camptothecin analogues in a mitoxantrone-resistant human breast carcinoma cell line is not due to DNA topoisomerase I alterations. *Cancer Res.*, **55**, 4004–4009 (1995).

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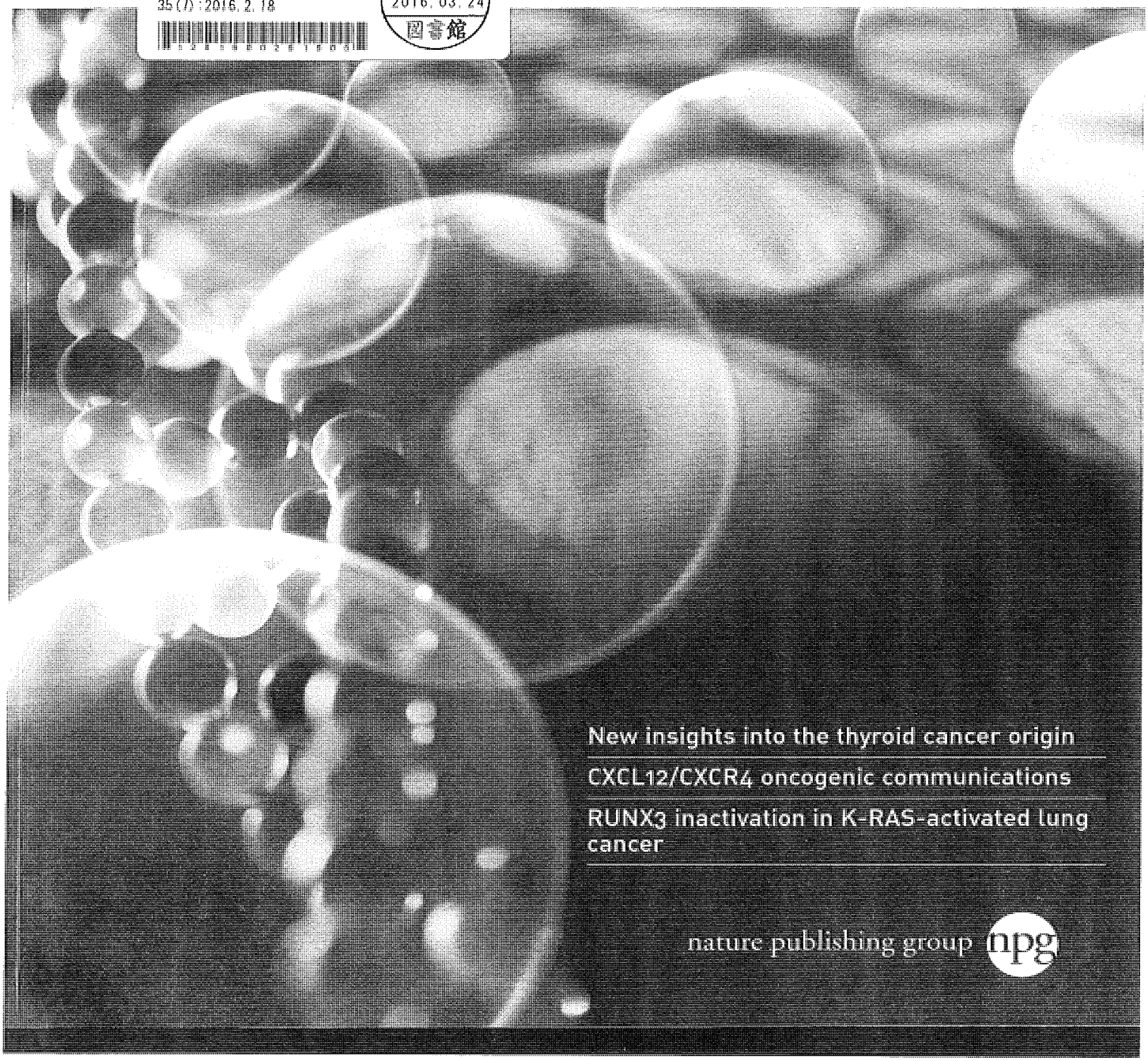

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ORIGINAL ARTICLE

Anti-HER3 monoclonal antibody patritumab sensitizes refractory non-small cell lung cancer to the epidermal growth factor receptor inhibitor erlotinib

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Human epidermal growth factor receptor (HER) 3 is aberrantly overexpressed and correlates with poor prognosis in non-small cell lung cancer (NSCLC). Patritumab is a monoclonal antibody against HER3 that has shown promising results in early-phase clinical trials, but an optimal target population for the drug has yet to be identified. In the present study, we examined whether heregulin, a HER3 ligand that is also overexpressed in a subset of NSCLC, can be used as a biomarker to predict the antitumorigenic efficacy of patritumab and whether the drug can overcome the epidermal growth factor receptor tyrosine kinase inhibitor (EGFR TKI) resistance induced by heregulin. Patritumab sensitivity was associated with heregulin expression, which, when abolished, resulted in the loss of HER3 and AKT activation and growth arrest. Furthermore, heregulin overexpression induced EGFR TKI resistance in NSCLC cells harbouring an activating EGFR mutation, while HER3 and AKT activation was maintained in the presence of erlotinib in heregulin-overexpressing, EGFR-mutant NSCLC cells. Sustained HER3-AKT activation was blocked by combining erlotinib with either anti-HER2 or anti-HER3 antibody. Notably, heregulin was upregulated in tissue samples from an NSCLC patient who had an activating EGFR mutation but was resistant to the TKI gefitinib. These results indicate that patritumab can overcome heregulin-dependent EGFR inhibitor resistance in NSCLC *in vitro* and *in vivo* and suggest that it can be used in combination with EGFR TKIs to treat a subset of heregulin-overexpressing NSCLC patients.

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INTRODUCTION

Members of the human epidermal growth factor receptor (HER) family of proteins—which includes HER1 (also known as epidermal growth factor receptor (EGFR)), HER2, HER3 and HER4—are receptor tyrosine kinases (RTKs)[1–3] that homodimerize or heterodimerize when activated by ligand binding. Aberrant HER signalling, which is caused by overexpression or activating mutations, has a critical role in the oncogenesis of some cancer types.[4,5] *HER3* is occasionally amplified and aberrantly upregulated in cancers such as non-small cell lung cancer (NSCLC),[6,7] and the latter is associated with a poor prognosis.[7] Owing to structural features that limit its intrinsic kinase activity, HER3 cannot be auto-phosphorylated but can be transphosphorylated through heterodimerization or higher-order clustering with other family members, such as HER2.[1–3,8] HER3 is a major mediator of the phosphoinositide 3-kinase/AKT cell survival signalling pathway.[9] The HER3 ligand heregulin is locally secreted and induces HER3 activation through an autocrine mechanism.[10] Alternatively, HER3 can also be activated independently of ligand binding by the dysregulation of other tyrosine kinase receptors.[9,11] For example, in NSCLC cases harbouring an activating EGFR mutation, EGFR can activate HER3 in *trans* through heterodimerization.[9] EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib can prevent HER3 phosphorylation in this subset of NSCLC.

HER3 is a promising oncotherapeutic target.[12] Monoclonal antibodies targeting HER3 have been investigated preclinically and clinically.[13–16] One of these, patritumab, is a novel, fully human monoclonal antibody directed against the extracellular domain of HER3. Early clinical trials designed to evaluate patritumab monotherapy revealed that it was well tolerated and limited tumour growth in some cancers, including NSCLC.[17] Despite these promising results, the drug's mechanism of action is incompletely understood, and a biomarker has not yet been identified for the selection of an optimal target patient subpopulation who can benefit from patritumab treatment.

EGFR is also a drug target in NSCLC, and somatic mutations in the tyrosine kinase domain cause constitutive activation of EGFR in a subset of NSCLC;[18,19] this activity can be blocked by EGFR-TKIs.[20] Although EGFR-TKIs show robust effects on NSCLC with activating EGFR mutations, the cells eventually acquire resistance; in approximately half of such cases, this is attributable to a secondary mutation in the EGFR kinase domain, most frequently a T790M substitution in exon 20.[21,22] In addition, HER3 has a key role in EGFR-TKI resistance.[23,24] Additionally, amplification of the *MET* oncogene can activate HER3, thereby switching on downstream phosphoinositide 3-kinase/AKT survival mechanisms, even in the presence of an EGFR-TKI.[25] Thus targeting HER3 may help

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prevent the development of cancer cell resistance to EGFR-TKIs. To investigate this hypothesis, we evaluated the antitumorigenic efficacy of patritumab in NSCLC as a single agent and in combination with established clinical EGFR inhibitors.

RESULTS

NSCLC cell lines exhibit differential sensitivity to patritumab

To evaluate the efficacy of patritumab *in vitro*, cell proliferation was assessed in 48 NSCLC cell lines with the soft agar colony-formation assay. Cells were treated with patritumab for 7 days at 100 μg/ml, which is comparable to the concentration in the plasma of cancer patients in phase II clinical trials.[13,26] The efficacy of patritumab varied across cell lines, with growth inhibition rates ranging from 0% to 40% (Figure 1a). Although patritumab had a marginal antiproliferative effect on most cell lines, in six of these—QG56, HARA, SW900, H1944, H358 and A549—a growth inhibition rate of > 25% was observed (Supplementary Table S1). These lines were defined as patritumab-sensitive, while the other lines were classified as patritumab-refractory. Cell viability was reduced in a dose-dependent manner in HARA, QG56, A549 and SW900 cells treated with patritumab, while in refractory cell lines such as H1437, PC3, H1573 and H520, patritumab did not inhibit cell growth even at the highest concentration (Figure 1b).

Patritumab sensitivity is associated with heregulin expression level in NSCLC

We hypothesized that the differential sensitivity to patritumab was due to molecular differences in the HER3 signalling pathway. Indeed, *heregulin* and *HER3* mRNA expression levels varied across the 48 NSCLC cell lines (Supplementary Figure S1). Cell lines were stratified into three groups ($n = 16$ each) based on their *heregulin* mRNA expression level. According to the *HER3* mRNA expression level, cell lines were divided into high and low expression groups ($n = 24$ each); growth inhibition rates upon treatment with 100 μg/ml patritumab were compared between groups. Cell lines with high *heregulin* expression were more sensitive to patritumab than those with medium or low *heregulin* expression (16.5% vs 1.1% vs 3.8%; $P < 0.002$, Figure 1c), and all six patritumab-sensitive cell lines had high *heregulin* expression. In contrast, there were no differences in the growth inhibition rates between the low and high *HER3* expression groups (7.5% vs 5.2%; $P = 0.500$). Notably, A549 and HARA cells had the lowest *HER3* expression among cell lines but were nonetheless sensitive to patritumab due to high *heregulin* expression (Supplementary Figure S1).

Activating EGFR mutations were identified in 10 cell lines (PC9, HCC827, H1650, HCC4006, Ma70, Ma70GR, 11-18, H1975, PC9ZD and HCC827GR5 cells; Supplementary Table S2). Five of these lines (PC9, HCC827, HCC4006, Ma70 and 11-18) were sensitive to EGFR-TKI (data not shown), and all cell lines were refractory to patritumab (Figure 1c, Supplementary Table S1). To verify whether

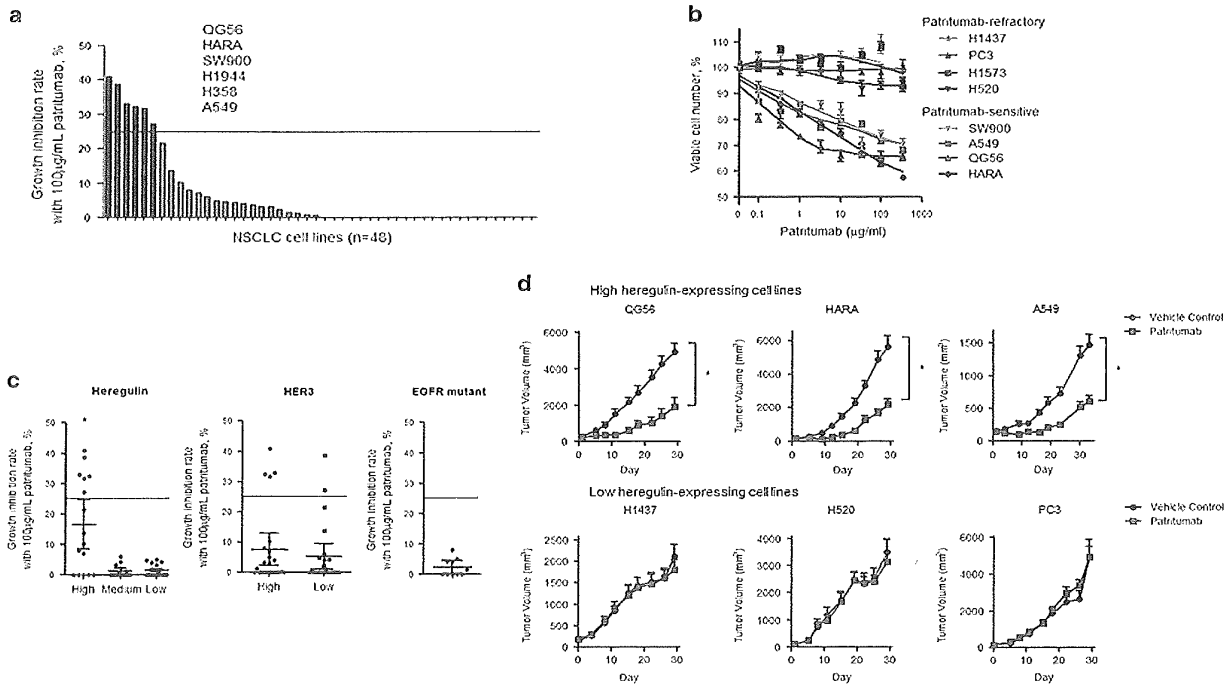

Figure 1. Patritumab sensitivity depends on heregulin expression in NSCLC *in vitro*. (**a**) Patritumab sensitivity was evaluated using a soft agar colony-formation assay. NSCLC cell lines ($n = 48$) were treated with 100 μg/ml patritumab, and the growth inhibition rate was calculated relative to untreated control cells. (**b**) Patritumab-sensitive or -refractory cell lines ($n = 8$) were treated with the indicated concentrations of patritumab for 3 days; cell viability was evaluated using the CellTiter-Glo viability assay and shown relative to untreated control cells (mean ± s.d. of six independent experiments). (**c**) Relationship between growth inhibition rate at 100 μg/ml patritumab and heregulin and HER3 expression levels among 48 NSCLC cell lines classified as high, medium or low expression ($n = 16$ each) for heregulin and high or low expression ($n = 24$ each) for HER3. Also shown are the growth inhibition rates for 10 NSCLC cell lines with activating EGFR mutations treated with 100 μg/ml patritumab. Patritumab-sensitive cell lines are highlighted in red. Data represent mean ± s.d. $^*P < 0.002$, unpaired *t*-test, high vs medium or low heregulin groups. (**d**) In a xenograft NSCLC model, cells expressing high (QG56, HARA and A549) or low (H1437, H520 and PC3) heregulin were treated with patritumab or vehicle. Each treatment group consisted of 10 mice. Data represent mean ± s.e.m. $^*P < 0.05$, unpaired *t*-test.

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our *in vitro* findings could be extended *in vivo*, high (QG56, HARA and A549) and low (H1437, H520 and PC3) heregulin-expressing NSCLC cell lines were implanted subcutaneously in mice, which were then treated with patritumab or vehicle for 4 weeks. Xenograft tumours arising from cells expressing high levels of heregulin were reduced in size by patritumab treatment (Figure 1d), while tumours originating from cells with low heregulin expression continued to grow. Taken together, these results suggest that heregulin contributes to patritumab sensitivity both in NSCLC cell lines and in a tumour model.

Heregulin-dependent NSCLC proliferation *in vitro* is inhibited by patritumab

Depending on the context, heregulin can stimulate or inhibit the proliferation of various cancer cells.[27,28] To evaluate the role of heregulin on NSCLC growth, A549 cells were cultured in medium containing exogenous heregulin at concentrations of 1 or 4 ng/ml, which was replaced every 3 days over 15 days. The growth rate steadily increased from day 8 onwards relative to control cells grown in the absence of heregulin, and this effect was concentration-dependent (Figure 2a). Similar effects were observed in seven other NSCLC cell lines treated with exogenous heregulin at 1 ng/ml for 8 days (Figure 2b).

To determine whether patritumab could inhibit heregulin-induced proliferation, A549 cells were cultured in medium with various concentrations of exogenous heregulin and treated with 100 μg/ml patritumab for 3 days. In these cells, the growth rate steadily increased relative to cells grown in medium without heregulin and in the absence of patritumab (Figure 2c). In contrast, proliferation was inhibited by patritumab, even in the presence of high levels of heregulin.

Finally, to examine whether heregulin knockdown would reduce proliferation in patritumab-sensitive cell lines, we transfected HARA, QG56 and A549 cells (which express high endogenous levels of heregulin) with small interfering RNA (siRNA) against heregulin (siHRG). Compared with siMock cells, siHRG significantly reduced proliferation of these cells, indicating that heregulin overexpression stimulates the growth of NSCLC cells.

Patritumab prevents heregulin-induced HER2/HER3 interaction

To elucidate the molecular mechanisms underlying patritumab sensitivity, the expression of HER3 and the downstream effector AKT was assessed in NSCLC cells expressing high (HARA, A549 and QG56) and low (PC3, H1437 and H520) levels of heregulin following treatment with 100 μg/ml patritumab for 6 h. In cell lines with high heregulin levels, patritumab treatment resulted in the dephosphorylation of HER3 and AKT (Figure 3a), without altering the total HER3 level. In contrast, in cells with low heregulin expression, HER3 phosphorylation was not observed before or after patritumab treatment, and the drug had no effects on AKT phosphorylation in these cells. Patritumab had no effect on the phosphorylation status of ERK in any of the cell lines examined (Supplementary Figure S2). These results suggest that patritumab can abolish heregulin-induced HER3 activation and that cell survival associated with AKT signalling is predominantly driven by HER3 activation in cells that express high levels of heregulin.

Additionally to determine whether HER3-AKT signalling activity depends on heregulin expression, A549 cells were treated with siHRG. Activation of HER3 as well as AKT was prevented by siHRG transfection due to lack of heregulin binding (lane 1 vs lane 3, Supplementary Figure S3). These results suggest that HER3-AKT signalling was activated by heregulin.

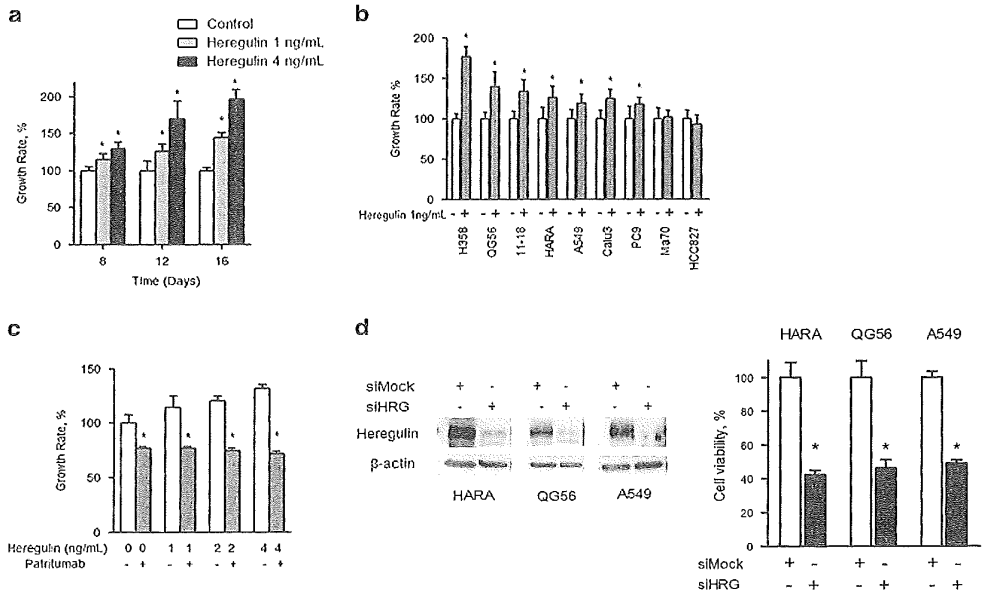

Figure 2. Heregulin stimulates proliferation in NSCLC *in vitro*, which is inhibited by patritumab. (**a**) A549 cells were treated with 1 or 4 ng/ml heregulin or vehicle for 16 days, and the growth rate was determined on days 8, 12 and 16 (mean ± s.d. of four independent experiments) relative to the control on the respective days. Cell viability was evaluated using the CellTiter-Glo viability assay. *$P < 0.05$ vs control, unpaired *t*-test. (**b**) Growth rates were determined for nine NSCLC cell lines treated with 1 ng/ml heregulin or vehicle for 8 days (mean ± s.d. of six independent experiments). *$P < 0.05$ vs control, unpaired *t*-test. (**c**) Growth rate was determined for A549 cells cultured in medium containing 0, 1, 2 or 4 ng/ml heregulin and 100 μg/ml patritumab for 3 days (mean ± s.d. of six independent experiments). *$P < 0.05$ vs untreated cells, unpaired *t*-test. (**d**) Heregulin-overexpressing HARA, QG56 and A549 cells were transfected with siRNA against heregulin (siHRG) or a non-targeting siRNA (siMock); the indicated proteins were detected by western blotting. Cell viability was evaluated using the CellTiter-Glo viability assay and calculated relative to siMock (mean ± s.d. of five independent experiments). *$P < 0.05$ vs siMock, unpaired *t*-test.

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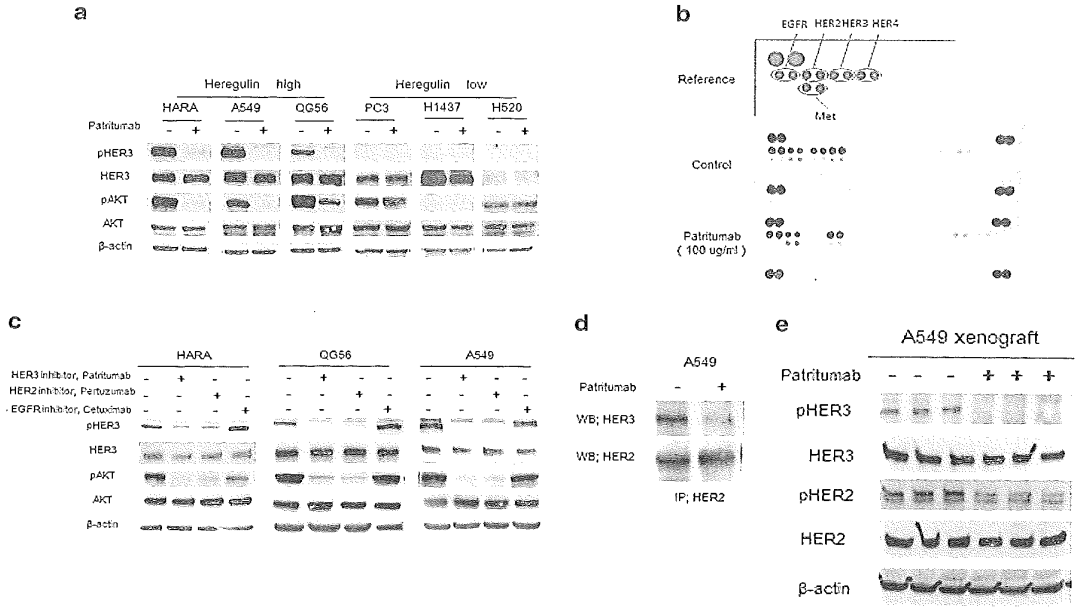

Figure 3. Patritumab blocks heregulin-induced heterodimerization of HER2 and HER3. (**a**) Cells expressing high (HARA, A549, QG56) or low (PC3, H1437, H520) levels of heregulin were treated with 100 μg/ml patritumab for 6 h and then probed for the indicated proteins. (**b**) HARA cells were treated with 100 μg/ml patritumab or vehicle for 6 h, and whole cell lysates were analysed using a phospho-RTK array. (**c**) HARA, QG56 and A549 cells were treated with patritumab, pertuzumab, cetuximab or vehicle for 6 h, and whole cell lysates were probed for the indicated proteins. (**d**) A549 cells were treated with 100 μg/ml patritumab or vehicle for 6 h, and whole cell lysates were immunoprecipitated with an antibody against HER2, which was detected along with HER3 by western blotting. (**e**) A549 cell xenograft mice were treated with vehicle or patritumab for 4 weeks; cell lysates from tumours were probed for the indicated proteins.

Previous reports indicate that HER3 activation occurs preferentially through heterodimerization with EGFR, HER2, HER4 or MET.[1,6,8] To determine whether patritumab inactivates multiple receptors, heregulin-overexpressing HARA cells were treated with 100 μg/ml patritumab or vehicle for 6 h, and the phosphorylation status of EGFR, HER2, HER3, HER4 and MET was evaluated using an RTK array. All of the receptors were phosphorylated in control cells; exposure to patritumab potently inhibited HER3 phosphorylation and moderately inhibited EGFR and HER2 phosphorylation, but it had no effects on MET and HER4 phosphorylation (Figure 3b, Supplementary Figure S4).

We also examined whether EGFR or HER2 inhibition prevents HER3 phosphorylation in heregulin-overexpressing cell lines. To this end, HARA, QG56 and A549 were treated with cetuximab, pertuzumab or patritumab or antibodies against EGFR, HER2 or HER3. Patritumab and pertuzumab, but not cetuximab, blocked the phosphorylation of HER3 and AKT in these cells (Figure 3c), suggesting that HER3 may interact with HER2 in cell lines that express high levels of heregulin. To test this possibility, HER2 was immunoprecipitated from A549 cells and the immune complex was probed for HER3. In untreated control cells, HER3 was detected in complex with HER2 (Figure 3d); however, patritumab-treated cells had a lower level of HER3 than control cells, indicating that the HER3 interaction with HER2 is abolished by patritumab treatment.

To examine whether these findings were relevant *in vivo*, the phosphorylation status of HER2 and HER3 was examined in A549 cells implanted subcutaneously in mice treated with patritumab or vehicle for 4 weeks. Both HER2 and HER3 were phosphorylated in A549 xenografts of untreated animals. However, HER3 was completely and HER2 was partially dephosphorylated in tumours from patritumab-treated mice, (Figure 3e), providing further evidence that HER3 preferentially interacts with HER2.

Heregulin causes EGFR-TKI resistance in NSCLC cells harbouring an activating EGFR mutation

We previously reported that heregulin-dependent resistance to the anti-EGFR antibody cetuximab in colorectal cancer is due to activation of HER2 and HER3 via heterodimerization, as this engenders persistent downstream proliferation signals even in the presence of cetuximab.[29] To determine whether heregulin expression results in EGFR-TKI resistance in NSCLC, the EGFR-TKI-sensitive NSCLC cell lines PC9 and HC827—which harbour an activating EGFR mutation—were stably transfected with heregulin and treated with erlotinib for 3 days (Figure 4a). In contrast to mock-transfected control cells, heregulin overexpression rendered both cell lines refractory to erlotinib. To explore the mechanistic basis for this change, transfected PC9 cells were treated with 1 μM erlotinib for 24 h. Erlotinib inhibited phosphorylation of EGFR in both cell lines (Figure 4b) and reduced HER3 transphosphorylation, as well as phosphorylation of the downstream effectors AKT and ERK in mock-transfected cells. In contrast, in heregulin-overexpressing cells, erlotinib caused HER3 phosphorylation in a time-dependent manner along with an increase in total HER3, despite EGFR inactivation (Supplementary Figure S5). Erlotinib initially prevented AKT and ERK phosphorylation following 3 h of exposure, whereas both proteins were hyperphosphorylated at later time points. These results suggest that HER3 can be activated in a heregulin-dependent manner in heregulin-overexpressing PC9 cells treated with erlotinib and that HER3 activation is amplified due to the increase in total HER3 upon erlotinib treatment. These findings were confirmed *in vivo* in mice implanted with PC9 mock- or heregulin-overexpressing cells and treated with erlotinib daily for 3 weeks; xenograft tumours arising from mock-transfected cells shrank following erlotinib treatment, whereas those overexpressing heregulin continued to grow in the presence of erlotinib (Figure 4c).

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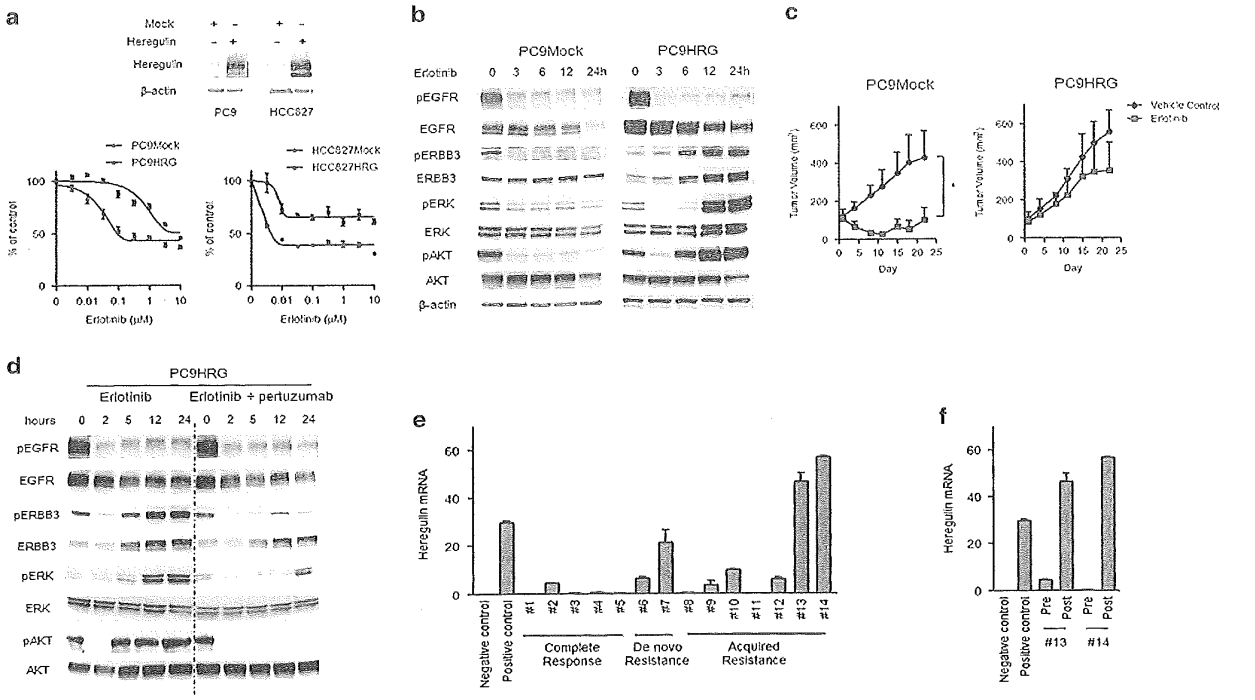

Figure 4. Heregulin induces EGFR TKI resistance in NSCLC cells harbouring activating EGFR mutations. (**a**) EGFR mutant NSCLC PC9 and HCC827 cells were mock-transfected (PC9Mock) or stably transfected with heregulin (PC9HRG) and then treated with the indicated concentrations of erlotinib; cell viability was measured 3 days later using the CellTiter-Glo viability assay, and values are plotted relative to untreated control cells (mean ± s.d., $n = 6$). Whole cell lysates were probed for heregulin expression. (**b**) PC9Mock and PC9HRG cells were treated with 1 μM erlotinib for the indicated times and then probed for the indicated proteins. (**c**) PC9Mock or PC9HRG cells xenograft mice were treated with erlotinib or vehicle for 3 weeks. Each treatment group consisted of eight mice. Data represent mean ± s.e.m. $^*P < 0.05$ vs vehicle, unpaired *t*-test. (**d**) PC9HRG cells were treated with either 1 μM erlotinib alone or 1 μM erlotinib combined with 100 μg/ml pertuzumab and then probed for the indicated proteins. (**e**) Tumour samples from 14 NSCLC patients with activating EGFR mutations were analysed for *heregulin* mRNA expression. Five patients achieved complete response, and three patients had primary resistance and six had acquired resistance to EGFR-TKI. *y* Axis, fold changes compared with HCT116 cell line (mean ± s.d. of three independent experiments). (**f**) Tumour samples were obtained from two NSCLC patients prior to and after EGFR-TKI treatment, and *heregulin* mRNA expression was analysed. *y* Axis, fold changes compared with HCT116 cell line (mean ± s.d. of three independent experiments).

To explore the functional mechanisms for this resistance, we treated PC9HRG cells with either erlotinib alone or in combination with the anti-HER2 antibody pertuzumab for 24 h. Erlotinib monotherapy elevated the total HER3 expression level and reactivated HER3 as well as AKT and ERK signalling in PC9HRG cells in a time-dependent manner (Figure 4d, Supplementary Figure S6). In contrast, treatment of erlotinib plus pertuzumab could inhibit re-activation of HER3 as well as AKT and ERK despite the increase in total HER3 expression. These results suggest that HER3 can be reactivated due to HER2/HER3 interaction in PC9HRG cells following erlotinib treatment. In addition, AKT and ERK lie downstream of the HER2/HER3 complex; presumably, AKT is downstream of HER3 and ERK is downstream of HER2.

To assess the clinical relevance of the observed heregulin-induced resistance to EGFR-TKI, *heregulin* mRNA expression was evaluated in clinical samples from 14 NSCLC patients harbouring activating EGFR mutations (Supplementary Table S3). Five specimens were from patients who showed a complete response to gefitinib, and two specimens were from patients resistant to gefitinib who discontinued treatment within a few months because of disease progression. The other seven specimens were from patients who initially responded to gefitinib and then later acquired resistance; tumour samples were obtained by re-biopsy upon disease progression. Heregulin upregulation comparable to the positive control (heregulin-overexpressing PC9 cells) was observed in samples from three NSCLC patients (Figure 4e). One (#7) had an *EGFR* exon 19 deletion and received erlotinib but discontinued treatment after 1 month because of disease progression; two other patients (#13 and #14) showed a partial response but then acquired EGFR-TKI resistance accompanied by an upregulation of heregulin expression, which was negligible prior to EGFR-TKI treatment (Figure 4f). Notably, all three patients were EGFR T790M-negative. DNA sequencing was also performed to investigate genomic amplification of *MET* and *HER2*. None of these genomic alterations were detected in samples #13 and #14,[25,30] and histological examination revealed no evidence of changes such as epithelial-to-mesenchymal transition or small cell lung cancer, which has been observed in some cases of acquired resistance.[22] Heregulin upregulation was not observed in specimens from the five patients who showed a complete response.

Patritumab overcomes erlotinib resistance induced by heregulin in NSCLC

The results described above suggested that patritumab could block HER3 activation in a heregulin-dependent manner in NSCLC. We therefore hypothesized that cells treated with patritumab could overcome erlotinib resistance induced by heregulin. To test this, heregulin-overexpressing PC9 cells were treated with erlotinib or patritumab alone or in combination, and the effect

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on proliferation was evaluated. Cells were refractory to erlotinib, with an IC50 $>1\,\mu M$ (Figure 5a). Strikingly, the cells were also resistant to patritumab, despite the high level of heregulin expression. However, a combination of both agents potently inhibited proliferation. Similar effects were observed in heregulin-overexpressing HARA cells (Figure 5b). The combination treatment was also evaluated in a panel of cell lines that were refractory to EGFR-TKIs due to various underlying mechanisms. This included PC9ZD cells with a T790M secondary EGFR mutation and HC827GR5 cells with MET amplification.[25,31] In contrast to the heregulin-overexpressing PC9 cells, these two cell lines were resistant to the erlotinib/patritumab combination, suggesting that patritumab could overcome EGFR-TKI resistance only when it is heregulin-dependent (Supplementary Table S4). Additionally, we examined whether erlotinib/patritumab combination could overcome erlotinib resistance in PC9ZD and HCC827GR5 cells when exposed to the medium containing high concentration of exogenous heregulin. Parent PC9 cells developed the resistance to erlotinib in the presence of exogenous 10 ng/ml heregulin, and this resistance could be overcome by treatment with erlotinib/patritumab combination (Figure 5c). In contrast to the effect observed in parent PC9 cells, erlotinib/patritumab combination therapy could not overcome erlotinib resistance in PC9ZD and HCC827GR5 cells despite the presence of 10 ng/ml heregulin in the medium. These results suggest that erlotinib/patritumab combination might not overcome EGFR-TKI resistance due to a T790M secondary EGFR mutation or MET amplification irrespective of the concomitant presence of heregulin.

To explore the mechanistic basis for the efficacy of patritumab in combination treatments, heregulin-overexpressing PC9 cells were treated with erlotinib or patritumab alone or in combination for 24 h. Treatment with patritumab alone minimally inhibited HER3 phosphorylation, and treatment with erlotinib alone caused re-activation of HER3 as well as AKT and ERK in a time-dependent manner. In contrast, the combination treatment inhibited the re-activation of HER3 as well as AKT and ERK, despite an increase in total HER3 level in PC9HRG cells (Figure 5d, Supplementary Figure S7). These results suggest that co-administration of patritumab can inhibit the heregulin-dependent HER3 activation that occurs following erlotinib treatment. Additionally, we performed an immunoprecipitation assay with anti-HER2 antibody, and the immune complex was probed for HER3 in PC9HRG cells. HER3 was detected in complex with HER2 in cells treated with erlotinib alone, compared with untreated controls cells. However, those complexes were disrupted with patritumab combination therapy (Figure 5e). This in turn provides a potential mechanistic explanation for the antiproliferative effects of patritumab during combination treatment.

The antitumorigenic potential of this drug combination was evaluated in mice bearing heregulin-overexpressing PC9 xenografts. Neither erlotinib nor patritumab alone affected tumour growth, whereas both drugs used in combination potently suppressed tumour growth compared with control mice (Figure 5f). However, after the treatment was discontinued, the tumours began to grow rapidly.

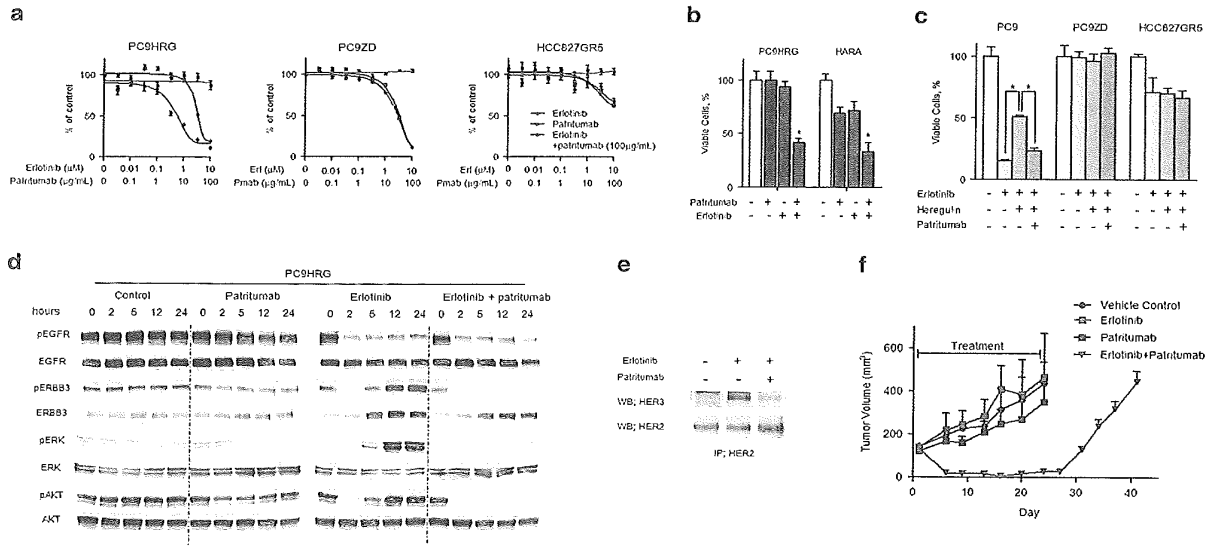

Figure 5. Patritumab overcomes heregulin-induced erlotinib resistance. (**a**) PC9HRG, PC9ZD and HCC827GR5 cells were treated with erlotinib, patritumab or both at the indicated concentrations (in the combination treatment, patritumab concentration was fixed at 100 μg/ml), and cell viability was measured 3 days later using the CellTiter-Glo viability assay. Data represent mean ± s.d. of six independent experiments. (**b**) PC9HRG and HARA cells were treated with patritumab (100 μg/ml), erlotinib (1 μM) or both; cell viability relative to untreated control cells was quantified 3 days later. Data represent mean ± s.d. of six independent experiments. $^*P < 0.05$ vs other treatments, unpaired *t*-test. (**c**) Parent PC9, PC9ZD and HCC827GR5 cells were treated with patritumab (100 μg/ml), erlotinib (1 μM), erlotinib (1 μM) in the presence of 10 ng/ml heregulin or erlotinib (1 μM) combined with patritumab (100 μg/ml) in the presence of 10 ng/ml heregulin. Cell viability relative to untreated control cells was quantified 3 days after treatment. Data represent mean ± s.d. of six independent experiments. $^*P < 0.05$ vs. other treatments, unpaired *t*-test. (**d**) PC9HRG cells were treated with control, 100 μg/ml patritumab alone, 1 μM erlotinib alone or 1 μM erlotinib combined with 100 μg/ml patritumab for the indicated times and then probed for the indicated proteins. (**e**) PC9HRG cells were treated with control, erlotinib (1 μM) alone or erlotinib (1 μM) combined with patritumab (100 μg/ml) for 24 h, and whole cell lysates were immunoprecipitated with an antibody against HER2, which was detected along with HER3 by western blotting. (**f**) PC9HRG cell xenograft mice were treated with vehicle or erlotinib, patritumab or both for 3 weeks. Each treatment group consisted of six mice. Data represent mean ± s.e.m. $^*P < 0.05$, combination therapy vs other treatments, unpaired *t*-test.

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DISCUSSION

The anti-HER3 monoclonal antibody patritumab is currently under clinical development and investigations are on-going to identify a biomarker that could be used to select the beneficiary patient subpopulation. Here we showed that *heregulin* expression level correlates with patritumab sensitivity in a preclinical NSCLC model (Figure 1). Mechanistically, this is most likely explained by the patritumab-dependent inhibition of heregulin binding to HER3 and concomitant dephosphorylation of the receptor. Alternatively, patritumab may act as a dimerization inhibitor, in a manner similar to the HER2 antibody pertuzumab or to LJM716, another HER3 monoclonal antibody.[14] Other anti-HER3 antibodies can reportedly inhibit proliferation via inactivation of HER3 in heregulin-overexpressing cancers.[13–16] Furthermore, our data suggest that heregulin expression may predict the efficacy of HER2 inhibitors such as pertuzumab or lapatinib. This is since HER2 plays a key role for HER3 activation in heregulin-overexpressing NSCLC cells. Head and neck cancer cells overexpressing heregulin are sensitive to lapatinib, even in the absence of *HER2* gene amplification, and tissue specimens from head and neck cancer patients have a similar level of *heregulin* mRNA expression as lapatinib-sensitive cell lines.[32]

In contrast to heregulin, sensitivity to patritumab did not correlate with HER3 expression level in the NSCLC cell lines examined. This discrepancy may be explained by the inactivation of HER3 in the absence of ligand binding (Figure 3). Unlike HER3, HER2 is constitutively active without ligand binding, and thus the efficacy of the anti-HER2 antibody trastuzumab depends on the HER2 expression level.[1,12]

Antitumorigenic effects of patritumab monotherapy were not detected in EGFR-mutant NSCLC cell lines. According to a previous study, HER3 is activated independently of ligand binding in EGFR-mutant cell lines.[9,25] HER3 depletion inhibited proliferation in HCC827 NSCLC cells that harbour an activating EGFR mutation.[25] However, in the current study, patritumab did not inhibit proliferation in EGFR-mutant NSCLC cell lines nor did it decrease HER3 phosphorylation or HER3 expression in these cells (Supplementary Figure S8). These results imply that patritumab does not abolish EGFR binding with HER3. Instead, we suggest that the inability of patritumab to internalize and degrade HER3 could limit cell sensitivity to the drug.

Heregulin overexpression induced EGFR-TKI resistance in NSCLC cell lines harbouring activating EGFR mutations, which was also confirmed in clinical specimens (Figure 4). Unlike mock-transfected PC9 cells, those overexpressing heregulin reactivated HER3 as well as AKT and ERK, even in the presence of erlotinib. Moreover, all these heregulin-dependent effects were prevented by treatment with the anti-HER2 antibody, pertuzumab (Figure 4d). These results suggest that the HER2/HER3 complex induces EGFR-TKI resistance in PC9 cells overexpressing heregulin. In addition, total HER3 expression increased in a time-dependent manner in PC9HRG cells treated with erlotinib. This might contribute to re-activation of HER3, although further experimental evidence is required to support this point.

Combination patritumab treatment abrogated heregulin-induced EGFR-TKI resistance (Figure 5). Strikingly, this effect was not found in other EGFR-mutant NSCLC cell lines that have different resistance mechanisms, suggesting that new therapeutic strategies designed to overcome or prevent resistance must be highly targeted.[21,22,25] In an on-going Japanese clinical trial of patritumab combined with erlotinib, 10 of the 12 NSCLC patients with activating EGFR mutations showed prolonged stabilization of tumours that had acquired EGFR-TKI resistance. These are encouraging results compared with the effects of erlotinib alone.[33,34] Thus a combination treatment of patritumab and an EGFR-TKI may improve the clinical outcome in NSCLC cases of acquired resistance.

A limitation of this study is the selection bias in the cell lines used and the *in vitro* approach that did not take into consideration host factors such as circulating heregulin or the tumour microenvironment, which could influence patritumab efficacy. Nonetheless, the latest results of a randomized phase II study of patritumab combined with erlotinib in NSCLC have shown that *heregulin* mRNA expression in tumours is significantly correlated with progression-free survival.[35]

The current study suggests that patritumab can overcome heregulin-induced EGFR inhibitor resistance in NSCLC both *in vitro* and *in vivo*. We recommend that patritumab combination with EGFR TKI should be examined in a clinical setting on a cohort of NSCLC patients with TKI-refractory tumours that have heregulin overexpression and EGFR-activating mutations.

MATERIALS AND METHODS

Cell culture and reagents

The 48 NSCLC cell lines used have been previously characterized[20,25,36,37] (Supplementary Table S1) and obtained from American Type Culture Collection (Manassas, VA, USA) except QG56, PC14, PC1, PC10 (IBL Co., Ltd, Gunma, Japan), HARA (Japan Human Science Foundation, Tokyo, Japan), EBC1, Sq-1 and RERF-LCA1 (Riken Cell Bank, Ibaraki, Japan). Ma70 was established in Kinki University School of Medicine, Osaka, Japan. PC9ZD, Ma70GR and HCC827GR5 are gefitinib-refractory clones of a gefitinib-sensitive EGFR-mutant parent cell line. Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS). We used cell lines within 6 months of the initial culture. Patritumab was provided by Daiichi-Sankyo Co. Ltd. (Tokyo, Japan). Erlotinib, cetuximab and pertuzumab were obtained from commercial sources.

Patients

Tumour specimens from EGFR TKI-treated patients were obtained from the Kinki University School of Medicine, with approval from the Institutional Review Board and with written informed consent from each patient.

Antibodies and western blotting

Cells were seeded at a density of 1×10^6 cells/plate in Prime Surface 60-mm plates (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) and allowed to grow overnight in medium containing 0.5% FBS before the addition of drugs to the medium. Cells were incubated for various times and then harvested. Western blotting was performed as previously described.[20] Proteins were transferred to nitrocellulose membranes, which were probed with antibodies against phospho-AKT, AKT, phospho-EGFR, EGFR, phospho-HER3 and heregulin (all from Cell Signaling Technology, Danvers, MA, USA) and phospho-ERK 1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Soft agar colony-formation assay

To screen the 48 NSCLC cell lines for patritumab sensitivity, colony formation was assessed using the CytoSelect 96-well cell transformation assay (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's protocol. Briefly, a base agar layer containing 0.6% agar, Dulbecco's modified Eagle's medium and 0.5% FBS was poured into each well of a 96-well plate and was covered with a cell agar layer with the same composition at a density of 5×10^3 cells/well, with patritumab added to a final concentration of 100 μg/ml. After 7 days, the agar was solubilized and the mixture was incubated with 3,(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl-tetrazolium bromide solution, and fluorescence was measured using a 96-well microplate reader at 570 nm. The growth inhibition rate was calculated relative to untreated control cultures.

Cell proliferation and growth inhibition assays

The sensitivity to patritumab, erlotinib and heregulin (HRG1-beta 1 extracellular domain, R&D Systems, Minneapolis, MN, USA) was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol.

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Quantitative real-time PCR

Total RNA was isolated from cells and paraffin-embedded tissues using the RNeasy Mini Kit and RNeasy FFPE Kit, respectively (both from Qiagen, Valencia, CA, USA), according to the manufacturer's specifications. cDNA was synthesized using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA, USA) and used for real-time PCR to measure heregulin (*NRG1*) and *HER3* expression, with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression used as an internal standard. Expression levels relative to the HCT116 cancer cell line were determined using a standard method with the ABI 7900 HT system SDS software (Applied Biosystems).

siRNA transfection

siRNA against heregulin (siHRG) from the ON-TARGET Plus SMARTpool (no. L-004608-01; Dharmacon, Lafayette, CO, USA) was a mixture of four sets of 21-nucleotide sense and antisense strands. Non-targeting siRNA (siMock) was used as a non-specific control. Cells were plated at 50% confluence in six-well plates and incubated for 24 h in RPMI-1640 medium supplemented with 0.5% FBS and then transfected with siRNAs using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA).

Establishment of a heregulin-overexpressing cell line

A full-length cDNA fragment encoding human heregulin (NRG1, NM_13956) was obtained from Origene (Rockville, MD, USA). The amplified fragment was cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and transferred to the pQCXIH retroviral vector (Clontech Laboratories, Inc., Mountain View, CA, USA). Retroviruses encoding heregulin were used to infect PC9 cells.

DNA sequencing

For multiplex PCR amplification, 10 ng DNA was processed using the Ion AmpliSeq Library Kit and Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies, Carlsbad, CA, USA). The Ion Xpress Barcode Adapters (Life Technologies) were ligated onto the PCR products and purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). Purified libraries were pooled and sequenced on an Ion Torrent PGM (Life Technologies) using the Ion PGM 200 Sequencing Kit v2 and the Ion 318 vs Chip Kit.

DNA sequencing data was accessed through Torrent Suite v3.4.2 (Life Technologies). Reads were aligned against the hg19 human reference genome, and variants were called using Variant Caller v3.6 (Life Technologies). Raw variant calls were filtered out with the following annotation: homozygous and heterozygous variants, QUAL of < 100, and depth of cover < 19.

Xenograft studies

NSCLC cells were implanted into female athymic nude mice and allowed to grow to at least 150 mm^3 before mice were treated with patritumab (100 μg/mouse), administered twice weekly by intraperitoneal injection, or erlotinib (25 mg/kg), administered daily by oral gavage. Mice were matched for tumour size in each experiment arm and treated without blinded. Studies were performed in accordance with the standards of the Institutional Animal Care and Use Committee under a protocol approved by the Animal Care and Use Committee at Kinki University.

Statistical analyses

Statistical analyses were performed using the StatView v.5.01 software (SAS Institute, Cary, NC, USA). All statistical tests were two-sided, unpaired *t*-tests, and a *P*-value < 0.05 was considered statistically significant. Data were graphically displayed using GraphPad Prism v.5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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REFERENCES

1 Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005; **5**: 341–354.
2 Zhang Q, Park E, Kani K, Landgraf R. Functional isolation of activated and unilaterally phosphorylated heterodimers of ERBB2 and ERBB3 as scaffolds in ligand-dependent signaling. *Proc Natl Acad Sci USA* 2012; **109**: 13237–13242.
3 Jura N, Shan Y, Cao X, Shaw DE, Kuriyan J. Structural analysis of the catalytically inactive kinase domain of the human EGF receptor 3. *Proc Natl Acad Sci USA* 2009; **106**: 21608–21613.
4 Ohsaki Y, Tanno S, Fujita Y, Toyoshima E, Fujiuchi S, Nishigaki Y *et al.* Epidermal growth factor receptor expression correlates with poor prognosis in non-small cell lung cancer patients with p53 overexpression. *Oncol Rep* 2000; **7**: 603–607.
5 Ji H, Li D, Chen L, Shimamura T, Kobayashi S, McNamara K *et al.* The impact of human EGFR kinase domain mutations on lung tumorigenesis and *in vivo* sensitivity to EGFR-targeted therapies. *Cancer Cell* 2006; **9**: 485–495.
6 Yi ES, Harclerode D, Gondo M, Stephenson M, Brown RW, Younes M *et al.* High c-erbB-3 protein expression is associated with shorter survival in advanced non-small cell lung carcinomas. *Mod Pathol* 1997; **10**: 142–148.
7 Cappuzzo F, Toschi L, Domenichini I, Bartolini S, Ceresoli GL, Rossi E *et al.* HER3 genomic gain and sensitivity to gefitinib in advanced non-small-cell lung cancer patients. *Br J Cancer* 2005; **93**: 1334–1340.
8 Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. *Proc Natl Acad Sci USA* 2010; **107**: 7692–7697.
9 Engelman JA, Jänne PA, Mermel C, Pearlberg J, Mukohara T, Fleet C *et al.* ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. *Proc Natl Acad Sci USA* 2005; **102**: 3788–3793.
10 Zhou B, Peyton M, He B, Liu C, Girard L, Caudler E *et al.* Targeting ADAM-mediated ligand cleavage to inhibit HER3 and EGFR pathways in non-small cell lung cancer. *Cancer Cell* 2006; **10**: 39–50.
11 Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP *et al.* A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. *Cancer Res* 2008; **68**: 5878–5887.
12 Baselga J, Swain S. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nat Rev Cancer* 2009; **9**: 463–475.
13 Schoeberl B, Faber AC, Li D, Liang MC, Crosby K, Onsum M *et al.* An ErbB3 antibody, MM-121, is active in cancers with ligand-dependent activation. *Cancer Res* 2010; **70**: 2485–2494.
14 Garner A, Bialucha C, Sprague E, Garrett JT, Sheng Q, Li S *et al.* An antibody that locks HER3 in the inactive conformation inhibits tumor growth driven by HER2 or neuregulin. *Cancer Res* 2013; **73**: 6024–6035.
15 Schaefer G, Haber L, Crocker LM, Shia S, Shao L, Dowbenko D *et al.* A two-in-one antibody against HER3 and EGFR has superior inhibitory activity compared with monospecific antibodies. *Cancer Cell* 2011; **20**: 472–486.
16 Mirschberger C, Schiller CB, Schräml M, Dimoudis N, Friess T, Gerdes CA *et al.* RG7116, a therapeutic antibody that binds the inactive HER3 receptor and is optimized for immune effector activation. *Cancer Res* 2013; **73**: 5183–5194.
17 LoRusso P, Jänne PA, Oliveira M, Rizvi N, Malburg L, Keedy V *et al.* Phase I study of U3-1287, a fully human anti-HER3 monoclonal antibody, in patients with advanced solid tumors. *Clin Cancer Res* 2013; **19**: 3078–3087.
18 Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497–1500.
19 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; **350**: 2129–2139.
20 Yonesaka K, Zejnullahu K, Lindeman N, Homes AJ, Jackman DM, Zhao F *et al.* Autocrine production of amphiregulin predicts sensitivity to both gefitinib and cetuximab in EGFR wild-type cancers. *Clin Cancer Res* 2008; **14**: 6963–6973.
21 Kobayashi S, Boggon T, Dayaram T, Jänne PA, Kocher O, Meyerson M *et al.* EGFR mutation and resistance of non-small cell lung cancer to gefitinib. *N Engl J Med* 2005; **352**: 786–792.
22 Sequist L, Waltman B, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P *et al.* Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* 2011; **3**: 75ra26.

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23 Noto A, Vitis CD, Roscilli G, Fattore L, Malpicci D, Marra E *et al.* Combination therapy with anti-ErbB3 monoclonal antibodies and EGFR TKIs potently inhibits non-small cell lung cancer. *Oncotarget* 2013; **4**: 1253–1265.
24 Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM *et al.* Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. *Nature* 2007; **445**: 437–441.
25 Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO *et al.* MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007; **316**: 1039–1043.
26 Wakui H, Yamamoto N, Nakamichi S, Tamura Y, Nokihara H, Yamada Y *et al.* Phase 1 and dose-finding study of patritumab (U3-1287), a human monoclonal antibody targeting HER3, in Japanese patients with advanced solid tumors. *Cancer Chemother Pharmacol* 2014; **73**: 511–516.
27 Aguilar Z, Akita R, Finn R, Ramos BL, Pegram MD, Kabbinavar FF *et al.* Biologic effects of heregulin/neu differentiation factor on normal and malignant human breast and ovarian epithelial cells. *Oncogene* 1999; **18**: 6050–6062.
28 Guerra-Vladusic F, Vladusic E, Tsai M, Lupu R. Signaling molecules implicated in heregulin induction of growth arrest and apoptosis. *Oncol Rep* 2001; **8**: 1203–1214.
29 Yonesaka K, Zejnullahu K, Okamoto I, Satoh T, Cappuzzo F, Souglakos J *et al.* Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. *Sci Transl Med* 2011; **3**: 99ra86.
30 Takezawa K, Pirazzoli V, Arcila ME, Nebhan CA, Song X, de Stanchina E *et al.* HER2 amplification: a potential mechanism of acquired resistance to EGFR inhibition in EGFR-mutant lung cancers that lack the second-site EGFRT790M mutation. *Cancer Discov* 2012; **2**: 922–933.
31 Ercan D, Zejnullahu K, Yonesaka K, Xiao Y, Capelletti M, Rogers A *et al.* Amplification of EGFR T790M causes resistance to an irreversible EGFR inhibitor. *Oncogene* 2010; **29**: 2346–2356.
32 Wilson TR, Lee DY, Berry L, Shames DS, Settleman J. Neuregulin-1-mediated autocrine signaling underlies sensitivity to HER2 kinase inhibitors in a subset of human cancers. *Cancer Cell* 2011; **20**: 158–172.
33 Murakami H, Yamamoto N, Kudo K, Nishio M, Kaneda H, Nakagawa K *et al.* Phase I study of Patritumab (U3-1287) in combination with erlotinib in Japanese patients with non-small cell lung cancer. *Ann Oncol* 2013; **24**(Suppl 9).
34 Cho BC, Im CK, Park MS, Kim SK, Chang J, Park JP *et al.* Phase II study of erlotinib in advanced non-small-cell lung cancer after failure of gefitinib. *J Clin Oncol* 2007; **25**: 2528–2533.
35 Von Pawel J, Tseng J, Dediu M, Schumann C, Moritz B, Mendell J *et al.* Phase 2 HERALD study of patritumab (P) with erlotinib (E) in advanced NSCLC subjects. *J Clin Oncol* 2014; **32**: 5s (abstract 8045).
36 Okabe T, Okamoto I, Tamura K, Terashima M, Yoshida T, Satoh T *et al.* Differential constitutive activation of the epidermal growth factor receptor in non-small cell lung cancer cells bearing EGFR gene mutation and amplification. *Cancer Res* 2007; **67**: 2046–2053.
37 Takezawa K, Okamoto I, Yonesaka K, Hatashita E, Yamada Y, Fukuoka M *et al.* Sorafenib inhibits non-small cell lung cancer growth by targeting B-RAF in KRAS wild-type cells and C-RAF in KRAS mutant cells. *Cancer Res* 2009; **69**: 6515–6521.

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Engineering multivalent antibodies to target heregulin-induced HER3 signaling in breast cancer cells

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Abbreviations: TKI, tyrosine kinase inhibitor; scFv, single chain variable fragment; ADCC, antibody dependent cell-mediated cytotoxicity; ADCP, antibody dependent cell-mediated phagocytosis

The use of antibodies in therapy and diagnosis has undergone an unprecedented expansion during the past two decades. This is due in part to innovations in antibody engineering that now offer opportunities for the production of "second generation" antibodies with multiple specificities or altered valencies. The targeting of individual components of the human epidermal growth factor receptor (HER)3-PI3K signaling axis, including the preferred heterodimerization partner HER2, is known to have limited anti-tumor effects. The efficacy of antibodies or small molecule tyrosine kinase inhibitors (TKIs) in targeting this axis is further reduced by the presence of the HER3 ligand, heregulin. To address these shortcomings, we performed a comparative analysis of two distinct approaches toward reducing the proliferation and signaling in HER2 overexpressing tumor cells in the presence of heregulin. These strategies both involve the use of engineered antibodies in combination with the epidermal growth factor receptor (EGFR)/HER2 specific TKI, lapatinib. In the first approach, we generated a bispecific anti-HER2/HER3 antibody that, in the presence of lapatinib, is designed to sequester HER3 into inactive HER2-HER3 dimers that restrain HER3 interactions with other possible dimerization partners. The second approach involves the use of a tetravalent anti-HER3 antibody with the goal of inducing efficient HER3 internalization and degradation. In combination with lapatinib, we demonstrate that although the multivalent HER3 antibody is more effective than its bivalent counterpart in reducing heregulin-mediated signaling and growth, the bispecific HER2/HER3 antibody has increased inhibitory activity. Collectively, these observations provide support for the therapeutic use of bispecifics in combination with TKIs to recruit HER3 into complexes that are functionally inert.

Introduction

Multiple cancers are driven by aberrant signaling through the human epidermal growth factor receptor (HER), also known as ErbB, family members. Recent studies have indicated that HER2-HER3 heterodimers can play a central role in tumorigenesis.[1-5] HER3 is a preferred dimerization partner for HER2, which has no known ligand and is constitutively active.[5-7] Although HER3 has very low intrinsic kinase activity, there are six phosphorylation-dependent binding sites for PI3K on the cytosolic tail of this receptor.[8] Consequently, HER2-HER3 heterodimers are the most effective activators identified to date of the PI3K/Akt pathway through both ligand-independent and ligand-dependent signaling.[5,9] Ligand-dependent activation of HER3 involves the binding of heregulin or other ligands to induce a conformational switch in the dimerization arm, driving heterodimer formation with kinase competent partners such as HER2 or EGFR.[5,6]

Consistent with HER3 as a driver of tumorigenesis, loss of HER3 expression in breast cancer cells results in reductions in both PI3K/Akt signaling and proliferation.[1,2] Further, modeling studies demonstrate that HER3 represents a central node in PI3K/Akt signaling.[4] In conjunction with the limited efficacy of solely targeting HER2 with monotherapies such as trastuzumab,[10-12] these observations have motivated the development of therapeutics targeting HER3 or HER2-HER3 heterodimers.[3,13-15] Recent data indicate that the targeting of this axis with antibodies is less effective in the presence of heregulin,[14] which is expressed in either autocrine or paracrine fashion in many tumor types.[16-18] Thus, there is a need for the generation

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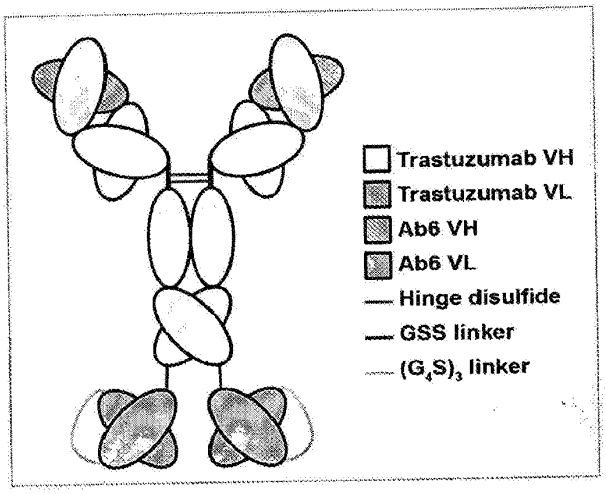

Figure 1. Schematic representation of the bispecific antibody (TAb6) comprising trastuzumab and a scFv derived from the anti-HER3 antibody, Ab6, used in the current study.

of improved therapeutics directed toward ligand-dependent activation of HER3. The current study involves a comparison of two distinct approaches using bispecific HER2/HER3 specific antibodies or multivalent HER3-specific antibodies to target HER3 in the presence of the HER3 ligand, heregulin.

As an alternative to targeting HER family members with antibodies, the use of small molecule inhibitors of the tyrosine kinase activity of EGFR or HER2, or of the downstream kinase, PI3K, has attracted much interest.[19-25] However, these inhibitors can lead to tumor escape due to upregulation of compensatory signaling pathways and complex cross-regulatory networks involving negative feedback loops. For example, the delivery of lapatinib, a tyrosine kinase inhibitor (TKI) that targets both EGFR and HER2, results in upregulation of HER2 and HER3 expression and PI3K/Akt signaling.[26-29] Lapatinib resistance pathways can also include activating mutations of PI3K,[30] and the inhibitory effects of lapatinib can be dampened by the presence of the HER3 ligand, heregulin.[14,31-33] This suggests that lapatinib in combination with antibodies or bispecifics that bind to HER3 might provide an effective route for therapy, particularly for tumors involving autocrine or paracrine heregulin loops that can result in limited efficacy of monotherapies.

Related to the limited therapeutic efficacy of TKIs such as lapatinib that inhibit EGFR and HER2 activation, HER3 can associate with other activating receptors such as cMET and insulin-like growth factor type I receptor I/insulin receptor substrate-1.[5,7,34,35] A possible strategy to extinguish HER3-PI3K signaling would therefore be to inhibit multiple potential HER3 partners. This provides the impetus for directly targeting HER3, which would avoid the complexity of identifying and globally inhibiting all HER3 partners. Here, we have taken the approach of investigating the ability of a bispecific anti-HER2/HER3 antibody to drive HER3 into HER2-HER3 heterodimers that, through combination treatment with lapatinib, are "kinase-dead". Locking HER3 into such inactive complexes is expected to sequester this receptor from interactions with other signaling competent partners and, as such, have anti-tumor effects.

A second route toward extinguishing HER3 signaling is to develop strategies to induce the efficient internalization and degradation of this receptor. In support of this approach, several studies have demonstrated that reduction of HER3 expression has anti-proliferative effects.[1,2] HER3 is constitutively internalized into the endolysosomal pathway[36] and it is well documented that receptor crosslinking leads to downregulation in multiple different cellular systems. We therefore reasoned that a multivalent (tetrameric) anti-HER3 antibody would induce more efficient HER3 internalization or degradation relative to its bivalent counterpart, thereby enhancing clearance from the cell surface. The efficacy of this approach has been compared with that of recruiting HER3 into kinase-inactivated HER2-HER3 heterodimers. These comparative studies have also been extended to microscopy analyses of the trafficking behavior of the different antibodies within cells, which relates to both drug delivery for antibody-drug conjugates and Fc-mediated cytotoxicity.[37,38]

Our study has led to several observations that are of relevance to targeting HER3. First, we show that in the presence of heregulin, combination treatment with antibodies and lapatinib is necessary to achieve inhibition of signaling and growth. Under these conditions, a tetravalent HER3-specific antibody induces increased degradation of HER3 and has more potent anti-tumor effects relative to its bivalent counterpart. Importantly, when used in combination with lapatinib, the bispecific anti-HER2/HER3 antibody is a more effective inhibitor of heregulin-driven signaling and growth compared with anti-HER3 antibodies, tetravalent anti-HER3 antibodies or mixtures of individual antibodies specific for HER2 and HER3. Our observations are consistent with a model in which the bispecific antibody recruits HER3 into HER2-HER3 heterodimers that are inactive in the presence of lapatinib. Collectively, these studies provide support for the combined use of multimeric anti-growth factor receptor antibodies with small molecule TKIs for the therapy of cancer.

Results

Antibodies specific for HER2 and HER3 have differential effects on signaling and proliferation

The anti-HER3 antibody, Ab6 (MM-121[3]), which competes with heregulin for binding to HER3, was generated in our laboratory as a biosimilar and used throughout these studies. To investigate whether a tetravalent anti-HER3 antibody is more effective than its bivalent counterpart in inhibiting cell growth and proliferation, we fused the Ab6 single chain variable fragment (scFv) to the CH3 domains of Ab6 via a Gly-Ser-Ser linker (Ab6tet). In addition, a bispecific trastuzumab (anti-HER2)-Ab6 antibody (TAb6) and a bispecific pertuzumab (anti-HER2)-Ab6 antibody (PAb6) were generated by fusing scFv fragments corresponding to Ab6 to the CH3 domains of trastuzumab and pertuzumab, respectively, using an analogous design (Fig. 1). All antibodies were expressed in transfected Chinese hamster ovary (CHO) cells. The expression yields

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of the bispecific antibodies were 4.5 mg/L, 2.5 mg/L, and 8 mg/L for Ab6tet, PAb6 and TAb6, respectively. Size exclusion and serum stability analyses of the bispecific proteins are presented in the Supplementary Materials (**Fig. S1 and S2**). For comparative purposes, size exclusion studies of Ab6, trastuzumab and pertuzumab are shown (**Fig. S1**). Ab6 and the bispecific antibodies also bound to immobilized, recombinant Fc-HER2 (TAb6, PAb6) or HER3 (Ab6, Ab6tet, TAb6, PAb6) in surface plasmon resonance experiments (BIAcore; data not shown). The in vivo half-lives (β-phase) of Ab6tet and TAb6 were also determined in BALB/c SCID mice and were 228 ± 14 (n = 4 mice) and 215 ± 11 (n = 5 mice) hours, respectively.

We initially assessed the effect of targeting HER3 and HER2/HER3 with several different antibody formats in the absence of the HER3 ligand, heregulin. For comparative purposes we also included the anti-HER2 antibodies, trastuzumab and pertuzumab, which bind to domain IV and the dimerization arm (domain II), respectively, of HER2.[39,40] The anti-HER3 antibody, Ab6, and its tetrameric form, Ab6tet, reduced proliferation in the HER2-overexpressing cancer cell lines, SK-BR-3 and HCC1419 (**Fig. 2A**). Although Ab6 and Ab6tet treatment resulted in a trend toward reduced proliferation in BT-474 cells, the effects were not significant. By contrast with SK-BR-3 and HCC1419 cells, BT-474 cells express a gain of function variant of PI3K[41,42] which could account for the reduced efficacy of anti-HER3 antibodies. Consistent with the observations of others,[43] trastuzumab has higher anti-proliferative activity on ligand-independent growth of SK-BR-3 and BT-474 cells relative to pertuzumab (**Fig. 2A**). A mixture of pertuzumab and Ab6 is also less effective than trastuzumab and Ab6.

Although combinations of anti-HER2 and anti-HER3 antibodies (Ab6 combined with trastuzumab or pertuzumab) had anti-proliferative activities, exposure of cells to the bispecific, TAb6, comprising trastuzumab plus Ab6, resulted in increased proliferation (**Fig. 2A**). Further, the bispecific antibody comprising Ab6 and pertuzumab (PAb6) induced proliferation (**Fig. 2A**). The effects of both PAb6 and TAb6, which bind to different sites of HER2,[39,40] indicate that proximity of HER2 and HER3 is sufficient for proliferative signaling, rather than a need for the receptors to dimerize in a specific configuration. This proximity model is also consistent with the observation that exposure of cells to a mixture of trastuzumab or pertuzumab and Ab6, which would not be expected to drive formation of HER2-HER3 heterodimers, results in reduced proliferation (**Fig. 2A**).

Analyses of the effects of the antibodies on the phosphorylation of HER3, Akt and Erk demonstrated that the anti-proliferative effects are associated with decreased pAkt levels in SK-BR-3 and BT-474 cells (**Fig. 2B**). Although pErk levels were lower following treatment of cells for one hour with Ab6, Ab6tet, or Ab6 combined with anti-HER2 antibodies than for cells treated with trastuzumab, pertuzumab, TAb6 or PAb6, these differences were not sustained at the 24 h time point (**Fig. 2B**). Exposure of SK-BR-3 or BT-474 cells to TAb6 or PAb6 resulted in increased pAkt (S473) levels that persisted for at least 24 h, consistent with the pro-proliferative effects of these bispecific antibodies. By contrast, the levels of pAkt at 24 h were decreased in cells treated with any of the other antibodies or antibody combinations (**Fig. 2B**). Pertuzumab as a single agent, or in combination with Ab6, was less effective than trastuzumab (with or without Ab6) in reducing pAkt (S473) phosphorylation, which is congruent with the lower anti-proliferative activity of this antibody (**Fig. 2A**).

Earlier studies have shown that relief of feedback inhibition of the FoxO1/3a transcription factors can lead to upregulation of multiple receptor tyrosine kinases such as HER3 and insulin-like growth factor-1 receptor (IGF-1R) and subsequent reactivation of Akt.[27,44,45] However, the possibility of pAkt reactivation occurring in the current study following treatment of cells with the bispecific antibodies, TAb6 or PAb6, can be excluded by the relatively rapid kinetics of pAkt induction observed (1 h, **Fig. 2B**). To further support this, we observed phosphorylation of Akt following 15 min of exposure of SK-BR-3 and BT-474 cells to the bispecific antibodies (**Fig. S3**).

Total HER3 levels in the cells following treatment with anti-HER3 antibodies were also analyzed. In general, HER3 levels were reduced by treatment with anti-HER3 antibodies, Ab6 and Ab6tet, whereas exposure of cells to the bispecific antibodies, TAb6 and PAb6, resulted in less HER3 degradation ($P < 0.05$; **Fig. 2B**; **Fig. S4**). Reduced HER3 degradation following TAb6 or PAb6 treatment is consistent with the inhibitory effects of HER2 expression on the internalization of ligand-activated EGFR or HER3.[46,47] The increased HER3 degradation induced by Ab6tet relative to Ab6 was more marked for SK-BR-3 than BT-474 cells, although in both cases the differences were statistically significant ($P < 0.05$; **Fig. 2B**).

Microscopy analyses were used to further investigate the intracellular trafficking pathways taken by Ab6, Ab6tet and TAb6 (**Fig. 3**). These studies demonstrate that Ab6tet is internalized into EEA-1 positive early endosomes more rapidly than Ab6, and enters these compartments within 5 min of treatment. Following 15 min of treatment, both Ab6 and Ab6tet are internalized into early endosomes, although the levels of Ab6 remaining on the plasma membrane are greater than for Ab6tet (**Fig. 3**). By contrast, the majority of TAb6 is present on the plasma membrane following 5–60 min of treatment (**Fig. 3**; **Fig. S5**). Within 60 min of treatment, Ab6 and Ab6tet are present in LAMP-1+ lysosomes (**Fig. S5**). Multivalent antibody binding to HER3 therefore enhances the rate of HER3 internalization into the endolysosomal pathway, consistent with the increased degradation of HER3 in the presence of Ab6tet relative to Ab6.

Targeting HER2/HER3 with antibodies is ineffective in the presence of heregulin

Heregulin is frequently present in tumors due to autocrine or paracrine production,[16-18] motivating an investigation of the effects of the antibodies on tumor cell growth in the presence of this HER3 ligand. HCC1419 cells showed increased proliferation in the presence of heregulin, whereas reduced proliferation was observed for SK-BR-3 cells (**Fig. 4A**). Although BT-474 cells showed a trend toward heregulin-stimulated proliferation, differences between vehicle- and heregulin-treated cells were not always significant. Reduced proliferation of SK-BR-3 cells in response to heregulin has been described previously.[48] Heregulin exposure ablated the inhibitory effects of Ab6, Ab6tet,

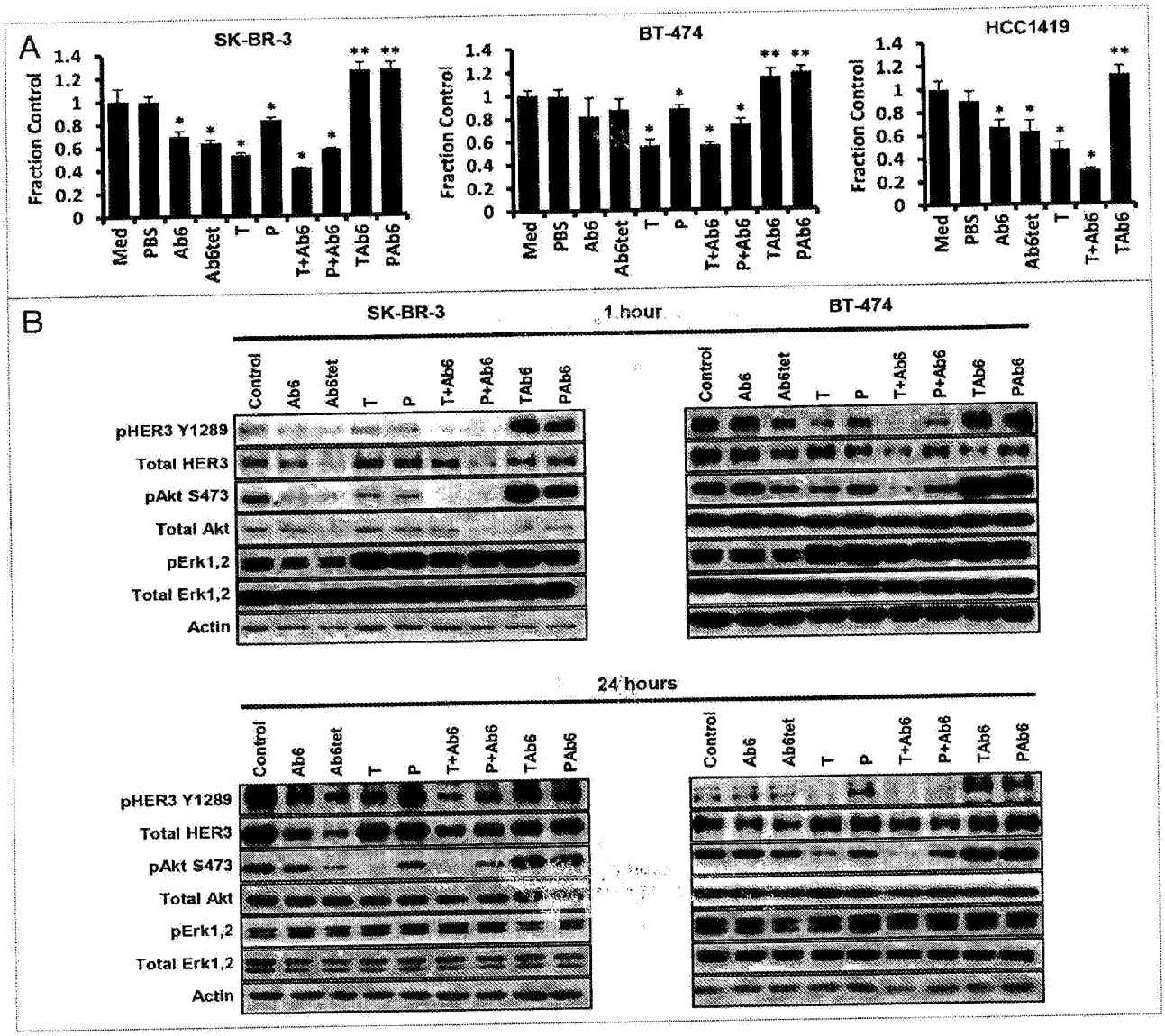

Figure 2. Effects of antibodies specific for HER2 and/or HER3 on HER2-overexpressing breast cancer cells. (**A**) Cells were incubated with 50 nM anti-HER3 (Ab6), tetrameric anti-HER3 (Ab6tet), trastuzumab (T), pertuzumab (P), trastuzumab or pertuzumab and Ab6 (T + Ab6 or P + Ab6), bispecific trastuzumab with anti-HER3 Ab6 scFv (TAb6), or bispecific pertuzumab with anti-HER3 Ab6 scFv (PAb6) for 5 d. Proliferative responses were assessed using the MTS reagent and were normalized against the proliferation of cells incubated in medium (Med) only. Data shown are means of triplicates ± standard deviation. The symbols * and ** indicate significantly lower or higher proliferative responses, respectively, between cells treated with antibody and PBS vehicle (Student's *t*-test; $P < 0.05$). (**B**) SK-BR-3 or BT-474 cells were treated with anti-HER2/HER3 antibodies (50 nM) for 1 or 24 h, and cell lysates analyzed by immunoblotting. Data shown are representative of at least two independent experiments.

trastuzumab or trastuzumab plus Ab6 on ligand-independent proliferation of BT-474 or HCC1419 cells (Fig. 2A and 4A). Slightly higher proliferation of HCC1419 cells was observed following TAb6 relative to trastuzumab plus Ab6 treatment, but differences for these two treatments were not significant for SK-BR-3 and BT-474 cells.

The reduced efficacy of the antibodies in the presence of heregulin was accompanied by either no change (SK-BR-3 cells with trastuzumab and TAb6 24 h following treatment and BT-474 cells with all treatments), or a reduction (SK-BR-3 cells with Ab6, Ab6tet, or a mixture of trastuzumab plus Ab6 at 24 h) in pAkt levels (Fig. 4B). Collectively, the data indicate that

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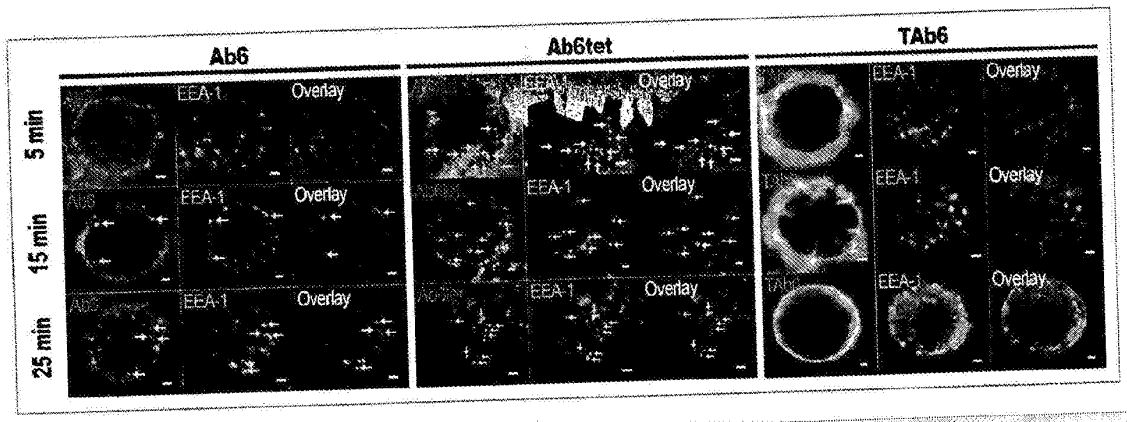

Figure 3. Ab6tet internalizes into SK-BR-3 cells more rapidly than Ab6. Cells were pulsed with 50 nM anti-HER3 (Ab6), tetrameric anti-HER3 (Ab6tet) or bispecific trastuzumab with anti-HER3 Ab6 scFv (TAb6) for 5 min at 37°C, chased for 0, 10 or 20 min, fixed, permeabilized and stained with anti-EEA-1 antibody. The combined pulse plus chase times are indicated on the left margin. Anti-HER3 or HER2/HER3 antibodies were detected with Alexa 555-labeled secondary antibody (pseudocolored red in overlay) and anti-EEA-1 antibody with Alexa 647-labeled secondary antibody (pseudocolored green in overlay). Yellow arrows in the images for Ab6tet-treated cells indicate examples of internalized Ab6tet that is associated with EEA-1 positive endosomes. Scale bars = 2 μm.

antibody targeting of HER2 and HER3 has limited efficacy in the presence of intratumoral HER3 ligands.

Lapatinib combined with antibodies specific for HER2/HER3 overcomes heregulin-mediated resistance

The induction of proliferative signaling by HER2-HER3 dimerization "forced" by TAb6 treatment suggested that the combination of this bispecific antibody with the small molecule inhibitor of HER2 (and EGFR) kinase activity, lapatinib, might stabilize HER2-HER3 heterodimers in an inactive state. Further, we hypothesized that such complexes would preclude the interaction of HER3 with signaling competent partners. Lapatinib treatment is known to result in upregulation of HER2 and HER3 levels on the plasma membrane,[26-29] and we confirmed these observations under the conditions of our assay (except that HER2 was not upregulated on BT-474 cells; Fig. S6). Treatment of SK-BR-3 and BT-474 cells with lapatinib at doses ranging from 50 nM–1 μM resulted in inhibition of proliferation (Fig. 5A). However, this growth inhibition was ablated by exposure of cells to heregulin (Fig. 5A). Although lapatinib treatment alone resulted in potent inhibition of HER3, Akt and Erk phosphorylation, this was partially reversed by incubation of cells with lapatinib plus heregulin (Fig. 5B).

The observation that lapatinib alone has limited efficacy in blocking cell proliferation in the presence of heregulin prompted us to investigate the effect of combination treatment of heregulin-exposed SK-BR-3, BT-474 and HCC1419 cells with lapatinib and HER3-targeting antibodies. Addition of Ab6 or Ab6tet to lapatinib resulted in anti-proliferative effects compared with the effects of lapatinib alone (Fig. 6A). In addition, for SK-BR-3 and HCC1419 cells, Ab6tet was more effective than Ab6 (Fig. 6A). Consistent with the inability of trastuzumab to inhibit ligand-dependent HER2-HER3 signaling,[43] this HER2-specific antibody did not reduce proliferation in the presence of heregulin and lapatinib. By contrast, of the antibodies/bispecifics tested, the anti-HER2/HER3 bispecific, TAb6, was the most potent of the antibodies tested in combination with lapatinib in reducing proliferation of heregulin-treated cells (Fig. 6A). Importantly, this bispecific also had increased anti-proliferative effects relative to a mixture of trastuzumab and Ab6 (Fig. 6A). Trastuzumab has superior activity over pertuzumab in inhibiting ligand-independent activity[43] (Fig. 2), suggesting that TAb6 would more effectively target both ligand-dependent (heregulin) and ligand-independent signaling than PAb6. In combination with the lower expression yield of PAb6 relative to that of TAb6 (2.5 mg/L vs. 8 mg/L), we therefore focused on the use of TAb6 in the lapatinib/bispecific combination studies. In the absence of heregulin, none of the antibodies further reduced the anti-proliferative activity of lapatinib (Fig. S7).

In the presence of lapatinib and heregulin, Ab6tet, trastuzumab plus Ab6 or TAb6 treatment resulted in lower levels of pHER3, pErk and pAkt (S473, T308) for SK-BR-3 and HCC1419 cells (Fig. 6B; Fig. S8). The improved activity of Ab6tet compared with Ab6 in SK-BR-3 and HCC1419 cells is consistent with the increased degradation of HER3 in the presence of the tetrameric antibody ($P < 0.05$; Fig. S8 and S9). TAb6 was the most potent antibody in reducing downstream signaling in SK-BR-3, BT-474 and HCC1419 cells, and was more inhibitory than mixtures of trastuzumab and Ab6 (Fig. 6B; Fig. S8). Further, TAb6 was the only antibody in combination with lapatinib that reduced heregulin-induced pAkt and pErk in BT-474 cells. Collectively, the data indicate that in the presence of lapatinib the bispecific antibody, TAb6, is the most potent inhibitor of ligand-induced activation.

Discussion

The limitations of targeting individual components of the HER3/PI3K/Akt signaling axis, including the activity of the preferred dimerization partner HER2, is well documented. Several factors contribute to the poor efficacy of this approach: first,

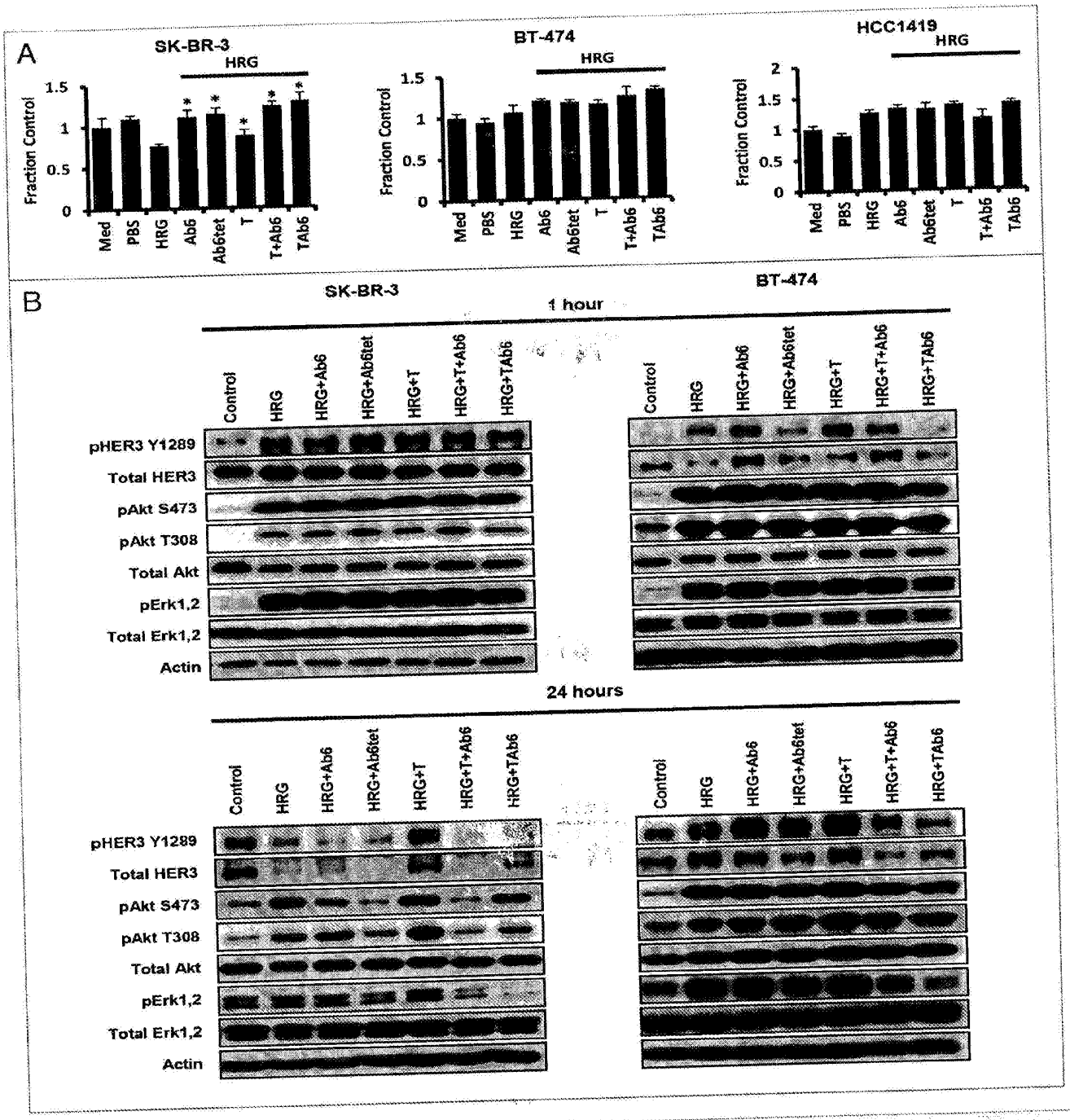

Figure 4. Antibodies specific for HER2 and/or HER3 have reduced efficacy in inhibiting proliferation and PI3K/Akt signaling in the presence of heregulin. (**A**) Cells were incubated with heregulin (HRG, 6.25 nM) and 50 nM anti-HER3 (Ab6), tetrameric anti-HER3 (Ab6tet), trastuzumab (T), trastuzumab and Ab6 (T + Ab6) or bispecific trastuzumab with anti-HER3 Ab6 scFv (TAb6) for 5 d. Proliferative responses were assessed using the MTS reagent and were normalized against the proliferation of cells incubated in medium (Med) only. Data shown are means of triplicates ± standard deviation. * indicates statistically significant differences between proliferative responses for cells treated with antibody in the presence of heregulin and cells treated with heregulin only (Student *t*-test; $P < 0.05$). (**B**) SK-BR-3 or BT-474 cells were treated with anti-HER2/HER3 antibodies (50 nM) in the presence of 6.25 nM heregulin for 1 or 24 h, and cell lysates analyzed by immunoblotting. Data shown are representative of at least two independent experiments.

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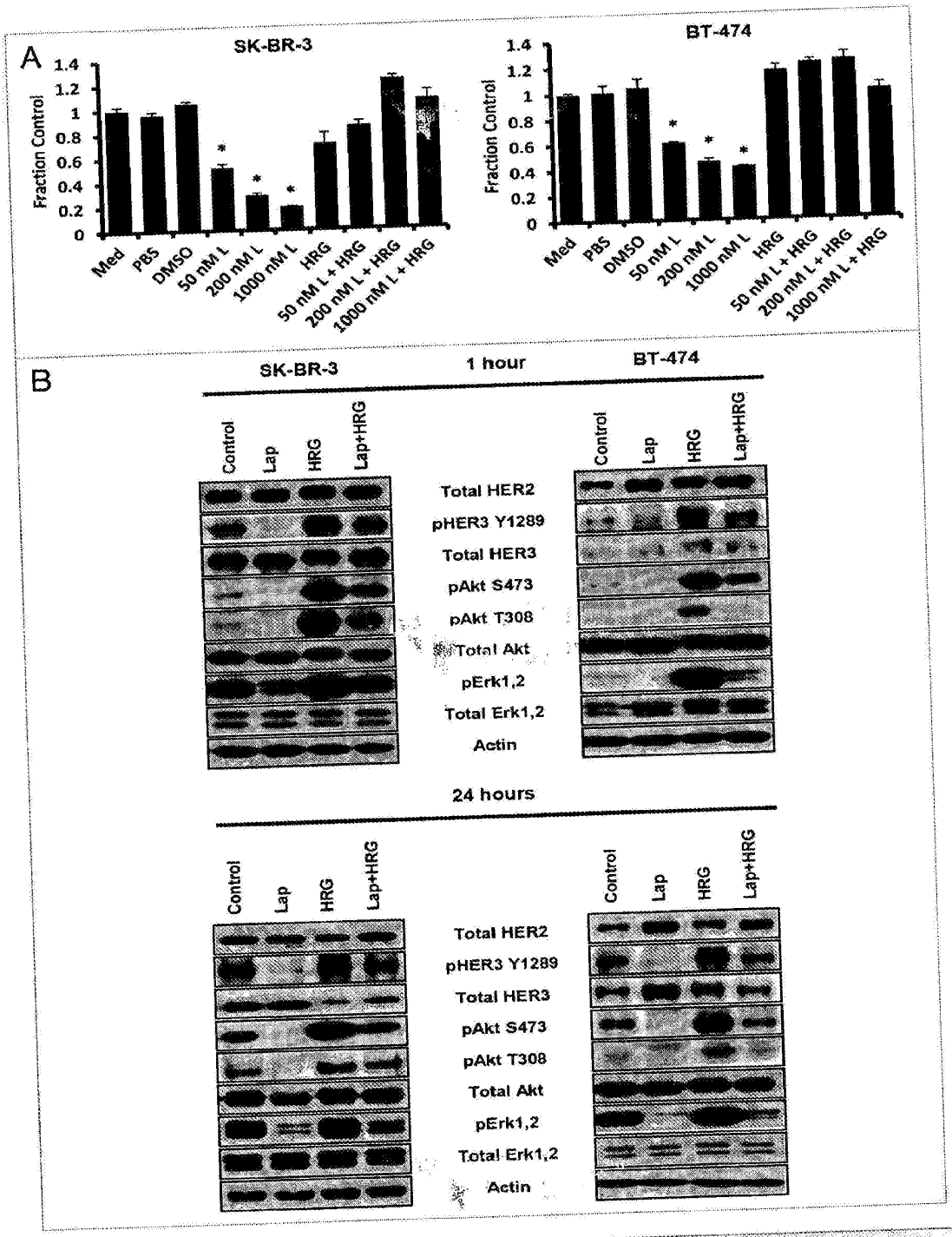

Figure 5. Heregulin treatment reverses the anti-proliferative effects of lapatinib in HER2 overexpressing cell lines. (**A**) Cells were incubated with different concentrations of lapatinib (L) in the presence and absence of heregulin (HRG; 6.25 nM) for 5 d. Proliferative responses were assessed using the MTS reagent and were normalized against the proliferation of cells incubated in medium (Med) only. Data shown are means of triplicates ± standard deviation. * indicates statistically significant differences between proliferative responses for cells treated with lapatinib and DMSO vehicle (Student *t*-test; $P < 0.001$). (**B**) SK-BR-3 or BT-474 cells were treated with 1 μM lapatinib (Lap) in the presence and absence of heregulin (6.25 nM) for 1 or 24 h, and cell lysates analyzed by immunoblotting. Data shown are representative of at least two independent experiments.

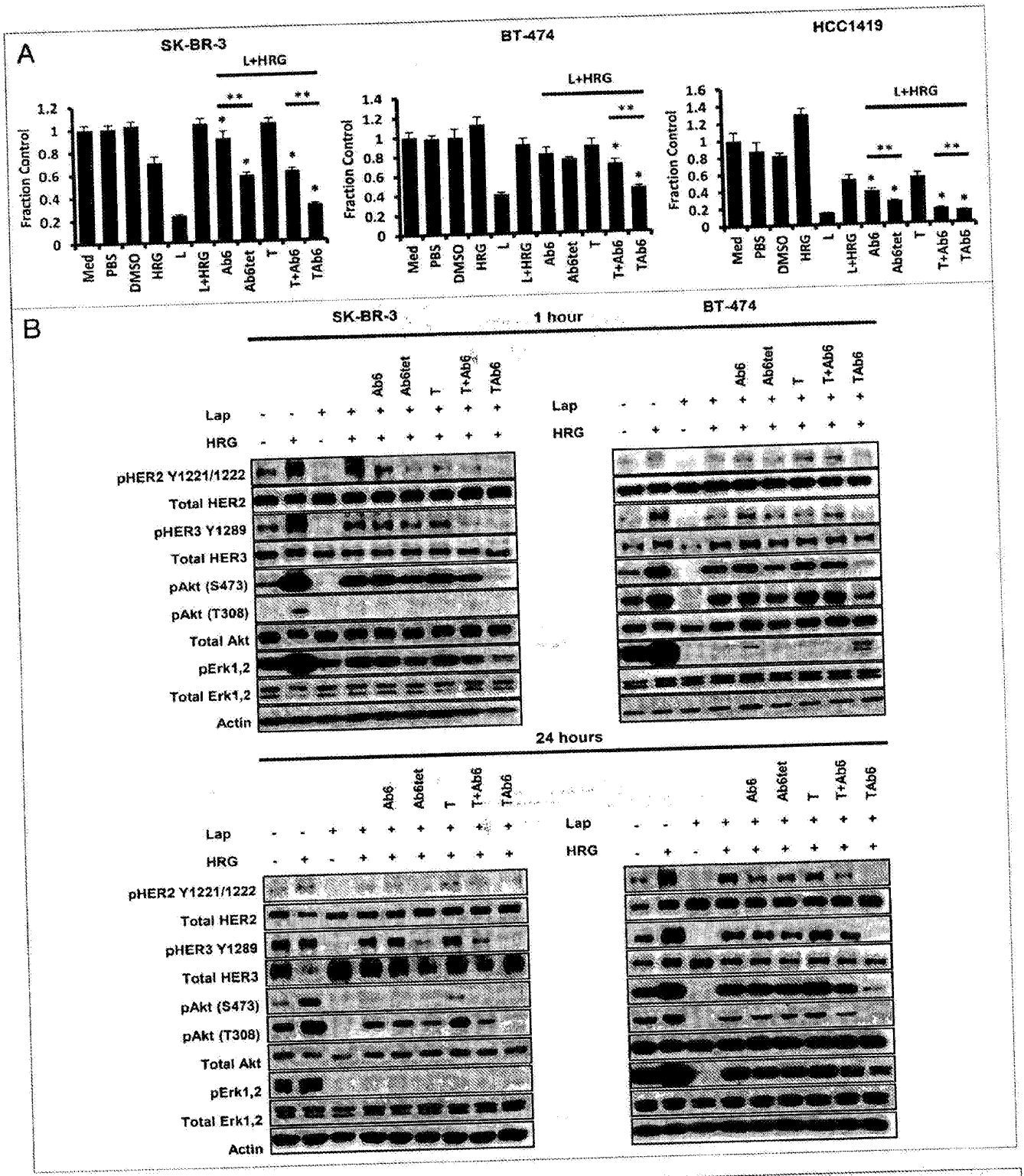

Figure 6. For figure legend, see page 348.

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Figure 6 (See previous page). The bispecific anti-HER2/HER3 antibody, TAb6, has the highest activity in reducing cell proliferation and PI3K/Akt signaling in the presence of heregulin and lapatinib. (**A**) Cells were incubated with 1 μM lapatinib (L) in the presence of heregulin (HRG; 6.25 nM) and treated with 50 nM anti-HER3 (Ab6), tetrameric anti-HER3 (Ab6tet), trastuzumab (T), trastuzumab and Ab6 (T + Ab6) or bispecific trastuzumab with anti-HER3 Ab6 scFv (TAb6) for 5 d. Proliferative responses were assessed using the MTS reagent and were normalized against the proliferation of cells incubated in medium (Med) only. Data shown are means of triplicates ± standard deviation. * indicates statistically significant differences between proliferative responses for cells treated with antibodies vs. vehicle in the presence of lapatinib and heregulin (Student *t*-test; $P < 0.05$). ** indicates statistically significant differences between proliferative responses for the pairwise comparison of the two indicated treatments (horizontal bars). (**B**) SK-BR-3 or BT-474 cells were treated with lapatinib (Lap), heregulin (6.25 nM) and anti-HER2/HER3 antibodies (50 nM) as indicated for 1 or 24 h, and cell lysates analyzed by immunoblotting. Data shown are representative of at least two independent experiments.

antibodies specific for HER2 and HER3 have reduced activity in inhibiting proliferation and signaling in the presence of the HER3 ligand heregulin[14,31,32] (this study), which is expressed in multiple tumor types.[16-18] Second, the use of small molecule TKIs such as lapatinib or gefitinib results in compensatory HER2 and HER3 upregulation and signaling through either incomplete blockade of HER2 kinase activity or HER3 association with other signaling competent partners.[5,7,26-29,34,35] Third, these compensatory effects are exacerbated by the presence of heregulin.[14,31-33] Consequently, TKIs such as lapatinib are ineffective as single agents in inhibiting breast tumor cell proliferation, indicating a need for the development of combination therapies.[14,26-29]

In the current study, we analyzed the efficacy of two HER3-focused strategies directed toward reducing breast cancer cell signaling and proliferation. These approaches also have broader relevance to the targeting of other cell surface receptors, and involve the use of distinct antibody designs to recruit HER3 into kinase-inhibited HER2-HER3 complexes or induce HER3 degradation. We demonstrate that the recruitment of HER3 into lapatinib-inactivated HER2-HER3 complexes by a bispecific anti-HER2/HER3 antibody (TAb6, comprising trastuzumab and an anti-HER3 scFv) is efficacious in both ablating PI3K/Akt signaling and reducing tumor cell proliferation in the presence of heregulin. Importantly, and of direct relevance to therapy, the bispecific antibody is a more potent inhibitor of signaling and growth than either HER2- or HER3-specific antibodies alone or a mixture of antibodies of both specificities. In a second approach, we compared the effect on tumor cell signaling and growth of a multivalent anti-HER3 antibody (Ab6tet) that is designed to enhance HER3 degradation with its bivalent counterpart, Ab6. In the presence of heregulin and lapatinib, Ab6tet has higher activity. By comparison with bivalent Ab6, this multivalent construct induces more rapid HER3 internalization into early endosomes. By contrast, the bispecific TAb6 induces less HER3 degradation than either Ab6 or Ab6tet, and yet is more effective in reducing cell signaling and proliferation. This indicates that the internalization resistance or recycling behavior of HER2[46,47] has a dominant effect on HER3 in the forced HER2-HER3 heterodimers. Further, the internalization resistance is consistent with earlier studies in which endocytic uptake of EGFR or HER3 is reduced by dimerization with HER2.[46,47] Importantly, our data indicate that the locking of HER3 into inactive HER2-HER3 complexes, even if they remain on the plasma membrane, is a more effective strategy for the ablation of heregulin-mediated signaling than induction of increased HER3 degradation.

The question arises as to why the anti-HER3 antibody, which competes for heregulin binding,[3] is not as effective either alone or in combination with trastuzumab as the bispecific antibody, TAb6, in reducing HER3-PI3K signaling. In addition to locking HER3 into dimers, TAb6 would be expected to enhance the avidity of binding of Ab6 to HER3 through bridging of trastuzumab-HER2 complexes. The trastuzumab-HER2 interaction is of very high affinity (100 pM[49]), and this, combined with high HER2 expression levels on the cell surface, should increase avidity effects. Further, although Ab6tet is expected to bind with increased avidity over Ab6 through increased valency, the Ab6-HER3 interaction is of lower affinity (0.8 nM[4]). Thus, the improved efficacy of Ab6tet relative to Ab6 could be due to both increased degradation of HER3 and higher avidity binding, but the avidity enhancement resulting from multivalent binding would be lower than that for TAb6.

The use of bispecific antibodies of different formats is an active area in the development of cancer therapeutics.[50] For example, strategies to target both HER2 and HER3 using several different bispecific antibody formats or mixtures of antibodies have been described.[13-15] Of relevance to the use of bispecifics, however, we observe that in the absence of laparinib (and heregulin), exposure of SK-BR-3, BT-474 or HCC1419 cells to bispecific antibodies comprising either trastuzumab or pertuzumab and the anti-HER3 scFv, Ab6 (TAb6 or PAb6, respectively), results in increased PI3K/Akt signaling and proliferation. The possibility that this activation is due to relief of feedback inhibition of the FoxO1/3a transcription factors, leading to upregulation of multiple receptor tyrosine kinases such as HER3 and IGF-1R,[27,44,45] is excluded by the rapid kinetics of pAkt induction (15 min). The observation that bispecifics containing either trastuzumab (TAb6) or pertuzumab (PAb6) are both active in inducing HER3 phosphorylation and PI3K/Akt signaling suggests that proximity rather than a specific configuration of HER2 and HER3 is sufficient for HER3 transphosphorylation. This is consistent with our observation that bispecific HER2/HER3 specific antibodies can sequester HER3 into HER2-HER3 heterodimers that, if inhibited by HER2-specific TKIs, effectively silence HER3.

We also characterized the effect of using antibodies of different designs on intracellular trafficking. The increased internalization and trafficking to lysosomes induced by using a multivalent anti-HER3 antibody (Ab6tet) suggests that this approach could be used to enhance the activity of antibody-drug conjugates for which lysosomal delivery is required.[38] By contrast, a high proportion of the bispecific, TAb6, persists on the plasma membrane where it is exposed for recognition by FcγRs or complement receptors, allowing antibody dependent cell-mediated cytotoxicity/phagocytosis (ADCC/ADCP) or complement-mediated cytotoxicity by appropriate effector cells.[37]

Earlier studies have demonstrated that dual targeting of HER2 and HER3, using either bispecific scFvs or mixtures of individual antibodies specific for HER2 and HER3, reduces tumor cell growth in the presence of lapatinib.[14,15] Significantly, we demonstrate that TAb6 is more effective than mixtures of trastuzumab and Ab6. This is consistent with the concept that TAb6 anchors HER3 into lapatinib-inactivated HER2-HER3 dimers, thereby sequestering it from interactions with other kinase competent partners and enabling Ab6 to bind to HER3 with increased avidity. By analogy with our observations, the use of a bispecific scFv-human albumin fusion (MM-111) has been shown to have anti-tumor effects in the presence of heregulin that were increased by the addition of lapatinib,[14] although the efficacy of MM-111 was not directly compared with that of anti-HER2 or anti-HER3 antibodies of the same specificities as those present in MM-111. Further, and by contrast with this earlier report, we observe lower levels of heregulin-induced proliferation and, for most conditions or cell lines, no significant anti-proliferative effects of monotherapy with lapatinib or trastuzumab in the presence of heregulin. These apparent discrepancies may be due to differences in assay conditions, such as the addition of heregulin following antibody treatment[14] rather than simultaneous addition as used in the current study.

An important difference between the activity of TAb6 and MM-111 is that, in the absence of heregulin and lapatinib, MM-111 does not induce proliferation.[14] The design of TAb6 and MM-111 differs in several respects: first, the scFvs specific for HER2 and HER3 in MM-111 are distinct from those in TAb6. Although both the anti-HER3 scFv ($H3^{51}$) and Ab6 compete with heregulin for binding to HER3, this does not exclude the possibility that the two antibodies recognize distinct epitopes. The HER2-specific scFv, B1D2, binds to an epitope that does not overlap with trastuzumab.[14,52] Second, TAb6 has two anti-HER2 and two anti-HER3 Fabs/Fvs per molecule, whereas MM-111 has one scFv of each specificity. Tetravalency is expected to enhance the avidity of the interaction with HER2/HER3. Third, TAb6 has an antibody Fc region whereas the scFvs in MM-111 are linked to albumin, resulting in variations in the "span" distance of the Fv components of the two constructs. One or more of these factors could contribute to the different activities of MM-111 compared with TAb6. Significantly, the presence of an Fc region in TAb6 confers effector function activities such as ADCC. The surface retention of targeting antibodies such as TAb6 is expected to enhance ADCC and other cell-mediated cytotoxicity pathways, which in turn could enhance anti-tumor activity.[37]

Other multivalent antibody-based or designed ankyrin repeat proteins (DARPins) that target HER2 or HER3 have been recently described.[53-55] Specifically, a tetravalent antibody (MM-141), with a similar design to TAb6 in the current study, that targets both HER3 and IGF-1R has been shown to have anti-proliferative effects both in vitro and in vivo.[54] Analogously to our observations using TAb6 in combination with lapatinib, MM-141 is more effective in reducing tumor cell signaling and growth than mixtures of the individual antibodies of the corresponding specificities. However, MM-141 may not be as effective as TAb6 in targeting HER2-overexpressing tumors, given the importance of the HER2/HER3 signaling axis in such malignancies. In the context of targeting HER2, a divalent anti-HER2 DARPin has been optimized to "lock" HER2 into an inactive dimeric configuration on the cell surface.[55] This DARPin-based construct represents a notable expansion to the possible types of molecular approaches for targeting HER2-addicted tumors.

Future experiments will be directed toward analyzing the anti-tumor activity of a combination of lapatinib and our lead antibody, TAb6, in tumor-bearing mice. Earlier studies using lapatinib with the bispecific anti-HER2/HER3 antibody, MM-111, indicated that weekly dosing of antibody with daily lapatinib was unexpectedly more effective than MM-111 delivery every three days with daily lapatinib.[14] This indicates that the dosing regimen of lapatinib and TAb6, which has a longer in vivo persistence than MM-111,[14] will require optimization.

Collectively, we compared the effects of targeting HER2, HER3 or both receptors with several different antibody formats on the proliferation and signaling of breast cancer cell lines. These studies demonstrate that in the presence of the TKI inhibitor, lapatinib, a bispecific anti-HER2/HER3 antibody has higher activity than individual (multivalent) antibodies specific for HER2 and HER3 in inhibiting heregulin-induced signaling through the PI3K/Akt pathway and cell proliferation. These observations suggest that the use of such bispecifics in combination therapy with lapatinib to sequester HER3 into inactive heterodimers may provide an effective pathway for the treatment of cancer.

Materials and Methods

Cell lines and reagents

The human breast cancer cell lines BT-474 and SK-BR-3 were obtained from the American Type Culture Collection (ATCC, catalog nos. HTB-20 and HTB-30, respectively) and cultured in Hybricare Medium (ATCC, catalog no. 46-X) and McCoy 5a (Gibco, catalog no. 12330–031/Hyclone, catalog no. SH30200.01), with 1% penicillin/streptomycin and 10% FCS, respectively. The human breast cancer cell line HCC1419 (a generous gift of Drs Adi Gazdar, John Minna, and Kenneth Huffman, Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas) was cultured in RPMI 1640 with 1% penicillin/streptomycin and 5% FCS.

For imaging experiments, cells were cultured in phenol-red free DMEM (Gibco, catalog no. 31053–028) supplemented with 1% penicillin/streptomycin, 1% L-Glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate, 100 nM MEM non-essential amino acids, 55 nM 2-mercaptoethanol and 10% FCS.

Polyclonal antibodies specific for phospho-Akt-T308 (catalog no. 9275S), Akt, phospho-Erk1,2 (catalog no. 9101S), Erk1,2 (catalog no. 9102S), phospho-HER2 Y1221/1222 (catalog no. 2249S), and monoclonal antibodies against phospho-Akt-S473 (D9E) (catalog no. 4060L), and phospho-HER3 Y1289 (D1B5) (catalog no. 2842) were obtained from Cell Signaling

Technologies. Polyclonal anti-HER3 antibody (C-17) (catalog no. SC-285) was from Santa Cruz Biotechnology and monoclonal anti-c-erbB2 antibody (Ab-3 3B5) (catalog no. OP15L) was from Millipore. The monoclonal anti-actin antibody (Ab-5) (catalog no. 612656) was from BD Bioscience. Horseradish peroxidase-labeled goat anti-rabbit and anti-mouse IgG (H+L) (catalog nos. 111-035-003 and 115-035-003, respectively) were purchased from Jackson ImmunoResearch Laboratories. Lapatinib (catalog no. L-4804) was obtained from LC Laboratories and recombinant human heregulin-β1 (HRG-β1; EGF-like domain, catalog no. 100–03) was obtained from Peprotech.

Recombinant antibodies

Clinical grade trastuzumab and pertuzumab were obtained from the UT Southwestern Pharmacy. For comparative purposes, trastuzumab and pertuzumab were also expressed in recombinant form using the same expression host as the other antibodies used in this study. Expression plasmids for the production of antibodies in stably transfected CHO cells were generated as follows: the genes encoding the heavy and light chain variable domains (VH and VL, respectively) of trastuzumab,[49] pertuzumab,[40] and Ab6 (US patent 20100266584A1; MM-121[3]) were synthesized commercially (Integrated DNA Technology, Genscript, or Thermo Fisher) and used to generate full-length human IgG1 and human kappa genes with the leader peptide (MGWSCIILFLVATATGVHS) from the anti-lysozyme antibody, Hulys10,[56] using standard methods of molecular biology. The vectors pcDNA 3.3 TOPO and pOptiVEC-TOPO (OptiCHO Ab Express Kit, Life Technologies, catalog nos. K8300–01 and 12744017, respectively) were used for the expression of the light and heavy chain genes, respectively.

To generate expression constructs for bispecific, tetravalent antibodies, a similar design to that described previously was used.[57] A linker sequence containing a unique *Xho*I site was inserted at the 3′ end of the heavy chain genes (trastuzumab and pertuzumab) using a designed oligonucleotide and the PCR. A scFv gene encoding the Ab6 scFv with codons encoding a $(Gly_4Ser)_3$ linker peptide between the VH (JH) and VL gene was generated using standard methods of molecular biology. *Xho*I sites and Gly-Ser-Ser codons to connect the CH3 domain to the VH gene, were appended to the 5′ and 3′ ends of the scFv gene using the PCR. This scFv gene was cloned into the *Xho*I sites at the 3′ ends of the trastuzumab and pertuzumab heavy chain genes to generate full-length heavy chains linked to the Ab6 scFv. Complete sequences of all expression plasmids are available upon request.

Transfections and expression of recombinant antibodies

Light chain expression constructs were transfected into CD/DG44 CHO cells (Life Technologies, catalog no. A11000-01) using electroporation. Desired clones were selected in CD/DG44 CHO medium (Life Technologies, catalog no. 12610010) containing 500 μg/ml geneticin without the HT supplement. The clone expressing the highest levels of light chain was identified by screening culture supernatants with ELISAs using goat anti-human kappa light chain antibody for detection (Sigma-Aldrich, catalog no. A7164). Heavy chain expression constructs were then transfected into their respective stably transfected light chain expressing CD/DG44 CHO clones via electroporation and selected with Opti-CHO Medium (Life Technologies, catalog no. 12681-011) containing 500 μg/ml geneticin. Supernatants of clones were screened by sandwich ELISA using goat anti-human IgG (Fab-specific, Sigma-Aldrich, catalog no. 15260) as capture antibody and goat anti-human IgG (Fc-specific) conjugated to horseradish peroxidase (Sigma-Aldrich, catalog no. A0170) as detection antibody. The clones expressing the highest levels of antibody were expanded and cultured in increasing concentrations of methotrexate (MTX, 50 nM–4 μM), to induce gene amplification. Clones were further expanded in shake flasks (130 rpm) in 8% CO_2 and antibody purified from culture supernatants using protein G-Sepharose (GE Healthcare, catalog no. 17-0618-05). Several antibodies were also scaled up and purified by BioXCell.

Surface plasmon resonance analyses

Surface plasmon resonance experiments were performed using a BIAcore 2000 and previously described methods.[58,59] Recombinant Fc-HER2 (R&D Systems, catalog no. 1129-ER-050) and HER3 (Acro Biosystems, catalog no. ER3-H5223) were coupled to flow cells of CM5 sensor chips to densities of 758 and 467 RU, respectively. Antibodies at a concentration of 50 nM were injected in phosphate buffered saline (PBS) plus 0.01% Tween at a flow rate of 10 μl/min. At the end of an 800 s dissociation phase, bound antibody was stripped from the flow cells using 0.1 M glycine, 0.9 M NaCl, pH 3.8. Data were analyzed using BIAevaluation software.

Pharmacokinetics

The in vivo half-lives of ^{125}I labeled Ab6tet and TAb6 were analyzed following intravenous or intraperitoneal delivery in BALB/c SCID mice (Jackson Laboratories) using previously described methods and whole body counting.[60]

Proliferation assays

Cells were plated in 96-well plates at a density of 2,500 cells per well and incubated overnight. Cells were treated with lapatinib (1 μM), HRG-β1 (6.25 nM), anti-HER3 antibody Ab6 or the tetrameric form, Ab6tet, trastuzumab (anti-HER2), pertuzumab (anti-HER2), or the bispecific anti-HER2/ HER3 antibodies TAb6 and PAb6 as indicated in the figure legends. Antibodies were used at a concentration of 50 nM unless otherwise indicated. Dimethyl sulfoxide or PBS were used as vehicle controls for lapatinib or HRG-β1/antibodies, respectively. After 5 d of incubation in a 37 °C 5% CO_2 incubator, cell proliferation was quantitated using CellTiter 96 AQueous One Solution Proliferation Assay kit (Promega, catalog no. G3580) according to the manufacturer's instructions.

Immunoblotting

Cells cultured to near confluence in 6-well plates were treated with lapatinib, HRG-β1 or antibodies as for proliferation assays. Following 15 min, 1 h, or 24 h incubation, cells were lysed using RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 μg/mL pepstatin A, and 0.4 mg/mL pefabloc SC PLUS). Lysates were centrifuged for 20 min at 14,600 rpm at 4 °C and supernatants were collected. Protein concentrations in each lysate were determined using

the BCA protein assay reagent (Pierce, catalog no. 23223). Total lysates were fractionated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, catalog no. IPVH00010) or nitrocellulose membranes (Bio-Rad, catalog no. 162-0145). Membranes were incubated with 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween (TBST, pH 8.0) for 1 h following transfer to block non-specific binding sites. Blocked membranes were incubated with primary antibodies overnight at 4 °C with agitation. Prior to incubation with the secondary antibodies conjugated to horseradish peroxidase, blots were washed three times (10 min per wash) with TBST. Following incubation with secondary antibodies for 1 h and washing, bound secondary antibody was detected using chemiluminescent detection regent (GE Healthcare, catalog no. RPN 2209/AF).

Fluorescence microscopy

SK-BR-3 cells incubated overnight at a density of 50,000 cells per dish were treated with 50 nM Ab6, Ab6tet, or TAb6 for 5 or 15 min at 37 °C and then either immediately washed and fixed or chased in medium at 37 °C for 10, 20, or 45 min prior to washing and fixation. For fixation, cells were treated with 1.7% (w/v) paraformaldehyde for 10 min at 37 °C and then permeabilized with 0.05% (v/v) saponin for 10 min at room temperature in PBS. A pre-block with 4% BSA/PBS was performed prior to staining with 2 μg/mL of polyclonal rabbit anti-LAMP-1 antibody (Abcam, catalog no. AB24170) and 12.5 μg/mL monoclonal mouse anti-EEA-1 (clone 14) from BD Bioscience (catalog no. 610456) for 30 min at room temperature. After blocking for 30 min with goat serum (Sigma-Aldrich, catalog no. G6767), bound primary antibodies were detected by incubating cells with the following secondary antibody conjugates for 25 min at room temperature: Alexa 555-labeled goat anti-human IgG (H+L) (Life Technologies, catalog no. A21433), Alexa 647-labeled goat anti-mouse IgG (H+L) (Life Technologies, catalog no. A21236) and Alexa 488-labeled goat anti-rabbit IgG (H+L) (Life Technologies, catalog no. A11034). Cells were washed twice with PBS between each incubation step and were stored at 4 °C in 1% BSA/PBS.

Images were acquired using a Zeiss Axiovert 200M inverted fluorescence microscope with a 63X Plan Apochromat objective as described previously.[61,62] Data was processed using custom written software in MatLab (MIAtool/LABSoft; http://www.wardoberlab.com/software/miatool/).

Statistical analyses

Statistical significance for differences in mean values of triplicate samples (immunoblots, ELISAs) was determined by Student *t* test. P values < 0.05 were considered to be significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

Kang JC, Poovassery JS, Ober RJ, and Ward ES designed the research; Kang JC, Poovassery JS, Bansal P, You S, and Manjarres IM performed the research; Kang JC, Poovassery JS, You S, Manjarres IM, Ober RJ, and Ward ES analyzed the data; Kang JC, Poovassery JS, Ober RJ, and Ward ES wrote the paper.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/mabs/article/27658

References

1. Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, Sliwkowski MX, Stern HM. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. Cancer Res 2008; 68:5878-87; PMID:18632642; http://dx.doi.org/10.1158/0008-5472.CAN-08-0380
2. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF 3rd, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci U S A 2003; 100:8933-8; PMID:12853564; http://dx.doi.org/10.1073/pnas.1537685100
3. Schoeberl B, Faber AC, Li D, Liang MC, Crosby K, Onsum M, Burenkova O, Pace E, Walton Z, Nie L, et al. An ErbB3 antibody, MM-121, is active in cancers with ligand-dependent activation. Cancer Res 2010; 70:2485-94; PMID:20215504; http://dx.doi.org/10.1158/0008-5472.CAN-09-3145
4. Schoeberl B, Pace EA, Fitzgerald JB, Harms BD, Xu L, Nie L, Linggi B, Kalra A, Paragas V, Bukhalid R, et al. Therapeutically targeting ErbB3: a key node in ligand-induced activation of the ErbB receptor-PI3K axis. Sci Signal 2009; 2:ra31; PMID:19567914; http://dx.doi.org/10.1126/scisignal.2000352
5. Baselga J, Swain SM. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. Nat Rev Cancer 2009; 9:463-75; PMID:19536107; http://dx.doi.org/10.1038/nrc2656
6. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001; 2:127-37; PMID:11252954; http://dx.doi.org/10.1038/35052073
7. Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, Ratzkin BJ, Yarden Y. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. Mol Cell Biol 1996; 16:5276-87; PMID:8816440
8. Hellyer NJ, Kim MS, Koland JG. Heregulin-dependent activation of phosphoinositide 3-kinase and Akt via the ErbB2/ErbB3 co-receptor. J Biol Chem 2001; 276:42153-61; PMID:11546794; http://dx.doi.org/10.1074/jbc.M102079200
9. Campbell MR, Amin D, Moasser MM. HER3 comes of age: new insights into its functions and role in signaling, tumor biology, and cancer therapy. Clin Cancer Res 2010; 16:1373-83; PMID:20179223; http://dx.doi.org/10.1158/1078-0432.CCR-09-1218
10. Murphy CG, Modi S. HER2 breast cancer therapies: a review. Biologics 2009; 3:289-301; PMID:19707416
11. Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nat Clin Pract Oncol 2006; 3:269-80; PMID:16683005; http://dx.doi.org/10.1038/ncponc0509
12. Hurvitz SA, Hu Y, O'Brien N, Finn RS. Current approaches and future directions in the treatment of HER2-positive breast cancer. Cancer Treat Rev 2013; 39:219-29; PMID:22658319; http://dx.doi.org/10.1016/j.ctrv.2012.04.008
13. Robinson MK, Hodge KM, Horak E, Sundberg AL, Russeva M, Shaller CC, von Mehren M, Shchaveleva I, Simmons HH, Marks JD, et al. Targeting ErbB2 and ErbB3 with a bispecific single-chain Fv enhances targeting selectivity and induces a therapeutic effect in vitro. Br J Cancer 2008; 99:1415-25; PMID:18841159; http://dx.doi.org/10.1038/sj.bjc.6604700

14. McDonagh CF, Huhalov A, Harms BD, Adams S, Paragas V, Oyama S, Zhang B, Luus L, Overland R, Nguyen S, et al. Antitumor activity of a novel bispecific antibody that targets the ErbB2/ErbB3 oncogenic unit and inhibits heregulin-induced activation of ErbB3. Mol Cancer Ther 2012; 11:582-93; PMID:22248472; http://dx.doi.org/10.1158/1535-7163.MCT-11-0820

15. Garrett JT, Sutton CR, Kuba MG, Cook RS, Arteaga CL. Dual blockade of HER2 in HER2-overexpressing tumor cells does not completely eliminate HER3 function. Clin Cancer Res 2013; 19:610-9; PMID:23224399; http://dx.doi.org/10.1158/1078-0432.CCR-12-2024

16. Li Q, Ahmed S, Loeb JA. Development of an autocrine neuregulin signaling loop with malignant transformation of human breast epithelial cells. Cancer Res 2004; 64:7078-85; PMID:15466203; http://dx.doi.org/10.1158/0008-5472.CAN-04-1152

17. Montero JC, Rodríguez-Barrueco R, Ocaña A, Díaz-Rodríguez E, Esparís-Ogando A, Pandiella A. Neuregulins and cancer. Clin Cancer Res 2008; 14:3237-41; PMID:18519747; http://dx.doi.org/10.1158/1078-0432.CCR-07-5133

18. Dunn M, Sinha P, Campbell R, Blackburn E, Levinson N, Rampaul R, Bates T, Humphreys S, Gullick WJ. Co-expression of neuregulins 1, 2, 3 and 4 in human breast cancer. J Pathol 2004; 203:672-80; PMID:15141382; http://dx.doi.org/10.1002/path.1561

19. Kaufman B, Trudeau M, Awada A, Blackwell K, Bachelot T, Salazar V, DeSilvio M, Westlund R, Zaks T, Spector N, et al. Lapatinib monotherapy in patients with HER2-overexpressing relapsed or refractory inflammatory breast cancer: final results and survival of the expanded HER2+ cohort in EGF103009, a phase II study. Lancet Oncol 2009; 10:581-8; PMID:19394894; http://dx.doi.org/10.1016/S1470-2045(09)70087-7

20. Gomez HL, Doval DC, Chavez MA, Ang PC, Aziz Z, Nag S, Ng C, Franco SX, Chow LW, Arbushites MC, et al. Efficacy and safety of lapatinib as first-line therapy for ErbB2-amplified locally advanced or metastatic breast cancer. J Clin Oncol 2008; 26:2999-3005; PMID:18458039; http://dx.doi.org/10.1200/JCO.2007.14.0590

21. Burstein HJ, Sun Y, Dirix LY, Jiang Z, Paridaens R, Tan AR, Awada A, Ranade A, Jiao S, Schwartz G, et al. Neratinib, an irreversible ErbB receptor tyrosine kinase inhibitor, in patients with advanced ErbB2-positive breast cancer. J Clin Oncol 2010; 28:1301-7; PMID:20142587; http://dx.doi.org/10.1200/JCO.2009.25.8707

22. Gradishar WJ. Emerging approaches for treating HER2-positive metastatic breast cancer beyond trastuzumab. Ann Oncol 2013; 24:2492-500; PMID:23827380; http://dx.doi.org/10.1093/annonc/mdt217

23. Baselga J, Albanell J, Ruiz A, Lluch A, Gascón P, Guillém V, González S, Sauleda S, Marimón I, Tabernero JM, et al. Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. J Clin Oncol 2005; 23:5323-33; PMID:15939921; http://dx.doi.org/10.1200/JCO.2005.08.326

24. Folkes AJ, Ahmadi K, Alderton WK, Alix S, Baker SJ, Box G, Chuckowree IS, Clarke PA, Depledge P, Eccles SA, et al. The identification of 2-(1H-indazol-4-yl)-6-{4-methanesulfonyl-piperazin-1-ylmethyl}-4-morpholin-4-yl-thieno[3,2-d]pyrimidine (GDC-0941) as a potent, selective, orally bioavailable inhibitor of class I PI3 kinase for the treatment of cancer. J Med Chem 2008; 51:5522-32; PMID:18754654; http://dx.doi.org/10.1021/jm800295d

25. Brachmann SM, Hofmann I, Schnell C, Fritsch C, Wee S, Lane H, Wang S, Garcia-Echeverria C, Maira SM. Specific apoptosis induction by the dual PI3K/mTor inhibitor NVP-BEZ235 in HER2 amplified and PIK3CA mutant breast cancer cells. Proc Natl Acad Sci U S A 2009; 106:22299-304; PMID:20007781; http://dx.doi.org/10.1073/pnas.0905152106

26. Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM, Moasser MM. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature 2007; 445:437-41; PMID:17206155; http://dx.doi.org/10.1038/nature05474

27. Chakrabarty A, Sánchez V, Kuba MG, Rinehart C, Arteaga CL. Feedback upregulation of HER3 (ErbB3) expression and activity attenuates antitumor effect of PI3K inhibitors. Proc Natl Acad Sci U S A 2012; 109:2718-23; PMID:21368164; http://dx.doi.org/10.1073/pnas.1018001108

28. Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, Smith DJ, Landolfi S, Ramon y Cajal S, Arribas J, et al. Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene 2009; 28:803-14; PMID:19060928; http://dx.doi.org/10.1038/onc.2008.432

29. Amin DN, Sergina N, Ahuja D, McMahon M, Blair JA, Wang D, Hann B, Koch KM, Shokat KM, Moasser MM. Resiliency and vulnerability in the HER2-HER3 tumorigenic driver. Sci Transl Med 2010; 2:ra7; PMID:20371474; http://dx.doi.org/10.1126/scitranslmed.3000389

30. Eichhorn PJ, Gili M, Scaltriti M, Serra V, Guzman M, Nijkamp W, Beijersbergen RL, Valero V, Seoane J, Bernards R, et al. Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235. Cancer Res 2008; 68:9221-30; PMID:19010894; http://dx.doi.org/10.1158/0008-5472.CAN-08-1740

31. Wilson TR, Fridlyand J, Yan Y, Penuel E, Burton L, Chan E, Peng J, Lin E, Wang Y, Sosman J, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. Nature 2012; 487:505-9; PMID:22763448; http://dx.doi.org/10.1038/nature11249

32. Xia W, Petricoin EF 3rd, Zhao S, Liu L, Osada T, Cheng Q, Wulfkuhle JD, Gwin WR, Yang X, Gallagher RI, et al. An heregulin-EGFR-HER3 autocrine signaling axis can mediate acquired lapatinib resistance in HER2+ breast cancer models. Breast Cancer Res 2013; 15:R85; PMID:24044505; http://dx.doi.org/10.1186/bcr3480

33. Sato Y, Yashiro M, Takakura N. Heregulin induces resistance to lapatinib-mediated growth inhibition of HER2-amplified cancer cells. Cancer Sci 2013; 104:1618-25; PMID:24112719; http://dx.doi.org/10.1111/cas.12290

34. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007; 316:1039-43; PMID:17463250; http://dx.doi.org/10.1126/science.1141478

35. Knowlden JM, Gee JM, Barrow D, Robertson JF, Ellis IO, Nicholson RI, Hutcheson IR. erbB3 recruitment of insulin receptor substrate 1 modulates insulin-like growth factor receptor signalling in oestrogen receptor-positive breast cancer cell lines. Breast Cancer Res 2011; 13:R93; PMID:21939528; http://dx.doi.org/10.1186/bcr3018

36. Sak MM, Breen K, Rønning SB, Pedersen NM, Bertelsen V, Stang E, Madshus IH. The oncoprotein ErbB3 is endocytosed in the absence of added ligand in a clathrin-dependent manner. Carcinogenesis 2012; 33:1031-9; PMID:22436610; http://dx.doi.org/10.1093/carcin/bgs128

37. Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. Nat Rev Immunol 2010; 10:301-16; PMID:20414204; http://dx.doi.org/10.1038/nri2761

38. Sievers EL, Senter PD. Antibody-drug conjugates in cancer therapy. Annu Rev Med 2013; 64:15-29; PMID:23043493; http://dx.doi.org/10.1146/annurev-med-050311-201823

39. Cho HS, Mason K, Ramyar KX, Stanley AM, Gabelli SB, Denney DW Jr., Leahy DJ. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. Nature 2003; 421:756-60; PMID:12610629; http://dx.doi.org/10.1038/nature01392

40. Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell 2004; 5:317-28; PMID:15093539; http://dx.doi.org/10.1016/S1535-6108(04)00083-2

41. Gymnopoulos M, Elsliger MA, Vogt PK. Rare cancer-specific mutations in PIK3CA show gain of function. Proc Natl Acad Sci U S A 2007; 104:5569-74; PMID:17376864; http://dx.doi.org/10.1073/pnas.0701005104

42. Weigelt B, Warne PH, Downward J. PIK3CA mutation, but not PTEN loss of function, determines the sensitivity of breast cancer cells to mTOR inhibitory drugs. Oncogene 2011; 30:3222-33; PMID:21358673; http://dx.doi.org/10.1038/onc.2011.42

43. Junttila TT, Akita RW, Parsons K, Fields C, Lewis Phillips GD, Friedman LS, Sampath D, Sliwkowski MX. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. Cancer Cell 2009; 15:429-40; PMID:19411071; http://dx.doi.org/10.1016/j.ccr.2009.03.020

44. Chandarlapaty S, Sawai A, Scaltriti M, Rodrik-Outmezguine V, Grbovic-Huezo O, Serra V, Majumder PK, Baselga J, Rosen N. AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell 2011; 19:58-71; PMID:21215704; http://dx.doi.org/10.1016/j.ccr.2010.10.031

45. Serra V, Scaltriti M, Prudkin L, Eichhorn PJ, Ibrahim YH, Chandarlapaty S, Markman B, Rodriguez O, Guzman M, Rodriguez S, et al. PI3K inhibition results in enhanced HER signaling and acquired ERK dependency in HER2-overexpressing breast cancer. Oncogene 2011; 30:2547-57; PMID:21278786; http://dx.doi.org/10.1038/onc.2010.626

46. Haslekås C, Breen K, Pedersen KW, Johannessen LE, Stang E, Madshus IH. The inhibitory effect of ErbB2 on epidermal growth factor-induced formation of clathrin-coated pits correlates with retention of epidermal growth factor receptor-ErbB2 oligomeric complexes at the plasma membrane. Mol Biol Cell 2005; 16:5832-42; PMID:16207817; http://dx.doi.org/10.1091/mbc.E05-05-0456

47. Sak MM, Szymanska M, Bertelsen V, Hasmann M, Madshus IH, Stang E. Pertuzumab counteracts the inhibitory effect of ErbB2 on degradation of ErbB3. Carcinogenesis 2013; 34:2031-8; PMID:23698633; http://dx.doi.org/10.1093/carcin/bgt173

48. Le XF, McWatters A, Wiener J, Wu JY, Mills GB, Bast RC Jr. Anti-HER2 antibody and heregulin suppress growth of HER2-overexpressing human breast cancer cells through different mechanisms. Clin Cancer Res 2000; 6:260-70; PMID:10656457

49. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci U S A 1992; 89:4285-9; PMID:1350088; http://dx.doi.org/10.1073/pnas.89.10.4285

50. Reichert JM, Dhimolea E. The future of antibodies as cancer drugs. Drug Discov Today 2012; 17:954-63; PMID:22561895; http://dx.doi.org/10.1016/j.drudis.2012.04.006

51. Horak E, Heitner T, Robinson MK, Simmons HH, Garrison J, Russeva M, Furmanova P, Lou J, Zhou Y, Yuan QA, et al. Isolation of scFvs to in vitro produced extracellular domains of EGFR family members. Cancer Biother Radiopharm 2005; 20:603-13; PMID:16398612; http://dx.doi.org/10.1089/cbr.2005.20.603

52. Schier R, Marks JD, Wolf EJ, Apell G, Wong C, McCartney JE, Bookman MA, Huston JS, Houston LL, Weiner LM, et al. In vitro and in vivo characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. Immunotechnology 1995; 1:73-81; PMID:9373335; http://dx.doi.org/10.1016/1380-2933(95)00007-0

53. Xu L, Kohli N, Rennard R, Jiao Y, Razlog M, Zhang K, Baum J, Johnson B, Tang J, Schoeberl B, et al. Rapid optimization and prototyping for therapeutic antibody-like molecules. MAbs 2013; 5:237-54; PMID:23392215; http://dx.doi.org/10.4161/mabs.23363

54. Fitzgerald JB, Johnson BW, Baum J, Adams S, Jadevaia S, Tang J, Rimkunas V, Xu L, Kohli N, Rennard R, et al. MM-141, an IGF-1R and ErbB3 directed bispecific antibody, overcomes network adaptations that limit activity of IGF-1R inhibitors. Mol Cancer Ther 2013; PMID:24282274; http://dx.doi.org/10.1158/1535-7163.MCT-13-0255

55. Jost C, Schilling J, Tamaskovic R, Schwill M, Honegger A, Plückthun A. Structural basis for eliciting a cytotoxic effect in HER2-overexpressing cancer cells via binding to the extracellular domain of HER2. Structure 2013; 21:1979-91; PMID:24095059; http://dx.doi.org/10.1016/j.str.2013.08.020

56. Foote J, Winter G. Antibody framework residues affecting the conformation of the hypervariable loops. J Mol Biol 1992; 224:487-99; PMID:1560463; http://dx.doi.org/10.1016/0022-2836(92)91010-M

57. Coloma MJ, Morrison SL. Design and production of novel tetravalent bispecific antibodies. Nat Biotechnol 1997; 15:159-63; PMID:9035142; http://dx.doi.org/10.1038/nbt0297-159

58. Zhou J, Mateos F, Ober RJ, Ward ES. Conferring the binding properties of the mouse MHC class I-related receptor, FcRn, onto the human ortholog by sequential rounds of site-directed mutagenesis. J Mol Biol 2005; 345:1071-81; PMID:15644205; http://dx.doi.org/10.1016/j.jmb.2004.11.014

59. Vaccaro C, Zhou J, Ober RJ, Ward ES. Engineering the Fc region of immunoglobulin G to modulate in vivo antibody levels. Nat Biotechnol 2005; 23:1283-8; PMID:16186811; http://dx.doi.org/10.1038/nbt1143

60. Vaccaro C, Bawdon R, Wanjie S, Ober RJ, Ward ES. Divergent activities of an engineered antibody in murine and human systems have implications for therapeutic antibodies. Proc Natl Acad Sci U S A 2006; 103:18709-14; PMID:17116867; http://dx.doi.org/10.1073/pnas.0606304103

61. Ober RJ, Martinez C, Vaccaro C, Zhou J, Ward ES. Visualizing the site and dynamics of IgG salvage by the MHC class I-related receptor, FcRn. J Immunol 2004; 172:2021-9; PMID:14764666

62. Gan Z, Ram S, Vaccaro C, Ober RJ, Ward ES. Analyses of the recycling receptor, FcRn, in live cells reveal novel pathways for lysosomal delivery. Traffic 2009; 10:600-14; PMID:19192244; http://dx.doi.org/10.1111/j.1600-0854.2009.00887.x

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Antibody-Drug Conjugates in Cancer Therapy

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immunotherapy, chemotherapeutic, monoclonal antibody, tumor, drug delivery, targeting

Abstract

An antibody-drug conjugate (ADC) provides the possibility of selectively ablating cancer cells by combining the specificity of a monoclonal antibody (mAb) for a target antigen with the delivery of a highly potent cytotoxic agent. ADC target antigens are typically highly expressed on the surface of cancer cells compared to normal cells. The tumor target, the cytotoxic agent, and the manner in which the agent is attached to the antibody are key determinants of clinical activity and tolerability. Recently, several clinical trials have demonstrated that ADCs achieve higher clinical response rates than unconjugated mAbs targeting the same cell surface antigen. Brentuximab vedotin represents one such ADC that has recently been approved for the treatment of relapsed Hodgkin and systemic anaplastic large cell lymphomas—both characterized by high expression of the target antigen, CD30, on the surface of malignant cells. This review summarizes key characteristics of current, clinically active ADCs and highlights recent clinical data illustrating the benefit of antibody-targeted delivery of cytotoxic agents to cancer cells.

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INTRODUCTION

HL: Hodgkin lymphoma

ALCL: anaplastic large-cell lymphoma

Clinically useful, unconjugated monoclonal antibodies (mAbs) selectively recognize antigens that are preferentially expressed on or near tumor cells and exert their cytotoxic effects through mechanisms such as cell signaling, antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, and complement-dependent cytotoxicity (1). However, the majority of these mAbs are used in combination with chemotherapy, and many others have demonstrated insufficient clinical activity. Therefore, significant effort has been devoted to empowering mAbs through various modifications (2). One approach by which the activities of these mAbs have been enhanced is through conjugation with cytotoxic drugs, generating antibody-drug conjugates (ADCs) capable of antigen-specific delivery of highly potent cytotoxic drugs to tumor cells.

DESIGN PRINCIPLES

In order to improve efficacy without compromising safety, an ADC must preferentially deliver the cytotoxic payload to tumor cells expressing the target antigen rather than to healthy tissue. This is accomplished by exploiting the specificity of a mAb targeting an antigen that is highly expressed on the surface of malignant cells. After binding the target antigen, the ADC-antigen complex is typically internalized and transported to intracellular organelles where release of the attached drug can occur. Upon release, the cytotoxic drug can interfere with various cellular mechanisms, leading to cell death (**Figure 1**). The development of ADCs has relied on several key areas of research including the choice of an appropriate antigen target, development of novel highly potent cytotoxic drugs, development of stable linkers that can release the cytotoxic payload upon internalization, and conjugation technology. This review discusses recent advances in these areas of research along with the application of these technologies to ADCs currently in the clinic.

Select an Appropriate Target

Target selection is arguably the most important contributor to the antitumor activity and tolerability of an ADC. As a general rule, cancer cells do not display novel immunogenic antigens because such behavior would lead to rapid clearance by the immune system. However, cancer cells occasionally express normal cell surface antigens at levels that are distinguishable from their healthy normal cellular counterparts. Ideally, the target antigen should be expressed with substantially higher copy numbers on tumor cells, as the efficacy and tolerability of an ADC appear to depend, in part, on its relative expression on normal tissue compared to tumor cells (3, 4). Two ADCs that have shown pronounced clinical activity at tolerated doses, brentuximab vedotin (ADCETRIS®) and trastuzumab emtansine (T-DM1), target antigens with preferential expression on tumor cells. The normal cell surface antigen targeted by brentuximab vedotin, CD30, is strongly expressed on the Reed-Sternberg cells of Hodgkin lymphoma (HL) and on systemic anaplastic large-cell lymphoma (ALCL) tumor cells (5). CD30 expression is otherwise thought to be largely limited to activated T and B cells in the physiologic state (6, 7). Similarly, the HER2 antigen, targeted by T-DM1, is highly overexpressed on some metastatic breast cancer tumors compared to normal tissue (8–10). Both of these ADCs have shown meaningful clinical activities with manageable safety profiles (11–13).

Upon binding of the ADC, many antigens undergo rapid internalization along with the bound ADC through receptor-mediated endocytosis (**Figure 1**). The efficiency of internalization can vary between an ADC and the unconjugated antibody; in some cases both species internalize at the same rate, whereas in other cases the ADC internalizes more efficiently (14, 15). The rate and extent of internalization are important because it influences uptake of the drug as well as release of the drug in tumor and normal cells (16, 17).

The expression level of the target antigen appears to be another important factor

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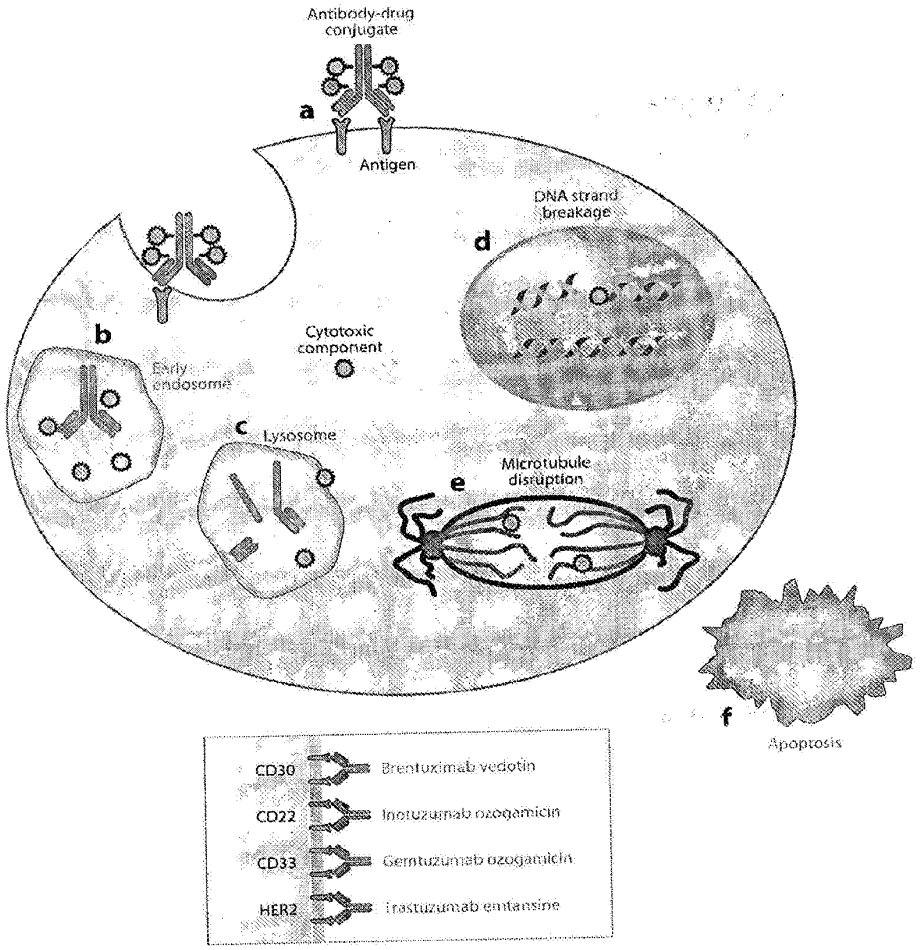

Figure 1

Generalized mechanism of action of antibody-drug conjugates (ADCs). (*a*) The ADC binds to the antigen on the target cell. (*b*) The antigen-ADC complex is internalized through receptor-mediated endocytosis and, in most cases, is transported from early endosomes to lysosomes. (*c*) In the lysosome, internal conditions may destabilize the linker or mAb backbone, causing the cytotoxic component to dissociate and circulate freely in the cytoplasm. Most cytotoxic components of current ADCs either bind to the minor groove of DNA and induce strand breakage (*d*) or bind to tubulin, resulting in microtubule disruption (*e*). (*f*) Both processes lead to apoptosis. *Inset*: Four advanced-stage ADCs are brentuximab vedotin, inotuzumab ozogamicin, gemtuzumab ozogamicin, and trastuzumab emtansine. The antigen targets for each ADC are shown.

in targeting tumors with ADCs; however, ADCs have shown efficacy over a wide range of antigen expression levels. For example, while CD33-positive acute myelogenous leukemia tumors express relatively low levels of target antigen (5,000–10,000 receptors per cell), meaningful clinical responses have been observed with gemtuzumab ozogamicin

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(Mylotarg®, GO), an ADC that targets the CD33 antigen (18, 19). Effective treatment of tumors with such low antigen expression levels requires an ADC with a highly potent cytotoxic component, such as calicheamicin in GO. Conversely, HER2 can be expressed on metastatic breast cancer tumor cells with very high copy numbers (in some cases, >3 million copies per cell) in some patients (20). Significant clinical activity against HER2-positive metastatic breast cancer has been reported for T-DM1, an ADC composed of trastuzumab conjugated to a cytotoxic maytansinoid, mertansine (DM1) (10, 21). These and other ADCs are effective over a wide range of antigen expression levels, although preclinical studies have shown that higher antigen expression levels do in fact predict greater ADC potency in cell culture (22). Taken together, these observations suggest that although expression level is an important factor in determining ADC potency, even antigen targets with lower copy numbers can be effectively targeted with a sufficiently potent ADC.

Minimize Systemic Drug Release

The linker that connects the cytotoxic drug to the mAb is a key determinant of ADC activity. These linkers covalently couple the cytotoxic drug to the antibody, producing an ADC that should be relatively stable in circulation (3). Stability of the linker is important because it prevents damage to nontarget tissue through spontaneous release of the cytotoxic drug while maximizing tumor drug exposure (3). Upon internalization, however, the linkers should facilitate efficient drug release. Intracellular conditions such as the low-pH environment in lysosomes or the reducing environment of the cytosol can destabilize acid-labile hydrazone linkers or disulfide-based linkers, respectively, resulting in drug release (**Figure 1**) (23). For example, the ADC GO contains an acid-labile hydrazone linking the calicheamicin-derived cytotoxic component to the CD33 mAb (24). However, both acid-labile hydrazone linkers and disulfide-based linkers are relatively unstable in plasma, with half-lives in plasma of approximately 48–72 hours and 1 day, respectively (2, 25).

Dipeptide linkers and thioether linkers have also been used in ADC development. Dipeptide linkers such as citrulline-valine linkers are predominantly cleaved by specific lysosomal proteases, imparting greater stability in plasma and increased exposure to the conjugated drug after intravenous delivery (26). Brentuximab vedotin is an ADC that uses a cleavable citrulline-valine dipeptide linker to facilitate release of the cytotoxic agent, monomethylauristatin E (MMAE) (27). There are also noncleavable thioether linkers that depend on degradation of the mAb component in the lysosome. Antibody degradation leads to the release of the cytotoxic drug still bound to the linker and the amino acid residue of the antibody to which it was attached (28, 29). T-DM1 is an ADC that uses this type of linker to tether the maytansinoid cytotoxic component to the mAb (21).

Tailor Conjugation Technology to Optimize Drug Effect

The specific method of attachment of the cytotoxic drug and linker to the mAb has been shown to play a key role in ADC activity and tolerability. The most common methods of antibody-drug conjugation are alkylation of reduced interchain disulfides, acylation of lysine residues, and alkylation of genetically engineered cysteine residues. The number of drugs per mAb and drug position have been shown to influence ADC aggregation, antigen binding, clearance from the circulation, potency, and tolerability (2, 30). Underconjugation can decrease potency of the ADC mixture, whereas the conjugation of too many drugs per mAb can lead to decreased circulation half-life, reduced tolerability, and impaired antigen binding (2, 30). The ideal number of drug molecules per antibody for most current ADCs appears to be about four. This ratio minimizes the presence of unconjugated antibody, limits the circulation half-life to that of the unmodified

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Table 1 Antibody-drug conjugates in clinical development

Drug	Status	Therapeutic area	Target	Cytotoxic class
brentuximab vedotin	approved	HL, ALCL	CD30	auristatin
gemtuzumab ozogamicin	withdrawn	AML	CD33	calicheamicin
inotuzumab ozogamicin	phase III	NHL, ALL	CD22	calicheamicin
trastuzumab emtansine	phase III	MBC	HER2	maytansinoid
BT062	phase II	multiple myeloma	CD138	maytansinoid
CDX-011	phase II	breast, melanoma	GPNMB	auristatin
milatuzumab-dox	phase II	multiple myeloma	CD74	anthracycline (doxorubicin)
SAR3419	phase II	DLBCL, ALL	CD19	maytansinoid
AGS-16M8F	phase I	RCC	AGS-16	auristatin
ASG-22ME	phase I	solid tumors	nectin-4	auristatin
ASG-5ME	phase I	prostate, pancreatic	SLC44A4	auristatin
BAY 79-4620	phase I	solid tumors	carbonic anhydrase 9	auristatin
BAY 94-9343	phase I	solid tumors	mesothelin	maytansinoid
BIIB015	phase I	solid tumors	cripto	maytansinoid
IMGN529	phase I	NHL, CLL	CD37	maytansinoid
IMMU-130	phase I	colorectal	CEACAM5	camptothecin analog (SN-38)
lorvotuzumab mertansine	phase I	SCLC, multiple myeloma	CD56	maytansinoid
MDX-1203	phase I	NHL, RCC	CD70	alkylating agent (duocarmycin)
PSMA ADC	phase I	prostate	PSMA	auristatin
RG7593	phase I	hematologic malignancies	CD22	auristatin
SAR566658	phase I	solid tumors	CA6	maytansinoid
SGN-75	phase I	NHL, RCC	CD70	auristatin

Abbreviations: ALCL, anaplastic large-cell lymphoma; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; HL, Hodgkin lymphoma; MBC, metastatic breast cancer; NHL, non-Hodgkin lymphoma; RCC, renal cell carcinoma; SCLC, small-cell lung cancer.

antibody, and does not significantly disrupt antigen binding (2).

Employ Highly Potent Cytotoxic Agents

Early ADC development focused on the use of readily available, clinically approved drugs such as doxorubicin, methotrexate, mitomycin, flurouracil, and vinca alkaloids (2). These drugs have relatively low potencies, so access to solid tumors by macromolecules was inefficient and accumulation of the cytotoxic drug in target cells was poor. The low clinical activity exhibited by these early ADCs prompted the development of ADCs employing much more potent cytotoxic drugs, which, though too toxic to use in an untargeted manner, have sufficient potency to be used in a more targeted manner (2).

The majority of highly potent cytotoxic agents used in current ADCs are auristatins, maytansinoids, or calicheamicins (**Table 1**). The cytotoxicities of auristatins and maytansinoids arise from binding to tubulin and the inhibition of polymerization, causing cell cycle arrest and subsequent apoptosis of the target cell (**Figure 1**) (2, 31, 32). MMAE is an auristatin that is used in several ADCs,

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conjugated directly to native cysteine residues using maleimide for conjugation and a dipeptide linker (valine and citrulline—vcMMAE) to facilitate drug release (33). MMAE is the cytotoxic component of the only ADC that is currently commercially available, brentuximab vedotin. The most advanced clinical ADC containing a maytansinoid derivative is T-DM1.

DNA-binding cytotoxic agents used in ADCs include duocarmycin and calicheamicin analogs. These agents exert their cytotoxic effects by binding to DNA in the double-helix minor groove (3). For example, binding of calicheamicin analogs induces double-strand breaks in DNA, resulting in cell death (**Figure 1**) (34). These molecules are very potent, but their lack of selectivity has prevented their use as single-agent therapeutics (3). Calicheamicins in particular have been integrated into inotuzumab ozogamicin, an ADC in late-stage clinical development, as well as the previously commercially available ADC GO, which has been voluntarily withdrawn from the market.

CLINICAL DEVELOPMENT

Considerable attention to the selection of (*a*) optimal tumor targets, (*b*) highly potent cytotoxic payloads, and (*c*) stabilizing linker technologies has yielded promising new treatment options for cancer patients. ADCs consistently demonstrate better response rates than unconjugated mAbs that target the same cell surface antigen and have been clinically evaluated in similar advanced cancer patient populations. **Table 2** presents a selection of tumor antigens that have been targeted by both unconjugated antibodies and ADCs in the context of the objective response rates observed in similar patient populations. For all antigen targets, more robust antitumor activity was observed in clinical trials incorporating treatment with a single-agent ADC. The patient populations compared were relatively similar and all studies examined single-agent therapy, but it is important to note that none of the reported studies directly compared an ADC with its unconjugated mAb counterpart. In addition, in the case of CD22, CD33, and one of the unconjugated HER2 antibodies, the unconjugated mAb and the conjugated mAb recognize different epitopes of the target antigen. Despite these differences, the clinical trial data support the hypothesis that antibodies can be successfully employed to deliver potent cytotoxic agents to cancer cells, resulting in clinically meaningful activity exceeding that of unconjugated mAbs with manageable toxicity. This section of the review focuses on ADCs that are or have been commercially available and those in late-stage development. Earlier-stage compounds are also briefly discussed. **Table 1** lists some of these ADCs.

Brentuximab Vedotin

Brentuximab vedotin is a next-generation ADC that is approved for use in the United States and is the first drug approved for HL in more than 30 years. It is composed of the chimeric mAb cAC10, which is specific for human CD30, as mentioned above, and the auristatin MMAE, a cytotoxic agent. The antibody is covalently attached to an average of four MMAE molecules per mAb through a protease-cleavable dipeptide linker. The ADC binds to the CD30 antigen and undergoes rapid internalization and proteolysis, leading to the efficient release of MMAE inside the target cells (35). Brentuximab vedotin embodies the important ADC design principles mentioned above: (*a*) an internalizing tumor-associated antigen that is minimally expressed on normal tissues, (*b*) a linker that minimizes systemic drug release, (*c*) a conjugation technology that preserves the pharmacokinetic properties of the mAb carrier, and (*d*) a potent cytotoxic component that is rapidly released upon exposure to intracellular proteases.

In 2011, brentuximab vedotin received accelerated approval from the US Food and Drug Administration (FDA) for the treatment of HL after failure of autologous stem cell transplant or after at least two prior multi-agent chemotherapy regimens in patients who are not autologous stem cell transplant candidates.

Table 2 Clinical efficacy of unconjugated antibodies and related antibody-drug conjugates

Target (indication)	Unconjugated antibody			Conjugated antibody		
	Species	ORR (%)	Reference	Species	ORR (%)	Reference
CD22 (NHL)	epratuzumab[a]	18	68[b]	inotuzumab ozogamicin[a]	39–53	52,[b] 69[b]
CD30 (HL)	cAC10	0	70[b]	brentuximab vedotin	75	11[b]
CD30 (ALCL)	cAC10	17	70[b]	brentuximab vedotin	86	12[b]
CD33 (AML)	lintuzumab[a]	15	71[c]	gemtuzumab ozogamicin[a]	26–30	42,[d] 72,[d]
HER2 (MBC)	trastuzumab	15–26	73,[b] 74,[e] 75[e]	trastuzumab emtansine	26–64	13,[b] 39,[e] 76,[b] 77,[b]
	pertuzumab[a]	7	78[b]			

Abbreviations: ALCL, anaplastic large-cell lymphoma; AML, acute myeloid leukemia; HL, Hodgkin lymphoma; MBC, metastatic breast cancer; NHL, non-Hodgkin lymphoma; ORR, objective response rate.

[a]Unconjugated and conjugated antibodies recognize the same antigen target but different epitopes.

[b]Relapsed or refractory disease.

[c]Previously untreated (or treated with palliative therapy) or in relapse after standard induction chemotherapy.

[d]Untreated first recurrence.

[e]Previously untreated disease.

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Accelerated approval was also granted for the treatment of systemic ALCL after failure of at least one prior multi-agent chemotherapy regimen. The approved dose of brentuximab vedotin is 1.8 mg/kg intravenously once every three weeks for up to 16 treatment cycles. In a pivotal single-arm phase II trial in relapsed or refractory HL patients, brentuximab vedotin demonstrated an objective response rate of 75% and a durable complete remission rate of 34% (11). In the subset of patients who obtained a complete remission, the median progression-free survival was 29.0 months (36). Similarly, in a pivotal single-arm phase II trial in relapsed or refractory systemic ALCL patients, brentuximab vedotin demonstrated an objective response rate of 86% and a durable complete remission rate of 57%. Patients who obtained a best response of complete remission experienced a median progression-free survival of 14.6 months (12). The robust clinical efficacy of the ADC brentuximab vedotin in patients with relapsed or refractory HL or systemic ALCL contrasts with the modest clinical activity exhibited by the unconjugated antibody in clinical trials (**Table 2**), highlighting the observation that ADCs can greatly enhance the activity of the parent antibody. In both studies, adverse events were generally grade 1 or 2 in severity and were largely manageable with either supportive care or dose reductions and/or delays. Adverse events of grade 3 or higher in both studies included peripheral sensory neuropathy, neutropenia, thrombocytopenia, and anemia. Peripheral sensory neuropathy was the most common adverse event in both studies, occurring in more than half of patients who received brentuximab vedotin (11, 12).

A total of four randomized phase III registrational trials will evaluate the potential efficacy of brentuximab vedotin in a variety of clinical settings, including earlier lines of treatment for HL and systemic ALCL as well as new indications such as cutaneous T cell lymphoma (CTCL) and other mature T cell lymphomas that express the CD30 target antigen. One unmet clinical need is represented by those patients with HL whose lymphoma recurs after autologous stem cell transplant. This typically young patient population has a median survival of only 2.4 years (37). To address this pronounced unmet need, a randomized placebo-controlled phase III trial is testing the hypothesis that post-transplant administration of brentuximab vedotin will prevent disease progression and improve outcomes (AETHERA trial; NCT01100502). To

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improve long-term outcomes in patients with newly diagnosed CD30-positive lymphomas, two planned international randomized phase III trials will evaluate the inclusion of brentuximab vedotin in the multi-agent treatment of newly diagnosed advanced-stage HL and mature T cell lymphoma. Additionally, a fourth randomized phase III trial is under way comparing the clinical activity of brentuximab vedotin with methotrexate or bexarotene (physician's choice) in patients with CD30-positive CTCL (NCT0158499).

Trastuzumab Emtansine

Trastuzumab emtansine (T-DM1), as described above, is an ADC formed by the conjugation of the maytansinoid DM1 with the anti-HER2 mAb trastuzumab through a noncleavable linker. Currently, T-DM1 is the only ADC in late-stage clinical development for metastatic breast cancer that overexpresses the HER2 antigen (38). The unconjugated antibody trastuzumab, available commercially as Herceptin®, was originally developed for the treatment of the 15%–25% of breast cancer cases that exhibit protein overexpression of HER2. Typically, trastuzumab is used in combination with conventional chemotherapy for first-line treatment of these breast cancers. However, trastuzumab has also been used as a single agent in the treatment of HER2-positive metastatic breast cancer, demonstrating objective response rates ranging from 15% to 26%. Conjugation of the cytotoxic agent DM1 to trastuzumab offered improvements in objective response rates, with single-agent efficacy in HER2-positive metastatic breast cancer ranging from 26% to 64% (**Table 2**). It is important to note that in these trials, T-DM1 was active against metastatic breast cancer that was previously resistant to trastuzumab-based therapy, highlighting the importance of the cytotoxic DM1 component.

In addition to improved efficacy, the targeting of chemotoxic agents using a mAb in ADCs should provide an improved safety profile compared to traditional chemotherapy. A head-to-head randomized phase II study was conducted in previously untreated metastatic breast cancer patients comparing the safety and efficacy of single-agent T-DM1 with trastuzumab in combination with the chemotherapeutic agent docetaxel, the standard of care in the front-line setting. Preliminary topline results from this study demonstrated a somewhat higher objective response rate (64% versus 58%) and median progression-free survival (14.2 months versus 9.2 months) in the single-agent T-DM1 group, but a lower rate of grade 3 or 4 adverse events (46.4% versus 89.4%) and treatment discontinuations due to adverse events (7.2% versus 28.8%). Furthermore, many side effects associated with conventional chemotherapy, such as diarrhea, neutropenia, rash, neuropathy, and alopecia, were less prominent in patients treated with single-agent T-DM1 compared with trastuzumab in combination with conventional chemotherapy (39). This study illustrates how antigen binding in ADCs may help improve the therapeutic index by focusing exposure to the cytotoxic agent on tumor cells.

Building on results from phase I and phase II clinical trials, EMILIA, a randomized open-label phase III trial, explored the safety and efficacy of single-agent T-DM1 in patients with HER2-positive locally advanced or metastatic breast cancer. Either single-agent T-DM1 or a combination of capecitabine and lapatinib was administered to 978 patients. The primary endpoints were progression-free survival, overall survival, and safety. Patients treated with T-DM1 showed significantly improved progression-free survival compared to those treated with lapatinib plus capecitabine (9.6 months versus 6.4 months; $p < 0.0001$). Median overall survival was not reached in the T-DM1 arm, but the one-year and two-year overall survival rates showed a trend to be higher in the T-DM1 arm (one-year: 84.7% versus 77.0%; two-year: 65.4% versus 47.5%). Single-agent T-DM1 was well tolerated, and the most common adverse events with severity of at least grade 3 were thrombocytopenia (12.9%), increased aspartate amino transferase levels (4.3%), and increased alanine amino

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transferase levels (2.9%) (40). Two other phase III studies are currently under way: MARIANNE (NCT01120184), which compares the safety and efficacy of single-agent T-DM1 with trastuzumab/pertuzumab or trastuzumab/taxane combination therapy in the first-line setting, and TH3RESA (NCT01419197), which compares T-DM1 with treatment of the physician's choice in metastatic breast cancer patients who have already received two prior regimens of HER2-directed therapy.

Gemtuzumab Ozogamicin

Gemtuzumab ozogamicin (CMA-676; GO) is an ADC composed of a DNA-binding calicheamicin derivative coupled to a humanized IgG4 mAb directed against CD33, a normal myeloid cell surface antigen that is present on leukemia tumors in ~90% of patients with acute myeloid leukemia (AML) (41). It was the first ADC to receive marketing authorization in the United States, receiving accelerated approval from the FDA in 2000 (see above) for the treatment of patients with CD33-positive AML in first relapse who are 60 years of age or older and who are not considered candidates for other cytotoxic chemotherapy (18). Approval was granted on the basis of results from three open-label single-arm phase II trials in a total of 142 patients in which 30% of patients obtained complete remission or complete remission with incomplete platelet recovery (42). However, clinically meaningful GO-related hepatotoxicity was reported in some patients (43, 44). This toxic effect led to the addition of a boxed warning for hepatotoxicity in the US label for GO in 2001.

In association with the accelerated approval, the sponsor and FDA agreed to the design and execution of a randomized phase III trial (SWOG 106) in order to confirm evidence of clinical benefit. This study compared GO-containing induction therapy (45 mg/m^2 daunorubicin, cytarabine, and GO) with standard induction therapy (60 mg/m^2 daunorubicin and cytarabine) in first-line AML patients under the age of 61. Unfortunately, no apparent benefit of GO was observed with regard to achievement of complete remission (GO-containing arm: 66%; control arm: 69%) or relapse-free survival (hazard ratio = 1.00, p = 0.50). Additionally, patients in the GO-containing arm experienced a higher induction death rate (GO-containing arm: 5.8%; control arm: 0.8%; p = 0.002) (45). Critics of the study point out that the dose of daunorubicin was lower in patients in the GO-containing arm, which may have masked further benefit of GO in induction. In addition, the induction death rate in the control arm (0.8%) was much lower than the rate seen in other chemotherapy trials of patients under age 61, suggesting that the trial's findings might not represent a realistic picture of the clinical benefit or risks of GO (46, 47). Despite these uncertainties raised in the medical literature, Pfizer voluntarily withdrew GO from the market at the request of the FDA in June 2010.

Results of recent phase III trials suggest GO may confer meaningful clinical benefit for certain patient subpopulations. Together, the open-label trials AML15 and AML16 randomized 2,228 patients to receive induction therapy with or without 3 mg/m^2 GO. In both trials, induction therapy containing GO was well tolerated without unacceptable increases in toxicity. The AML15 trial showed a significant survival benefit for GO-containing induction therapy in younger AML patients with favorable-risk cytogenetics (five-year overall survival: 79% with GO, 51% without GO; odds ratio = 0.32) (46). AML16 showed a significant survival benefit in older AML patients regardless of cytogenetic status (two-year overall survival: 35% with GO, 29% without GO; p = 0.04) (48). The phase III open-label ALFA-0701 trial investigated induction therapy with or without lower, fractionated doses of GO (3 mg/m^2 on days 1, 4, and 7). The rationale for the lower 3 mg/m^2 dose was based on the responses to lower doses of GO in the initial phase I studies, and dose fractionation is supported by the rapid re-expression of CD33 molecules observed after first exposure. This approach

NHL: non-Hodgkin lymphoma(s)

ALL: acute lymphocytic leukemia(s)

DLBCL: diffuse large B cell lymphoma

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was remarkably successful. Time-to-endpoint assessments including event-free survival (15.6 months versus 9.7 months), relapse-free survival (28.1 months versus 11.4 months), and overall survival (34.0 months versus 19.2 months) were significantly more favorable for patients in the GO-containing arm (49). The significant survival benefits observed in these phase III studies suggest that induction regimens containing GO may have an important future role in the treatment of patients with good- or intermediate-risk AML (47).

Inotuzumab Ozogamicin

Inotuzumab ozogamicin (CMC-544) consists of a humanized anti-CD22 IgG4 mAb conjugated to a calicheamicin derivative by an acetyl butyrate linker that targets the normal B cell surface antigen CD22, which is expressed on the majority of non-Hodgkin lymphomas (NHL) and acute lymphocytic leukemias (ALL) of B cell derivation (41, 50, 51). Akin to the relatively restricted maturational expression of the normal myeloid antigen CD33, the B cell antigen CD22 does not appear to be meaningfully expressed by either lymphocyte progenitors or memory B cells—possibly resulting in lower chronic immunosuppression than might be observed in the context of comprehensive B cell ablation obtained by other agents (52, 53).

Promising preliminary evidence of inotuzumab ozogamicin antitumor activity was observed in several single-arm trials. In the first dose-escalation trial, inotuzumab ozogamicin was administered to 79 patients with multiple-relapsed or treatment-refractory B cell lymphomas as an intravenous infusion every three or four weeks at doses ranging from 0.4 to 2.4 mg/m^2. Thrombocytopenia and neutropenia were observed more commonly at higher dose levels, and the maximum tolerated dose was defined as 1.8 mg/m^2 delivered every three weeks. Thrombocytopenia not associated with clinically meaningful episodes of hemorrhage was the most notable recurrent adverse event. With the intention of allowing greater time for platelet recovery, an expansion cohort increased the dosing interval to four weeks. The overall response rate was 39% across various lymphoma subtypes and dose levels (52). Similarly, a 57% overall response rate was observed in an exploratory phase II trial in 49 adults and children with relapsed ALL (54). Because allogeneic hematopoietic stem cell transplantation is particularly useful as a means of securing long-term leukemia control among patients with relapsed ALL who achieve remission, it is also notable that a strategy employing inotuzumab ozogamicin consolidated by allograft was reasonably well tolerated and appeared to enable a proportion of patients to achieve and maintain durable remissions (55).

On the basis of these collective findings, multiple randomized trials in diffuse large B cell lymphoma (DLBCL) and ALL are likely to help define the utility of this novel ADC (NCT01232556 and NCT01564784). Data from these randomized trials should enable regulators and clinicians to determine if inotuzumab ozogamicin provides greater clinical benefit than existing treatment approaches in patients with relapsed or refractory B cell cancers.

Early-Stage ADCs

In addition to the later-stage and commercially used ADCs discussed above, many ADCs are in early-stage clinical testing (**Table 1**). CDX-011 (glembatumumab vedotin) is an ADC in phase II testing that consists of the auristatin MMAE conjugated to a mAb that recognizes GPNMB, a novel glycoprotein often expressed at higher levels in melanoma and breast tumors relative to normal tissue. In a phase I/II trial in patients with unresectable late-stage melanoma, CDX-011 showed overall response rates ranging from 14% to 19% depending on dosing frequency and GPNMB expression levels (56, 57). In breast cancer, GPNMB expression is associated with poor outcomes including reduced progression-free survival and overall survival. This is consistent with results in a clinical trial of heavily pretreated breast cancer patients, which showed an association

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between GPNMB expression and improved outcomes following CDX-011 treatment, with an objective response rate of 29% in GPNMB-positive patients and 0% in GPNMB-negative patients (56, 58). A randomized phase II study is currently under way in advanced breast cancer patients comparing the safety and efficacy of chemotherapy of the investigator's choice to CDX-011 (NCT01156753).

Another ADC in early-stage clinical testing is SAR3419, a maytansinoid-based ADC targeting CD19 in B cell malignancies. In a phase I/II study in patients with relapsed or refractory CD19-expressing B cell NHL, including follicular lymphoma and DLBCL, 32% of patients achieved an objective response, including six complete remissions. SAR3419 exhibited a manageable safety profile with no evidence of myelosuppression (59). SAR3419 is currently being tested in phase II trials in patients with relapsed or refractory DLBCL (NCT01472887) and ALL (NCT01440179).

FUTURE DEVELOPMENTS

The use of ADCs as therapeutics is a new field driven by innovation at all levels of development. At the preclinical level, novel antigen targets for drug delivery are being discovered (2, 32). These include antigens found on both hematologic and solid tumors. A great deal of research is also under way to define new highly potent drugs that are appropriate for ADC applications. These include the DNA-alkylating agents duocarmycins and pyrrolobenzodiazepine dimers, as well as the RNA polymerase inhibitor amanitin (60–62). These drugs may complement the activities of the auristatins and other drugs that are currently in advanced clinical trials. Other advancements being made in the field include site-directed mAb mutations with both natural and unnatural amino acids that permit specific drug attachment and more uniform production of ADCs with specific drug/mAb ratios (63, 64). Besides having more predictable pharmacokinetics, such conjugates may provide advantages in terms of tolerability (63) and stability (65). Finally, a considerable amount of research has been directed toward antibody alternatives that might penetrate solid tumors more efficiently than mAbs (66). Localization studies with an antibody fragment–drug conjugate derived from a diabody (molecular mass 26 kDa) demonstrated tumor localization. The disadvantages of this construct were higher-than-normal tissue exposure and rapid blood clearance. Despite these shortcomings, the diabody-based ADC showed antitumor activity at tolerated doses in preclinical models (67). Further studies are needed to establish the potential of non-IgG scaffolds for ADC drug delivery.

The use of ADCs has ushered in a new era of immune-based treatments for cancer. The coupling of novel highly potent cytotoxic agents with carefully engineered antibodies has provided meaningful improvements in clinical activity over unconjugated antibodies. The design of novel linkers and new conjugation technologies have enabled further improvements in stability and tolerability. Further honing of ADC technology along with active clinical development programs will likely provide new treatments for patients with previously unmet needs.

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LITERATURE CITED

1. Reichert JM, Rosensweig CJ, Faden LB, et al. 2005. Monoclonal antibody successes in the clinic. *Nat. Biotechnol.* 23:1073–78
2. Senter PD. 2009. Potent antibody drug conjugates for cancer therapy. *Curr. Opin. Chem. Biol.* 13:235–44
3. Teicher BA, Chari RV. 2011. Antibody conjugate therapeutics: challenges and potential. *Clin. Cancer Res.* 17:6389–97
4. Chari RV. 2008. Targeted cancer therapy: conferring specificity to cytotoxic drugs. *Acc. Chem. Res.* 41:98–107
5. Wahl AF, Klussman K, Thompson JD, et al. 2002. The anti-CD30 monoclonal antibody SGN-30 promotes growth arrest and DNA fragmentation in vitro and affects antitumor activity in models of Hodgkin's disease. *Cancer Res.* 62:3736–42
6. Stein H, Foss HD, Durkop H, et al. 2000. CD30$^+$ anaplastic large cell lymphoma: a review of its histopathologic, genetic, and clinical features. *Blood* 96:3681–95
7. Falini B, Pileri S, Pizzolo G, et al. 1995. CD30 (Ki-1) molecule: a new cytokine receptor of the tumor necrosis factor receptor superfamily as a tool for diagnosis and immunotherapy. *Blood* 85:1–14
8. Francisco JA, Cerveny CG, Meyer DL, et al. 2003. cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. *Blood* 102:1458–65
9. Oflazoglu E, Kissler KM, Sievers EL, et al. 2008. Combination of the anti-CD30-auristatin-E antibody-drug conjugate (SGN-35) with chemotherapy improves antitumour activity in Hodgkin lymphoma. *Br. J. Haematol.* 142:69–73
10. Lewis Phillips GD, Li G, Dugger DL, et al. 2008. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer Res.* 68:9280–90
11. Younes A, Gopal AK, Smith SE, et al. 2012. Results of a pivotal phase II study of brentuximab vedotin for patients with relapsed or refractory Hodgkin's lymphoma. *J. Clin. Oncol.* 30:2183–89
12. Pro B, Advani R, Brice P, et al. 2012. Brentuximab vedotin (SGN-35) in patients with relapsed or refractory systemic anaplastic large cell lymphoma: results of a phase 2 study. *J. Clin. Oncol.* 30:2190–96
13. Krop IE, Beeram M, Modi S, et al. 2010. Phase I study of trastuzumab-DM1, an HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. *J. Clin. Oncol.* 28:2698–704
14. Law CL, Cerveny CG, Gordon KA, et al. 2004. Efficient elimination of B-lineage lymphomas by anti-CD20-auristatin conjugates. *Clin. Cancer Res.* 10:7842–51
15. Smith LM, Nesterova A, Alley SC, et al. 2006. Potent cytotoxicity of an auristatin-containing antibody-drug conjugate targeting melanoma cells expressing melanotransferrin/p97. *Mol. Cancer Ther.* 5:1474–82
16. Alley SC, Zhang X, Okeley NM, et al. 2009. The pharmacologic basis for antibody-auristatin conjugate activity. *J. Pharmacol. Exp. Ther.* 330:932–38
17. Thurber GM, Schmidt MM, Wittrup KD. 2008. Antibody tumor penetration: transport opposed by systemic and antigen-mediated clearance. *Adv. Drug Deliv. Rev.* 60:1421–34
18. Bross PF, Beitz J, Chen G, et al. 2001. Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. *Clin. Cancer Res.* 7:1490–96
19. Sievers EL. 2003. Antibody-targeted chemotherapy of acute myeloid leukemia using gemtuzumab ozogamicin (Mylotarg). *Blood Cells Mol. Dis.* 31:7–10
20. Slamon DJ, Leyland-Jones B, Shak S, et al. 2001. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* 344:783–92
21. Junttila TT, Li G, Parsons K, et al. 2010. Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer. *Breast Cancer Res. Treat.* 128:347–56
22. Mao W, Luis E, Ross S, et al. 2004. EphB2 as a therapeutic antibody drug target for the treatment of colorectal cancer. *Cancer Res.* 64:781–88
23. Erickson HK, Widdison WC, Mayo MF, et al. 2010. Tumor delivery and in vivo processing of disulfide-linked and thioether-linked antibody-maytansinoid conjugates. *Bioconjug. Chem.* 21:84–92
24. Hamann PR, Hinman LM, Hollander I, et al. 2002. Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjug. Chem.* 13:47–58

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25. Boghaert ER, Khandke KM, Sridharan L, et al. 2008. Determination of pharmacokinetic values of calicheamicin-antibody conjugates in mice by plasmon resonance analysis of small (5 microl) blood samples. *Cancer Chemother. Pharmacol.* 61:1027–35
26. Sanderson RJ, Hering MA, James SF, et al. 2005. In vivo drug-linker stability of an anti-CD30 dipeptide-linked auristatin immunoconjugate. *Clin. Cancer Res.* 11:843–52
27. Senter P, Sievers EL. 2012. The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. *Nat. Biotechnol.* 30:631–37
28. Erickson HK, Park PU, Widdison WC, et al. 2006. Antibody-maytansinoid conjugates are activated in targeted cancer cells by lysosomal degradation and linker-dependent intracellular processing. *Cancer Res.* 66:4426–33
29. Doronina SO, Mendelsohn BA, Bovee TD, et al. 2006. Enhanced activity of monomethylauristatin F through monoclonal antibody delivery: effects of linker technology on efficacy and toxicity. *Bioconjug. Chem.* 17:114–24
30. Hamblett KJ, Senter PD, Chace DF, et al. 2004. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin. Cancer Res.* 10:7063–70
31. Carter D. 2008. The neuroendocrine tumors of the lung, 1926–1998: some historical observations. *Semin. Diagn. Pathol.* 25:154–65
32. Alley SC, Okeley NM, Senter PD. 2010. Antibody-drug conjugates: targeted drug delivery for cancer. *Curr. Opin. Chem. Biol.* 14:529–37
33. Doronina SO, Toki BE, Torgov MY, et al. 2003. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol.* 21:778–84
34. Thorson JS, Sievers EL, Ahlert J, et al. 2000. Understanding and exploiting nature's chemical arsenal: the past, present and future of calicheamicin research. *Curr. Pharm. Des.* 6:1841–79
35. Katz J, Janik JE, Younes A. 2011. Brentuximab vedotin (SGN-35). *Clin. Cancer Res.* 17:6428–36
36. Smith S, Chen R, Gopal A, et al. 2012. Long-term follow-up results of an ongoing pivotal study of brentuximab vedotin in patients with relapsed or refractory Hodgkin lymphoma (HL). *Eur. Hematol. Assoc. Annu. Meet. Abstr.*, Abstr. 1109
37. Horning S, Fanale M, deVos S, et al. 2008. Defining a population of Hodgkin lymphoma patients for novel therapeutics: an international effort. *Ann. Oncol.* 19:118
38. LoRusso PM, Weiss D, Guardino E, et al. 2011. Trastuzumab emtansine: a unique antibody-drug conjugate in development for human epidermal growth factor receptor 2-positive cancer. *Clin. Cancer Res.* 17:6437–47
39. Hurvitz S, Dirix L, Kocsis J, et al. 2011. ORAL trastuzumab emtansine (T-DM1) vs trastuzumab plus docetaxel (H+T) in previously-untreated HER2-positive metastatic breast cancer (MBC): primary results of a randomized, multicenter, open-label phase II study (TDM4450g/BO21976). *Eur. Multidiscip. Cancer Congr.*, Abstr. 5001
40. Blackwell KL, Miles D, Gianni L, et al. 2012. Primary results from EMILIA, a phase III study of trastuzumab emtansine (T-DM1) versus capecitabine (X) and lapatinib (L) in HER2-positive locally advanced or metastatic breast cancer (MBC) previously treated with trastuzumab (T) and taxane. *J. Clin. Oncol. Meet. Abstr.*, Abstr. LBA1
41. Ricart AD. 2011. Antibody-drug conjugates of calicheamicin derivative: gemtuzumab ozogamicin and inotuzumab ozogamicin. *Clin. Cancer Res.* 17:6417–27
42. Sievers EL, Larson RA, Stadtmauer EA, et al. 2001. Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. *J. Clin. Oncol.* 19:3244–54
43. Giles FJ, Kantarjian HM, Kornblau SM, et al. 2001. Mylotarg (gemtuzumab ozogamicin) therapy is associated with hepatic venoocclusive disease in patients who have not received stem cell transplantation. *Cancer* 92:406–13
44. Rajvanshi P, Shulman HM, Sievers EL, et al. 2002. Hepatic sinusoidal obstruction after gemtuzumab ozogamicin (Mylotarg) therapy. *Blood* 99:2310–14
45. Petersdorf S, Kopecky K, Stuart RK, et al. 2009. Preliminary results of Southwest Oncology Group Study S0106: an international intergroup phase 3 randomized trial comparing the addition of gemtuzumab

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Her-2/*neu* and Breast Cancer

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Breast cancer is the most common malignancy in women in the United States in the year 2000. The proto-oncogene Her-2/*neu* (c-erb-B2) has become an increasingly important prognostic and predictive factor in breast cancer. Overexpression/amplification of the Her-2/*neu* has been associated with a worse outcome in patients with breast cancer. Herceptin, a "humanized" murine monoclonal antibody directed against the extracellular domain of the Her-2/*neu* protein, is being used to treat breast cancer that overexpresses Her-2/*neu*. The status of Her-2/*neu* in the tumor has become a critical factor in the management strategy of a breast cancer patient. The objective of this article is to provide a comprehensive review of all aspects of Her-2/*neu* in breast cancer, including biology, prognostic and predictive value, targeted Herceptin therapy, and the laboratory testing of Her-2/*neu*.

Key Words: Breast carcinoma—Her-2/*neu*—c-erb-B2—Herceptin—Trastuzumab.

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Breast cancer is the most common malignancy in women and is estimated to account for 182,800 or 30% of the new cancer cases in the United States in the year 2000 (50). Although the number of new breast cancer cases has been increasing (representing a lifetime probability of 1 in 8 women developing breast cancer), the death rate has been steadily decreasing an average of 1.8% per year; it now represents the second leading cause of death (41,943 or 15% of the total) from cancer in women (50). This trend may be attributable to earlier diagnosis resulting from increased mammographic screening, and/or increased survival resulting from the use of adjuvant therapy (88). In fact, in 1996, approximately 57% of all reported cases were diagnosed as stage 0 or I compared with 42.5% in 1985 (36,48). Moreover, according to consensus statements published by the Early Breast Cancer Trialists Group, the use of systemic adjuvant therapy has been found to reduce the odds of recurrence and/or death from breast cancer by 10% to 50% (42,43,59).

Clinical outcome is affected by prognostic factors (56) and predictive factors (59). Prognostic factors are associated with either the metastatic or the growth potential of the primary tumor. Predictive factors are associated with the relative sensitivity and/or resistance to specific therapies. Routinely available prognostic indicators include tumor size, type, and grade, axillary lymph node status, estrogen (ER) and progesterone (PR) receptor status, as well as markers of proliferative activity (4,56,59, 94,121). ER status and PR status also serve as predictive factors for expected response to hormone therapy (59). Many other molecular markers are being investigated for their clinical usefulness (108). One of the major molecular prognostic and predictive markers in breast cancer is the amplification status of the proto-oncogene Her-2/*neu* (c-erb-B2) (17,56,66,120–122).

BASIC BIOLOGY OF HER-2/*neu*

The Her-2/*neu* (c-erb-B2) proto-oncogene is a component of a four-member family of closely related growth factor receptors that includes the epidermal growth factor

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receptor (EGFR or Her-1/c-erb-B1), Her-3 (c-erb-B3), and Her-4 (c-erb-B4) (9,17,52,66,67,108,120–122). This proto-oncogene was discovered in the 1980s through three independent lines of investigation (132): the *neu* oncogene was identified as a transforming gene present in neuroblastomas in fetal rats (131), while c-erb-B2 (127) and Her-2 (31) are human genes discovered by their homology to the retroviral gene v-erb-B. Subsequent sequence analysis and chromosomal mapping studies found that *neu*, Her-2, and c-erb-B2 represent the same gene (31,125). This gene was found to be amplified in a mammary carcinoma cell line (79), and further studies identified the oncogenic potential of overexpressed Her-2/*neu* protein on transfection of the gene into cell lines (35,65).

The human gene is located on chromosome 17q21 and encodes a 185-kDa protein with tyrosine kinase activity (1) that is also known by the designation p185 (67,132). Structurally, the protein has extracellular, transmembrane, and cytoplasmic domains, the latter of which contains the tyrosine kinase domain (9,56,67) and shares significant homology, although is distinct, from EGFR (10,56,73,155). Her-2/*neu* is thought to function as a growth factor receptor and plays a role in cell differentiation, adhesion, and motility (34,42,56). Under normal circumstances, low levels of Her-2/*neu* expression are detectable immunohistochemically in a variety of fetal and adult epithelial cells throughout the gastrointestinal, respiratory, and genitourinary tracts (110). Amplification of the Her-2/*neu* proto-oncogene or overexpression of the p185 protein, which generally correlate with one another (67,135) has been identified in 10% to 34% of breast cancers as well as in gastrointestinal, pulmonary, and genitourinary tumors (66,67,108,121,122). Investigational studies suggest that overexpressed Her-2/*neu* plays a direct role in the pathogenesis and aggressiveness of tumors through several lines of experimental evidence: transfection of Her-2/*neu* into non-neoplastic cells effects malignant transformation (39,64); transgenic mice expressing Her-2/*neu* develop mammary tumors (54); and the presence of Her-2/*neu* overexpression may be associated with the development of metastatic disease (54). The mechanism by which overexpressed Her-2/*neu* leads to a neoplastic phenotype has been associated with at least two factors: its ability to induce resistance to tumor necrosis factor-α, a protein that has been shown to play an important role in tumor cell killing in vitro as well as in vivo via cytotoxicity mediated by macrophages and natural killer cells (63), and its activation of several different signaling pathways that lead to gene activation, ultimately resulting in cell proliferation (67). Although the mechanism of activation of Her-2/*neu* has not been completely elucidated, it is thought to involve the formation of heterodimers with other members of the epidermal growth factor (EGF) family of receptors or spontaneous homodimerization (5,9,17,44). A ligand that directly binds the Her-2/*neu* receptor has not been identified, although a family of peptide ligands named *neu* differentiation factors (NDF) or heregulins have been identified that bind to the Her-3 and Her-4 receptors, inducing heterodimerization with the Her-2 receptor, and thereby inducing transphorylation and thus activation of the Her-2 receptor (9,44,56,66,67,73). Heterodimers between the EGFR (Her-1) and Her-2/*neu* can also form on binding of ligand such as EGF or transforming growth factor-α (TGF-α) to the EGFR (44).

PROGNOSTIC ROLE OF HER-2/*neu* STATUS

The association between Her-2/neu gene amplification and poor prognosis was first determined in 1987 by Slamon et al. (134), whose results showed that amplification of the Her-2/*neu* gene was strongly correlated with time to relapse (disease-free survival [DFS]) and overall survival (OS). In this study, 28% of the primary breast cancers studied showed a 2- to 20-fold gene amplification as assessed by Southern blot analysis. On multivariate analysis, amplification of Her-2/*neu* retained its independent prognostic significance when compared with size of tumor, hormone receptor status, and number of involved lymph nodes (114,134). A subsequent study performed by the same group (135) confirmed the independent prognostic significance of Her-2/*neu* amplification in predicting DFS and OS in a subgroup of node-positive patients, but not in a subgroup of node-negative patients. In addition, a strong and direct correlation was found between Her-2/*neu* gene amplification as assayed by Southern blot analysis and overexpression as assayed by Northern blot analysis for RNA expression, and Western blot analysis and immunohistochemistry on frozen sections for protein overexpression. Complete concordance between all of the tests was present in 90% of the samples that were found to be amplified. However, a minority of cases (10% of the samples) showed overexpression at the RNA and protein levels without amplification of the Her-2/*neu* gene (135), indicating that in a minority of instances mechanisms other than gene amplification may be responsible for Her-2/*neu* overexpression.

Since these initial studies, at least 52 publications evaluating >16,975 patients have explored the prognostic significance of Her-2/*neu* gene amplification (as assessed by Southern blot analysis, slot blot testing, polymerase chain reaction [PCR], fluorescence in situ hybridization [FISH] and chromagenic in situ hybridization [CISH]), and/or overexpression (as assessed by Western blot analysis, immunohistochemistry [IHC] on frozen and fixed tissues, and enzyme-linked immunosorbent assays [ELISA]) (10). Overall, 46 of the 52 studies (88%) have shown that Her-2/*neu* amplification or overexpres-

sion predicted a worse outcome in either univariate or multivariate analysis, whereas 6 of the 52 studies (12%) showed no correlation between Her-2/*neu* status and outcome. Of the 46 studies that showed a positive correlation between Her-2/*neu* status and outcome, multivariate analyses were performed in 39. Thirty-three of these 39 studies showed that Her-2/*neu* was an independent prognostic indicator in multivariate analysis; 6 studies reported prognostic significance in univariate analysis alone (10).

These differences in study results are thought to be attributable to issues of study design, differences in patient populations studied, length of follow-up, and most importantly methodologic variability regarding the testing of Her-2/*neu* status (8,29,61,92,109). One recent review (120) made the interesting observation that five of six studies (83%) that failed to show Her-2/*neu* status as an independent prognosticator and four of six studies (67%) that showed no correlation between Her-2/*neu* status and prognosis were performed using IHC. This is consistent with recent data indicating that Her-2 results obtained by this testing method are somewhat less well correlated with clinical and biologic parameters than Her-2 results obtained by FISH (see below), because of the greater degree of technical variability present in IHC methods, and/or the inclusion of specimens overexpressing Her-2 in the absence of amplification.

Her-2/*neu* in Node-Positive Breast Cancer

The prognostic importance of Her-2/*neu* has also been analyzed in the context of patient subgroups with node-positive and node-negative disease. Many of the studies that examined the prognostic importance of Her-2/*neu* in patients with positive lymph nodes have shown that Her-2/*neu* amplification/overexpression is associated with a worse outcome in either univariate or multivariate analysis (6,22,24,53,57,83,92,93,96,113,128,133–135,139, 142,146,152,153), and many have shown that it maintains independent prognostic significance in determining shorter DFS and/or OS in multivariate analysis (6,53,83,93,96,113,128,133–135,139,142,146,152,153). A few studies, however, have not shown statistically significant correlation (39,149).

Her-2/*neu* in Node-Negative Breast Cancer

The prognostic importance of Her-2/*neu* amplification/overexpression in patients with negative lymph nodes has shown mixed results. Although many studies have shown that Her-2/*neu* amplification/overexpression is associated with a worse outcome in these patients (7,41,53,109,112,113,117,128,152,153) or in subsets of these patients (3,21,96), an equal number of studies have not shown any significant correlation (8,22,24,39,83,92,93,119,133,135,139,144,146). Moreover, only a few of the studies that showed a prognostic importance for Her-2/*neu* amplification/overexpression in patients with negative lymph nodes have shown that it maintains an independent significance for DFS and/or OS in multivariate analysis (7,53,109,113,128,152). These conflicting results are attributed to the differences in sample size and length of follow-up among the various studies, which would affect their ability to detect a significant correlation between prognosis and Her-2/*neu* status in this setting where a fewer number of events and recurrences are expected (61,109,112,114). Nonetheless, examination of the studies that involved >100 patients with >3 years follow-up (114) shows equally conflicting results. Thus, although it is generally accepted that patients with positive lymph nodes and Her-2/*neu* amplification/overexpression have a worse prognosis, the prognostic significance of Her-2/*neu* status in patients with negative lymph nodes is unresolved.

Her-2/*neu* and Other Prognostic Factors in Breast Cancer

Many attempts have been made to correlate Her-2/*neu* amplification/overexpression with other prognostic factors in breast cancer, but these have also found inconsistent results. Although many studies have shown an association between Her-2/*neu* amplification/overexpression and the absence of ER and PR (6,7,16,22,24,53,57,60,88,93,128,134,139,142,153), others have not (2,11,39,49,51,109,117,144,145). Many studies have correlated Her-2/*neu* amplification/overexpression with poor histologic or nuclear grade of the primary tumor (6,11,19,22,51,53,57,96,117,134, 142,146,147,153), whereas others have not (2,7,21,49, 60,93,109,144,149). Likewise, the association with positive lymph node status found in some studies (6,22,57, 92,128,134,139,142,145) has not been confirmed by others (2,11,24,39,49,51,53,60,93,113,146,149,153). Most studies found no association with tumor size (6,7, 16,21,49,51,92,93,109,113,117,128,134,139,142,146, 153), although a few did find an association with larger tumors (>2 cm) (22,24,149). Other studies have associated Her-2/*neu* amplification/overexpression with a higher tumor cell proliferation rate as shown by more mitoses (60,146,147) or a higher S-phase fraction (6,22).

PREDICTIVE ROLE OF HER-2/*neu* STATUS IN RESPONSE TO THERAPY

Combination Chemotherapy

Her-2/*neu* has also been examined for its role as a predictive factor in response to therapy. These studies

evaluated the effectiveness of chemotherapeutic regimens containing cyclophosphamide, methotrexate, and fluorouracil (CMF) in patients with or without amplification/overexpression of Her-2/*neu* (87). Allred et al. (3) randomized a group of 306 patients with node-negative disease and high-risk tumors (ER negative or ER positive and large [>3 cm]) to treatment with adjuvant chemotherapy with CMFP (with prednisone) versus no chemotherapy after surgery. Patients with Her-2/*neu*-negative tumors who were treated showed a significantly longer DFS than those who were untreated, showing that patients with Her-2/*neu*-negative tumors benefit from adjuvant therapy. In contrast, patients with Her-2/*neu*-positive tumors who received adjuvant therapy showed no significant difference in DFS from those who did not, implying a relative resistance of Her-2/*neu*-positive tumors to CMF. However, the small number of patients in the Her-2/*neu*-positive subgroup and the small number of relapse events in this subgroup suggest that this study may have been underpowered and thus unable to detect a significant difference (101). Gusterson et al. (53) studied a group of patients who were treated in the International (Ludwig) Breast Cancer Study Group Trial V, which included both node-positive ($n = 46$) and node-negative ($n = 60$) subgroups. Patients with node-negative disease were randomized to receive a single cycle of perioperative CMF versus no therapy after surgery, whereas the node-positive subgroup was randomized to a single cycle of perioperative CMF versus perioperative CMF with six additional cycles of CMFp (including tamoxifen for postmenopausal patients). For the node-negative subgroup, Her-2/*neu* status had no effect on DFS or OS in patients who did not receive any therapy, but patients who received perioperative CMF had a worse outcome if their tumors were Her-2/*neu* positive. In the node-positive subgroup, patients who had Her-2/*neu*-positive tumors had a worse outcome in both treatment arms, suggesting that Her-2/*neu* overexpression is correlated with a decreased response to CMF therapy. However, because the number of patients in the Her-2/*neu*-positive subgroups was again small, a beneficial effect of treatment may have been missed. As well, the addition of tamoxifen may have confounded the results (101). Several additional studies have also suggested a relative resistance of Her-2/*neu*-positive tumors to CMF (5,101,122,142). However, a recent study (88) found that patients found to be Her-2/*neu* positive or negative by IHC both benefit from CMF treatment.

Anthracyclines

In contrast to the relationship between positive Her-2/*neu* status and resistance to CMF therapy, studies examining the association between Her-2/*neu* status and response to anthracycline-containing therapeutic regimens show that Her-2/*neu*-positive tumors have increased sensitivity to anthracyclines. In a study reported by Muss et al. (89), 442 random tumor samples from 1572 patients with node-positive breast cancer enrolled in a Cancer and Leukemia Group B (CALGB) trial were evaluated for the predictive role of Her-2/*neu* status. Patients were randomized to receive high, moderate, or low doses of cyclophosphamide, doxorubicin (Adriamycin), and fluorouracil (CAF) chemotherapy. Patients randomly assigned to receive high-dose CAF had a significantly longer DFS and OS if their tumors were Her-2/*neu* positive than those whose tumors were Her-2/*neu* negative. Moreover, patients with Her-2/*neu*-positive tumors showed a dose-response relationship to the adjuvant chemotherapy compared with the Her-2/*neu*-negative tumors, which did not show any dose-response effect. This study showed that Her-2/*neu*-positive tumors are sensitive to anthracycline-based chemotherapy in a dose-dependent manner. A follow-up study reported by Thor et al. (143) using a second cohort of patients from the original study showed similar results. Another study reported by Paik et al. (95) retrospectively studied patients who had lymph node-positive, hormone receptor-negative breast cancer enrolled in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-11 trial who were randomized to receive either L-phenylalanine mustard and fluorouracil (PF) or PF with doxorubicin (PAF). Results showed a significant benefit from PAF with respect to DFS in patients whose tumors were Her-2/*neu* positive (but not Her-2/*neu* negative). In vitro studies have shown that amplification/overexpression of Her-2/*neu* in breast cancer cells is frequently correlated with coamplification/expression of topoisomerase IIα (5,74, 89,137). Topoisomerase IIα is an enzyme involved in DNA replication that acts by generating and resealing double-stranded DNA breaks (74,137). Topoisomerase IIα is a target for several anticancer drugs, including doxorubicin (5,74,89,137), which inactivate this enzyme, leading to the accumulation of double-stranded DNA breaks, which are lethal to the cell (74). Sensitivity to these chemotherapeutic agents is correlated with the level of expression of this enzyme (137). This may explain the sensitivity of Her-2/*neu*-amplified/overexpressing tumors to anthracycline-containing chemotherapeutic regimens (5,89).

Tamoxifen

The predictive effect of Her-2/*neu* amplification/overexpression has also been studied in relationship to tamoxifen therapy. Expression of hormone receptors is associated with increased response (70–80%) to tamoxifen therapy among patients with advanced breast cancer, although up to 50% of patients with receptor-positive tumors will not benefit from such therapy and approxi-

mately 10% of those with receptor-negative tumors will respond (42). Many of the studies addressing this question conclude that patients with tumors with amplification/overexpression of Her-2/*neu* do not respond or may even do worse with tamoxifen therapy (5,33,42,122). The first report of an association between Her-2/*neu* and poor response to tamoxifen therapy was that of Nicholson et al. (91). They showed that the response rate of tumors overexpressing Her-2/*neu* in patients with metastatic breast cancer treated with tamoxifen as first-line endocrine therapy was 7% compared with a response rate of 38% in tumors that did not overexpress Her-2/*neu* (91). Moreover, five of the six patients who were positive for both ER and Her-2/*neu* also did not respond to tamoxifen. A subsequent study performed by Wright et al. (154) showed that the presence of Her-2/*neu* overexpression reduced the response rate of ER-positive tumors from 48% to 20% and of ER-negative tumors from 27% to 0% ($P < 0.01$). Klijn et al. (80) observed that 79% of patients with Her-2/*neu*-amplified metastatic tumors treated with tamoxifen as first-line therapy showed progressive disease compared with 47% in patients without Her-2/*neu*-amplified tumors ($P = 0.05$). In the adjuvant setting, Borg et al. (23) studied the 5-year survival in a group of nonrandomized patients who were treated with surgery alone or surgery with tamoxifen. Among patients treated with adjuvant tamoxifen, 5-year OS was significantly worse in patients with Her-2/*neu*-positive tumors (46%) compared with Her-2/*neu*-negative tumors (67%) ($P < 0.0001$). However, in the untreated group there was no significant difference in 5-year OS between patients with Her-2/*neu*-positive tumors (59%) versus those with Her-2/*neu*-negative tumors. Among patients with hormone receptor-positive tumors and node-positive disease, patients who received tamoxifen once again showed a significantly worse 5-year OS if their tumors were Her-2/*neu* positive (34%) versus negative (73%) ($P < 0.0001$). In the untreated group, there was no significant difference in 5-year OS between patients with Her-2/*neu*-positive tumors (54%) versus Her-2/*neu*-negative tumors (66%). Although a direct comparison between the tamoxifen-treated and untreated groups cannot be made on the basis of the nonrandomized design of the study, the results suggest that tamoxifen treatment in patients with Her-2/*neu*-positive tumors shows a decreased OS compared with patients with Her-2/*neu*-positive tumors who are not treated (80). The first randomized clinical study that examined the association between Her-2/*neu* overexpression and tamoxifen treatment was reported by Carlomagno et al. (26), who retrospectively studied this association in a group of patients with node-negative disease who were enrolled in the GUN-1 trial during a mean follow-up of 12 years. Patients were randomly assigned to receive 2-year treatment with adjuvant tamoxifen or no adjuvant therapy. Positive Her-2/*neu* status was detected in 30% of patients and was significantly associated with larger tumor size and decreased ER expression ($P = 0.01$). Among the Her-2/*neu*-negative patients, adjuvant tamoxifen prolonged DFS and showed a trend toward longer OS. In contrast, in Her-2/*neu*-positive patients, not only did tamoxifen treatment not affect DFS, but it also appeared to have a detrimental effect on OS, although the authors cautioned that this result was based on a small cohort of patients (26). In addition, the course of tamoxifen therapy was 2 years instead of the 5 years commonly used in the United States. In contrast, Elledge et al. (45) found no significant association between resistance to tamoxifen or worse outcome on treatment with tamoxifen in patients with metastatic tumors that were positive for both ER and Her-2/*neu*. They concluded that other studies showing such an association might have derived that result by failing to strictly exclude ER-negative tumors, as they would normally show a decreased responsiveness to tamoxifen and would be more likely to have higher levels of Her-2/*neu*. The decreased ER expression might be a contributing factor in some of the above studies but is unlikely to be a major contributor for the different outcomes of tamoxifen treatment between Her-2/*neu*-positive and -negative patients.

Mechanism of Hormone Resistance in Her-2/*neu*-Positive Tumors

This has been the focus of much laboratory investigation. Experimental studies used MCF-7 cells, a breast cancer cell line that requires estrogen for growth, has a single copy of the Her-2/*neu* gene, and lacks heregulin expression (33). Transfection of MCF-7 cells with the Her-2/*neu* gene induces estrogen-independent growth of these breast cancer cells (33,82,106) and resistance to the growth-inhibiting effect of tamoxifen in both in vitro (18,106) and in vivo models (18,82), an effect that can be reversed by the addition of anti-Her-2 monoclonal antibodies in vitro (106). Further evidence of a connection between the ER and Her-2/*neu* pathways comes from experiments that show that exposure of ER-positive/Her-2/*neu*-positive cells to estrogen induces a dose-dependent decrease in Her-2/*neu* expression at the mRNA and protein levels (115,124). This effect is probably mediated through an estrogen-dependent part of the Her-2/*neu* promoter (124) and can be reversed by the addition of antiestrogens (115). Moreover, in MCF-7 cells, both stimulation of Her-2/*neu* by addition of heregulin or overexpression of Her-2/*neu* protein by transfection of the Her-2/*neu* gene induces both a downregulation of the ER as well as activation of the ER pathway. Activation of the ER pathway consists of phosphorylation of ER on tyrosine residues, nuclear binding of the ER, and activation of ER-induced transcription of specific proteins such

as PR (106). After prolonged activation, the cellular content of PR is also reduced, leading to a cellular phenotype that is steroid receptor negative and therefore less sensitive to hormonal manipulation (106).

Taxanes

The effect of Her-2/*neu* overexpression in relationship to sensitivity to taxanes has also been examined. Paclitaxel (Taxol), one of the first taxanes examined in clinical trials, has been found to be active against many cancers deemed refractory to conventional chemotherapy. This drug acts to inhibit microtubule disassembly and promote tubulin bundling, leading to cell death by disrupting cell division (123). Early trials of paclitaxel sensitivity in women with metastatic breast cancer have shown response rates ranging from 17% to 62% (123). In a study performed at Memorial Sloan-Kettering Cancer Center (13), sensitivity to taxanes was studied in a group of women with metastatic breast cancer. The overall response rate to the taxane was 47%. Among the patients with Her-2/*neu*-positive tumors (37% overall), a significant response rate of 65% was observed versus a response rate of 36% in patients with Her-2/*neu*-negative tumors. The observed sensitivity to taxanes in Her-2/*neu*-positive tumors remained even after controlling for confounding variables that were correlated with Her-2/*neu* overexpression (13). Paclitaxel is thought to act via activation of the Her-2/*neu* signal transduction pathway, which is thought to lead to drug-induced apoptosis (13). Nonetheless, these clinical results are in conflict with experimental data that suggests that Her-2/*neu* overexpression in transfected mammary cells leads to resistance to taxanes in vitro (28,156).

HER-2/*neu* AS A TARGET FOR THERAPY

Although numerous studies have suggested that Her-2/*neu* amplification/overexpression may predict responsiveness to conventional chemotherapeutic regimens, the presence of an overexpressed oncoprotein also provides a target for specifically designed alternative therapeutic strategies.

Experimental Validation of Her-2/*neu* Protein as a Therapeutic Target

Experimental studies suggested that Her-2/*neu* overexpression plays a direct role in the pathogenesis and aggressiveness of the neoplastic phenotype, and that this tumorigenic potential is reversible on exposure of Her-2/*neu*-overexpressing cells to monoclonal antibodies directed at p185 (39,64). These findings suggested that the Her-2/*neu* oncoprotein might be a useful therapeutic target. Several laboratory groups produced many different monoclonal antibodies directed against p185 (40,47,58, 138). Antibodies directed at p185 were found to inhibit the neoplastic phenotype of Her-2/*neu*-overexpressing tumor cells in vitro, as evidenced by the inhibition of anchorage-independent growth (38,58), monolayer tumor growth (58), and growth of colonies in soft agar (30,37). In addition, anti-p185 monoclonal antibodies inhibited the tumorigenic growth of Her-2/*neu*-overexpressing tumor cells in transgenic mice (in vivo model) (39,58,138) and sensitized Her-2/*neu*-overexpressing cells to tumor necrosis factor-α (64). Sensitivity to the growth inhibitory effects of the monoclonal antibody was found to be directly related to the level of expression of Her-2/*neu*, with the highest expressors showing the greatest inhibition of cell growth (64,81). This effect appeared to be specific because untransformed cells were not affected by application of the antibody (47). Examination of the potency of the various monoclonal antibodies generated found that the murine monoclonal antibody 4D5 directed against the extracellular domain of the Her-2/*neu* receptor showed the maximum inhibition of cellular proliferation in vitro and in vivo (64,81,130). However, because murine antibodies are limited clinically as a result of their immunogenicity, the 4D5 antibody was "humanized" by constructing a fusion antibody that combined the murine antigen binding loops with the human variable region framework and the IG1 constant domains (27). The resulting antibody had a high affinity for p185 and markedly inhibited the growth of breast tumor cells exhibiting high levels of Her-2/*neu* overexpression (27). This antibody-induced growth inhibition of Her-2/*neu*-overexpressing cancer cells has been associated with a reduction in the number of tumor cells undergoing S phase, a down-modulation of the Her-2/*neu* receptor, a reduction in vascular endothelial growth factor production, and an increased induction of antibody-dependent cell-mediated cytotoxicity against these tumor cells (136).

Phase 1/2 Trials of Herceptin

Based on these preclinical data, the recombinant humanized anti-Her-2/*neu* monoclonal antibody trastuzumab (Herceptin) was tested in several phase 1 trials and subsequently in a phase 2 clinical trial reported by Baselga et al. (14,15). This study evaluated the safety and efficacy of Herceptin in 46 patients with metastatic breast cancer whose tumors overexpressed Her-2/*neu* as evaluated by IHC using the monoclonal antibody 4D5. Patients enrolled in the trial had extensive metastatic disease, with 35% exhibiting three or more metastatic sites. Most patients had received prior chemotherapy, with 63% having received two or more regimens for metastatic disease. Moreover, 80% of the tumors exhibited high levels of Her-2/*neu* overexpression, as defined

by the presence of positive membrane staining in >50% of the cells on IHC. Toxicity was minimal, and no recipient antibodies were developed to the drug. The overall response rate was 11.6%, which included one complete remission and five partial remissions.

A second trial evaluating the safety and efficacy of Herceptin as a single agent was recently reported by Cobleigh et al. (30). This multinational study involved 222 women with Her-2/*neu*-overexpressing metastatic breast cancer whose disease progressed after chemotherapy. Her-2/*neu* status was determined by IHC using the monoclonal antibodies 4D5 and CB11. Once again, the study group involved patients with advanced metastatic disease and extensive prior therapy. An overall response rate of 15% was achieved, which included 8 complete and 26 partial remissions, with a median duration of response of 8.1 months.

Herceptin as Single-Agent Therapy

Preliminary results from an additional clinical trial examining the safety and efficacy of single-agent Herceptin in the first-line treatment of Her-2/*neu*-overexpressing metastatic breast cancer in patients who have not been previously treated with chemotherapy for metastatic disease have been recently reported (129). Patients enrolled in this trial had received extensive adjuvant chemotherapy previously and were randomized as to the dose and schedule of Herceptin. An overall response rate of 23% was reported, which included 6 complete and 20 partial responses.

Combination of Herceptin and Cisplatin

Evidence from preclinical trials showed that the combination of anti-Her-2/*neu* receptor monoclonal antibodies and the DNA-damaging drug cisplatin showed a synergistically greater growth inhibition of Her-2/*neu*-overexpressing cells and xenografts (55,107). Based on this data, a clinical trial conducted by Pegram et al. (100,102) was designed to examine the safety and response to treatment of the combination of Herceptin and cisplatin in a group of 39 patients with advanced and previously treated metastatic breast cancer that had progressed during standard chemotherapy and overexpressed Her-2/*neu* as determined by IHC using the monoclonal antibody 4D5. Overall, a 24% response rate was obtained, with a median response duration of 5.3 months. This was a significantly higher response rate than had been previously reported for cisplatin (7%) or Herceptin (11%) alone. The synergistic effect of this combination was attributed to a marked decrease in DNA synthesis and a reduction in the repair of cisplatin-induced DNA adducts seen in the Her-2/*neu*-overexpressing cells, which led to an enhanced drug-induced cytotoxicity (107).

Combination of Herceptin and Other Agents

Similar preclinical studies were performed using the combination of Herceptin with many other chemotherapeutic agents in both in vitro and in vivo models (12,99). These experiments showed that the combination of Herceptin with doxorubicin or paclitaxel, among others, resulted in a greater inhibition of tumor cell growth compared with that obtained using either agent alone. These data formed the basis for a randomized, controlled, multinational trial aimed at determining the safety and efficacy of Herceptin in combination with either doxorubicin or paclitaxel. Patients with Her-2/*neu*-overexpressing metastatic breast cancer without prior treatment of metastatic disease were randomized to receive Herceptin with doxorubicin/cyclophosphamide (AC) or AC alone, if they had not previously received anthracyclines in the adjuvant setting, or Herceptin with paclitaxel or paclitaxel alone if they had (129). Preliminary results showed an overall response rate of 65% in patients receiving Herceptin and AC compared with 42% in patients receiving AC alone. Similarly, the overall response rate for patients receiving Herceptin and paclitaxel was 57% compared with 25% for patients receiving paclitaxel alone (129).

METHODS USED FOR HER-2/*neu* STATUS DETERMINATION

The increasingly important prognostic, predictive, and therapeutic role of Her-2/*neu* status in breast cancer treatment today has highlighted the need for validated and standardized testing methods. The use of many different testing methods in prior studies is regarded as one of the major causes for the lack of consensus among studies and a major contributor to the difficulty in comparing the results from different studies (56,98,111).

Her-2/*neu* status has been determined by many different assays in the past, which include methods directed at evaluating oncoprotein overexpression or gene amplification. Methods by which p185 overexpression can be determined include IHC on frozen or formalin-fixed paraffin-embedded tissue, ELISA analysis on tumor cytosols or serum, and Western blot analysis. Overexpression can also be detected by evaluation of mRNA expression by Northern blot analysis. Gene amplification can be determined by Southern blot, slot blot, CISH, FISH, and PCR (56,120–122).

Technical Comparison of Methods

There are many advantages and disadvantages in the techniques used for determining Her-2/*neu* status. The

solid matrix blotting techniques, which include Southern, slot blot, Northern, and Western blot analyses, are limited by the dilutional artifacts that arise from the variability in the content of tumor to stroma present in a given sample. This is particularly important in breast cancer, where inflammatory, vascular, and stromal cells and in situ carcinoma may constitute a significant portion of the sample (56,101,135). This dilutional artifact is most influential in Western blot analysis because the large amounts of noncellular stroma can contribute substantial amounts of extracellular matrix proteins to the lysate, whereas the poor cellularity will contribute only minimally to the total DNA or RNA extracted from the specimen (135). Moreover, the use of these methods requires large amounts of tissue for analysis; this may be problematic (135), especially today, because the average tumor size is smaller as a result of intensive screening programs (56). The ELISA technique performed on tumor cytosols also suffers from the same disadvantages as the solid matrix blotting techniques: it is adversely affected by dilutional artifacts and requires large amounts of tissue for analysis (120,121). Although PCR is a very sensitive technique that allows the evaluation of small amounts of tumor (17,101), it is also affected by dilutional artifacts. Moreover, its application as a quantitative technique requires a more demanding procedure that includes the construction of internal controls (101). Further, none of the previously described methods allows simultaneous morphologic assessment of the tissue to be tested. Thus, unless microdissection is used to isolate the area to be studied, the results from all of these tests may be affected by the inclusion of in situ carcinoma, which may express high levels of Her-2/*neu* (120,121,149). In contrast, assays that involve the use of tissue sections, such as IHC and FISH, are advantageous in that they require less tissue for evaluation and preserve the histologic architecture of the examined tissue. This latter characteristic not only allows evaluation of the specific area of interest, but also alleviates the problem of dilutional artifacts (101). Moreover, both IHC and FISH can be fully automated, allowing for their practical usefulness in the clinical laboratory setting (70,120).

Regardless of the method used, an overall high concordance has been found between gene amplification, RNA expression, and protein overexpression as determined by Southern, Northern, and Western blot analyses and IHC, consistent with the view that Her-2/*neu* overexpression is predominantly due to gene amplification (111,119,135,151). Of the specimens that showed Her-2/*neu* gene amplification by Southern blot analysis, Slamon et al. (135) found discordance (no overexpression) in 6% of specimens tested by Western blot, 2% of those tested by Northern blot, and 1% of those tested by frozen-section IHC.

Her-2/*neu* IHC: Impact of Fixation and Antigen Retrieval

Although IHC has been the predominant technique used to assess Her-2/*neu*, it is affected by many technical variables. The performance of this test on fixed, paraffin-embedded tissue as opposed to frozen tissue results in a reduction of staining because of the loss of antigen reactivity resulting from the fixation process. This problem is most significant in tumor samples containing moderate amounts of Her-2/*neu* overexpression (135), which may not be detectable when the tissue is fixed (135). In addition, the type of fixative used (103) and the storage duration of the fixed material (20,71) will affect the staining intensity obtained. All of these factors could contribute to false-negative results. However, the method of antigen retrieval used to counterbalance the fixation-induced loss of antigen reactivity will influence the degree of nonspecific staining obtained, possibly leading to false-positive results (120). Although the performance of IHC on frozen tissue is an effective, reliable technique (56,101) that circumvents the problem of fixation-induced reduction of antigen integrity (101,135), it has practical limitations due to the availability of fresh or frozen tissue on which to perform the test (120). This limitation is particularly relevant when the test is performed in reference laboratories or when archived material is tested retrospectively.

Her-2/*neu* IHC: Impact of Primary Antibody

One of the most important variables that affects the results obtained from IHC is the type of antibody used in the assay, because each antibody varies considerably in its ability to detect overexpression after tissue processing for paraffin embedding (25,56,111). One study that addresses this question was performed by Press et al. (111), who evaluated the staining characteristics of 7 polyclonal and 21 monoclonal antibodies on a panel of formalin-fixed paraffin-embedded tissue samples. These tumors had been previously characterized as to their Her-2/*neu* status as determined by Southern, Northern, and Western blot analysis as well as by IHC performed on frozen tissue. Depending on the antibody used, IHC analysis performed on fixed, paraffin-embedded tissue showed a sensitivity ranging from 6% to 80% (111).

Her-2/*neu* IHC: Significance of Staining Pattern

Although most studies evaluating Her-2/*neu* overexpression by IHC have considered only membranous staining intensity and pattern, many different studies noted that cytoplasmic staining may also be present. However, because p185 is located on the plasma membrane, only membranous staining can be considered a reliable sign of overexpression (unless the cytoplasmic

localization can be explained by internalization of the membrane-bound receptor) (34). Moreover, cytoplasmic staining has not been found to correlate with gene amplification (34,111,141) and is currently considered nonspecific. This view was corroborated by one study that found that cytoplasmic staining was not significantly associated with prognosis in a subset of patients with node-positive disease (56,142), although another study showed that breast cancers with moderate to strong cytoplasmic staining did correlate with poor prognosis (56,78).

Her-2/*neu* IHC: Impact of Scoring System

Variability in IHC testing results is also due to a lack of agreement on the scoring system to be used, which results in a high degree of interobserver variability in the interpretation of staining (77), a topic that has been the focus of a recent consensus conference (150). Because the qualitative assessment of tumor staining obtained by IHC leads to subjectivity in staining interpretation and contributes to interobserver variability, recent attempts have been made to quantitate the amount of Her-2/*neu* protein visualized by IHC. This issue was addressed in a recent study that used the relative absorption of cells with defined levels of expression on densitometric scanning as calibration material with which to compare the absorption of tumor cells produced by a computerized image of the IHC tissue assay. This comparison resulted in a quantitative determination of the amount of oncoprotein product present in the tissue specimen (112).

Federally Approved Her-2/*neu* Testing Kits

Four tests using two different methods have received approval from the Food and Drug Administration (FDA) for use in Her-2/*neu* testing: the HercepTest (DAKO, Carpinteria, CA), an IHC-based assay using a goat polyclonal antibody approved in 1998 for use in determining which patients should be treated with Herceptin; the INFORM Her-2/*neu* test (Oncor/Ventana, Tucson, AZ), a FISH-based assay for use along with other prognostic variables in patients with primary breast cancer with negative lymph nodes; the Path Vysion Her-2 DNA Probe Kit (Vysis, Downers Grove, IL), another FISH-based assay approved in 1998 for use in determining the response of patients with stage 2, node-positive breast cancer to therapy with CAF (90); and the CB11 IHC test (Ventana, Tucson, AZ), approved in 2000, which uses the monoclonal antibody CB11 in determining which patients should be treated with Herceptin.

Although the DAKO HercepTest has been approved by the FDA for determining Her-2/*neu* status for the purpose of identifying patients who would benefit from Herceptin therapy, the actual correlation of Her-2/*neu* status based on the HercepTest results to clinical response from Herceptin therapy has not been established (46). All of the patients enrolled in the clinical trials that established the efficacy of Herceptin as a single or combined therapeutic agent in patients with stage 4 disease were tested with an investigational immunohistochemical assay called the clinical trial assay (CTA), which used the 4D5 and CB11 monoclonal antibodies. Entry into these trials (and therefore eligibility for Herceptin treatment) required a positive Her-2/*neu* test result as determined by the CTA (2+ or 3+ score). The DAKO HercepTest was developed to provide an alternative to this research assay.

The performance of the HercepTest was evaluated in an independent study comparing the results of the HercepTest to the CTA on 548 breast tumor specimens that were independent of the Herceptin trials. The overall concordance of the DAKO HercepTest to the CTA was 79% (32,86). A 3+ reading on the HercepTest was found to correlate highly with a positive test result on the CTA (2+ or 3+) (32,86). However, a finding of 2+ on the HercepTest did not correlate as well with the CTA results. Approximately 42% of the positive 2+ HercepTest results corresponded to a negative (0 or 1+) test result by the CTA (32). Thus, 42% of patients whose breast tumors were found to be 2+ by the HercepTest would not have been eligible to participate in the Herceptin clinical trials and receive Herceptin treatment (32,62). Moreover, because subgroup analyses of the results from these studies suggested that patients whose tissues were strongly positive for Her-2/*neu* protein expression benefited more from Herceptin therapy than those whose tissues were weakly positive, and because none of the patients in the Herceptin trials were selected using the HercepTest, the correlation between the degree of positivity on the HercepTest and the likelihood of clinical benefit from Herceptin treatment has not been rigorously established (32, 46,62). Other studies have also highlighted the apparently low specificity of the HercepTest (12,69,118), which some have attributed to the use of the FDA-approved scoring system (69) that does not control for the intensity of background staining present in normal breast epithelium when evaluating the intensity of staining present in the invasive tumor (69). Other studies have also noted the apparently high sensitivity of the DAKO HercepTest in staining formalin-fixed paraffin-embedded tissue, which is manifested by the increased amount of weakly staining (2+) tumor cells characterized by this test (84).

Her-2/*neu* FISH

The FISH technique represents the newest methodologic approach for the evaluation of gene amplification (76,98,104,105,109). This technique, as well as the more recently developed chromogenic in situ hybridization

(CISH) (140), allows simultaneous assessment of morphology and gene amplification. Her-2/*neu* status can thus be determined specifically in the invasive tumor. The technique does not suffer from the dilutional artifacts that affect the other methods for detecting gene amplification (120). This approach is more sensitive than the solid matrix blotting procedures used to determine DNA amplification (120) and has been shown to have a sensitivity of 98% and a specificity of 100% (109). Moreover, the Vysis FISH test allows for the distinction between increased copy number of Her-2/*neu* due to gene amplification versus chromosomal duplication (85). This method has been found to be highly reproducible and reliable (85). Similar to IHC, FISH testing can also be automated for use in clinical laboratories (72), although, according to one study (68), the increased cost and increased intensity of technical and professional time needed for the performance and interpretation of the test can make it difficult to justify its routine use in low-volume settings.

Comparison of FISH and IHC

Results obtained from FISH analysis have been highly concordant with those obtained by IHC, particularly in the 3+ category, although the concordance in the 2+ category has been less than acceptable (68,75,76,81,85, 86,116). Among the IHC assays, CB11 appears to correlate most specifically with FISH results (126). Some studies have found that a small percentage of specimens overexpress Her-2/*neu* by IHC in the absence of gene amplification as assayed by FISH (75,111,135), although others have shown this not to be the case (148). A recent study has shown FISH analysis to be a better method than IHC in predicting overall survival and, significantly, found that the outcome of patients with Her-2/*neu* protein overexpression without gene amplification is similar to that of patients without protein overexpression (97). Because Her-2/*neu* gene amplification by FISH appears to be a better predictor of response to Herceptin-based therapy than Her-2/*neu* overexpression by IHC (86), and considering the much higher cost of Herceptin therapy and the associated morbidity compared with FISH testing itself, FISH analysis should be performed at least in the IHC 2+ group for better selection of patients who would most likely benefit from Herceptin treatment. At Memorial Sloan-Kettering Cancer Center, Her-2 status is taken into account when selecting patients for Herceptin-based or anthracycline therapy, and patients with invasive carcinoma showing either 3+ staining by Herce-Test or gene amplification by FISH when IHC is 2+, 1+, or 0 are considered candidates for the above treatment.

CONCLUSION

Given the central importance of Her-2/*neu* status in determining prognosis, response to conventional chemotherapy, and response to targeted monoclonal antibody therapy, the overall lack of technical standardization and clinical validation of the currently available testing methods remains an area of legitimate concern for pathologists and clinicians alike, as well as an area of intense laboratory investigation.

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REFERENCES

1. Akiyama T, Sudo C, Ogawara H, et al. The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science* 1986;232:1644–46.
2. Ali IU, Campbell G, Lidereau R, Callahan R. Lack of evidence for the prognostic significance of *c-erb*B-2 amplification in human breast carcinoma. *Oncogene Res* 1988;3:139–146.
3. Allred DC, Clark GM, Tandon AK, et al. Her-2/neu in node-negative breast cancer: prognostic significance of overexpression influenced by the presence of in situ carcinoma. *J Clin Oncol* 1992;10:599–605.
4. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998;11:155–168.
5. Allred DC, Swanson PE. Testing for erbB-2 by immunohistochemistry in breast cancer. *Am J Clin Pathol* 2000;113:171–175.
6. Anbazhagan R, Gelber RD, Bettelheim R, et al. Association of *c-erb*B-2 expression and S-phase fraction in the prognosis of node positive breast cancer. *Ann Oncol* 1991;2:47–53.
7. Andrulis IL, Bull SB, Blackstein ME, et al., for the Toronto Breast Cancer Study Group. *Neu/erb*B-2 amplification identifies a poor-prognosis group of women with node-negative breast cancer. *J Clin Oncol* 1998;16:1340–1349.
8. Babiak J, Hugh J, Poppema S. Significance of c-erbB-2 amplification and DNA aneuploidy. *Cancer* 1992;70:770–776.
9. Bacus SS, Zelnick CR, Plowman G, Yarden Y. Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers. Implication for tumor biology and clinical behavior. *Am J Clin Pathol* 1994;102(Suppl 1):S13–24.
10. Bargmann CI, Hung M-C, Weinberg RA. The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature* 319:226–230.
11. Barnes DM, Lammie GA, Millis RR, et al. An immunohistochemical evaluation of *c-erb*B-2 expression in human breast carcinoma. *Br J Cancer* 1988;58:448–452.
12. Baselga J, Norton L, Albanell J, et al. Recombinant humanized anti-HER2 antibody (Herceptin™) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/*neu* overexpressing human breast cancer xenografts. *Cancer Res* 1998;58:2825–2831.
13. Baselga J, Seidman AD, Rosen PP, Norton L. HER2 overexpression and paclitaxel sensitivity in breast cancer: therapeutic implications. *Oncology* 1997;11(Suppl 2):43–48.
14. Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/*neu*-overexpressing metastatic breast cancer. *Semin Oncol* 1999;26(Suppl 12):78–83.
15. Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous recombinant humanized anti-p185^{HER2} monoclonal antibody in patients with HER2/*neu*-overexpressing metastatic breast cancer. *J Clin Oncol* 1996;14:737–44.
16. Battifora H, Gaffey M, Esteban J, et al. Immunohistochemical assay of *neu/c-erb*B-2 oncogene product in paraffin-embedded tissues in early breast cancer: retrospective follow-up study of 245 stage I and II cases. *Mod Pathol* 1991;4:466–474.
17. Benz CC, Brandt BH, Zanker KS. Gene diagnostics provide new

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insights into breast cancer prognosis and therapy. *Gene* 1995;159: 3–7.
18. Benz CC, Scott GK, Sarup JC, et al. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/*neu*. *Breast Cancer Res Treat* 1992;24:85–95.
19. Berger MA, Locher GW, Saurer S, et al. Correlation of c-*erb*B-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. *Cancer Res* 1988; 48:1238–1243.
20. Bertheau P, Cazals-Hatem D, Meignin V, et al. Viability of immunohistochemical reactivity on stored paraffin slides. *J Clin Pathol* 1998;51:370–4.
21. Bianchi S, Paglierani M, Zampi G, et al. Prognostic significance of c-*erb*B-2 expression in node negative breast cancer. *Br J Cancer* 1993;67:625–9.
22. Borg A, Baldetorp B, Fernö M, et al. erbB2 amplification in breast cancer with a high rate of proliferation. *Oncogene* 1991; 6:137–43.
23. Borg A, Baldetorp B, Fernö M, et al. ERBB2 amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. *Cancer Lett* 1994;81:137–44.
24. Borg A, Tandon AK, Sigurdsson H, et al. Her-2/neu amplification predicts poor survival in node-positive breast cancer. *Cancer Res* 1990;50:4332–7.
25. Busmanis I, Feleppa F, Jones A, et al. Analysis of cerbB2 expression using a panel of 6 commercially available antibodies. *Pathology* 1994;26:261–7.
26. Carlomagno C, Perrone F, Gallo C, et al. c-*erb*B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. *J Clin Oncol* 1996;14:2702–8.
27. Carter P, Presta L, Gorman CM, et al. Humanization of an anti-p185 ^{HER2} antibody for human cancer therapy. *Proc Natl Acad Sci USA* 1992;89:4285–9.
28. Ciardiello F, Caputo R, Pomatico G, et al. Resistance to taxanes is induced by c-erbB-2 overexpression in human MCF-10A mammary epithelial cells and is blocked by combined treatment with an antisense oligonucleotide targeting type 1 protein kinase A. *Int J Cancer* 2000;85:710–5.
29. Clark GM, McGuire WL. Follow-up study of her-2/*neu* amplification in primary breast cancer. *Cancer Res* 1991;51:944–8.
30. Cobleigh MA, Vogel CL, Tripathy D, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have Her2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 1999;17:2639–48.
31. Coussens L, Yang-Feng TL, Liao Y-C, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. *Science* 1985;230:1132–9.
32. Dako HercepTest for immunoenzymatic staining. Handbook produced by Dako AS, Glostrup, Denmark.
33. De Placido S, Carlomagno C, De Laurentiis M, Bianco AR. c-*erb*B2 expression predicts tamoxifen efficacy in breast cancer patients. *Breast Cancer Res Treat* 1998;52:55–64.
34. De Potter CR, Scheldhout A-M. The neu-protein and breast cancer. *Virchows Arch* 1995;426:107–15.
35. Di Fiore PP, Pierce JH, Kraus MH, et al. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science* 1987; 237:178–82.
36. Digman JJ. Differences in breast cancer prognosis among African-American and Caucasian women. *CA Cancer J Clin* 2000; 50:50–64.
37. Drebin JA, Link VC, Greene MI. Monoclonal antibodies specific for the *neu* oncogene product directly mediate anti-tumor effects in vivo in vivo. *Oncogene* 1988;2:387–94.
38. Drebin JA, Link VC, Stern DF, et al. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell* 1985;41:695–706.
39. Drebin JA, Link VC, Weinberg RA, Greene MI. Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. *Proc Natl Acad Sci USA* 1986;83: 9129–33.
40. Drebin JA, Stern DF, Link VC, et al. Monoclonal antibodies identify a cell-surface antigen associated with an activated cellular oncogene. *Nature* 1984;312:545–8.
41. Dykins R, Corbett IP, Henry JA, et al. Long-term survival in breast cancer related to overexpression of the c-*erb*B-2 oncoprotein: an immunohistochemical study using monoclonal antibody NCI-CB11. *J Pathol* 1991;163:105–10.
42. Early Breast Cancer Trialists Collaborative Group. Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. *N Engl J Med* 1988;319:1681–92.
43. Early Breast Cancer Trialists Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy: 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet* 1992; 339:1–15.
44. Earp HS, Dawson TL, Li X, Yu H. Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. *Breast Cancer Res Treat* 1995;35:115–32.
45. Elledge RM, Green S, Ciocca D, et al. HER-2 expression and response to tamoxifen in estrogen receptor-positive breast cancer: a Southwest Oncology Group Study. *Clin Cancer Res* 1998;4: 7–12.
46. Espinoza F, Anguiano A. The HercepTest assay: another perspective. *J Clin Oncol* 1999;17:2293–4.
47. Fendly BM, Winget M, Hudziak RM, et al. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/*neu* gene product. *Cancer Res* 1990;50:1550–8.
48. Fremgen AM, Bland KI, McGinnis LS Jr, et al. Clinical highlights from the National Cancer Database, 1999. *CA Cancer J Clin* 1999;49:145–58.
49. Gasparini G, Gullick WJ, Bevilacqua P, et al. Human breast cancer: prognostic significance of the c-erbB-2 oncoprotein compared with epidermal growth factor receptor, DNA ploidy, and conventional pathologic features. *J Clin Oncol* 1992;10:686–95.
50. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics 2000. *Ca Cancer J Clin* 2000;50:7–34.
51. Gullick WJ, Love SB, Wright C, et al. C-*erb*B-2 protein expression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. *Br J Cancer* 1991;63:434–8.
52. Gullick WJ, Srinivasan R. The type 1 growth factor receptor family: new ligands and receptors and their role in breast cancer. *Breast Cancer Res Treat* 1998;52:43–53.
53. Gusterson BA, Gelber RD, Goldhirsch A, et al. Prognostic importance of c-*erb*B-2 expression in breast cancer. *J Clin Oncol* 1992;10:1049–1056.
54. Guy CT, Webster MA, Schaller M, et al. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci USA* 1992;89: 10578–82.
55. Hancock MC, Langton BC, Chan T, et al. A monclonal antibody against the c-*erb*B-2 protein enhances the cytotoxicity of cis-Diamminedichloroplatinum against human breast and ovarian tumor cell lines. *Cancer Res* 1991;51:4575–80.
56. Hanna W, Kahn HJ, Trudeau M. Evaluation of HER-2/*neu* (erbB-2) status in breast cancer: from bench to bedside. *Mod Pathol* 1999;12:827–34.
57. Hartmann LC, Ingle JN, Wold LE, et al. Prognostic value of c-*erb*B2 overexpression in axillary lymph node positive breast cancer. Results from a randomized adjuvant treatment protocol. *Cancer* 1994;74:2956–63.
58. Harwerth I-M, Wels W, Schlegel J, et al. Monoclonal antibodies directed to the *erb*B-2 receptor inhibit in vivo tumor cell growth. *Br J Cancer* 1993;68:1140–5.
59. Hayes DF, Trock B, Harris AL. Assessing the clinical impact of prognostic factors: when is "statistically significant" clinically useful? *Breast Cancer Res Treat* 1998;52:305–19.
60. Heintz NH, Leslie KO, Rogers LA, Howard PL. Amplification of the c-*erb* B-2 oncogene and prognosis of breast adenocarcinoma. *Arch Pathol Lab Med* 1990;114:160–3.
61. Henderson IC, Patek AJ. The relationship between prognostic and predictive factors in the management of breast cancer. *Breast Cancer Res Treat* 1998;52:261–88.

62. Herceptin® trastuzumab. Informational insert perpared by Genentech, Inc., South San Francisco, CA, USA.
63. Hudziak RM, Lewis GD, Shalaby MR, et al. Amplified expression of the HER2/erbB2 oncogene induces resistance to tumor necrosis factor α in NIH 3T3 cells. *Proc Natl Acad Sci USA* 1988;85:5102–6.
64. Hudziak RM, Lewis GD, Winget M, et al. $p185^{HER2}$ monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. *Mol Cell Biol* 1989;9:1165–72.
65. Hudziak RM, Schlessinger J, Ullrich A. Increased expression of the putative growth factor receptor $p185^{HER2}$ causes transformation and tumorigenesis of NIH 3T3 cells. *Proc Natl Acad Sci USA* 1987;84:7159–63.
66. Hung M-C, Lau Y-K. Basic science of HER-2/*neu*: a review. *Semin Oncol* 1999;26(Suppl 12):51–9.
67. Hynes NE, Stern DF. The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta* 1994;1198:165–84.
68. Jacobs TW, Gown AM, Yaziji H, et al. Comparison of fluorescence in situ hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. *J Clin Oncol* 1999;17:1974–82.
69. Jacobs TW, Gown AM, Yaziji H, et al. Specificity of HercepTest in determining HER-2/neu status of breast cancers using the United States Food and Drug Administration-approved scoring system. *J Clin Oncol* 1999;17:1983–7.
70. Jacobs TW, Gown AM, Yaziji H, et al. HER-2/neu protein expression in breast cancer evaluated by immunohistochemistry. A study of interlaboratory agreement. *Am J Clin Pathol* 2000;113:251–8.
71. Jacobs TW, Prioleau JE, Stillman IE, Schnitt SJ. Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. *J Natl Cancer Inst* 1996;88:1054–9.
72. Jacobson K, Thompson A, Browne G, et al. Automation of fluorescence in situ hybridization pretreatment: a comparative study of different sample types. *Mol Diagnosis* 2000;5:209–20.
73. Jardines L, Weiss M, Fowble B, Greene M. neu/c-erbB-2/HER2) and the epidermal growth factor receptor (EGFR) in breast cancer. *Pathobiology* 1993;61:268–82.
74. Järvinen TAH, Kononen J, Pelto-Huikko M, Isola J. Expression of topoisomerase IIα is associated with rapid cell proliferation, aneuploidy, and c-erbB2 overexpression in breast cancer. *Am J Pathol* 1996;148:2073–82.
75. Jimenez RE, Wallis T, Tabasczka P, Visscher DW. Determination of Her-2/*neu* status in breast carcinoma: comparative analysis of immunohistochemistry and fluorescent in situ hybridization. *Mod Pathol* 2000;13:37–45.
76. Kakar S, Puangsuvan N, Stevens JM, et al. Her-2/neu assessment in breast cancer by immunohistochemistry and fluorescence in situ hybridization: comparision of results and correlation with survival. *Mol Diagnosis* 2000;5:199–207.
77. Kay EW, Walsh CJB, Cassidy M, et al. c-*erb*B-2 immunostaining: Problems with interpretation. *J Clin Pathol* 1994;47:816–22.
78. Keshgegian AA, Cnaan A. erbB-2 oncoprotein expression in breast carcinoma. Poor prognosis associated with high degree of cytoplasmic positivity using CB-11 antibody. *Am J Clin Pathol* 1997;108:456–63.
79. King CR, Kraus MH, Aaronson SA. Amplification of a novel v-*erb*B-related gene in a human mammary carcinoma. *Science* 1985;229:974–6.
80. Klijn JGM, Berns EMJJ, Bontenbal M, Foekens J. Cell biologic factors associated with the response of breast cancer to systemic treatment. *Cancer Treat Rev* 1993;19:45–63.
81. Lebeau A, Deimling D, Kaltz C, et al. Her-2/*neu* analysis in archival tissue samples of human breast cancer: comparison of immunohistochemistry and fluorescence in situ hybridization. *J Clin Oncol* 2001;19:354–63.
82. Liu Y, El-Ashry D, Chen D, et al. MCF-7 breast cancer cells overexpressing transfected c-*erb*B-2 have an in vitro growth advantage in estrogen-depleted conditions and reduced estrogen-dependence and tamoxifen-sensitivity in vivo. *Breast Cancer Res Treat* 1995;34:97–117.
83. Lovekin C, Ellis IO, Locker A, et al. C-*erb*B-2 oncoprotein expression in primary and advanced breast cancer. *Br J Cancer* 1991;63:439–43.
84. Maia DM. Immunohistochemical assays for *HER2* overexpression. *J Clin Oncol* 1999;17:1650.
85. Masood S, Bui MM, Yung J-F, et al. Reproducibility of LSI Her-2/neu SpectrumOrange and CEP 17 SpectrumGreen dual color deoxyribonucleic acid probe kit. *Ann Clin Lab Sci* 1998;28:215–23.
86. Mass R, Sanders C, Kasiam C, et al. The concordance between the clinical trials assay (CTA) and fluorescence in situ hybridization (FISH) in the Herceptin pivotal trials. *Proc ASCO* 2000;19:76a.
87. Menard S, Valagussa P, Pilotti S, et al. Response to cyclophosphamide, methotrexate, and fluorouracil in lymph node-positive breast cancer according to HER2 overexpression and other tumor biologic variables. *J Clin Oncol* 2001;19:329–35.
88. Mettlin C. Global breast cancer mortality statistics. *CA Cancer J Clin* 1999;49:138–44.
89. Muss HB, Thor AD, Berry DA, et al. c-*erb*B-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. *N Engl J Med* 1994;330:1260–6.
90. Nelson NJ. Experts debate value of HER2 testing methods. *J Natl Cancer Inst* 2000;92:292–4.
91. Nicholson S, Wright C, Sainsbury JRC, et al. Epidermal growth factor receptor (EGFR) as a marker for poor prognosis in node-negative breast cancer patients: Neu and tamoxifen failure. *J Steroid Biochem Molec Biol* 1990;37:811–4.
92. Noguchi M, Koyasaki N, Ohta N, et al. C-*erb*B-2 oncoprotein expression versus internal mammary lymph node metastases as additional prognostic factors in patients with axillary lymph node-positive breast cancer. *Cancer* 1992;69:2953–60.
93. O'Reilly SM, Barnes DM, Camplejohn RS, et al. The relationship between c-*erb*B-2 expression, S-phase fraction and prognosis in breast cancer. *Br J Cancer* 1991;63:444–6.
94. Page DL, Jensen RA, Simpson JF. Routinely available indicators of prognosis in breast cancer. *Breast Cancer Res Treat* 1998;51:195–208.
95. Paik S, Bryant J, Park C, et al. erbB-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer. *J Natl Cancer Inst* 1998;90:1361–70.
96. Paik S, Hazan R, Fisher ER, et al. Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: prognostic significance of *erb*B-2 protein overexpression in primary breast cancer. *J Clin Oncol* 1990;8:103–12.
97. Pauletti G, Dandekar S, Rong HM, et al. Assessment of methods for tissue-based detection of the Her-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. *J Clin Oncol* 2000;18:3651–64.
98. Pauletti G, Godolphin W, Press MF, Slamon DJ. Detection and quantitation of HER-2/*neu* gene amplification in human breast cancer archival material using fluorescence in situ hybridization. *Oncogene* 1996;13:63–72.
99. Pegram M, Hsu S, Lewis G, et al. Inhibitory effects of combinations of HER-2/*neu* antibody and chemotherapeutic agents used for treatment of human breast cancers. *Oncogene* 1999;18:2241–51.
100. Pegram MD, Lipton A, Hayes DF, et al. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-$p185^{HER2/neu}$ monoclonal antibody plus cisplatin in patients with HER2/*neu*-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J Clin Oncol* 1998;16:2659–71.
101. Pegram MD, Pauletti G, Slamon DJ. Her-2/neu as a predictive marker of response to breast cancer therapy. *Breast Cancer Res* 1998;52:65–77.
102. Pegram MD, Slamon DJ. Combination therapy with trastuzumab (Herceptin) and cisplatin for chemoresistant metastatic breast cancer: evidence for receptor-enhanced chemosensitivity. *Semin Oncol* 1999;26(Suppl 12):89–95.
103. Penault-Llorca F, Adelaide J, Houvenaeghel G, et al. Optimization of immunohistochemical detection of ERBB2 in human breast cancer: impact of fixation. *J Pathol* 1994;173:65–75.
104. Persons DL, Borelli KA, Hsu PH. Quantitation of HER-2/*neu* and

c-myc gene amplification in breast carcinoma using fluorescence in situ hybridization. *Mod Pathol* 1997;10:720–7.
105. Persons DL, Bui MM, Lowery MC, et al. Fluorescence in situ hybridization (FISH) for detection of HER-2/neu amplification in breast cancer: a multicenter portability study. *Ann Clin Lab Sci* 2000;30:41–8.
106. Pietras RJ, Arboleda J, Reese DM, et al. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. *Oncogene* 1995;10:2435–46.
107. Pietras RJ, Fendly BM, Chazin VR, et al. Antibody to HER-2/*neu* receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. *Oncogene* 1994;9:1829–38.
108. Porter-Jordan K, Lippman ME. Overview of the biologic markers of breast cancer. *Hematol Oncol Clin North Am* 1994;8:73–100.
109. Press MF, Bernstein L, Thomas PA, et al. Her-2/*neu* gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. *J Clin Oncol* 1997;15:2894–904.
110. Press MF, Cordon-Cardo C, Slamon DJ. Expression of the HER-2/*neu* proto-oncogene in normal human adult and fetal tissues. *Oncogene* 1990;5:953–62.
111. Press MF, Hung G, Godolphin W, Slamon DJ. Sensitivity of HER-2/*neu* antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. *Cancer Res* 1994;54:2771–7.
112. Press MF, Pike MC, Chazin VR, et al. Her-2/*neu* expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. *Cancer Res* 1993;53:4960–70.
113. Quénel N, Wafflart J, Bonichon F, et al. The prognostic value of c-erbB2 in primary breast carcinomas: a study of 942 cases. *Breast Cancer Res Treat* 1995;35:283–91.
114. Ravdin PM, Chamness GC. The c-*erb*B-s proto-oncogene as a prognostic and predictive marker in breast cancer: a paradigm for the development of other macromolecular markers—a review. *Gene* 1995;159:19–27.
115. Read LD, Keith D Jr, Slamon DJ, Katzenellenbogen BS. Hormonal modulation of HER-2/*neu* protooncogene messenger ribonucleic acid and p185 protein expression in human breast cancer cell lines. *Cancer Res* 1990;50:3947–51.
116. Ridolfi RL, Jameidor MR, Arbes JM. Her-2/neu testing in breast carcinoma: a combined immunohistochemical and fluorescence in situ hybridization approach. *Mod Pathol* 2000;13:866–73.
117. Ro J, El-Naggar A, Ro JY, et al. c-*erb*B-2 amplification in node-negative human breast cancer. *Cancer Res* 1989;49:6941–4.
118. Roche PC, Ingle JN. Increased HER2 with U.S. Food and Drug Administration-approved antibody. *J Clin Oncol* 1999;17:434–5.
119. Rosen PP, Lesser ML, Arroyo CD, et al. Immunohistochemical detection of HER-2/*neu* in patients with axillary lymph node negative breast carcinoma. A study of epidemiologic risk factors, histologic features, and prognosis. *Cancer* 1995;75:1320–6.
120. Ross JS, Fletcher JA. Her-2/*neu* (c-*erb*-B2) gene and protein in breast cancer. *Am J Clin Pathol* 1999;112(Suppl 1):S53–67.
121. Ross JS, Fletcher JA. The HER-2/*neu* oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. *Oncologist* 1998;3:237–52.
122. Ross JS, Fletcher JA. The Her-2/neu oncogene: prognostic factor, predictive factor and target for therapy. *Semin Cancer Biol* 1999; 9:125–38.
123. Rowinsky EK, Donehower RC. Paclitaxel (Taxol). *N Engl J Med* 1995;332:1004–14.
124. Russell KS, Hung M-C. Transcriptional repression of the *neu* protooncogene by estrogen stimulated esuogen receptor. *Cancer Res* 1992;52:6624–9.
125. Schechter AL, Hung M-C, Vaidyanathan L, et al. The neu gene: an erbB-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. *Science* 1985;229:976–8.
126. Seidman AD, Fornier M, Esteva FJ, et al. Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by Her-2 immunophenotype and gene amplification. *J Clin Oncol* 2001;19:2587–95.
127. Semba K, Kamata N, Toyoshima K, Yamamoto T. A v-*erb*B-related protooncogene, c-*erb*B-2, is distinct from the c-*erb*-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. *Proc Natl Acad Sci USA* 1985;82:6497–501.
128. Seshadri R, Firgaira FA, Horsfall DJ, et al., for the South Australian Breast Cancer Study Group P. Clinical significance of Her-2/*neu* oncogene amplification in primary breast cancer. *J Clin Oncol* 1993;11:1936–42.
129. Shak S, for the Herceptin Multinational Investigator Study Group. Overview of the trastuzumab (Herceptin) anti-HER2 monoclonal antibody clinical program in HER2-overexpressing metastatic breast cancer. *Semin Oncol* 1999;26(Suppl 12):71–7.
130. Shepard HM, Lewis GD, Sarup JC, et al. Monoclonal antibody therapy of human cancer: taking the HER2 protooncogene to the clinic. *J Clin Immunol* 1991;11:117–27.
131. Shih C, Padhy LC, Murray M, Weinberg RA. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* 1981;290:261–4.
132. Singleton TP, Strickler JG. Clinical and pathologic significance of the c-erbB-2 (HER-2/neu) oncogene. *Pathol Annu* 1992;27: 165–90.
133. Sjögren S, Inganäs M, Lindgren A, et al. Prognostic and predictive value of c-*erb*B-2 overexpression in primary breast cancer, alone and in combination with other prognostic markers. *J Clin Oncol* 1998;16:462–9.
134. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the Her-2/*neu* oncogene. *Science* 1987;235:177–82.
135. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989;244:707–12.
136. Sliwkowski MX, Lofgren JA, Lewis GD, et al. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). *Semin Oncol* 1999,26(Suppl 12):60–70.
137. Smith K, Houlbrook S, Greenall M, et al. Topoisomerase IIα co-amplification with *erb*B2 in human primary breast cancer and breast cancer cell lines: relationship to *m*-AMSA and mitoxantrone sensitivity. *Oncogene* 1993;8:933–938.
138. Stancovski I, Hurwitz E, Leitner O, et al. Mechanistic aspects of the opposing effects of monoclonal antibodies to the erbB2 receptor on tumor growth. *Proc Natl Acad Sci USA* 1991;88: 8691–5.
139. Tandon AK, Clark GM, Chamness GC, et al. Her-2/*neu* oncogene protein and prognosis in breast cancer. *J Clin Oncol* 1989;7: 1120–8.
140. Tanner M, Gancberg D, Di Leo A, et al. Chromogenic in situ hybridization: A practical alternative for fluorescence in situ hybridization to detect Her-2/neu oncogene amplification in archival breast cancer samples. *Am J Pathol* 2000;157:1467–72.
141. Taylor SL, Flatt-Higgins A, Rudland PS, et al. Cytoplasmic staining of c-erbB-2 is not associated with the presence of detectable c-erbB-2 mRNA in breast cancer specimens. *Int J Cancer* 1998; 76:459–63.
142. Tétu B, Brisson J. Prognostic significance of her-2/*neu* oncoprotein expression in node-positive breast cancer. The influence of the pattern of immunostaining and adjuvant therapy. *Cancer* 1994;73:3259–65.
143. Thor AD, Berry DA, Budman DR, et al. erbB-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer. *J Natl Cancer Inst* 1998;90:1346–60.
144. Thor AD, Schwartz LH, Koerner FC, et al. Analysis of c-*erb*B-2 expression in breast carcinomas with clinical follow-up. *Cancer Res* 1989;49:7147–52.
145. Tiwari RK, Borgen PI, Wong GY, et al. Her-2/neu amplification and overexpression in primary human breast cancer is associated with early metastasis. *Anticancer Res* 1992;12:419–26.
146. Toikkanen S, Helin H, Isola J, Joensuu H. Prognostic significance of Her-2 oncoprotein expression in breast cancer: a 30-year follow up. *J Clin Oncol* 1992;10:1044–8.
147. Tsuda H, Hirohashi S, Shimosato Y, et al. Correlation between histologic grade of malignancy and copy number of c-*erb*B-2 gene in breast carcinoma: A retrospective analysis of 176 cases. *Cancer* 1990;65:1794–800.

148. Tubbs RR, Pettay JD, Roche PC, et al. Discrepancies in clinical laboratory testing of eligibility for trastuzumab therapy: apparent immunohistochemical false-positives do not get the message. *J Clin Oncol* 2001;19:2714–21.
149. Van de Vijver MJ, Peterse JL, Mooi WJ, et al. Neu-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. *N Engl J Med* 1988;319:1239–45.
150. Van Diest PJ, van Dam P, Henzen-Logmans C, et al. A scoring system for immunohistochemical staining: Consensus report of the task force for basic research of the EORTC-GCCG. *J Clin Pathol* 1997;50:801–4.
151. Venter DJ, Tuzi NL, Kumar S, Gullick WJ. Overexpression of the c-*erb*B-2 oncoprotein in human breast carcinomas: immunohistologic assessment correlates with gene amplification. *Lancet* 1987;2(8550):69–71.
152. Winstanley J, Cooke T, Murray GD, et al. The long-term prognostic significance of c-erbB-2 in primary breast cancer. *Br J Cancer* 1991;63:447–50.
153. Wright C, Angus B, Nicholson S, et al. Expression of c-*erb*B-2 oncoprotein: a prognostic indicator in human breast cancer. *Cancer Res* 1989;49:2087–90.
154. Wright C, Nicholson S, Angus B, et al. Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. *Br J Cancer* 1992;65:118–21.
155. Yamamoto T, Ikawa S, Akiyama T, et al. Similarity of protein encoded by the human c-erB-2 gene to epidermal growth factor receptor. *Nature* 1986;319:230–4.
156. Yu D, Liu B, Jing T, et al. Overexpression of both $p185^{c\text{-}erbB2}$ and $p170^{mdr\text{-}1}$ renders breast cancer cells highly resistant to taxol. *Oncogene* 1998;16:2087–94.

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ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer

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Overexpression of the *erbB-2* gene contributes to aggressive behavior of various human adenocarcinomas, including breast cancer, through an unknown molecular mechanism. The *erbB-2*-encoded protein is a member of the ErbB family of growth factor receptors, but no direct ligand of ErbB-2 has been reported. We show that in various cells ErbB-2 can form heterodimers with both EGF receptor (ErbB-1) and NDF receptors (ErbB-3 and ErbB-4), suggesting that it may affect the action of heterologous ligands without the involvement of a direct ErbB-2 ligand. This possibility was addressed in breast cancer cells through either overexpression of ErbB-2 or by blocking its delivery to the cell surface by means of an endoplasmic reticulum-trapped antibody. We report that ErbB-2 overexpression enhanced binding affinities to both EGF and NDF, through deceleration of ligand dissociation rates. Likewise, removal of ErbB-2 from the cell surface almost completely abolished ligand binding by accelerating dissociation of both growth factors. The kinetic effects resulted in enhancement and prolongation of the stimulation of two major cytoplasmic signaling pathways, namely: MAP kinase (ERK) and c-Jun kinase (SAPK), by either ligand. Our results imply that ErbB-2 is a pan-ErbB subunit of the high affinity heterodimeric receptors for NDF and EGF. Therefore, the oncogenic action of ErbB-2 in human cancers may be due to its ability to potentiate *in trans* growth factor signaling.

Keywords: growth factors/oncogene/receptor dimerization/signal transduction/tyrosine kinase

Introduction

Many growth and differentiation signals are transmitted into the cell's interior by means of receptors whose cytoplasmic domains share an intrinsic tyrosine kinase catalytic activity (Fantl *et al.*, 1993). On the basis of structural determinants it is possible to classify the known receptors into subgroups that contain two to nine highly related receptors. This multiplicity does not exist in insect cells, suggesting that it has been evolved in order to accommodate increasing physiological requirements. While the mechanism of signal transduction by a single receptor is relatively well characterized and involves sequential steps of receptor activation and association with cytoplasmic effector proteins, the mechanisms that propagate signals from one receptor to other signaling systems remain poorly understood. One example is the ErbB subfamily of receptor tyrosine kinases. All of the four members of the group share a similar primary structure but they differ remarkably in their ligand specificity and kinase function (Peles and Yarden, 1993; Carraway and Cantley, 1994): ErbB-1 binds to several distinct ligands whose prototype is the epidermal growth factor (EGF), while both ErbB-3 and ErbB-4 bind all of the isoforms of a related factor called Neu differentiation factor (NDF, or heregulin). ErbB-2 binds no known ligand (Dougall *et al.*, 1994), but unlike ErbB-1 whose kinase activity is strictly ligand dependent, and ErbB-3, which is characterized by very low kinase activity (Kraus *et al.*, 1993; Guy *et al.*, 1994; Prigent and Gullick, 1994), the catalytic action of ErbB-2 is relatively high, even in the absence of a ligand (Lonardo *et al.*, 1990).

Although several ErbB ligands and receptors were implicated in cancer, most of the interest has been focused on ErbB-2 (Hynes and Stern, 1994). Whereas the rodent homolog of the gene can undergo oncogenic activation, as a result of a point mutation (Bargmann *et al.*, 1986), the unmutated human gene is frequently amplified or otherwise overexpressed in several types of human adenocarcinomas, including breast tumors (Slamon *et al.*, 1987; Varley *et al.*, 1987). Importantly, retrospective analyses of several types of human tumor correlated *erbB-2* overexpression with poor prognosis of breast, ovarian and lung cancer (Slamon *et al.*, 1989; Kern *et al.*, 1990). The gene is amplified in mammary carcinomas of all stages but not in benign breast tumors, and overexpression is maintained in the metastatic lesions (Igelhart *et al.*, 1990). The mechanism by which an overexpressed ErbB-2 contributes to the development of a more aggressive phenotype of cancer cells is currently unknown. The answer to this question may be related to another open issue, namely whether or not ErbB-2 has a ligand of its own. Several putative ligands that presumably interact directly with ErbB-2 were purified from breast cancer cells (Lupu *et al.*, 1992), bovine kidney (Huang and Huang, 1992), activated macrophages (Tarakhovsky *et al.*, 1991) and human T cells (Dobashi *et al.*, 1991). However, in no case was it proven by molecular cloning that the activity corresponds to a direct ErbB-2 ligand.

Even without a ligand of its own, ErbB-2 can undergo activation by heterologous ligands. EGF has been shown

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to elevate tyrosine phosphorylation of ErbB-2 in living cells through the formation of heterodimers between ErbB-1 (EGF receptor) and ErbB-2 (Goldman *et al.*, 1990; Wada *et al.*, 1990). Moreover, simultaneous overexpression of the two proteins synergistically affected malignant transformation of murine fibroblasts (Kokai *et al.*, 1989). This was accompanied by an increased binding affinity and mitogenic responsiveness to EGF (Wada *et al.*, 1990). Likewise, NDF elevates tyrosine phosphorylation of ErbB-2 through binding to its own receptors (Peles *et al.*, 1993; Plowman *et al.*, 1993; Carraway *et al.*, 1994; Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1994), and heterodimerization of ErbB-3 with ErbB-2 has been implicated in the elevation of binding affinity to NDF (Sliwkowski *et al.*, 1994). On the other hand, ErbB-1–ErbB-3 heterodimers presumably enable EGF to recruit phosphatidylinositol 3′-kinase through ErbB-3 (Soltoff *et al.*, 1994). It thus appears that ErbB-2 may participate in combinatorial receptor–receptor interactions, and thereby it may play an important role in signal transduction by both NDF and EGF, even without the involvement of an endogenous ligand. This possibility has been recently supported by the observation that blocking ErbB-2 expression at the cell surface impaired various aspects of cytoplasmic signaling by both NDF and EGF (Graus-Porta *et al.*, 1995). To further examine the role of ErbB-2 in signal transduction and oncogenesis by these two families of growth factors, we analyzed kinetic and signaling effects of either overexpression or complete removal of the protein from the surface of mammary tumor cells. Our results imply that ErbB-2 is an essential component of the high affinity receptors of NDF and EGF, and thereby it significantly affects signaling by the two growth factors.

Results

ErbB-2 heterodimerizes with ErbB-1, ErbB-3 and ErbB-4

To examine the effect of ErbB-2 on binding and responsiveness of mammary tumor cells to heterologous ErbB ligands, we used two human breast cancer cell lines, MCF-7 and T47D. Two derivatives of these cell lines were employed as model cellular systems. In the first one, the endogenous low level of *erbB-2* expression in MCF-7 cells was elevated through gene transfer, resulting in MCF-7/ErbB-2 cells (Peles *et al.*, 1993). The second cellular system comprised two derivatives of the T47D cell line, that originally has moderate *erbB-2* expression: T47D-puro, a control infected cell line, and T47D-5R cells that display no ErbB-2 at the cell surface as a result of ectopic expression, in the endoplasmic reticulum, of an engineered single chain monoclonal antibody to ErbB-2 (Beerli *et al.*, 1994). Immunoprecipitation of biosynthetically-labeled ErbB-2 from extracts of MCF-7/ErbB-2 cells showed that expression of the protein was elevated by 8-10-fold relative to the parental cell line (Figure 1A). Conversely, the level of ErbB-2 expression is the same in both derivatives of the T47D cell line. However, the corresponding protein band in the 5R cells displayed faster electrophoretic migration (Figure 1A) because of its retention in the endoplasmic reticulum (Graus-Porta *et al.*, 1995). In order to determine if the changes in ErbB-2 expression were accompanied by alterations in the

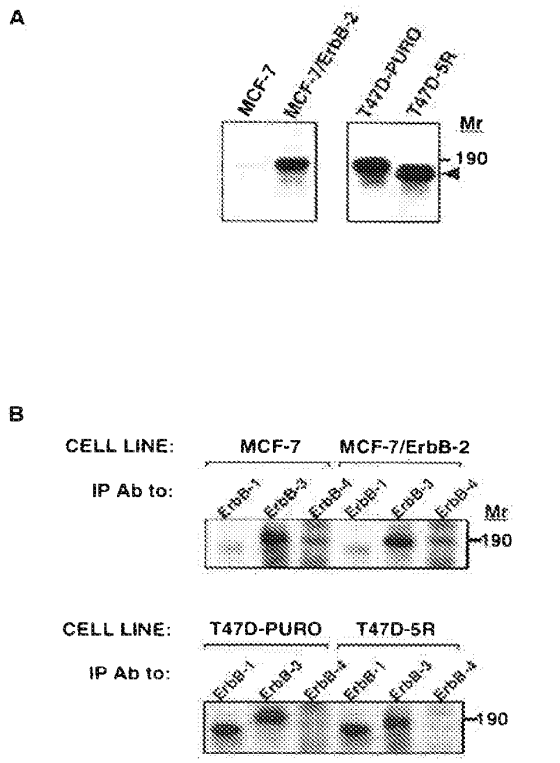

Fig. 1. Immunoprecipitation of biosynthetically-labeled ErbB proteins from MCF-7 and T47D cells. (A) The indicated cell lines were incubated for 16 h in [^{35}S]methionine-containing medium and their ErbB-2 proteins were subjected to immunoprecipitation by using rabbit antibodies to the carboxy-terminal peptide of ErbB-2. Note overexpression of ErbB-2 in MCF-7/ErbB-2 cells and the increased electrophoretic mobility of the endoplasmic reticulum-trapped ErbB-2 in T47D-5R cells (arrowhead). (B) Immunoprecipitates of the indicated ErbB-1, ErbB-3 and ErbB-4 proteins were prepared from the indicated pairs of biosynthetically-labeled cells by using monoclonal antibodies to ErbB-1 (Kawamoto *et al.*, 1983), ErbB-2 (Stancovski *et al.*, 1991) or ErbB-4, or polyclonal rabbit antibodies to a recombinant ectodomain of ErbB-3. This was followed by electrophoresis and autoradiography as described above. Equal amounts of acid-precipitable radioactivity in cell lysates were taken for each lane. The extensively washed immunoprecipitates were resolved by gel electrophoresis and autoradiography.

expression of other ErbB proteins, we performed additional immunoprecipitation analyses with antibodies to ErbB-1, ErbB-3 and ErbB-4. These experiments indicated that both MCF-7 cells and T47D cells express the three other members of the ErbB family, but their expression levels displayed no significant change in the derivative cell lines (Figure 1B). This conclusion was supported also by determination of cell surface expression of the three ErbB proteins by using specific monoclonal antibodies and a radiolabeled secondary antibody (data not shown). In both parental cell lines, ErbB-4 was the least abundant member and ErbB-3 displayed relatively high levels of expression.

Consistent with the presence of all ErbB proteins, radiolabeled NDF and EGF underwent covalent crosslinking to monomers and dimers of their receptors in the parental cell lines and in their derivatives (Figure 2). Significantly, immunoprecipitation of ErbB-2 from lysates

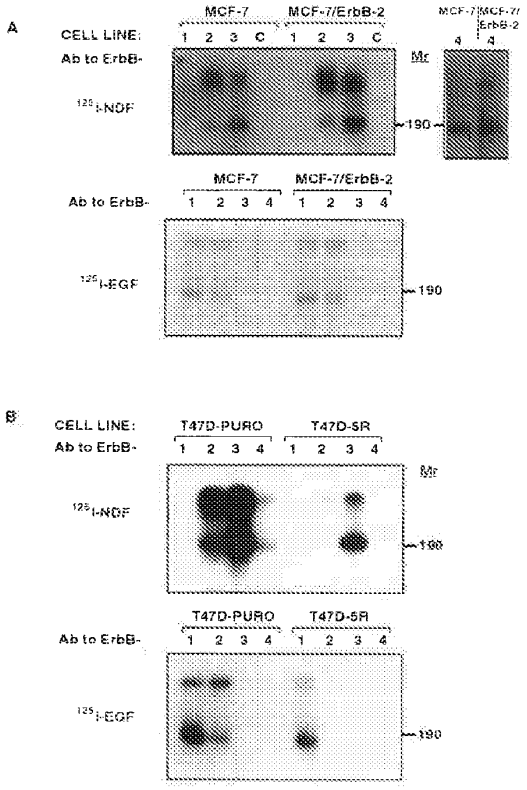

Fig. 2. Affinity labeling of NDF and EGF receptors and their co-immunoprecipitation with the ErbB-2 protein. Monolayers of either MCF-7 cells and their derivative line MCF-7/ErbB-2 (A) or T47D-puro cells and their 5R derivatives (B) were incubated with the indicated radiolabeled ligands and then subjected to covalent crosslinking analysis at 4°C as described in Materials and methods. Cell lysates were prepared and antibodies to the indicated ErbB proteins or control antibodies (labeled C) were used in immunoprecipitation. Autoradiograms of the gel-resolved immunoprecipitates are shown.

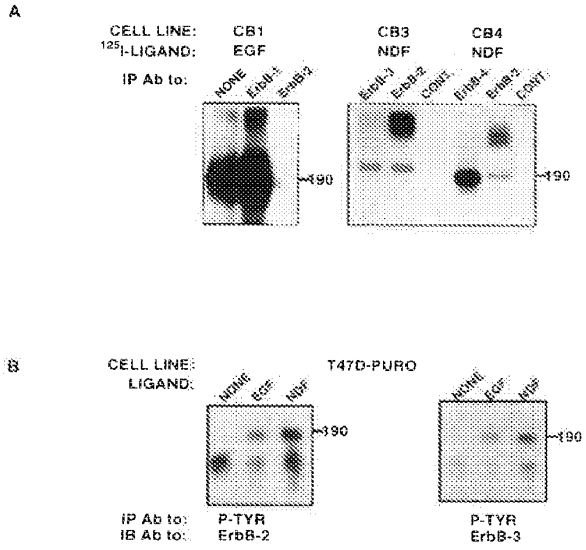

Fig. 3. Inter-receptor interactions and transphosphorylation. (A) CHO cell lines that overexpress either EGF receptor/ErbB-1 (CB1 cells), ErbB-3 (CB3 cells) or ErbB-4 (CB4 cells) were subjected to covalent crosslinking of radiolabeled NDF or EGF as described in the legend to Figure 2. The indicated receptors were immunoprecipitated (IP) by using specific antibodies. For control, we directly subjected whole cell lysates to gel electrophoresis (lane labeled NONE) or used a monoclonal antibody to the fibroblast growth factor receptor (lanes labeled CONT.). Immunoprecipitates were washed extensively and resolved by gel electrophoresis (6.5% acrylamide), which was followed by autoradiography. (B) Monolayers of T47D-puro cells were treated for 10 min at 37°C with NDF or EGF (each at 50 ng/ml), or left untreated (NONE). The cells were transferred to 4°C, washed in PBS and solubilized in solubilization buffer that contained phosphatase inhibitors. Phosphotyrosine-containing proteins were immunoprecipitated (IP) by using Sepharose–protein A-immobilized antibody to phosphotyrosine and the complexes resolved by gel electrophoresis. After electrophoretic transfer to nitrocellulose, the filters were immunoblotted (IB) with rabbit antibodies that are directed to the carboxy-terminal peptide of either ErbB-2 or ErbB-3.

of the four cell lines with a specific antiserum led to coprecipitation of monomeric and dimeric forms of affinity-labeled NDF and EGF receptors. This observation implied that in both MCF-7 and T47D cells, ErbB-2 physically interacts with the three other ErbB proteins. However, although ErbB-2 does not bind to any of the two ligands (Peles *et al.*, 1993), its presence enhanced affinity labeling of the other ErbB proteins. For example, overexpression of ErbB-2 in MCF-7 cells resulted in an increased covalent crosslinking of NDF to its receptors, namely ErbB-3 and ErbB-4 (Figure 2A). Similarly, the absence of ErbB-2 at the surface of T47D-5R cells caused reduced labeling of the two NDF receptors (Figure 2B). The effect of ErbB-2 on affinity labeling of the EGF receptor was similar but less prominent (compare for example the signals observed with T47D-puro cells to those of T47D-5R cells in the lower panel of Figure 2B). In contrast to the strong signals of [^{125}I]EGF and [^{125}I]NDF, that were precipitated with antibodies to ErbB-2, we observed only faint NDF signals in immunoprecipitates of ErbB-1/EGF receptor (compare for example the first two lanes in the upper panels of Figure 2A and B). Likewise, [^{125}I]EGF signals were hardly precipitable by ErbB-3- and ErbB-4-specific antibodies, suggesting that ErbB-2-containing heterodimers are preferred over heterodimeric complexes of ErbB-1 with ErbB-3, and probably also with the least abundant receptor, ErbB-4.

In order to further analyze the interactions of NDF and EGF receptors with ErbB-2, and also compare the relative stability of the various ErbB-2-containing heterodimers, we established another model cellular system, namely transfected Chinese hamster ovary (CHO) cells. These cells express a moderate amount of endogenous ErbB-2, but no other member of the family. Therefore, introduction of the three other ErbB proteins, by means of transfection of the corresponding expression vectors, should enable examination of individual heterodimers. To this end we established three stable CHO cell lines that express either ErbB-1 (denoted CB1), ErbB-3 (CB3 cell line) or ErbB-4 (CB4 cell line). Despite overexpression, we were unable to detect ligand-induced ErbB-2 heterodimers in these cell lines, by blotting gel-separated immunoprecipitates with antibodies to ErbB-2 or to the transfected receptor. How-

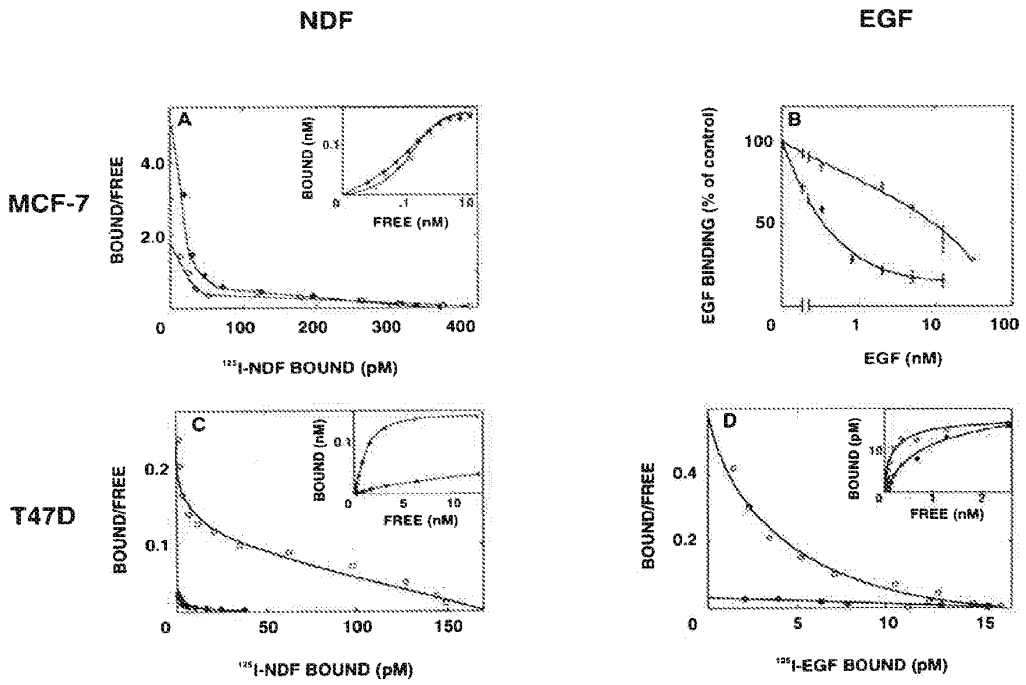

Fig. 4. Demonstration of ErbB-2-dependent changes in the cell surface binding of NDF and EGF. Monolayers ($1–2\times10^5$ cells/well in 48-well dishes) of the indicated cell lines were incubated for 2 h at 4°C with different concentrations of ^{125}I-labeled NDF-β1$_{177–246}$ or [^{125}I]EGF, as indicated (A, C and D), or with radiolabeled EGF (5ng/ml) in the presence of increasing concentrations of the unlabeled ligand (B). Cells were then washed three times with ice-cold binding buffer, lysed in 0.5 ml of 0.1N NaOH containing 0.1% SDS and the radioactivity was determined. Binding results are presented as Scatchard plots, saturation curves (insets) or displacement curves (B). Open circles refer to the control parental cell lines and closed circles indicate results obtained with the respective modified cell lines MCF-7/ErbB-2 and T47D-5R. Each data point represents the average and standard deviation (bars) of triplicate determinations, after substraction of non-specific ligand binding. The assays were performed at least twice.

ever, the more sensitive assay that uses co-immunoprecipitation of affinity-labeled NDF or EGF receptors together with the endogenous ErbB-2 protein of CHO cells, provided direct support for the conclusion that ErbB-2 can form heterodimers with ErbB-1, ErbB-3 and ErbB-4. Thus, by using CB1 cells, we detected the affinity-labeled EGF receptor in ErbB-2 immunoprecipitates (Figure 3A). In contrast to this relatively weak interaction, affinity labeling of the two NDF receptors in CB3 and CB4 cells revealed extensive interactions of these receptors with ErbB-2. Similar to the breast cancer cells (Figure 2), anti-ErbB-2 antibodies precipitated from CB3 and CB4 cell extracts, primarily a dimeric form of NDF receptors, most likely a heterodimer that contains ErbB-2 (Figure 3A). The reason why a larger amount of the presumed heterodimer is precipitated by antibodies to ErbB-2, as compared with anti-ErbB-3 and anti-ErbB-4 precipitates, may be related to better accessibility of ErbB-2 epitopes in the heterodimer. The interaction between ErbB-4 and ErbB-2 is especially important, because we could not rigorously establish it in MCF-7 and in T47D cells due to the very low expression level of ErbB-4 in these cells. In conclusion, ErbB-2 interacts with the three other members of the family in a manner that is independent of the specific cellular environment. In addition, ErbB-2 heterodimers that contain NDF receptors are apparently more stable than ErbB-1-ErbB-2 heterodimers.

Another aspect of heterodimer formation between ErbB-2 and either NDF or EGF receptors is the ability of these receptors to increase tyrosine phosphorylation of ErbB-2, possibly via an *in trans* mechanism. We chose to examine this aspect in T47D-puro cells, because they express relatively high levels of all four members of the ErbB family, unlike MCF-7 cells in which ErbB-1 is expressed at very low levels (Figure 1B). The results shown in Figure 3B indicate that in T47D cells, ErbB-2 underwent increased tyrosine phosphorylation after binding of either EGF or NDF. It is important to note that the latter ligand induced a stronger signal, in accordance with the superior ability of NDF receptors to heterodimerize with ErbB-2 (Figure 3A). Consistent with previous reports that documented interactions between EGF receptor and ErbB-3 (Soltoff *et al.*, 1994), we found that EGF, as well as NDF, increased phosphorylation of ErbB-3 in T47D cells (Figure 3B). Because ErbB-3 has very low, if any, tyrosine kinase activity (Prigent and Gullick, 1994), the observed phosphorylation should be mediated *in trans*, by either ErbB-2 or ErbB-1.

Binding of NDF and EGF to breast cancer cells depends on the presence of ErbB-2 at the cell surface and is enhanced by ErbB-2 overexpression

We next analyzed direct binding of radiolabeled NDF and EGF to the T47D-5R and MCF-7/ErbB-2 cell lines (Figure 4). Scatchard analyses of the binding data revealed that in the absence of ErbB-2 at the cell surface, binding of

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Table I. Summary of ligand binding parameters of *erbB-2*-expressing cells

	NDF			EGF		
	K_d (nM)		k_{off} ($s^{-1} \times 10^{-4}$)	K_d (nM)		k_{off} ($s^{-1} \times 10^{-4}$)
	High	Low		High	Low	
MCF-7	0.04	0.8	0.91	(9)		6.7
MCF-7/ErbB-2	0.02	0.6	0.26	(0.8)		3.9
T47D	0.05	1.2	0.5	0.01	0.08	2.6
T47D-5R	0.2	8.5	2.3	None	0.4	6.1

Listed are the parameters obtained from the data presented in Figure 4 (K_d equilibrium) and Figure 5 (k_{off}). The data in parentheses refer to displacement curves (Figure 4B). Ligand dissociation constants were derived from the initial (fast) components of the corresponding dissociation curves.

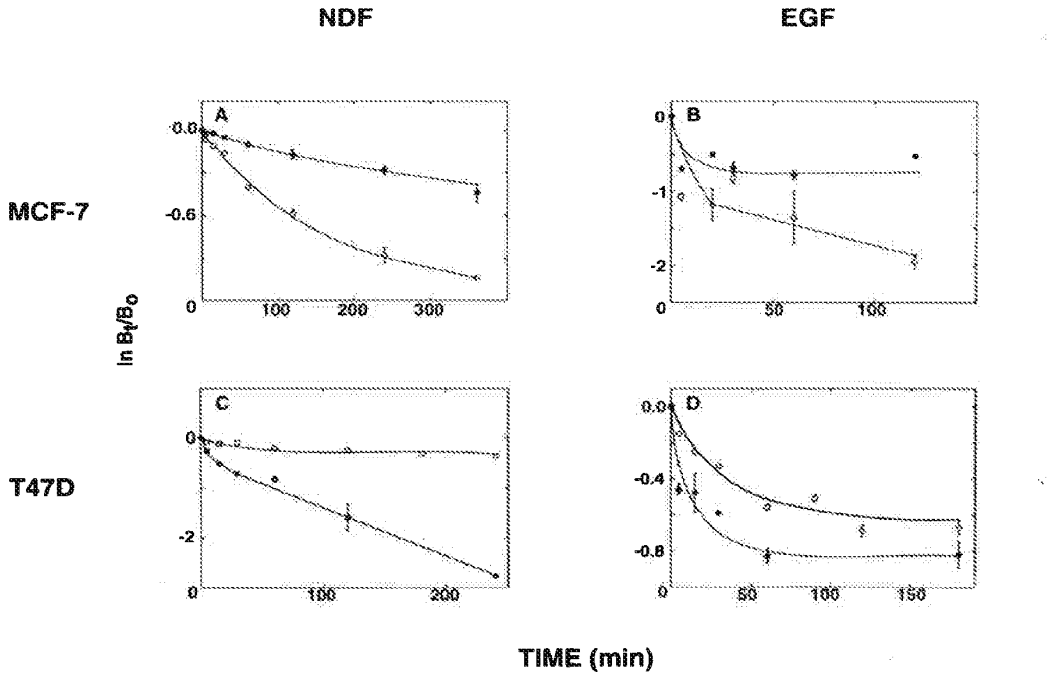

Fig. 5. ErbB-2 delays dissociation of NDF and EGF from their receptors. Dissociation kinetics of the indicated radiolabeled ligands were analyzed on confluent monolayers of MCF-7 cells and T47D cells, that were preincubated at 4°C with the corresponding ligand at saturating concentrations. After removal of unbound ligand, the cells were incubated with the unlabeled ligand and radioactivity that was released from cells, as well as the residual cell-bound ligand, was determined as a function of the time of incubation at 4°C. The results were expressed in terms of the ratio between the amount of ligand that remained bound at time t (B_t) and the initially bound radioactivity (B_o). Nonspecifically bound and released radioactivity was subtracted. Open circles refer to the parental cell lines and closed circles refer to the derivative lines MCF-7/ErbB-2 and T47D-5R. The experiments were repeated twice.

both ligands was dramatically reduced. The curvilinear Scatchard curve of NDF binding to the parental T47D-puro cells indicated the existence of two populations of NDF binding sites: a major population of low affinity receptors (~87% of the total number of sites), and a minor high affinity class of ligand binding sites, whose affinity was higher by 24-fold (Figure 4C). Both sites almost completely disappeared in T47D-5R cells, and their affinities were remarkably reduced (Table I). Likewise, in the absence of cell surface ErbB-2, all of the high affinity binding sites of EGF disappeared and the affinity of the major population of sites was reduced (Figure 4D and Table I). However, in contrast to the case of NDF receptors, the number of the low affinity EGF binding sites remained unchanged.

Consistent with the *trans*-regulatory effect of ErbB-2 in T47D cells, ErbB-2 overexpression in MCF-7 cells was associated with increased binding affinities to both NDF (Figure 4A) and EGF (Figure 4B), in agreement with previous observations that were made in rodent and insect cells (Wada *et al.*, 1990; Sliwkowski *et al.*, 1994). Due to the relatively low number of EGF receptors in MCF-7 cells, we were unable to perform Scatchard analysis. However, ligand displacement analysis indicated an 11-fold increase in EGF binding affinity (Figure 4B) as a result of the ~8-fold enhanced expression of ErbB-2 in

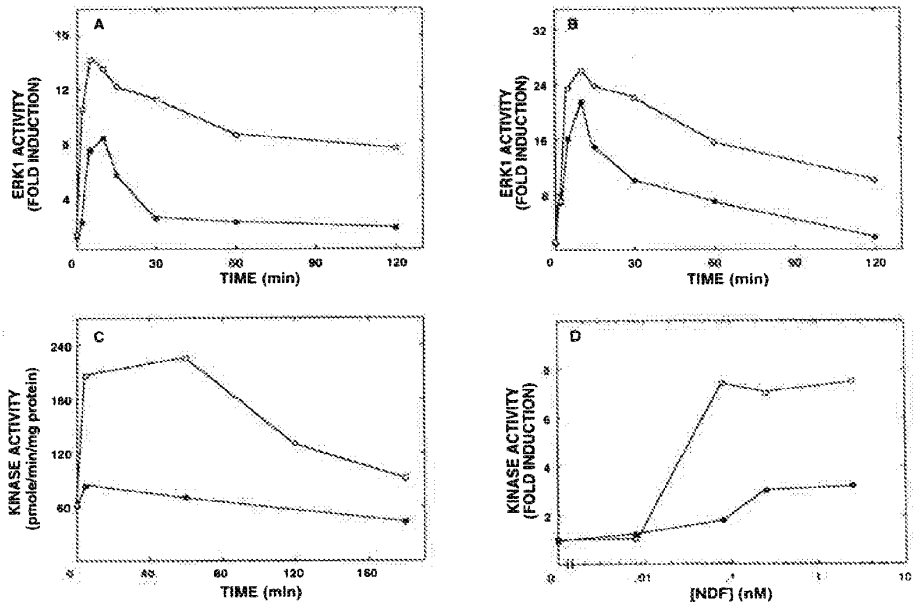

Fig. 6. Cell surface expression of ErbB-2 potentiates and prolongs the activation of MAP kinase by NDF and EGF. Monolayers of T47D-puro cells (open circles) or their 5R derivatives (closed circles) were serum-starved for 12 h and then treated with either NDF or EGF. Cell lysates were prepared and assayed for MAP kinase activity. (**A** and **B**) Cells were stimulated for the indicated time intervals with 0.5 nM NDF (A) or EGF (B) and the ERK1 isoform of MAP kinase was immunoprecipitated from cell lysates by using a specific antibody. Kinase activity was determined by using myelin basic protein (MBP) as a substrate, gel electrophoresis, and quantitation of MBP phosphorylation by image analysis. (**C**) Cells were stimulated with 0.5 nM NDF for the indicated time intervals. A cellular fraction that contains >85% of both ERK isoforms was isolated by ion-exchange chromatography on mini-columns (Seger *et al.*, 1994). MAP kinase activity was determined by using MBP as a substrate. (**D**) Cells were stimulated for 10 min with the indicated concentrations of NDF. Cell lysates were prepared and their MAP kinase activity was determined in MBP-containing polyacrylamide gels as described (Tobe *et al.*, 1991). Briefly, cell lysates were electrophoresed on an SDS–polyacrylamide gel that was polymerized with MBP (0.5 mg/ml). After electrophoresis, denaturation and renaturation, the gel was incubated for 1 h in phosphorylation buffer containing [γ-^{32}P]ATP. The amount of radioactivity that was incorporated into MBP in the gel region corresponding to 42–45 kDa bands, was quantitated after extensive washing.

MCF-7/ErbB-2 cells. Although the effect on NDF binding to MCF-7 cells was lower than that observed in T47D cells, in both cases ErbB-2 affected mostly the high affinity population of binding sites (Figure 4A), which has previously been attributed to receptor dimers and heterodimers (Wada *et al.*, 1990; Ben-Levy *et al.*, 1992).

ErbB-2 decelerates dissociation rates of its heterologous ligands

Because ligand binding is determined by both the rate of association with the receptor and by the rate of dissociation of the ligand–receptor complex, we examined the question concerning which of these processes is affected by ErbB-2. While analyses of the association rates of NDF and EGF revealed relatively small effects upon changes in ErbB-2 expression (data not shown), determination of the rates of dissociation of both ligands indicated that ErbB-2 significantly reduced the release of each ligand from its own receptor (Figure 5). The rates of dissociation of NDF and EGF from the parental MCF-7 cells were rapid and displayed biphasic kinetics (Figure 5A and B). Conversely, in the presence of an overexpressed ErbB-2 the rate of release of either ligand was slower, and in the case of NDF it displayed linear kinetics (Figure 5A and Table I). Similarly, blocking ErbB-2 delivery to the surface of T47D cells accelerated NDF dissociation and replaced the linear kinetics of release with a biphasic behavior (Figure 5C and Table I). It is important to note that the effect of ErbB-2 on the release of NDF from both MCF-7 and T47D cells was significantly more extensive than that observed with EGF (Figure 5D), consistent with our conclusion that the interaction of ErbB-2 with NDF receptor(s) is stronger than with the EGF receptor.

ErbB-2 potentiates and prolongs NDF- and EGF-induced activation of MAP kinase and c-Jun kinase

To examine the intracellular effect of the ErbB-2-mediated lengthening of the half lives of ligand–receptor complexes, we concentrated on the activation of two protein kinase cascades that funnel many extracellular signals to the nucleus (Marshall, 1994; Seger and Krebs, 1995): c-Jun kinase (JNK, also known as stress-activated protein kinase, SAPK) (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994), and mitogen-activated protein kinase (MAP kinase/ERK). Importantly, the latter mediates the oncogenic action of ErbB-2 (Ben-Levy *et al.*, 1994), and is included in a group of other signaling molecules (e.g. Shc and p70/p85^{S6K}), whose activation in T47D cells is potentiated by ErbB-2 (Graus-Porta *et al.*, 1995). We first concentrated on T47D cells and their 5R derivatives, in which ErbB-2 has been shown to enhance activation of the ERK2 isoform of MAP kinase (Graus-Porta *et al.*, 1995). Determination of kinase activity in immunoprecipitates of the ERK1 isoform, that were prepared from NDF- (Figure 6A) or EGF-

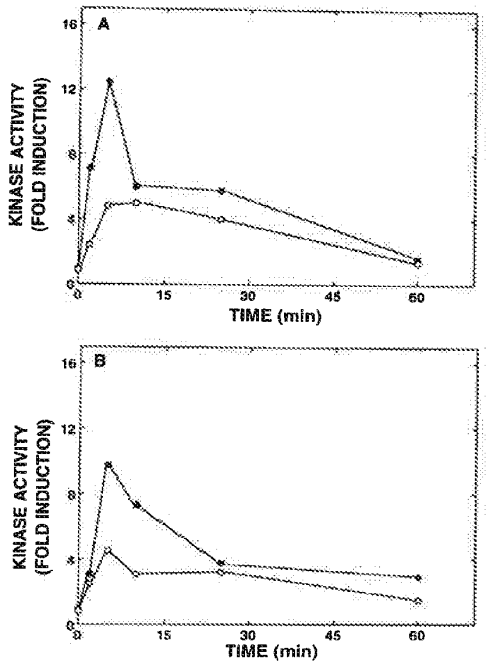

Fig. 7. Overexpression of ErbB-2 potentiates activation of MAP kinase in MCF-7 cells. Monolayers of MCF-7 cells (open circles) or MCF-7/ErbB-2 cells (closed circles) were incubated for different time intervals with 0.5 nM of either NDF (A) or EGF (B). MAP kinase activity in cell extracts was determined by using an in-gel kinase assay with MBP as a substrate, as described in the legend to Figure 6D.

treated cells (Figure 6B), revealed that the absence of ErbB-2 at the cell surface caused significant reduction in the extent and duration of kinase activation by either growth factor. The effect of ErbB-2 on the NDF-induced signal was larger than on the EGF-induced activation, in accordance with the results of ligand binding and dissociation analyses. We therefore concentrated on the effect of NDF and attempted to confirm it by using two alternative MAP kinase assays, that do not require the use of MAP kinase-specific antibodies, which may interfere with kinase activity *in vitro*. The assays involved fractionation of whole lysates from NDF-treated cells by using either column chromatography or gel electrophoresis, and was followed by phosphorylation of myelin basic protein (MBP) *in vitro*. Isolation of a fraction that contains >85% of ERK activity confirmed that T47D cells that express no ErbB-2 at the cell surface lost most of the effect of NDF on MAP kinase activation (Figure 6C). By using the in-gel assay of MAP kinase, we found that in the absence of ErbB-2 the concentration-dependency of MAP kinase activation by NDF was dramatically shifted to the right (Figure 6D). This means that the presence of ErbB-2 allowed T47D cells to respond to lower concentrations of NDF than cells that do not express this co-receptor, which is in accordance with observations made by using the two other assays of MAP kinase.

Comparison of the response of MCF-7 cells with that of their *erbB-2*-overexpressing derivatives, by using the in-gel assay of MAP kinase, indicated that ErbB-2 potentiated MAP kinase activation by either NDF (Figure 7A) or EGF (Figure 7B) and also prolonged the ligand-induced active state of the enzyme. Qualitatively similar results were obtained when we used the two other methods of MAP kinase assays (data not shown).

In order to examine JNK activation we prepared whole cell extracts from growth factor-stimulated cells and determined their ability to phosphorylate a recombinant c-Jun protein (Hibi *et al.*, 1993). The extent of c-Jun phosphorylation *in vitro* was determined after gel electrophoresis and autoradiography (insets in Figure 8) that was complemented by densitometry. Analysis of the T47D cell lines revealed that in the absence of a cell surface-localized ErbB-2, JNK activation by NDF was significantly reduced at all concentrations of the ligand (Figure 8A). In addition, in the presence of ErbB-2 at the cell surface, the kinetic of JNK activation was longer than in its absence and it returned to the base line only after 2 h (Figure 8B and data not shown). Kinetic analyses of the activation of c-Jun kinase in MCF-7 cells by either NDF (Figure 8C) or EGF (Figure 8D) similarly indicated that ErbB-2 overexpression augmented and extended the stimulatory action of both ligands. For example, whereas in the parental MCF-7 cells maximal JNK activation by NDF was 3-fold and it returned to base line after 1.5 h, in ErbB-2-overexpressing cells maximal activation reached 8-fold and it returned to the initial level after 3 h. The response of MCF-7 cells to EGF was relatively small (Figure 8D), probably because these cells express very low levels of EGF receptor. Nevertheless, overexpression of ErbB-2 augmented the JNK signals. Taken together, these results imply that ErbB-2 presence at the cell surface, and the extent of its overexpression, significantly affect the potency and duration of MAP kinase and JNK activation by either NDF or EGF, probably because ErbB-2 decelerates the rates of dissociation of the heterologous ligands.

Discussion

Despite extensive knowledge on the phenomenology of *erbB-2* overexpression in human cancer and detailed structure–function analyses of the putative receptor, the exact molecular mechanism that underlies transformability by the apparently normal gene product remained obscure (Hynes and Stern, 1994). This is due in part to the failure of many attempts to identify a direct ligand of ErbB-2 (Peles and Yarden, 1993). The results of our present study indicate that ErbB-2 may be considered an integral subunit of NDF and EGF receptors, and as such it may have no direct ligand. Consequently, we raise the possibility that at least part of the transforming ability of an overexpressed ErbB-2 is due to transactivation of growth factor signaling.

ErbB-2 is a pan-ErbB receptor subunit

The major support to our contention that ErbB-2 should be considered an essential common subunit of NDF and EGF receptors is based on the remarkable reduction in binding of the two ligands in the absence of ErbB-2 at the cell surface (Figure 4). The results of affinity labeling experiments are also supportive, but the effect of ErbB-2 expression is less extensive (Figure 2). We attribute this difference to the observation that ErbB-2 primarily affects the process of ligand dissociation (Figure 5), which is blocked by the procedure of covalent crosslinking. Because

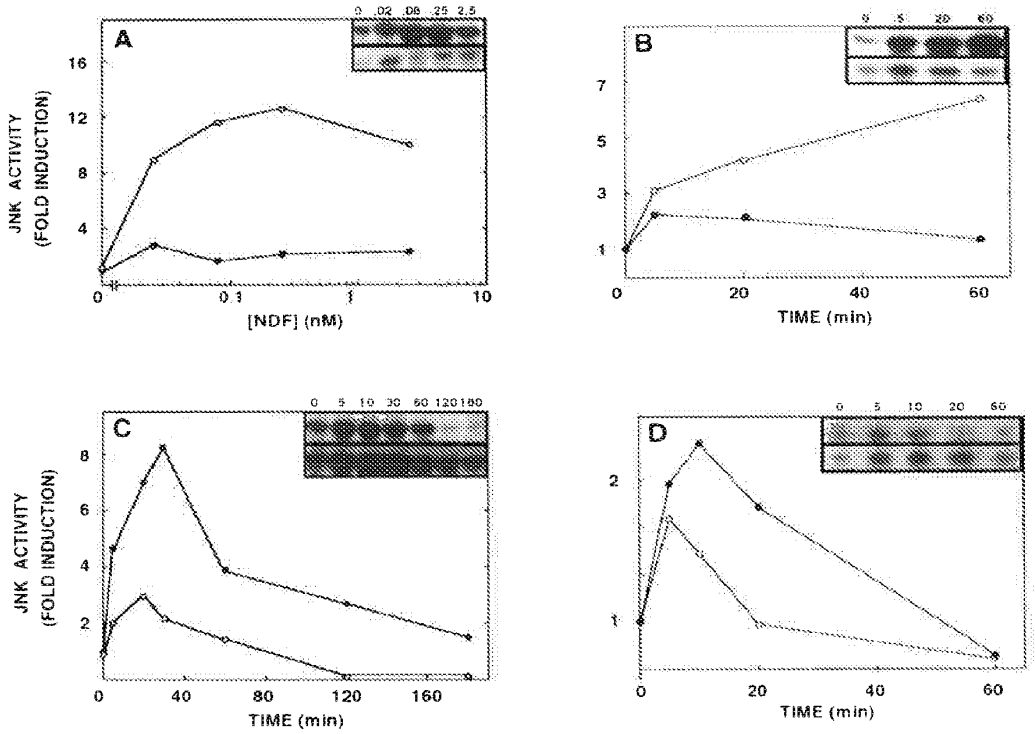

Fig. 8. Potentiation of ligand-induced activation of c-Jun kinase (JNK) by an overexpressed ErbB-2. (**A** and **B**) NDF-induced activation of c-Jun kinase was determined with either T47D-puro cells (open circles) or with their 5R derivative cells (closed circles). Cells were serum-starved for 12 h and then incubated for 10 min with different concentrations of NDF (A), or with a fixed concentration of NDF (1 nM) for the indicated time intervals (B). c-Jun kinase activity was determined in cell lysates by using recombinant GST-Jun fusion protein as a substrate and [γ-^{32}P]ATP, as described (Hibi *et al.*, 1993). Phosphorylation *in vitro* was followed by gel electrophoresis and quantitation of the amount of radioactivity that was incorporated into c-Jun. Insets show the corresponding autoradiograms of phosphorylated recombinant c-Jun. The upper parts of the insets refer to T47D-puro cells and the lower parts to T47D-5R cells. Each experiment was repeated twice. (**C** and **D**) Monolayers of MCF-7 cells (open circles) or MCF-7/ErbB-2 cells (closed circles) were incubated for the indicated time intervals with 0.5 nM of either NDF (C) or EGF (D). c-Jun kinase activity was quantitated as described above. The upper parts of the insets show the phosphorylated c-Jun band in MCF-7 cells and the lower parts refer to MCF-7/ErbB-2 cells. Each experiment was repeated thrice.

ErbB-2 can form heterodimers with the three other ErbB proteins (Figures 2 and 3), we favor the possibility that it affects binding of NDF and EGF through heterodimer formation with the respective receptors. Conceivably, both ligands bind with higher affinities to heterodimers that include ErbB-2 than they bind to the corresponding homodimers. According to this model, in a heterodimeric configuration ErbB-2 is able to inhibit ligand release from the single binding site of the heterodimer. Previous results have already indicated that homodimeric receptors that were stabilized by either a mutation or by a bivalent reagent, displayed higher ligand binding affinity as compared with their monomeric species, and this was due to slower rates of ligand dissociation (Ben-Levy *et al.*, 1992; Zhou *et al.*, 1993). In addition, the apparent forcing of heterodimer formation between ErbB-2 and either ErbB-1 (Wada *et al.*, 1990) or ErbB-3 (Sliwkowski *et al.*, 1994), by means of overexpression of ErbB-2, has been shown to augment binding of EGF and NDF respectively. We note that relatively moderate overexpression of ErbB-2 (<10-fold) is already sufficient for enhancement of ligand binding (Figure 4). In line with ErbB-2 being a shared subunit of the high affinity NDF and EGF receptors, we observed in various tumor cells an NDF-induced reduction in EGF binding affinity (Karunagaran *et al.*, 1995). Moreover, overexpression of ErbB-2 in these cells prevented the *trans*-inhibitory effect of NDF, as expected by a heterodimerization model. It is noteworthy that the function of ErbB-2 as a component of NDF and EGF receptors, that acts by slowing ligand dissociation, is analogous to non-binding components of interleukin (IL) receptors. For example, gp130 is shared by the receptors for IL-6, leukemia inhibitory factor, oncostatin M, IL-11 and ciliary neurotrophic factor (Hilton *et al.*, 1994, and references therein). Likewise, signaling by IL-2, IL-4, IL-7 and IL-9 depends on the formation of heterodimers between specific β subunits and a common γ subunit (Kawahara *et al.*, 1994). One important implication of our results is that the physiological receptors for NDF and EGF are in fact heterodimers that contain ErbB-2. This realization may explain why detergent solubilization of EGF receptors involves 10-fold reduction in binding affinity (Carpenter, 1987), and how mutants of the EGF receptor that are defective in kinase activity or tyrosine autophosphorylation sites are still able to transmit some biochemical signals (Selva *et al.*, 1993; Gotoh *et al.*, 1994). An inevitable

question is how this aspect has so far escaped detection. This may be due to the generally broader expression of ErbB-2, as compared with ErbB-1, ErbB-3 and ErbB-4. In other words, virtually all of the EGF or NDF receptor positive cell lines also express ErbB-2, so that analysis of ligand binding to the individual receptors in their natural cell context is practically impossible.

Signal amplification by ErbB-2 may explain its oncogenic activity

Removal of ErbB-2 from the cell surface has been previously shown to reduce various aspects of the signaling by NDF and EGF, including tyrosine phosphorylation of cytoplasmic substrates, and the activation of MAP kinase and ribosomal protein S6 kinase (Graus-Porta *et al.*, 1995). In the present study, we extended these observations to the activation of another protein kinase cascade, namely JNK/SAPK, and concentrated on the effect of a relatively moderate ErbB-2 overexpression. This level of overexpression is comparable to, or smaller than, the extent of overexpression that is frequently observed in breast and in other human adenocarcinomas (Stancovski *et al.*, 1994). In analogy to the trans-stimulatory action of an overexpressed ErbB-2 that we observed in mammary cells (Figure 7), it has been recently shown that overexpression of either EGF or insulin receptor can prolong MAP kinase activation by the respective ligands (Dikic *et al.*, 1994; Traverse *et al.*, 1994). Our studies with MCF-7 cells indicate that even small changes in the level of ErbB-2 expression can profoundly affect signaling by NDF and EGF. One explanation for the enhancement and prolongation effects on MAP kinase, as well as on c-Jun kinase, is that they are solely due to the ErbB-2-mediated extension of the half-lives of ligand–receptor complexes (Figure 5). However, because it has been previously shown that ErbB-2 efficiently stimulates the MAP kinase pathway (Ben-Levy *et al.*, 1994), it is likely that this receptor functions not only as a common binding component but also as a shared signaling subunit of NDF and EGF receptors. For example, ErbB-2, by means of its own set of docking sites for Src homology 2 (SH-2) proteins, may diversify the nature of the intracellular signals that are generated by NDF and EGF (Carraway and Cantley, 1994). Because sustained activation of MAP kinase leads to either transformation or differentiation (Cowley *et al.*, 1994), our observations may explain, on the one hand, how ErbB-2 alone (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987), or in combination with ErbB-1 (Kokai *et al.*, 1989), transforms fibroblasts and, on the other hand, how NDF affects both mitogenesis (Holmes *et al.*, 1992) and differentiation (Peles *et al.*, 1992) in different mammary tumor cells. Taken together, our results imply that by overexpression of ErbB-2, tumor cells may gain a selective advantage because they can now respond with higher potency to relatively low concentrations of EGF-like growth factors. In the light of this finding, various biological aspects of *erbB-2*-overexpressing tumors, such as high incidence in ductal carcinoma *in situ* (Barnes *et al.*, 1992) and the response of *erbB-2*-overexpressing tumors to chemotherapy (Gusterson *et al.*, 1992; Wright *et al.*, 1992; Muss *et al.*, 1994) may be considered as reflections of increased sensitivity to growth factors. In addition, the proposed model predicts that co-expression of ErbB-2 with other ErbB proteins, or with their corresponding ligands, may bear prognostic or therapeutic significance.

Lastly, lessons learned with the ErbB family may be relevant to other subgroups of growth factor receptors. Conceivably, the multiplicity of homologous receptors that preserved heterodimerizing capability can confer to mammalian cells an additional level of combinatorial complexity. Presumably, an internal hierarchy exists in each receptor family and this, in conjunction with spatial and temporal control of receptor co-expression, may determine the potency and diversity of intracellular signals.

Materials and methods

Materials and cell culture

EGF (human, recombinant) was purchased from Boehringer Mannheim and recombinant NDF preparations were from Amgen (Thousand Oaks, California). Radioactive materials were obtained from Amersham (Buckinghamshire, UK). Protein A coupled to Sepharose was obtained from Pharmacia (Uppsala, Sweden) or prepared in our laboratory. Molecular weight standards for gel electrophoresis were from Bio-Rad or from Sigma. Iodogen and BS^3 were from Pierce. All other chemicals were purchased from Sigma unless otherwise indicated. Polyclonal antibodies against the C-terminal portions of ErbB-1, ErbB-2 and ErbB-3 proteins (14 amino acid-long synthetic peptides) were generated in rabbits as described (Peles *et al.*, 1991) or were purchased from Santa Cruz Biotechnology (SC285, an anti-ErbB-3 antibody). A murine monoclonal antibody to ErbB-4 was generated in mice that were immunized with a recombinant extracellular portion of human ErbB-4, and it will be described elsewhere. As control we used antibodies to the fibroblast growth factor receptor (from Santa Cruz Biotechnology). Anti-ERK2 antiserum was raised in rabbits that were injected with a synthetic peptide (residues 346–358) as described (Marte *et al.*, 1995).

Establishment of ErbB-expressing CHO cell lines

CHO cells were transfected with expression vectors that direct expression of *erbB-1*, *erbB-3* and *erbB-4* as previously described (Tzahar *et al.*, 1994), except that the *erbB-1* expression vector that we used was pcDNA3/*erbB-1* (Karunagaran *et al.*, 1995). Clones of stably expressing cells were selected in the presence of G418 (Gibco) and assayed for EGF and NDF binding.

Buffered solutions

Binding buffer contained Dulbecco modified Eagle's medium (DMEM) with 0.1% bovine serum albumin (BSA). HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol. Solubilization buffer contained 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.5 mM EGTA, 1.5 mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (0.15 trypsin inhibitor unit/ml) and 10 μg/ml leupeptin.

Radiolabeling of ligands, covalent crosslinking and ligand binding analyses

Human recombinant EGF and human recombinant NDF-β$1_{177–246}$ were labeled with Iodogen (Pierce) as follows: 5 μg protein in phosphate buffered saline (PBS) was mixed in an Iodogen-coated (1μg reagent) tube with $Na^{125}I$ (1 mCi). Following 10 min at 23°C, tyrosine was added to a final concentration of 0.1 mg/ml and the mixture was separated on a column of Excellulose GF-5 (Pierce). The specific activity was determined by counting γ-radioactivity before and after separation on the column. The range of specific activity varied between 2 and 5 $\times 10^5$ c.p.m./ng. For ligand displacement analyses, monolayers of cells ($1–2\times10^5$ cells/well) in 48-well dishes were washed once with binding buffer and then incubated with 5 ng/ml of [^{125}I]EGF in the same buffer. The unlabeled ligand, at different concentrations, was co-incubated with the radiolabeled ligand for 2 h at 4°C, and the cells were washed three times with ice-cold binding buffer. Labeled cells were lysed in 0.5 ml of 0.1 N NaOH containing 0.1% SDS for 15 min at 37°C and the radioactivity was determined by using a γ-counter. For Scatchard analysis, the indicated cell lines were incubated for 2 h at 4°C with different concentrations of either ^{125}I-labeled NDF-β$1_{177–246}$ or [^{125}I]EGF. Non-specific binding was determined by the addition of 100-fold excess of

the unlabeled ligand together with the corresponding labeled ligand in the same experiment. Scatchard analysis was performed by using the computerized program LIGAND (Munson and Rodbard, 1980). For covalent crosslinking analysis, monolayers (10^6 cells) of cells were incubated on ice for 2 h with either [^{125}I]EGF (20 ng/ml) or [^{125}I]NDF-$\beta1_{177-246}$ (10 ng/ml). The chemical crosslinking reagent BS^3 was then added (1 mM) and after 45 min on ice cells were washed with PBS. Cell lysates were prepared and analyzed by gel electrophoresis.

Lysate preparation and immunoprecipitation

For analysis of total cell lysates, gel sample buffer was directly added to cell monolayers. For other experiments, solubilization buffer was added to the monolayer of cells on ice. Cells were scraped with a rubber policeman into 1 ml of the buffer, transferred to microtubes, vortexed harshly, and centrifuged (10 000 *g*, 10 min at 4°C). Rabbit antibodies were coupled directly to protein A–Sepharose while shaking for 20 min. Mouse antibodies were first coupled to rabbit anti-mouse immunoglobulin G and then to protein A–Sepharose by the same procedure. The proteins in the lysate supernatants were immunoprecipitated with aliquots of the protein A–Sepharose–antibody complex for 1 h at 4°C. Immunoprecipitates were then washed three times with HNTG (1 ml each wash).

Analysis of ligand dissociation

The cells were first incubated with 5 ng/ml [^{125}I]EGF or [^{125}I]NDF-$\beta1_{177-246}$ for 2 h at 4°C and then washed three times. Dissociation was monitored by incubating the cells in binding buffer with the corresponding unlabeled ligand (100 ng/ml) for various periods of time at 4°C. Non-specific binding and release of radiolabeled ligand were determined in a parallel experiment and subtracted from the total amount of bound ligand at each time point. The data were analyzed by plotting the natural logarithm of B_t/B_o versus time, where B_t is the concentration of ligand bound at time t and B_o is the same at the starting time of dissociation.

Biosynthetic labeling

Biosynthetic labeling was performed essentially as described (Ben-Levy *et al.*, 1994). Subconfluent cells in 10 cm dishes were washed with methionine-free DMEM and grown for 16 h in the same medium supplemented with 2% dialyzed calf serum containing 50 μCi/ml of [^{35}S]methionine. The cells were washed three times with PBS and then scraped into 1 ml of solubilization buffer. The lysates were spun for 10 min at 4°C (10 000 *g*) and the supernatants were used for immunoprecipitation as described earlier. Equal amounts of trichloroacetic acid precipitable radioactivity were subjected to immunoprecipitation in each lane.

Determination of MAP kinase activity

Cells were grown in DMEM with 10% calf serum, serum-starved for 1–2 days in 0.5% calf serum in DMEM, and then stimulated with growth factors. Three different assays were used to determine MAP kinase activity with MBP as a substrate.

Mini-columns. Cell lysates were prepared by sonication (7 s at 50 W, twice) in lysis buffer (40 mM HEPES at pH 7.5, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM benzamidine, 1 mM DTT, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 2 μg/ml pepstatin A) and cleared by centrifugation. The lysates were fractionated on DEAE cellulose minicolumns (0.4 ml, equilibrated in 50 mM β-glycerophosphate at pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT and 0.1 mM sodium orthovanadate) and the MAP kinase-containing fraction (0.22 M NaCl) assayed for phosphate incorporation into myelin basic protein as described (Seger *et al.*, 1994). The assayed fraction contained both ERK1 and ERK2 and >85% of MAP kinase activity.

Immunoprecipitation assay. The previously described method (Graus-Porta *et al.*, 1995) was used with no modifications.

Kinase assay in MBP-containing polyacrylamide gels. Cell lysates were prepared as described above and aliquots were subjected to electrophoresis in SDS–polyacrylamide gels that were polymerized in the presence of 0.5 mg/ml MBP. The protocol used for in-gel MAP kinase assay was essentially as described (Tobe *et al.*, 1991), with some modifications. SDS was removed from the gel by washing twice for 1 h in 100 ml of 20% isopropanol in 50 mM Tris–HCl (pH 8.0), followed by three washes in 50 ml buffer A (5 mM β-mercaptoethanol in 50 mM HEPES pH 7.6). Protein denaturation was performed by incubating the gel for 15 min in buffer A containing 6 M urea. This step was repeated and the gel subjected to renaturation by washing at 4°C in decreasing concentrations of urea (3 M, 1.5 M and 0.75 M, 15 min each) in buffer A that contained also 0.05% Tween-20. Finally, the gel was washed thrice in the same buffer, but without urea, and left overnight in the last washing solution. The next day the gel was incubated for 30 min at 30°C in 30 ml kinase buffer (20 mM HEPES pH 7.6, containing 20 mM $MgCl_2$). This buffer was replaced with kinase buffer that contained in addition 2 mM DTT, 20 μM ATP and 100 μCi [γ-^{32}P]ATP. After 2 h at 30°C, the gel was washed in washing solution [50 ml of trichloroacetic acid (5% w/v), 1% Na-pyrophosphate] until radioactivity of the solution became negligible. The washed gel was dried and then subjected to autoradiography.

Determination of c-Jun kinase activity

JNK activity was determined in cell lysates that were prepared as described above for MAP kinase (protocol 1). Lysates (0.15 ml) were mixed with 20 μl GSH–agarose suspension (Sigma) to which GST–Jun (10 μg) was bound and 30 μl of 10-fold concentrated binding buffer (220 mM HEPES pH 7.7, 20 mM $MgCl_2$, 1 mM EDTA, 0.75% Triton X-100, 400 mM β-glycerophosphate, 1 mM sodium orthovanadate and protease inhibitors) was added. The mixture (0.3 ml) was incubated at 4°C for 2 h (Hibi *et al.*, 1993). The beads were pelleted, washed in HNTG and resuspended in 30 μl of kinase buffer (20 mM HEPES buffer at pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate and 0.1 mM Na_3VO_4) containing 20 μM ATP and 2 μCi of [γ-^{32}P]ATP. The reaction was terminated after 20 min at 30°C by adding Laemmli sample buffer and electrophoresis on SDS–polyacrylamide gels (10% acrylamide). Autoradiograms were scanned after fixation and gel staining.

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References

Bargmann,C.I., Hung,M.C. and Weinberg,R.A. (1986) *Cell*, **45**, 649–657.
Barnes,D.M., Battkova,J., Camplejohn,R.S., Gullick,W.J., Smith,P.J. and Millis,R.R. (1992) *Eur. J. Cancer*, **28**, 644–648.
Beerli,R.R., Wels,W. and Hynes,N.E. (1994) *J. Biol. Chem.*, **269**, 23931–23936.
Ben-Levy,R., Peles,E., Goldman-Michael,R. and Yarden,Y. (1992) *J. Biol. Chem.*, **267**, 17304–17313.
Ben-Levy,R., Paterson,H.F., Marshall,C.J. and Yarden,Y. (1994) *EMBO J.*, **13**, 3302–3311.
Carpenter,G. (1987) *Annu. Rev. Biochem.*, **56**, 881–914.
Carraway,K.L. and Cantley,L.C. (1994) *Cell*, **78**, 5–8.
Carraway,K.L.I. *et al.* (1994) *J. Biol. Chem.*, **269**, 14303–14306.
Cowley,S., Paterson,H., Kemp,P. and Marshall,C.J. (1994) *Cell*, **77**, 841–852.
Derijard,B., Hibi,M., Wu,I.-H., Barrett,T., Su,B., Deng,T., Karin,M. and Davis,R.J. (1994) *Cell*, **76**, 1025–1037.
Di Fiore,P.P., Pierce,J., Kraus,M.H., Segatto,O., King,C.R. and Aaronson,S.A. (1987) *Science*, **237**, 178–182.
Dikic,I., Schlessinger,J. and Lax,I. (1994) *Curr. Biol.*, **4**, 702–708.
Dobashi,K., Davis,J.G., Mikami,Y., Freeman,J.K., Hamuro,J. and Greene,M.I. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 8582–8586.
Dougall,W.C., Qian,X., Peterson,N.C., Miller,M.J., Samanta,A. and Greene,M. (1994) *Oncogene*, **9**, 2109–2123.
Panti,W.J., Johnson,D.E. and Williams,L.T. (1993) *Annu. Rev. Biochem.*, **62**, 453–481.
Goldman,R., Ben-Levy,R., Peles,E. and Yarden,Y. (1990) *Biochemistry*, **29**, 11024–11028.
Gotoh,N., Tojo,A., Muroya,K., Hashimoto,Y., Hattori,S., Nakamura,S., Takenawa,T., Yazaki,Y. and Shibuya,M. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 167–171.
Graus-Porta,D., Beerli,R.R. and Hynes,N.E. (1995) *Mol. Cell. Biol.*, **15**, 1182–1191.
Gusterson,B.A. *et al.* (1992) *J. Clin. Oncol.*, **10**, 1049–1056.
Guy,P.M., Platko,J.V., Cantley,L.C., Cerione,R.A. and Carraway III,K.L. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 8132–8136.
Hibi,M., Lin,A., Smeal,T., Minden,A. and Karin,M. (1993) *Genes Dev.*, **7**, 2135–2148.

Hilton,D.J. *et al.* (1994) *EMBO J.*, **13**, 4765–4775.
Holmes,W.E. *et al.* (1992) *Science*, **256**, 1205–1210.
Huang,S.S. and Huang,J.S. (1992) *J. Biol. Chem.*, **267**, 11508–11512.
Hudziak,R.M., Schlessinger,J. and Ullrich,A. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 7159–7163.
Hynes,N.E. and Stern,D.F. (1994) *Biochim. Biophys. Acta*, **1198**, 165–184.
Igelhart,J.D., Kraus,M.H., Langton,B.C., Huper,G., Kers,B.J. and Marks,J.R. (1990) *Cancer Res.*, **50**, 6701–6707.
Karunagaran,D., Tzahar,E., Liu,N., Wen,D. and Yarden,Y. (1995) *J. Biol. Chem.*, **270**, 9982–9990.
Kawahara,A., Minami,Y. and Taniguchi,T. (1994) *Mol. Cell. Biol.*, **14**, 5433–5440.
Kawamoto,T., Sato,J.D., Le,A., Polikoff,J., Sato,G.H. and Mendelsohn,J. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 1337–1341.
Kern,J.A., Schwartz,D.A., Nordberg,J.E., Weiner,D.B., Greene,M.I., Torney,L. and Robinson,R.A. (1990) *Cancer Res.*, **50**, 5184–5187.
Kokai,Y., Myers,J.N., Wada,T., Brown,V.I., LeVea,C.M., Davis,J.G., Dobashi,K. and Greene,M.I. (1989) *Cell*, **58**, 287–292.
Kraus,H.M., Fedi,P., Starks,V., Muraro,R. and Aaronson,S.A. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 2900–2904.
Kyriakis,J.M., Banerjee,P., Nikolakaki,E., Dai,T., Rubie,E.A., Ahmad, M.F., Avruch,J. and Woodgett,J.R. (1994) *Nature*, **369**, 156–160.
Lonardo,F., Di Marco,E., King,C.R., Pierce,J.H., Segatto,O., Aaronson, S.A. and Di Fiore,P.P. (1990) *New Biol.*, **2**, 992–1003.
Lupu,R., Colomer,R., Kannan,B. and Lippman,M.E. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 2287–2291.
Marshall,C.J. (1994) *Curr. Opin. Gen. Dev.*, **4**, 82–89.
Marte,B.M., Graus-Porta,D., Jeschke,M., Fabbro,D., Hynes,N.E. and Taverna,D. (1995) *Oncogene*, **10**, 167–175.
Munson,P.J. and Rodbard,D. (1980) *Anal. Biochem.*, **107**, 220–239.
Muss,H.B. *et al.* (1994) *New Engl. J. Med.*, **330**, 1260–1266.
Peles,E. and Yarden,Y. (1993) *BioEssays*, **15**, 815–824.
Peles,E., Ben-Levy,R., Or,E., Ullrich,A. and Yarden,Y. (1991) *EMBO J.*, **10**, 2077–2086.
Peles,E., Bacus,S.S., Koski,R.A., Lu,H.S., Wen,D., Ogden,S.G., Ben-Levy,R. and Yarden,Y. (1992) *Cell*, **69**, 205–216.
Peles,E., Ben-Levy,R., Tzahar,E., Naili,L., Wen,D. and Yarden,Y. (1993) *EMBO J.*, **12**, 961–971.
Plowman,G.D., Green,J.M., Culouscou,J.-M., Carlton,G.W., Rothwell, V.M. and Sharon,B. (1993) *Nature*, **366**, 473–475.
Prigent,S.A. and Gullick,W.J. (1994) *EMBO J.*, **13**, 2831–2841.
Seger,R. and Krebs,E.G. (1995) *FASEB J.*, **9**, 726–735.
Seger,R. *et al.* (1994) *J. Biol. Chem.*, **269**, 25699–25709.
Selva,E., Raden,D.L. and Davis,R. (1993) *J. Biol. Chem.*, **268**, 2250–2254.
Slamon,D.J., Clark,G.M., Wong,S.G., Levin,W.J., Ullrich,A. and McGuire,W.L. (1987) *Science*, **235**, 177–182.
Slamon,D.J. *et al.* (1989) *Science*, **244**, 707–712.
Sliwkowski, M. X. *et al.* (1994) *J. Biol. Chem.*, **269**, 14661–14665.
Soltoff,S.P., Carraway,K.L.I., Prigent,S.A., Gullick,W.G. and Cantley, L.C. (1994) *Mol. Cell. Biol.*, **14**, 3550–3558.
Stancovski,I., Hurwitz,E., Leitner,O., Ullrich,A., Yarden,Y. and Sela,M. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 8691–8695.
Tarakhovsky,A., Zaichuk,T., Prassolov,V. and Butenko,Z.A. (1991) *Oncogene*, **6**, 2187–2196.
Tobe,K. *et al.* (1991) *J. Biol. Chem.*, **266**, 24793–24803.
Traverse,S., Seedorf,K., Paterson,H., Marshall,C.J., Cohen,P. and Ullrich,A. (1994) *Curr. Biol.*, **4**, 694–701.
Tzahar,E. *et al.* (1994) *J. Biol. Chem.*, **269**, 25226–25233.
Varley,J.M., Swallow,J.E., Brammar,W.J., Whittaker,J.L. and Walker,R.A. (1987) *Oncogene*, **1**, 423–430.
Wada,T., Qian,X. and Greene,M.I. (1990) *Cell*, **61**, 1339–1347.
Wright,C., Cairns,J., Cantwell,B.J., Catan,A.R., Hall,A.G., Harris,A.L. and Horne,C.H.W. (1992) *Br. J. Cancer*, **65**, 271–274.
Zhou,M., Felder,S., Rubinstein,M., Hurwitz,D.R., Ullrich,A., Lax,I. and Schlessinger,J. (1993) *Biochemistry*, **32**, 8193–8198.

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The anti-HER3 antibody patritumab abrogates cetuximab resistance mediated by heregulin in colorectal cancer cells

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ABSTRACT

We previously showed that tumor-derived heregulin, a ligand for HER3, is associated with both de novo and acquired resistance to cetuximab. We have now examined whether patritumab, a novel neutralizing monoclonal antibody to HER3, is able to overcome such resistance. Human colorectal cancer (DiFi) cells that are highly sensitive to cetuximab were engineered to stably express heregulin by retroviral infection, and the effects of cetuximab and patritumab on the resulting DiFi-HRG cells were examined. DiFi-HRG cells released substantial amounts of heregulin and showed resistance to cetuximab. Cetuximab alone inhibited EGFR and ERK phosphorylation in DiFi-HRG cells, but it had no effect on the phosphorylation of HER2, HER3, or AKT, suggesting that sustained AKT activation by HER2 and HER3 underlies cetuximab resistance in these cells. In contrast, patritumab in combination with cetuximab markedly inhibited the phosphorylation of EGFR, HER2, HER3, ERK, and AKT. The combination therapy also inhibited the growth of DiFi-HRG tumor xenografts in nude mice to a greater extent than did treatment with either drug alone. Activation of HER2-HER3 signaling associated with the operation of a heregulin autocrine loop confers resistance to cetuximab, and patritumab is able to restore cetuximab sensitivity through inhibition of heregulin-induced HER3 activation.

INTRODUCTION

Cetuximab, a chimeric human-mouse monoclonal antibody to the epidermal growth factor receptor (EGFR), has shown clinical efficacy in individuals with metastatic colorectal cancer (mCRC). However, a subset of mCRC patients fails to show an initial response (de novo resistance) to this agent, whereas others develop resistance after an initial response (acquired resistance). Well-established causes of de novo resistance to cetuximab include activating mutations in codon 12 or 13 of *KRAS* and in *BRAF* [1–4]. Various mechanisms responsible for acquired resistance to cetuximab in colorectal cancer have also been identified [5–7]. We previously established cetuximab-resistant cancer cells by exposing parental cells to increasing concentrations of cetuximab [8]. Analysis of these cells revealed that cell-derived heregulin confers cetuximab resistance through bypass signaling via HER2 (also known as ERBB2) and HER3 (also known as ERBB3). Heregulin is a ligand for HER3 and stabilizes the HER2-HER3 heterodimer [9]. We also found that high initial levels of serum heregulin protein and tumor heregulin mRNA were significantly associated with a poor clinical outcome in mCRC patients treated with cetuximab [8]. Furthermore, in patients who initially

achieved a partial response to cetuximab-based therapy, the serum concentration of heregulin after the development of clinical cetuximab resistance was significantly higher than that before treatment [8]. These preclinical and clinical data indicate that increased levels of heregulin are associated with both de novo and acquired resistance to cetuximab.

Patritumab (U3-1287) is a first-in-class, fully human monoclonal antibody directed to the extracellular domain (ECD) of HER3 that is currently in clinical development, as are other HER3-targeted antibodies such as MM-121 and LJM716 (MM-121 prevents ligand binding, whereas LJM716 specifically binds to an epitope formed by ECD domains II and IV in the closed conformation of HER3 [10]). Patritumab has been shown both to inhibit ligand-induced HER3 phosphorylation and to suppress the growth of pancreatic, non–small cell lung cancer, and colorectal cancer xenograft tumors [11, 12]. To identify strategies or agents capable of overcoming resistance to cetuximab induced by heregulin, we have now established sublines of the cetuximab-sensitive human colorectal cancer cell line DiFi that stably express heregulin derived from transfected cDNA. With the use of these cells, we investigated the effects of patritumab on cetuximab resistance mediated by cell-derived heregulin both *in vitro* and *in vivo*.

RESULTS

DiFi cells stably overexpressing heregulin show resistance to cetuximab

The human colorectal cancer cell line DiFi, which harbors wild-type alleles of *KRAS, BRAF*, and *PI3K*, is highly sensitive to cetuximab [13]. To investigate whether cell-derived heregulin might induce cetuximab resistance in DiFi cells, we established DiFi sublines that stably overexpress this protein (DiFi-HRG4, DiFi-HRG5, and DiFi-HRG6) or that stably harbor the corresponding empty vector (DiFi- Mock1) as a result of retroviral infection. Heregulin is a soluble growth factor that is synthesized as a transmembrane precursor molecule of 105 kDa. Cell surface proteases catalyze cleavage of the extracellular domain of this precursor, which is then released and functions as a ligand for HER3. Immunoblot analysis revealed the presence of the transmembrane form of heregulin in DiFi-HRG cells (with its abundance being greatest in DiFi-HRG4 cells), whereas no such band was detected in DiFi-Mock1 cells or the parental DiFi cells (Fig. 1A). Analysis of conditioned medium from these cell lines with an enzyme-linked immunosorbent assay (ELISA) also revealed the presence of substantial amounts of heregulin in the medium from all DiFi-HRG cell lines but not in that from DiFi-Mock1 or the parental cells (Fig. 1B). To assess the effect of cetuximab on cell growth, we exposed DiFi-HRG and DiFi-Mock1 cells to various concentrations of the drug for 5 days and then measured cell viability. All DiFi-HRG cell lines showed a reduced sensitivity to cetuximab compared with DiFi-Mock1 cells, with median inhibitory concentration (IC_{50}) values of > 100 μg/mL for the former cell lines and ~0.1 μg/mL for the latter (Fig. 1C). The DiFi-HRG cell lines also showed resistance to panitumumab, another antibody to EGFR (data not shown). These data thus suggested that DiFi-HRG cells are resistant to EGFR-targeted antibodies.

Heregulin maintains HER3 and AKT phosphorylation and survivin expression in the presence of cetuximab in DiFi-HRG cell lines

To investigate possible differences in signal transduction among the DiFi isogenic lines, we examined the effects of cetuximab (10 μg/mL) on EGFR, HER2, HER3, AKT, and extracellular signal–regulated kinase (ERK) phosphorylation (Fig. 2A). Immunoblot analysis revealed that cetuximab markedly inhibited the phosphorylation of all of these proteins in DiFi-Mock1 cells. In contrast, whereas cetuximab substantially reduced the level of EGFR and ERK phosphorylation in DiFi-HRG cells, it had little effect on the phosphorylation of HER2, HER3, or AKT. We next examined the effects of cetuximab on expression of the apoptosis-related proteins BIM (a proapoptotic BH3-only protein) and survivin (a member of the inhibitor of apoptosis, or IAP, family). We previously showed that inhibition of the MEK-ERK signaling pathway induces BIM expression, and that inhibition of the PI3K-AKT pathway suppresses survivin expression, with both of these effects being independently required for tyrosine kinase inhibitor (TKI)–induced apoptosis in lung cancer cells positive for *EGFR* mutation [14], breast cancer cells positive for *HER2* amplification [15], and gastric cancer cells positive for *MET* amplification [16]. Consistent with these observations, we found that cetuximab induced both up-regulation of BIM and down-regulation of survivin in DiFi-Mock1 cells, resulting in generation of the cleaved form of poly(ADP-ribose) polymerase (PARP), a characteristic of apoptosis (Fig. 2B). In contrast, in DiFi-HRG cell lines, whereas cetuximab induced BIM expression, it had little effect on the abundance of survivin or PARP cleavage (Fig. 2B), suggesting that sustained AKT signaling and survivin expression confer resistance to cetuximab in these cell lines.

The HER3 neutralizing antibody patritumab abrogates cetuximab resistance induced by heregulin

To investigate further the role of HER3 and heregulin in the resistance of DiFi-HRG cell lines to cetuximab, we exposed DiFi-HRG4 cells to cetuximab, the fully human HER3-targeted monoclonal antibody patritumab, or the combination of both agents. We found that neither antibody alone substantially affected cell proliferation, whereas the combination of both agents

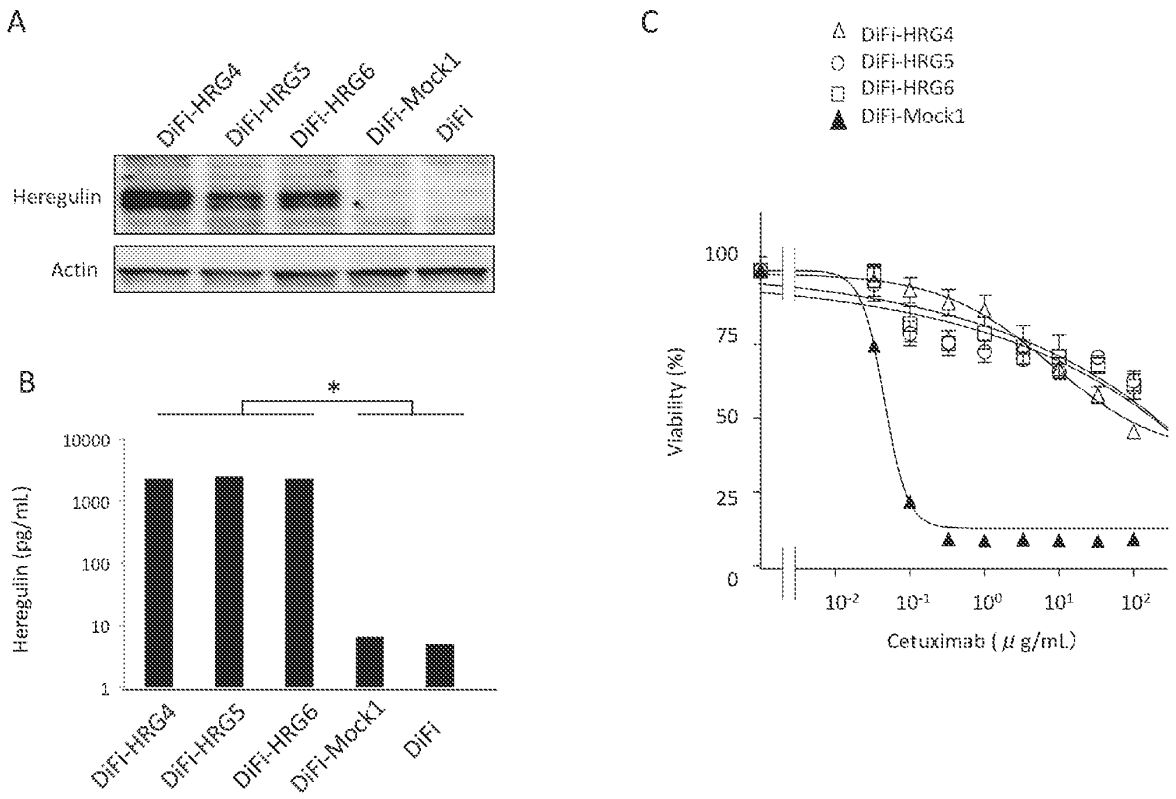

Figure 1: Characterization of DiFi isogenic cell lines. **(A)** DiFi isogenic cell lines (DiFi, DiFi-Mock1, DiFi-HRG4, DiFi-HRG5, and DiFi-HRG6) were cultured overnight in medium containing 10% serum and then incubated for 24 h in serum-free medium, after which the cells were lysed and subjected to immunoblot analysis with antibodies to heregulin and to β-actin (loading control). **(B)** Culture supernatants from cells cultured as described in Materials and Methods were assayed for heregulin with an ELISA. Data are means ± SE from three independent experiments. $^{*}P < 0.05$ (Student's *t* test) for comparison of each DiFi-HRG line with DiFi-Mock1 or DiFi cells. **(C)** Cells were treated with cetuximab at the indicated concentrations for 5 days, after which cell viability was assessed. Data are means ± SE from three independent experiments.

induced marked inhibition of cell growth (Fig. 3A). We next examined the effects of these antibodies on apoptosis in DiFi-Mock1 and DiFi-HRG4 cells. An annexin V binding assay revealed that cetuximab alone induced a substantial level of apoptosis in DiFi-Mock1 cells but not in DiFi-HRG4 cells (Fig. 3B, C), suggesting that the operation of a heregulin autocrine loop in these latter cells inhibits cetuximab-induced apoptosis. However, exposure of DiFi-HRG4 cells to the combination of patritumab (10 μg/mL) and cetuximab (10 μg/mL) resulted in a marked increase in the proportion of apoptotic cells (Fig. 3B, C), suggesting that patritumab sensitizes DiFi-HRG cells to cetuximab such that the extent of apoptosis induced by both antibodies in these cells is similar to that induced by cetuximab alone in DiFi-Mock1 cells.

We also examined the effects of patritumab alone or in combination with cetuximab on intracellular signaling. Immunoblot analysis showed that patritumab alone had little effect on such signaling in DiFi-Mock1 cells. In contrast, patritumab alone markedly inhibited the phosphorylation of HER3 and AKT, without affecting that of ERK, in DiFi-HRG4 cells (Fig. 3D). The combination of patritumab and cetuximab markedly attenuated the phosphorylation of EGFR, HER2, HER3, AKT, and ERK in DiFi-HRG4 cells (Fig. 3D). It also induced the cleavage of PARP in these cells to an extent similar to that observed in DiFi-Mock1 cells treated with cetuximab alone, and this effect was accompanied by both up-regulation of BIM and down-regulation of survivin expression (Fig. 3E). These results thus indicated that cetuximab resistance induced by heregulin is abrogated by patritumab through attenuation of AKT-suvivin signaling in DiFi-HRG4 cells.

Cell-derived heregulin induces cetuximab resistance and patritumab restores cetuximab sensitivity in tumor xenografts *in vivo*

To examine whether cell-derived heregulin induces cetuximab resistance as well as the efficacy of combined

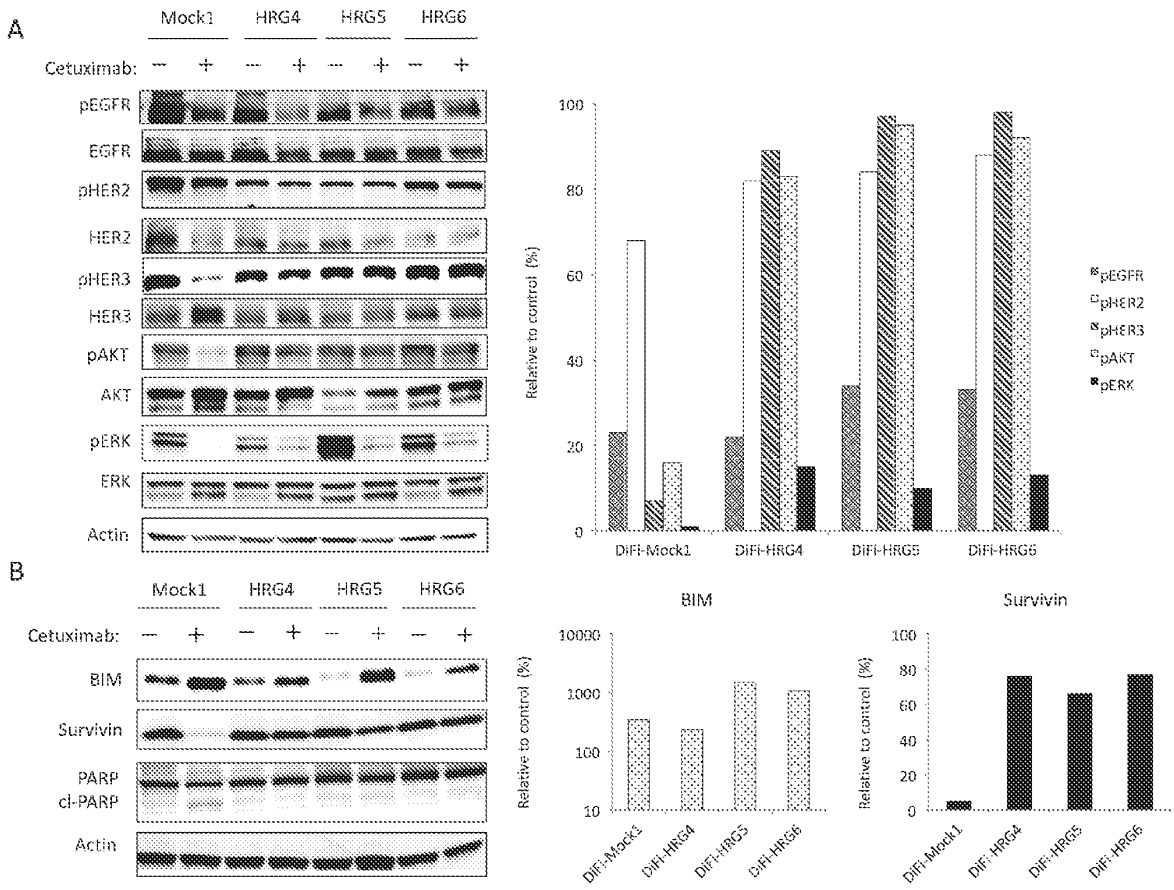

Figure 2: Effects of cetuximab on intracellular signaling and the expression of apoptosis-related proteins in DiFi isogenic cell lines. DiFi-Mock1, DiFi-HRG4, DiFi-HRG5, or DiFi-HRG6 cells were cultured overnight in medium containing 10% serum and then incubated for 6 h **(A)** or 24 h **(B)** in serum-free medium with or without cetuximab (10 μg/mL), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of the indicated proteins (left panels). A band corresponding to the cleaved (cl) form of PARP is indicated. The intensity of the bands corresponding to phosphorylated forms of EGFR, HER2, HER3, AKT, and ERK (A) or to BIM and survivin (B) was normalized by that of the corresponding total proteins or β-actin, respectively, and then expressed relative to the corresponding value for control cells not exposed to cetuximab (right panels).

treatment with patritumab and cetuximab *in vivo*, we injected nude mice with DiFi-Mock1 or DiFi-HRG4 cells to allow the formation of tumor xenografts. Whereas cetuximab alone markedly inhibited the growth of DiFi-Mock1 xenografts (Fig. 4A), DiFi-HRG4 xenografts were resistant to this drug (Fig. 4B). Patritumab alone had little effect on the growth of tumors formed by either cell line. However, the combination of cetuximab and patritumab induced substantial regression of DiFi-HRG4 xenografts (Fig. 4B). These results thus suggested that heregulin produced by colorectal cancer tumors harboring wild-type *KRAS* induces cetuximab resistance, and that combination therapy with cetuximab and patritumab overcomes such resistance *in vivo*.

DISCUSSION

Resistance to cetuximab is a major problem in the treatment of colorectal cancer. Although various mechanisms of cetuximab resistance have been identified [1–7, 17–20], the optimal treatment strategies for mCRC patients who show resistance to this drug remain unclear. We previously showed that tumor-derived heregulin mediates cetuximab resistance in preclinical models [8]. High levels of heregulin were also associated with a poor clinical outcome in mCRC patients treated with cetuximab-based regimens [8]. Moreover, increased heregulin levels were observed in such patients after the development of clinical resistance to cetuximab-based

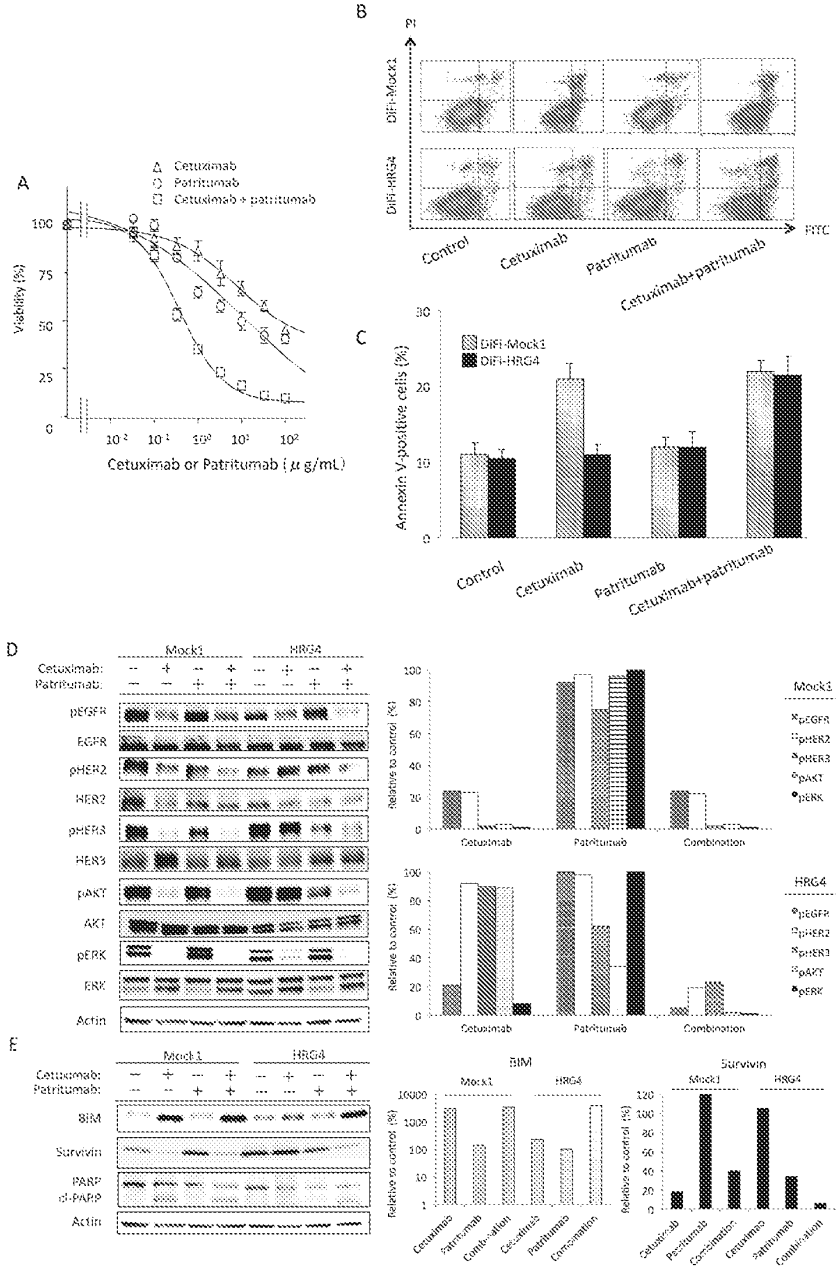

Figure 3: Effect of patritumab on heregulin-mediated cetuximab resistance in DiFi-HRG cells *in vitro*. **(A)** DiFi-HRG4 cells were incubated for 5 days with cetuximab alone, patritumab alone, or the combination of both drugs at the indicated concentrations, after which cell viability was assessed. Data are means ± SE from three independent experiments. **(B, C)** DiFi-Mock1 or DiFi-HRG4 cells were cultured overnight in medium containing 10% serum and then incubated for 48 h in the absence or presence of cetuximab alone (10 μg/mL), patritumab alone (10 μg/mL), or the combination of both drugs in serum-free medium, after which the number of apoptotic cells was determined by staining with propidium iodide (PI) and fluorescein isothiocyanate (FITC)–labeled annexin V followed by flow cytometry. Representative flow cytometric profiles are shown in (B), and quantitative data (means ± SE of three independent experiments) are shown in (C). **(D, E)** DiFi-Mock1 or DiFi-HRG4 cells were cultured overnight in medium containing 10% serum and then incubated for 6 h (D) or 48 h (E) in the absence or presence of cetuximab alone (10 μg/mL), patritumab alone (10 μg/mL), or the combination of both drugs in serum-free medium, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins (left panels). The intensity of the bands corresponding to phosphorylated forms of EGFR, HER2, HER3, AKT, and ERK (D) or to BIM and survivin (E) was normalized by that of the corresponding total proteins or β-actin, respectively, and then expressed relative to the corresponding value for control cells not exposed to drug (right panels).

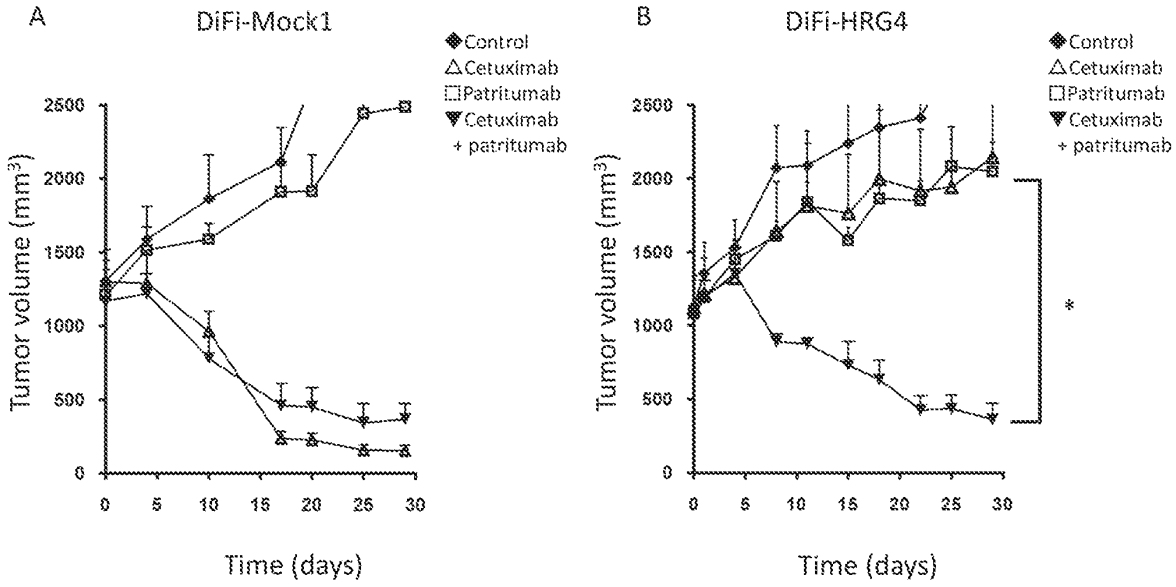

Figure 4: Effects of cetuximab, patritumab, and the combination of both drugs on the growth of DiFi-HRG tumor xenografts *in vivo*. Nude mice with tumor xenografts established by subcutaneous injection of DiFi-Mock1 **(A)** or DiFi-HRG4 **(B)** cells were treated for 4 weeks with vehicle (control), cetuximab (1.0 mg/body), patritumab (1.0 mg/body), or both drugs, as described in Materials and Methods. Tumor volume was determined at the indicated times after the onset of treatment. Data are means ± SE from six mice per group. *$P < 0.05$ for comparison of the combination of both drugs with cetuximab alone or patritumab alone (Student's *t* test).

therapy [8]. Effective treatment options to overcome cetuximab resistance mediated by heregulin are thus urgently required.

We have here established heregulin-overexpressing sublines of DiFi cells (DiFi-HRG cells) and shown that these cells are resistant to cetuximab both *in vitro* and *in vivo*. Whereas the amount of the transmembrane form of heregulin was substantially higher in DiFi-HRG4 cells than in DiFi-HRG5 or DiFi-HRG6 cells (Fig. 1A), these three cell lines appeared to release similar amounts of the soluble form of heregulin into the culture medium (Fig. 1B). This difference in the relative abundance of the transmembrane and soluble forms of heregulin among DiFi-HRG cell lines might reflect a difference in the activity of cell surface proteases among the cell lines. Alternatively, the production of the transmembrane form of heregulin in DiFi-HRG4 cells might exceed the capacity of such proteases. To investigate the mechanism responsible for cetuximab resistance in DiFi-HRG cells, we examined differences in signal transduction between these cells and DiFi-Mock1 cells. In DiFi-Mock1 cells, cetuximab inhibited the phosphorylation of EGFR, HER2, HER3, AKT, and ERK as well as up-regulated BIM expression and down-regulated survivin expression, resulting in the induction of apoptosis. By contrast, in DiFi-HRG cell lines, whereas cetuximab inhibited EGFR and ERK phosphorylation, leading to BIM induction, it did not affect HER2, HER3, or AKT phosphorylation or survivin expression. Given that down-regulation both of AKT signaling and of the expression of its downstream target survivin is required for apoptosis induced by inhibition of receptor tyrosine kinases [14–16], our data suggest that sustained AKT-survivin signaling in the presence of cetuximab is responsible for the resistance of DiFi-HRG cell lines to this drug. To investigate further the relation between AKT signaling and the operation of a heregulin autocrine loop, we examined the effects of patritumab, a neutralizing monoclonal antibody to HER3, in DiFi-HRG4 cells. We found that exposure of these cells to patritumab in combination with cetuximab resulted in inhibition of EGFR, HER2, HER3, AKT, and ERK phosphorylation as well as in both up-regulation of BIM expression and down-regulation of survivin expression, leading to the induction of apoptosis. These results indicate that AKT signaling is triggered by heregulin binding to HER3.

Given that HER3 (a kinase-dead receptor) manifests impaired kinase activity [21], it requires dimerization with other HER family members to activate signaling after ligand binding [22, 23]. HER2 has been implicated as a dimerization partner of HER3, and heregulin stabilizes the HER2-HER3 heterodimer [9]. In this context, we examined the effects of lapatinib, a TKI for EGFR and HER2, in DiFi-HRG4 cells. Inhibition of EGFR and HER2 by lapatinib resulted in down-regulation of HER3, AKT, and ERK phosphorylation as well as in the induction of BIM and suppression of survivin expression in these cells, thereby triggering apoptosis (Supplementary Fig. S1A, S1B). These

findings suggest that, in DiFi-HRG cells, HER3 is trans-phosphorylated by HER2 as a result of heregulin-induced HER2-HER3 heterodimerization, which in turn leads to the activation of AKT signaling [24] (Fig. 5A, 5B, 5C). The heterodimerization partner of HER3 is thus likely switched from EGFR to HER2 as a result of the overexpression of heregulin in DiFi-HRG cells. We also found that patritumab alone inhibited the phosphorylation of HER3 and AKT as well as down-regulated survivin expression in DiFi-HRG4 cells but not in DiFi-Mock1 cells (Fig. 3D, 3E). These results suggest that patritumab may prevent ligand-dependent HER3 phosphorylation by blocking HER2-HER3 heterodimerization, whereas it has little effect on ligand-independent HER3 phosphorylation.

In our DiFi-HRG xenograft model, we showed that combination therapy with patritumab and cetuximab inhibited tumor growth to the same extent as did cetuximab alone in the DiFi-Mock1 xenograft model. Antagonism of the heregulin-HER3 interaction by patritumab thus represents an effective strategy to abrogate cetuximab resistance induced by heregulin derived from tumor cells. Given that elevated circulating levels of heregulin are associated with both de novo and acquired cetuximab resistance in mCRC patients [8], our model systems based on stable overexpression of heregulin are clinically relevant and should prove useful for establishing strategies to overcome cetuximab resistance mediated by the heregulin autocrine loop. Indeed, a recent phase I/II study with refractory colorectal cancer patients revealed potential antitumor activity of the combination of cetuximab and pertuzumab, a HER2-targeted antibody that blocks ligand-dependent HER2-HER3 heterodimerization. However, this drug combination was not tolerable as a result of overlapping toxicities [25]. Given that the toxicity profile of patritumab differs from that of pertuzumab [26], the combination of cetuximab and patritumab warrants evaluation in the clinical setting. We also found that the IC_{50} value for the antiproliferative effect of the combination of cetuximab and patritumab in DiFi-HRG4 cells was ~10 times as high as that for cetuximab alone in DiFi-Mock1 cells (Fig. 1C, Fig. 3A). The discrepancy between these *in vitro* data and our *in vivo* findings may suggest that antibody-dependent cellular cytotoxicity [27] involving NK cell activation plays a role in tumor growth inhibition by the combination of both agents. Moreover, combination therapy with these two IgG1 antibodies may result in an enhanced antitumor activity mediated by cytotoxic T lymphocytes [28] in the clinical setting. Given the recent evidence implicating the importance of interactions between therapeutic antibodies and the immune system in the efficacy of antibody treatment [20], further investigation of such mechanisms is warranted.

In conclusion, we have shown that consecutive activation of HER2-HER3 and AKT by heregulin in an autocrine-dependent manner confers resistance to cetuximab, and that patritumab restores cetuximab sensitivity in tumors with heregulin-induced cetuximab resistance. Further studies of combination therapy with patritumab and cetuximab are thus warranted in mCRC patients with heregulin-induced resistance to EGFR-targeted antibodies.

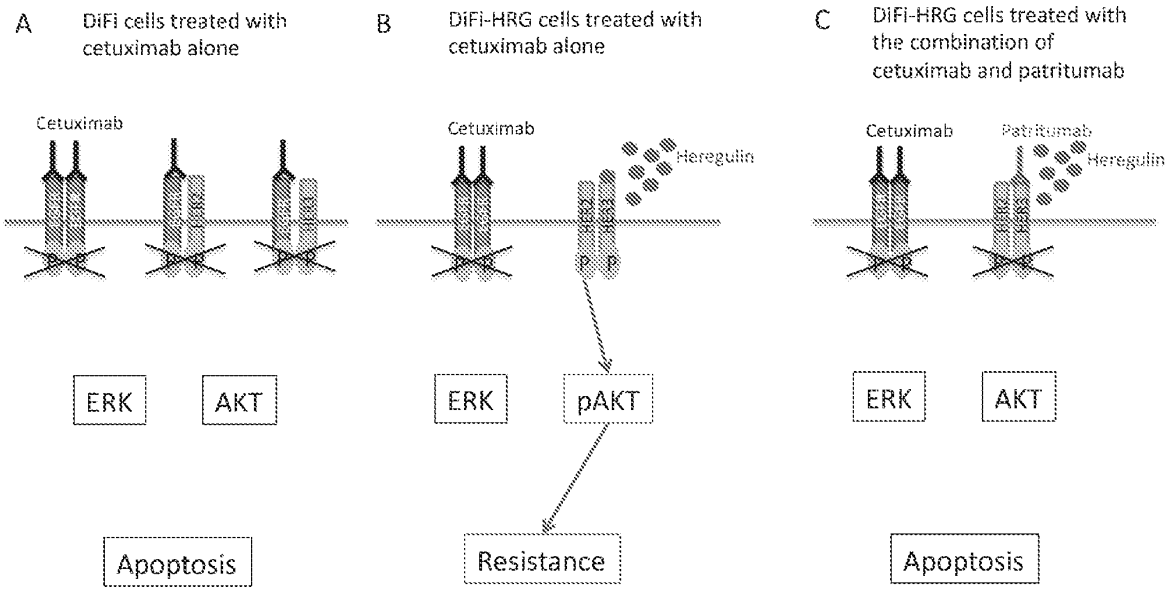

Figure 5: Model for intracellular signaling underlying the induction of apoptosis by cetuximab and patritumab in colorectal cancer cells. **(A)** DiFi colorectal cancer cells treated with cetuximab alone. **(B)** DiFi cells that stably overexpress heregulin (DiFi-HRG cells) are resistant to cetuximab as a result of HER2-HER3 heterodimerization and AKT activation induced by heregulin. **(C)** Patritumab abrogates cetuximab resistance mediated by the heregulin autocrine loop in DiFi-HRG cells.

MATERIALS AND METHODS

Cells and reagents

The DiFi human colorectal cancer cell line was kindly provided by P. A. Janne (Dana Farber Cancer Institute). DiFi cells were maintained under a humidified atmosphere of 5% CO_2 in air at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and supplemented with Ham's F-12 and 10% fetal bovine serum (FBS). Cetuximab was obtained from Merck Serono, and patritumab was kindly provided by Daiichi-Sankyo (Tokyo, Japan). Recombinant human heregulin (NRG1-β1/HRG1-β1 extracellular domain) was obtained from R&D Systems.

Establishment of cells stably overexpressing heregulin

A full-length cDNA encoding human heregulin (NRG1, GenBank accession no. NM_013956) was obtained from Origene (Rockville, MD). The amplification product was verified by sequencing after its cloning into the pCR-Blunt II-TOPO vector (Invitrogen). The heregulin cDNA was then excised from pCR-Blunt II-TOPO and transferred to the pQCXIH retroviral vector (Clontech), and retroviruses encoding heregulin were produced and used to infect DiFi cells as described [29]. Cells stably expressing heregulin were then isolated by selection with hygromycin (Invivogen) at 500 μg/mL.

Cell growth inhibition assay

Cells were transferred to 96-well flat-bottomed plates and cultured for 24 h before exposure to various concentrations of cetuximab or patritumab in medium containing 1% FBS for 120 h. Cell Counting Kit-8 solution (Dojindo, Kumamoto, Japan) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems). Absorbance values were expressed as a percentage of that for nontreated cells, and the IC_{50} of cetuximab for inhibition of cell growth was determined.

Heregulin ELISA

The concentration of heregulin in cell culture supernatants was measured with the use of a sandwich ELISA (NRG1-β1 DuoSet, R&D Systems) as previously described [30]. Cells were seeded in six-well plates at a density of 0.5×10^6 cells per well in DMEM supplemented with 10% FBS. After the cells had achieved confluence, the medium was replaced with 5 ml of DMEM supplemented with 0.1% FBS, the cells were incubated for 48 h, and the culture supernatants were collected for assay of heregulin.

Immunoblot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed with 1 × Cell Lysis Buffer (Cell Signaling Technology) consisting of 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA (disodium salt), 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, leupeptin (1 μg/mL), and 1 mmol/L phenylmethylsulfonyl fluoride. The protein concentration of the lysates was determined with a bicinchoninic acid assay kit (Thermo Fisher Scientific), and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis on a 7.5% gel (Bio-Rad). The separated proteins were transferred to a nitrocellulose membrane, which was then incubated with Blocking One solution (Nacalai Tesque) for 20 min at room temperature before incubation overnight at 4°C with primary antibodies. Antibodies to heregulin (NRG1-β1), to phosphorylated EGFR (phospho-Tyr^{1068}), to phosphorylated HER2 (phospho-Tyr^{1248}), to phosphorylated HER3 (phospho-Tyr^{1289}), to phosphorylated or total forms of AKT, to phosphorylated ERK, to PARP, and to BIM were obtained from Cell Signaling Technology; those to total HER3, to total ERK, and to survivin were from Santa Cruz Biotechnology; those to total EGFR were from Zymed/Invitrogen; those to total HER2 were from Millipore; and those to β-actin were from Sigma. The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare). Immune complexes were finally detected with ECL detection reagents (GE Healthcare).

Annexin V binding assay

The binding of annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche). Cells were harvested by exposure to trypsin-EDTA, washed with PBS, and centrifuged at 200 × *g* for 5 min. The cell pellets were resuspended in 100 μL of Annexin-V-FLUOS labeling solution, incubated for 10 to 15 min at 15° to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson).

Tumor growth inhibition assay *in vivo*

All animal experiments were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University. The ethical procedures followed conformed to the UKCCCR guidelines for the welfare and use of

animals in cancer research [31]. The study was also reviewed and approved by the Animal Ethics Committee of Kinki University (approval no. KAME-22-018). Cells were injected subcutaneously into the axilla of 5- to 6-week-old female athymic nude mice (BALB/c nu/nu; CLEA Japan), and treatment was initiated when tumors in each group of six mice achieved an average volume of 1000 to 1200 mm^3. Treatment groups consisted of vehicle control, cetuximab (1.0 mg/body), patritumab (1.0 mg/body), and the combination of both agents. Cetuximab and patritumab were administered by intraperitoneal injection twice a week for 4 weeks, with control animals receiving a 0.5% (w/v) aqueous solution of hydroxyl propylmethyl cellulose as vehicle. Tumor volume was determined from caliper measurements of tumor length (L) and width (W) according to the formula $LW^2/2$. Both tumor size and body weight were measured twice weekly. For ethical reasons, animals were removed from the study if the tumor volume exceeded 2500 mm^3.

Statistical analysis

Quantitative data are presented as means ± SE unless indicated otherwise. The significance of differences was evaluated with the unpaired two-tailed Student's t test. A P value of < 0.05 was considered statistically significant.

Conflict of interest statement

The authors declare no conflict of interest.

REFERENCES

1. Van Cutsem E, Kohne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, D'Haens G, Pinter T, Lim R, Bodoky G, Roh JK, Folprecht G, Ruff P, Stroh C, Tejpar S, Schlichting M, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med. 2009; 360:1408–1417.
2. Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de Braud F, Donea S, Ludwig H, Schuch G, Stroh C, Loos AH, Zubel A, Koralewski P. Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. J Clin Oncol. 2009; 27:663–671.
3. Tol J, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, Erdkamp FL, Vos AH, van Groeningen CJ, Sinnige HA, Richel DJ, Voest EE, Dijkstra JR, Vink-Borger ME, Antonini NF, Mol L, et al. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. N Engl J Med. 2009; 360:563–572.
4. Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, Bardelli A. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J Clin Oncol. 2008; 26:5705–5712.
5. Montagut C, Dalmases A, Bellosillo B, Crespo M, Pairet S, Iglesias M, Salido M, Gallen M, Marsters S, Tsai SP, Minoche A, Seshagiri S, Serrano S, Himmelbauer H, Bellmunt J, Rovira A, et al. Identification of a mutation in the extracellular domain of the Epidermal Growth Factor Receptor conferring cetuximab resistance in colorectal cancer. Nat Med. 2012; 18:221–223.
6. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, Valtorta E, Schiavo R, Buscarino M, Siravegna G, Bencardino K, Cercek A, Chen CT, Veronese S, Zanon C, Sartore-Bianchi A, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012; 486:532–536.
7. Bardelli A, Corso S, Bertotti A, Hobor S, Valtorta E, Siravegna G, Sartore-Bianchi A, Scala E, Cassingena A, Zecchin D, Apicella M, Migliardi G, Galimi F, Lauricella C, Zanon C, Perera T, et al. Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. Cancer Discov. 2013; 3:658–673.
8. Yonesaka K, Zejnullahu K, Okamoto I, Satoh T, Cappuzzo F, Souglakos J, Ercan D, Rogers A, Roncalli M, Takeda M, Fujisaka Y, Philips J, Shimizu T, Maenishi O, Cho Y, Sun J, et al. Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. Sci Transl Med. 2011; 3:99ra86.
9. Wallasch C, Weiss FU, Niederfellner G, Jallal B, Issing W, Ullrich A. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. EMBO J. 1995; 14:4267–4275.
10. Gala K, Chandarlapaty S. Molecular pathways: HER3 targeted therapy. Clin Cancer Res. 2014; 20:1410–1416.
11. Freeman D, Ogbagabreiel S, Rothe M, Radinsky R, Treder M. 2008; Fully human Anti-HER3 monoclonal antibodies (mAbs) have unique *in vitro* and *in vivo* functional and antitumor activities versus other HER family inhibitors (abstract). Proceedings of the Annual Meeting of the American Association for Cancer Research; San Diego, CA: AACR.
12. Treder M, Hartmann S, Ogbagabrei S, Borges E, Green L, Kang Jea (2008): Fully human Anti-HER3 monoclonal antibodies (mAbs) inhibit oncogenic signaling and tumor cell growth *in vitro* and vivo. Proceedings of the Annual Meeting of the American Association for Cancer Research; San Diego, CA.: AACR.
13. McDermott U, Sharma SV, Dowell L, Greninger P, Montagut C, Lamb J, Archibald H, Raudales R, Tam A, Lee D, Rothenberg SM, Supko JG, Sordella R, Ulkus LE, Iafrate AJ, Maheswaran S, et al. Identification of genotype-correlated sensitivity to selective kinase inhibitors by using high-throughput tumor cell line profiling. Proc Natl Acad Sci U S A. 2007; 104:19936–19941.
14. Okamoto K, Okamoto I, Okamoto W, Tanaka K, Takezawa K, Kuwata K, Yamaguchi H, Nishio K,

Nakagawa K. Role of survivin in EGFR inhibitor-induced apoptosis in non-small cell lung cancers positive for EGFR mutations. Cancer Res. 2010; 70:10402–10410.

15. Tanizaki J, Okamoto I, Fumita S, Okamoto W, Nishio K, Nakagawa K. Roles of BIM induction and survivin downregulation in lapatinib-induced apoptosis in breast cancer cells with HER2 amplification. Oncogene. 2011; 30:4097–4106.
16. Okamoto W, Okamoto I, Arao T, Kuwata K, Hatashita E, Yamaguchi H, Sakai K, Yanagihara K, Nishio K, Nakagawa K. Antitumor action of the MET tyrosine kinase inhibitor crizotinib (PF-02341066) in gastric cancer positive for MET amplification. Mol Cancer Ther. 2012; 11:1557–1564.
17. Siena S, Sartore-Bianchi A, Di Nicolantonio F, Balfour J, Bardelli A. Biomarkers predicting clinical outcome of epidermal growth factor receptor-targeted therapy in metastatic colorectal cancer. J Natl Cancer Inst. 2009; 101:1308–1324.
18. De Roock W, De Vriendt V, Normanno N, Ciardiello F, Tejpar S. KRAS, BRAF, PIK3CA, and PTEN mutations: implications for targeted therapies in metastatic colorectal cancer. Lancet Oncol. 2011; 12:594–603.
19. Bardelli A, Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. J Clin Oncol. 2010; 28:1254–1261.
20. Troiani T, Zappavigna S, Martinelli E, Addeo SR, Stiuso P, Ciardiello F, Caraglia M. Optimizing treatment of metastatic colorectal cancer patients with anti-EGFR antibodies: overcoming the mechanisms of cancer cell resistance. Expert Opin Biol Ther. 2013; 13:241–255.
21. Guy PM, Platko JV, Cantley LC, Cerione RA, Carraway KL, 3rd . Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc Natl Acad Sci U S A. 1994; 91:8132–8136.
22. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF, 3rd, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci U S A. 2003; 100:8933–8938.
23. Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME, Barrow D, Wakeling AE, Nicholson RI. Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. Endocrinology. 2003; 144:1032–1044.
24. Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, Ratzkin BJ, Yarden Y. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. Mol Cell Biol. 1996; 16:5276–5287.
25. Rubinson DA, Hochster HS, Ryan DP, Wolpin BM, McCleary NJ, Abrams TA, Chan JA, Iqbal S, Lenz HJ, Lim D, Rose J, Bekaii-Saab T, Chen HX, Fuchs CS, Ng K. Multi-drug inhibition of the HER pathway in metastatic colorectal cancer: results of a phase I study of pertuzumab plus cetuximab in cetuximab-refractory patients. Invest New Drugs. 2014; 32:113–122.
26. LoRusso P, Janne PA, Oliveira M, Rizvi N, Malburg L, Keedy V, Yee L, Copigneaux C, Hettmann T, Wu CY, Ang A, Halim AB, Beckman RA, Beaupre D, Berlin J. Phase I Study of U3-1287, a Fully Human Anti-HER3 Monoclonal Antibody, in Patients with Advanced Solid Tumors. Clin Cancer Res. 2013; 19:3078–3087.
27. Kimura H, Sakai K, Arao T, Shimoyama T, Tamura T, Nishio K. Antibody-dependent cellular cytotoxicity of cetuximab against tumor cells with wild-type or mutant epidermal growth factor receptor. Cancer Sci. 2007; 98:1275–1280.
28. Correale P, Botta C, Cusi MG, Del Vecchio MT, De Santi MM, Gori Savellini G, Bestoso E, Apollinari S, Mannucci S, Marra M, Abbruzzese A, Aquino A, Turriziani M, Bonmassar L, Caraglia M, Tagliaferri P. Cetuximab +/– chemotherapy enhances dendritic cell-mediated phagocytosis of colon cancer cells and ignites a highly efficient colon cancer antigen-specific cytotoxic T-cell response *in vitro*. Int J Cancer. 2012; 130:1577–1589.
29. Tanaka K, Arao T, Maegawa M, Matsumoto K, Kaneda H, Kudo K, Fujita Y, Yokote H, Yanagihara K, Yamada Y, Okamoto I, Nakagawa K, Nishio K. SRPX2 is overexpressed in gastric cancer and promotes cellular migration and adhesion. Int J Cancer. 2009; 124:1072–1080.
30. Yonesaka K, Zejnullahu K, Lindeman N, Homes AJ, Jackman DM, Zhao F, Rogers AM, Johnson BE, Janne PA. Autocrine production of amphiregulin predicts sensitivity to both gefitinib and cetuximab in EGFR wild-type cancers. Clin Cancer Res. 2008; 14:6963–6973.
31. Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham DA, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR, et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer. 2010; 102:1555–1577.

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REVIEW ARTICLE

非小細胞肺癌における抗 HER3 抗体 patritumab による EGFR 阻害剤耐性の克服について

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受付日
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Anti-HER3 Antibody Patritumab Overcomes Resistance to EGFR Inhibitor in Non-small Cell Lung Cancer

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ABSTRACT —— ***Objectives.*** In the present study, we elucidated the mechanism underlying EGFR inhibitor resistance and examined whether the anti-HER3 antibody patritumab can overcome this resistance. Furthermore, heregulin, a HER3 ligand, was assessed in regard to whether it could be used as a predictive biomarker of patritumab plus erlotinib therapy in patients with non-small cell lung cancer (NSCLC). ***Methods.*** The EGFR mutant NSCLC cell line PC9HRG with a high heregulin expression was used to evaluate the sensitivity of patritumab and erlotinib and the effects of these drugs on intracellular signaling. The heregulin expression was measured using tissue samples obtained from NSCLC patients treated with patritumab plus erlotinib or placebo plus erlotinib in a randomized phase II study (HERALD study). ***Results.*** PC9 cells were found to be sensitive to erlotinib, whereas PC9HRG cells were resistant to erlotinib, as HER3 was reactivated depending on the heregulin expression. The degree of susceptibility to patritumab also depended on the heregulin expression level, not the HER3 expression level, among 49 NSCLC cell lines. Resistance to erlotinib was overcome by the patritumab combination in PC9HRG cells. Therefore, patritumab plus erlotinib significantly prolonged the progression-free survival versus the placebo plus erlotinib in the NSCLC patients with a high heregulin expression in this randomized phase II clinical study. ***Conclusions.*** EGFR inhibitor resistance is induced by heregulin overexpression in NSCLC; however, this resistance is overcome by patritumab combination therapy.

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KEY WORDS —— Patritumab, Erlotinib, Heregulin, Non-small cell lung cancer, Human epidermal growth factor receptor

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要旨 —— **目的.** 非小細胞肺癌における EGFR 阻害剤の耐性機序を解明し，抗 HER3 抗体 patritumab（Pmab）と EGFR 阻害剤 erlotinib（Erl）の併用効果を確認する．そしてこの効果を予測するバイオマーカーとして HER3 受容体のリガンドであるヘレグリン（HRG）の有用性について評価する．**方法.** HRG 遺伝子を導入した EGFR 遺伝子変異型肺癌細胞株 PC9HRG を用いて Erl，Pmab 及び両薬剤の併用に対する感受性や細胞内シグナル伝達について評価する．非小細胞肺癌症例を対象とした Pmab と Erl 併用療法の第 II 相臨床試験において，HRG の発現を評価する．**結果.** Erl 感受性 PC9 細胞株は，HRG 遺伝子の導入によって同薬剤に対し耐性化し，これは HRG に依存した HER3 受容体の活性化によるものであった．そして PC9HRG 株は Pmab の併用によって Erl への耐性を克服された．第 II 相臨床試験の結果，HRG 高発現症例において Pmab と Erl の併用療法はプラセボと Erl の併用療法に比べ有意に無増悪生存期間を延長した．**結論.** 肺癌において HRG は EGFR 阻害剤への耐性化をもたらすが，この耐性は Pmab の併用療法により克服された．

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※第 55 回日本肺癌学会学術集会シンポジウム 7「分子標的治療の耐性克服～耐性メカニズム解明と治療の最前線～」

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索引用語——パトリツマブ，エルロチニブ，ヘレグリン，　非小細胞肺癌，上皮成長因子受容体

はじめに

進行期のEGFR遺伝子変異型非小細胞肺癌の治療では，EGFRチロシンキナーゼ阻害剤(EGFR-TKI)による薬物療法が標準治療である.[1-3] Gefitinibやerlotinibなどの EGFR-TKIの大規模な臨床試験によると，同薬剤はEGFR遺伝子変異型非小細胞肺癌症例に対しおよそ70%の奏効率，およそ10か月の無増悪生存期間をもたらした．これらの成績は，従来の標準治療である殺細胞性抗癌剤に比べ有意に優れたものである．しかしながらEGFR-TKIの継続投与によって，癌細胞はやがて同剤に対し耐性を獲得する(獲得耐性)．この獲得耐性は臨床上大きな問題となっており，今後克服しなければならない課題である．獲得耐性の機序には種々のものが報告されているが，未だ完全には解明されていない．我々は，EGFR-TKIへの耐性化をきたす機序の一つとして，HER3受容体のリガンドであるヘレグリンの過剰発現とそれに伴うHER3受容体の活性化を同定した.[4] またこの耐性の克服には抗HER3抗体であるpatritumabの併用療法が有用であることを前臨床試験から臨床試験を通じて確認した．本稿ではこれらについて紹介し，現在進行中の臨床試験についても言及したい．

HER3受容体の活性化によるEGFR-TKIへの耐性について

EGFR遺伝子変異型非小細胞肺癌におけるEGFR-TKIへの耐性化の機序は，これまでに複数報告されている．特に重要なものにEGFR遺伝子のエクソン20における二次的な変異T790Mが挙げられる.[5] 同遺伝子変異によりEGF受容体のチロシンキナーゼ部位は三次元構造の変化をきたし，ATPに対する親和性が高まり，結果として癌細胞はgefitinibなどのEGFR-TKIに耐性となる．現在T790M依存性EGFR-TKI耐性症例を対象とした第3世代のEGFR-TKI(AZD-9291，rociletinibなど)の臨床開発が進んでおり，優れた抗腫瘍効果を示している.[6,7] またこの他の耐性機序としてMET遺伝子増幅やHER2遺伝子増幅によるバイパスシグナルを介したものなどが報告されている.[8,9] 我々のグループはあらたなEGFR-TKIへの耐性化の機序として，EGF受容体と同じHERファミリーに属するHER3受容体のリガンドであるヘレグリンの過剰発現とそれに伴うHER3受容体の活性化を報告した.[4]

EGFR遺伝子変異型肺癌ではEGF受容体はHER3受容体と結合し，HER3受容体及びその下流シグナル(主にAKTタンパク)を強力に活性化することでアポトーシス抵抗性を保っている(Figure 1A).[8] そしてEGFR-TKIの投与下ではEGF受容体の不活性化に引き続き，HER3受容体，AKTの不活性化が起こるため癌細胞はアポトーシスをきたす(Figure 1B)．既報によるとEGFR-TKIに耐性である癌細胞の一部ではHER3受容体はEGFR非依存的に活性化されており，EGFR-TKI投与下でもアポトーシス抵抗性を保っている.[8,10] 従ってEGFR遺伝子変異型非小細胞肺癌では，HER3受容体はEGFR-TKIへの感受性あるいは耐性を規定する重要な役割を担っているといえる．

ヘレグリンはHER3受容体のリガンドであり，同受容体に結合するとその細胞外ドメインの三次元構造を変化させて受容体同士の二量体形成を促す．興味深いことに結腸・直腸癌における抗EGFR抗体cetuximab治療では，ヘレグリンの過剰発現症例は同薬剤に対し耐性であることが報告されていた.[11] すなわち結腸・直腸癌の腫瘍組織あるいは血清においてヘレグリンが高発現している症例では有意にcetuximab治療後の予後が不良であり，また同治療に耐性である症例では奏効例に比べ有意にヘレグリンの発現レベルが高かった．同様に今回の検討によって，EGFR遺伝子変異型非小細胞肺癌においてもヘレグリンがEGFR-TKIへの耐性に関与することが確認された.[4] すなわちEGFR-TKI感受性EGFR遺伝子変異型肺癌細胞株PC9にヘレグリン遺伝子を導入した株PC9HRGは，*in vitro*，*in vivo*のいずれの検討においてもEGFR-TKIであるerlotinibに対し耐性に変化した．また細胞内シグナル伝達の解析結果によるとヘレグリン導入株PC9HRGではerlotinibの曝露後3時間後までにはHER3受容体及びその下流のAKTの活性化は抑制されるが，3時間以降ではこれらは継時的に再活性化された.[4] そしてPC9HRG細胞株ではerlotinib曝露後，HER3受容体はHER2受容体と好んで結合することがわかった．これらの結果が示唆することは，ヘレグリン高発現癌細胞ではEGFR-TKIの曝露後，HER3受容体の活性化がEGFR依存性からヘレグリン(及びHER2受容体)依存性に移行することである(Figure 1C)．そしてこのヘレグリン(及びHER2受容体)依存的なHER3受容体の活性化によって，癌細胞はEGFR-TKIに対する耐性を獲得する．

実際にEGFR遺伝子変異型非小細胞肺癌のEGFR-TKI(gefitinib)治療例を対象にヘレグリンの発現を測定

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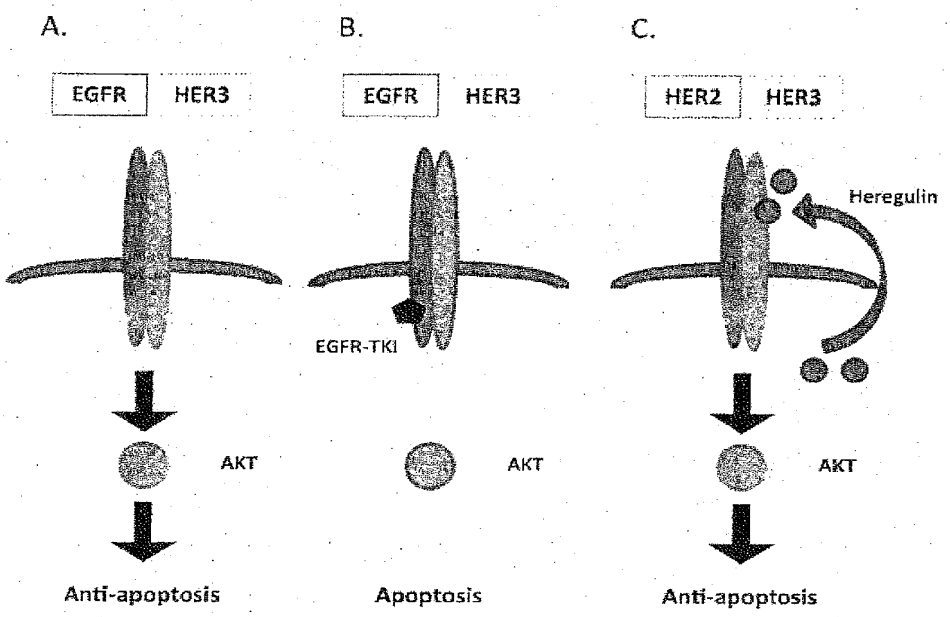

Figure 1. Mechanisms of EGFR-TKI resistance mediated by heregulin in EGFR mutant NSCLC. **A.** Intracellular EGFR-HER3-AKT signaling pathway under no treatment. **B.** Intracellular EGFR-HER3-AKT signaling pathway under EGFR-TKI treatment. **C.** Intracellular HER2-HER3-AKT signaling pathway in cancer cells with a high heregulin expression. EGFR-TKI: EGFR tyrosine kinase inhibitor.

した.[4] 7症例がgefitinib単剤により治療を行われ、いずれも奏効を達成したが、その後の継続投与によって同薬への耐性を獲得した. 耐性獲得後に腫瘍組織を採取し、治療前の組織とヘレグリンの発現量を定量的PCR法によって比較した. その結果, 7例中2例で有意に耐性獲得後にヘレグリンの発現が亢進していた. またこの2例について耐性後の腫瘍組織にEGFR遺伝子の二次変異T790MやHER2遺伝子増幅, MET遺伝子増幅といった既報の耐性因子がいずれも確認されず, ヘレグリンの発現亢進に依存したEGFR-TKIへの耐性であることが示唆された. また他のグループの報告においても, 非小細胞肺癌においてヘレグリンの高発現症例ではerlotinib治療に対し抵抗性であることが散見される.[12,13]

抗HER3抗体patritumabによるEGFR-TKIへの耐性克服治療とそのバイオマーカーとしてのヘレグリンについて

以上のようにヘレグリンによるHER3受容体の活性化は, EGFR遺伝子変異陽非小細胞肺癌におけるEGFR-TKIへの耐性に関与することが確認された. またこの他にHER3受容体の発現亢進は様々な癌種で予後不良因子となることが報告されている.[14,15] このためHER3受容体は悪性腫瘍の薬物療法の開発において重要な治療標的であると考えられ, 多くのHER3阻害剤が開発途上にある(Table 1). HER3受容体のユニークな特徴として他のHERファミリーの受容体と比べそのキナーゼ活性に乏しいため, HER3受容体の阻害剤はいずれもチロシンキナーゼ阻害剤でなく抗体薬である. これらの抗HER3抗体の一つであるpatritumabは, 完全ヒト化された抗体であり, HER3受容体の細胞外ドメインに結合しその作用を阻害する. 非小細胞肺癌細胞株(n＝49)を用いた同薬の*in vitro*での感受性試験では, 一部の細胞株に対し増殖抑制効果を認めた.[4] そしてこの感受性を規定する因子としてHER3発現量, (HER3リガンドの)ヘレグリン発現量, EGFR遺伝子変異の有無などが評価された. その結果, patritumabの直接の標的であるHER3受容体の発現量と同薬の細胞増殖抑制効果には有意な関連を認めず, むしろHER3受容体のリガンドであるヘレグリン発現量と効果は有意に相関した. そしてEGFR遺伝子変異型非小細胞肺癌株はいずれもpatrituniabに対し抵抗性であった. ヘレグリン高発現細胞株は*in vivo*のマウス移植モデルでの感受性試験においてもpatritumabに対し高い感受性を示し, 一方ヘレグリン低発現細胞株は同薬への感受性を示さなかった.[4] また細胞内シグナル伝達

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Table 1. Anti-HER3 Antibodies Under Research in Clinical Trials

Compound's name	Companies name	Clinical trial
Patritumab	Daiichi-Sankyo	Phase III
Duligotumab	Roche	Phase II
Seribantumab (MM-121)	Merrimack	Phase II
MM-111	Merrimack	Phase II
LJM716	Novartis	Phase I
RG-7116	Roche	Phase I
GSK2849330	GLaxoSmithKline	Phase I
REGN1400	Regeneration Pharmaceuticals	Phase I
AV-203	AVEO Pharmaceuticals	Phase I
KTN3379	Kolltan Pharmaceuticals	Phase I

の解析結果では，ヘレグリン高発現細胞株は，patritumab の曝露によって HER3 受容体及びその下流に存在する AKT の活性化が顕著に阻害された．一方ヘレグリン低発現細胞株では，patritumab 曝露後も HER3 受容体及び AKT の活性化の阻害は確認されなかった．以上の結果より，patritumab の効果予測バイオマーカーが HER3 受容体のリガンドであるヘレグリンであることが示唆された．

次に patritumab が EGFR-TKI への耐性克服において有用であるか前臨床試験にて確認した．[4] 前述のヘレグリン遺伝子導入 EGFR 遺伝子変異型肺癌細胞株 PC9HRG は，*in vitro* での細胞増殖抑制試験において erlotinib 単剤あるいは patritumab 単剤への治療に対し抵抗性であったが，両薬剤を併用することで強力に細胞増殖を抑制された．同様の結果が，*in vivo* のマウス移植モデルにおける感受性試験においても再現された（Figure 2）．[4] 一方，他の耐性機序を有する EGFR 遺伝子変異型肺癌細胞株，すなわち T790M 遺伝子変異を獲得した細胞株，あるいは MET 遺伝子増幅を獲得した細胞株は patritumab 単剤あるいは patritumab と erlotinib の併用療法に対し耐性であった．これらの結果は，patritumab がヘレグリン依存性の EGFR-TKI の耐性克服においてのみ有用であることを示唆している．

また細胞内シグナル伝達の解析では，ヘレグリン導入株 PC9HRG は erlotinib 単独曝露で曝露開始後 3 時間以降，HER3 受容体及びその下流の AKT の再活性化をきたしたが，erlotinib と patritumab を併用することで HER3 受容体及び AKT の再活性化は 24 時間以上にわたり抑制された．[4] また共免疫沈降法による検討の結果，PC9HRG 細胞では erlotinib 単剤治療によって HER3 受容体と HER2 受容体のヘテロ二量体の形成が促されたが，erlotinib と patritumab の併用治療では，このヘテロ二量体の形成は阻害された．[4]

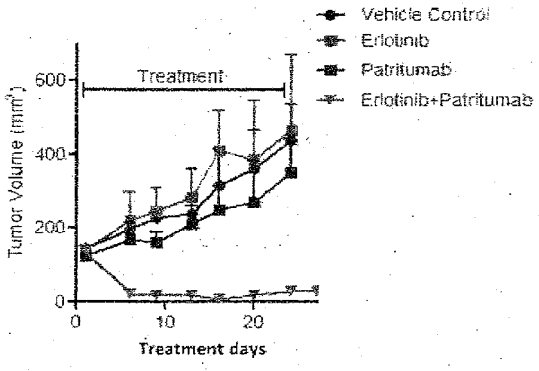

Figure 2. Heregulin-expressing EGFR mutant NSCLC cell line PC9HRG mouse xenograft model study evaluating the efficacy of erlotinib alone, patritumab alone and both drugs in combination. This figure is cited from reference 4.

以上をまとめるとヘレグリンを高発現している肺癌細胞（PC9HRG など）では，HER3 受容体はヘレグリン（及び HER2 受容体）に依存して活性化されており，EGFR-TKI 単独治療では HER3 受容体とその下流の AKT を阻害できない（Figure 3A）．一方，同細胞に EGFR-TKI と同時に抗 HER3 抗体 patritumab を投与すると，HER3 受容体はヘレグリン（及び HER2 受容体）に依存した活性化も阻害される（Figure 3B）．そしてこの両薬剤の併用は強力に AKT の活性化を阻害し，アポトーシスをきたす．

非小細胞肺癌症例を対象とした patritumab と erlotinib の併用療法の無作為化比較第 II 相臨床試験（HERALD 試験）とそのバイオマーカー解析について

以上の前臨床試験の結果，ヘレグリンの発現が patritumab の効果を予測するバイオマーカーであり，なおかつ patritumab はヘレグリン依存性の EGFR-TKI の耐性克服に有用であることが示唆された．これらについて臨床においても再現性が得られるか，ヘレグリンをあらかじめ定めたバイオマーカーとして erlotinib と patritumab の併用療法の臨床試験が行われた．[12] 試験の対象は，進行期非小細胞肺癌で EGFR-TKI を除く化学療法既治療症例で，登録適格基準を満たした症例は，以下の 3 群のいずれかの治療群に無作為に振り分けられた（Figure 4A）．すなわち高用量の patritumab（18 mg/kg）と erlotinib の併用療法群（高用量 patritumab 併用群），低用量の patritumab（9 mg/kg）と erlotinib の併用療法群（低用量 patritumab 併用群），そしてプラセボと erlotinib

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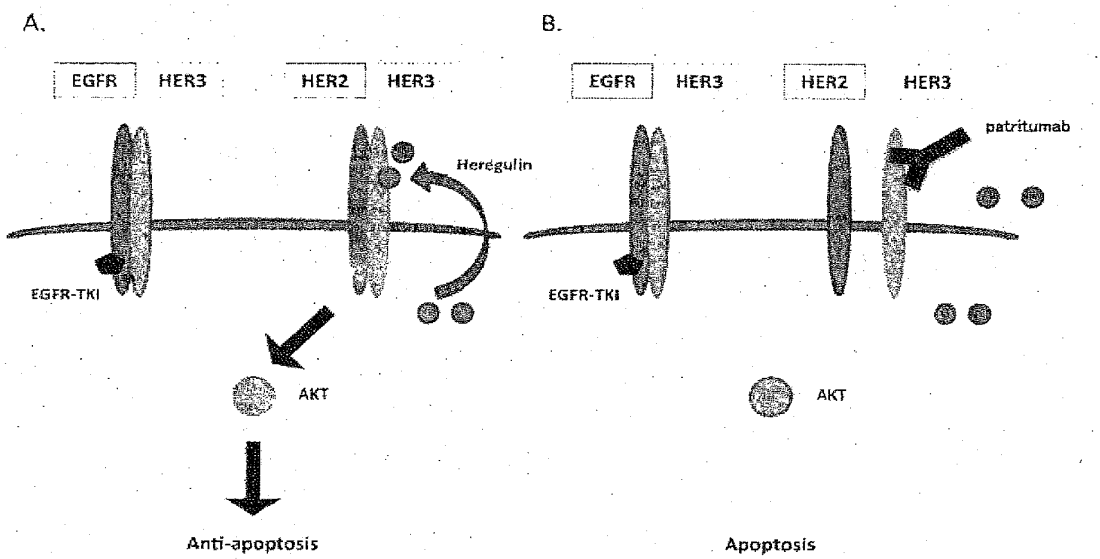

Figure 3. Anti-HER3 antibody patritumab in combination with EGFR-TKI alters the intracellular signaling pathway in heregulin-expressing EGFR mutant NSCLC. **A.** Intracellular signaling pathway in heregulin-expressing EGFR mutant NSCLC under erlotinib treatment. **B.** Intracellular signaling pathway in heregulin-expressing EGFR mutant NSCLC under patritumab combination treatment with erlotinib. EGFR-TKI, EGFR tyrosine kinase inhibitor.

の併用療法群（プラセボ併用群）である．いずれの群でもerlotinibは1日1回150 mgを経口投与し，patritumabあるいはプラセボを3週間隔で経静脈的に投与された．本試験の主要評価項目は無増悪生存期間と安全性であり，副次的にバイオマーカーについて評価された．バイオマーカーに関して，あらかじめ定めた定量的PCR法で腫瘍組織のヘレグリンmRNAの定量を行い，その発現量と治療効果との関係を評価された．215症例が欧米にて試験に登録の上，治療を施行された．

試験の結果，212症例がintent-to-treat（ITT）解析の対象となり（高用量patritumab併用群70名，低用量patritumab併用群71名，プラセボ併用群71名），各群間に患者背景の大きな偏りはみられなかった．[12] そしてこのITT解析対象例の3群間に有意な無増悪生存期間の差を認めなかった（高用量patritumab併用群vs低用量patritumab併用群vsプラセボ併用群，中央値1.4 vs 2.5 vs 1.6か月，ハザード比［95% 信頼区間］高用量patritumab併用群：0.98［0.67～1.42］，低用量patritumab併用群：0.77［0.52～1.13］）．同様に全生存期間についても3群間で有意な差を認めなかった（ハザード比［95% 信頼区間］高用量patritumab併用群：1.23［0.83～1.80］，低用量patritumab併用群：0.93［0.63～1.37］）．

次にバイオマーカー解析についてみると，腫瘍組織のヘレグリンmRNAの測定は215例中103例で可能であった．[12] ヘレグリンmRNA発現の分布は，正規分布パターンを示した（Figure 4B）．そしてヘレグリンmRNA発現のΔCT値の中央値（3.9）をカットオフ値として，カットオフ値以上をヘレグリン高発現症例（n＝51），カットオフ値以下をヘレグリン低発現症例（n＝50）と定めた．ヘレグリン高発現症例を対象として3群間の治療効果について比較検討したところ，高用量patritumab併用群，低用量patritumab併用群のいずれもプラセボ併用群に比べ有意に無増悪生存期間を改善した（ハザード比［95% 信頼区間］高用量patritumab併用群：0.37［0.16～0.85］，低用量patritumab併用群：0.29［0.13～0.66］，Figure 4C）．一方ヘレグリン低発現症例を対象とした3群間の検討では，patritumabの併用による無増悪生存期間の有意な延長はみられなかった．

以上の結果より前臨床試験で予測されたように，非小細胞肺癌症例においてヘレグリンmRNAはpatritumabとerlotinibの併用療法の効果予測因子であることが臨床的に確認された．一方HER3受容体の発現レベルとpatritumab併用療法の効果との関係も評価されたが，前臨床試験と同様に両者の間に有意な相関関係はみられなかった．[12]

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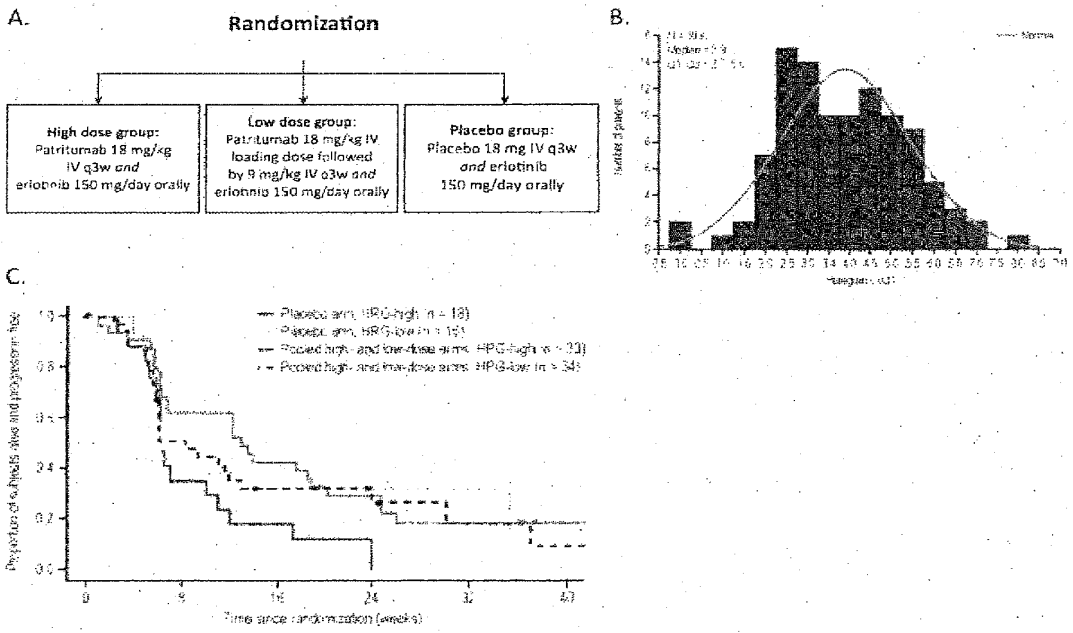

Figure 4. Phase II HERALD study of patritumab with erlotinib in advanced NSCLC subjects. A. Trial flow. B. Distribution of heregulin mRNA in the HERALD study. C. Kaplan-Meier survival curve for progression-free survival in the HRG-high and HRG-low patients in the placebo and patritumab arms. This figure is cited from reference 12. HRG: heregulin, ΔCt: median delta threshold cycle, Q: quartile.

ヘレグリン陽性非小細胞肺癌症例を対象としたpatritumabとerlotinibの併用療法の無作為化比較第III相臨床試験（HER3-Lung試験）について

以上のような非小細胞肺癌症例を対象としたpatritumabとerlotinibの併用療法の第II相臨床試験（HERALD試験）の結果に基づき，現在同治療法においては第III相臨床試験（HER3-Lung試験）にて生存期間の延長効果の検証がグローバルに進められている（Figure 5）．第II相臨床試験ではあらかじめヘレグリンmRNAの中央値をカットオフ値と定めて試験が行われたが，試験治療の効果を予測する最適なカットオフ値は異なる可能性が残る．そこで第III相試験は2-partに分かれており，Part Aではヘレグリン mRNAの発現レベルと治療効果の関係を追加で評価し，ヘレグリン mRNAの最適なカットオフ値が決定される．そしてPart Bではこのカットオフ値に基づきヘレグリン陽性例を選択し，patritumab（9 mg/kg）とerlotinibの併用治療とプラセボとerlotinibの併用治療の2群間で全生存期間の延長を検証する試験が行われる．

まとめ

肺癌治療は分子標的薬剤全盛の時代を迎え，有望な薬剤が多く見受けられる．同時にバイオマーカーを利用して治療効果を見込まれる症例を選別することが，効率的に薬剤の臨床開発を進める上で重要であり，何より効果に乏しい不要な治療を避けられることは個々の患者にとって大きな利点である．抗HER3抗体patritumabの開発においても，前臨床試験を通じて見出されたバイオマーカー・ヘレグリンの有用性が第II相臨床試験で確認されており，さらにヘレグリン陽性例に対象を絞った第III相臨床試験がグローバルで進行中である．同様に他の抗HER3抗体であるMM-121も非小細胞肺癌において臨床開発が進んでいる．興味深いことに非小細胞肺癌を対象としたMM-121とerlotinibの併用療法の第II相試験においてもヘレグリンmRNAは同治療法の効果予測因子であると明らかになり，現在MM-121の臨床開発もヘレグリン陽性症例を対象に進められている．[13] ヘレグリンをバイオマーカーとして利用することが，非小細胞肺癌においてさらなる個別化治療の進展につながると期待される．

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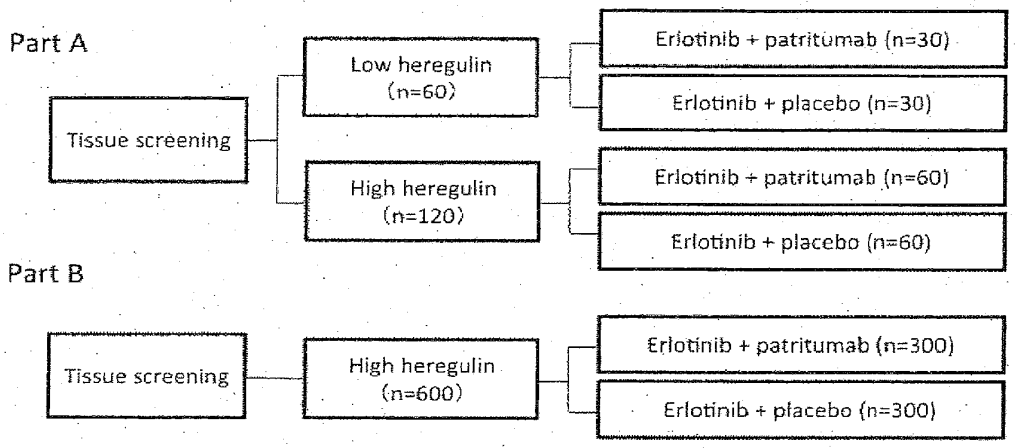

Figure 5. Study flow of patritumab combination therapy with erlotinib in a phase III clinical study of advanced NSCLC patients (HER3-Lung).

またEGFR遺伝子変異型肺癌の治療戦略，特にEGFR-TKIへの耐性獲得後の治療についても進歩しつつある．T790M陽性症例では第3世代EGFR-TKIが優れた抗腫瘍効果を示したが，他方T790M陰性例における治療戦略は定まっていない．自験例でgefitinibに対し耐性を獲得したEGFR遺伝子変異型非小細胞肺癌の一部の症例でヘレグリンが高発現しており，かつT790Mを含め他の耐性因子がみられなかった点は興味深く，今後EGFR-TKI耐性獲得後にも抗HER3抗体の有用性を検討する必要性を想起する．

本論文内容に関連する著者の利益相反：なし

REFERENCES

1. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med.* 2009;361:947-957.
2. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med.* 2010;362:2380-2388.
3. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol.* 2010;11:121-128.
4. Yonesaka K, Hirotani K, Kawakami H, Takeda M, Kaneda H, Sakai K, et al. Anti-HER3 monoclonal antibody patritumab sensitizes refractory non-small cell lung cancer to the epidermal growth factor receptor inhibitor erlotinib. *Oncogene.* 2015 [Epub ahead of print]
5. Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small cell lung cancer to gefitinib. *N Engl J Med.* 2005;352:786-792.
6. Jänne PA, Yang JC, Kim DW, Planchard D, Ohe Y, Ramalingam SS, et al. AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. *N Engl J Med.* 2015;372:1689-1699.
7. Sequist LV, Soria JC, Goldman JW, Wakelee HA, Gadgeel SM, Varga A, et al. Rociletinib in EGFR-mutated non-small-cell lung cancer. *N Engl J Med.* 2015;372:1700-1709.
8. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science.* 2007;316:1039-1043.
9. Takezawa K, Pirazzoli V, Arcila ME, Nebhan CA, Song X, de Stanchina E, et al. HER2 amplification: a potential mechanism of acquired resistance to EGFR inhibition in EGFR-mutant lung cancers that lack the second-site EGFRT790M mutation. *Cancer Discov.* 2012;2:922-933.
10. Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM, et al. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. *Nature.* 2007;445:437-441.
11. Yonesaka K, Zejnullahu K, Okamoto I, Satoh T, Cappuzzo F, Souglakos J, et al. Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. *Sci Transl Med.* 2011;3:99ra86.
12. Mendell J, Freeman DJ, Feng W, Hettmann T, Schneider M, Blum S, et al. Clinical translation and validation of a predictive biomarker for patritumab, an anti-human epidermal growth factor receptor 3 (HER3) monoclonal antibody, in patients with advanced non-small cell lung cancer. *EBioMedicine.* 2015;2:264-271.
13. Sequist LV, Lopez-Chavez A, Doebele RC, Gray JE, Harb WA, Modiano MR, et al. A randomized phase 2 trial of

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MM-121, a fully human monoclonal antibody targeting ErbB3, in combination with erlotinib in EGFR wild-type NSCLC patients. *J Clin Oncol.* 2014;32(Suppl):abstr 8051.
14. Yi ES, Harclerode D, Gondo M, Stephenson M, Brown RW, Younes M, et al. High c-erbB-3 protein expression is associated with shorter survival in advanced non-small cell lung carcinomas. *Mod Pathol.* 1997;10:142-148.
15. Cappuzzo F, Toschi L, Domenichini I, Bartolini S, Ceresoli GL, Rossi E, et al. HER3 genomic gain and sensitivity to gefitinib in advanced non-small-cell lung cancer patients. *Br J Cancer.* 2005;93:1334-1340.

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KOREANA SINCE 1970 PATENT FIRM

(Translation)

Issuance Date: May 1, 2018
Submission Due Date: July 1, 2018

THE KOREAN INTELLECTUAL PROPERTY OFFICE
NOTICE OF GROUNDS FOR REJECTION

Applicant : DAIICHI SANKYO COMPANY, LIMITED

Agent : Koreana Patent Firm

Application No. : Korean Patent Application No. 10-2016-7015961

Filing Date : June 15, 2016

Title of Invention: ANTI-HER2 ANTIBODY-DRUG CONJUGATE

This application shall be rejected on the following grounds pursuant to Article 63 of the Korean Patent Law. If you have any objection, please submit an Argument and/or an Amendment to the KIPO by **July 1, 2018**. (The term can be extended by one month each, but up to only four months. A request for an extension of 2 months or more (up to four months) may be filed, if necessary. If you wish to obtain an extension of time in excess of the allowed extension period (4 months) due to unavoidable circumstances (*refer to* the guidelines below), you would have to submit a Request for an Extension of the Time Limit to which a document explaining the reason therefor is attached.)

GROUNDS FOR REJECTION

[Examination Results]

□ Examined claims: Claims 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10
□ The parts having grounds for rejection and related laws:

No.	The parts having grounds for rejection	Related laws
1	Claims 1 and 4 to 10	Article 42(4)(ii) of the Patent Law
2	Claims 1 and 4 to 10	Article 42(4)(i) of the Patent Law
3	All claims	Article 29(2) of the Patent Law

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[Detailed grounds for rejection]

1. Claims 1 and 4 to 10 of the present application do not satisfy the description requirement of Article 42(4)(ii) of the Patent Law as indicated below, and thus this application cannot be patented.

-Below-

(1) **Claim 1** recites "anti-HER2 antibody" which is an unclear expression which is difficult to specify the structure, component, or sequence, etc. thereof.

(2) **Claims 6 and 9** claim drug or pharmaceutical composition, etc. but does not specify in detail the pharmaceutical use, and thus **claims 1 and 4 to 10** comprising said claims and claims referring thereto do not clearly describe the matter for which protection is sought.

2. Claims 1 and 4 to 10 of the present application do not satisfy the description requirement of Article 42(4)(i) of the Patent Law as indicated below, and thus this application cannot be patented.

-Below-

(1) **Claims 1, 4, and 5** linker and drug represented by the following formula, and the antibody-drug conjugate to which **anti-HER2 antibody** is conjugated, but reviewing the detailed description including the example, it only describes antibody-drug conjugate conjugated to mainly **trastuzumab** as an antibody and a preparation process thereof, and thus said claim is not supported by the detailed description.

(2) **Claims 6-10** claims a drug or pharmaceutical composition, etc., containing antibody-drug conjugate according to any one of claims 1 to 5, etc., but this includes the rejection ground substantially identical to the rejection ground identical to rejection ground 2-(1), and thus is not supported by the detailed description.

3. The invention recited in all claims of the present application can be easily invented by a person having ordinary skill in the art to which the present invention pertains as indicated below. Accordingly, this application cannot be patented pursuant to Article 29(2) of the Patent Law.

-Below-

[D1] **Korean Patent Laid-Open No. 10-2001-0052385 (June 25, 2001)**
[D2] **Korean Patent Laid-Open No. 10-2011-0044808 (April 29, 2011)**

(1) **Claim 1** relates to an antibody-drug conjugate, wherein a linker and a drug represented by the following formula and anti-HER2 antibody are connected: -(Succinimid-3-yl-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX). **D1** relates to a copolymer-linker-drug complex, **D2** relates to antibody-linker-drug complex, and the inventions can be compared as below.

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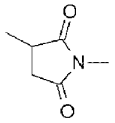
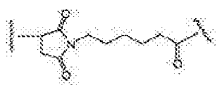
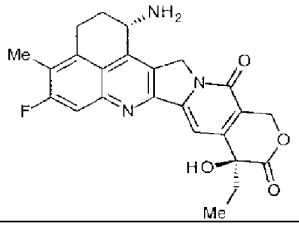
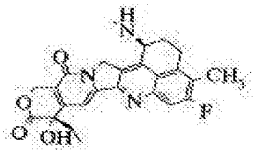
Gist of Technology	Claim 1	D1	D2	Comparison
Feature 1	 linker; ( )-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)	linker; GGFG-NH-p-C6H4-CH2-O-CO	linker;  -amino acid residues (see paragraph [0165], etc.)	Easily Derivable
Feature 2	drug (NH-DX) 	drug; DX-8951  (see page 9, etc.)	drug (see paragraph [0112], etc.)	Easily Derivable
Feature 3	anti-HER2 antibody	-	antibody	Easily Derivable
Feature 4	anti-HER2 antibody-linker-drug conjugate	copolymer-linker-drug conjugate	antibody-linker-drug conjugate	

Comparing claim 1 and D1, **D1** comprises amino acid residue (GGFG)-NH-p-C_6H_4-CH_2-O-CO as a linker, and there is a difference from claim 1 in that the linker includes -p-C_6H_4- and does not include an antibody. However, **D2** discloses comprising as a linker, and that the linker can include amino acid residues (see paragraphs [0164], [0165], etc.), and thus a skilled person can easily derive the linker of claim 1 by combining the linker comprising the amino acid residues of each of D1 and D2. Also, removing -p-C_6H_4- included in the linker of D1 or adding the antibody of D2 to the conjugate is a matter a skilled person can apply by selecting or altering as needed, and thus there is no particular difficulty in constitution.

Moreover, in view of the detailed description and Example of the present application, an antibody-drug conjugate combining a linker and a drug and mainly trastuzumab as antibody and a preparation process thereof are described, and thus, as to effect, it is not recognized that the conjugate of claim 1 has a remarkable effect which cannot be predicted by a skilled person from the combination of D1 and D2.

Thus, claim 1 can be easily invented by a skilled person from the combination of D1 and D2.

(2) **Claims 2 and 3** refer to claim 1 and describe the type of antibody and the amino acid sequence of the antibody, but **D2** discloses comprising anti-CD22 antibody (see claim 1, etc.). Since the type of antibody is a matter a skilled person can apply by selection as needed, there is no particular difficulty in constitution. Also, the amino acid sequence can also be easily derived by a skilled person according to the type of antibody selected.

Thus, claims 2 and 3 can be easily invented by a skilled person from the combination of D1 and D2.

(3) **Claims 4 and 5** refer to claim 1 and describe the average number of bonds per 1 antibody of the drug-linker structure, but this is a matter a skilled person can adjust by selecting or altering as needed, and thus there is no particular difficulty in constitution.

Thus, claims 4 and 5 can be easily invented by a skilled person from the combination of D1 and D2.

(4) **Claims 6 to 10** relate to a drug containing the antibody-drug conjugate according to any one of claims 1 to 9.

However, as can be seen in Rejection Grounds 3-(1) to 3-(3), the antibody-drug conjugate can be easily derived by a skilled person from the combination of D1 and D2.

Also, **D1** discloses a substantial pharmaceutical composition containing antitumor drug or anti-inflammatory drug, etc. (see Abstract, etc.), comprising antitumor drug as a pharmaceutical (see claim 9, etc.); and **D2** discloses a pharmaceutical composition comprising the conjugate and pharmaceutically acceptable carrier (see paragraph [0194], etc.).

Moreover, the types of cancers are described, but this is only listing the types of cancers widely known by the skilled person in the art, a skilled person could select the type of antitumor drug and further select the type of cancer to be treated, and thus there is no particular difficulty in constitution.

Thus, said claims can be easily invented by a skilled person from the combination of D1 and D2.

[Attachment]
Attached 1. A copy of Korean Patent Laid-Open No. 10-2001-0052385 (June 25, 2001)
Attached 2. A copy of Korean Patent Laid-Open No. 10-2011-0044808 (April 29, 2011)

Dated May 1, 2018

Pharmaceutical Examination Division
under Patent Examination Bureau 2
of Korean Intellectual Property Office

Examiner-in-charge Jae-Jeong LEE

KOREANA SINCE 1970 PATENT FIRM

[Guidelines]

1. Please note that in the case of a patent application filed on or after July 1, 2013, if more than one Amendments are submitted in response to a Notice of Preliminary Rejection within the time period for responding to the Notice, the Amendments except for the last Amendment are deemed withdrawn (Article 47(4) of the amended Patent Law proclaimed on March 22, 2013).

2. Guidelines on an extension of the designated time limit

If a Request for an Extension of the Time Limit to which an explanation document is attached is submitted in order to obtain an extension of time in excess of the allowed extension period (4 months), the examiner will determine whether the explanation falls within the following cases, and determine whether to grant an extension of time and how long the time limit can be extended, and then notify the applicant.

The cases where a further extension of time is allowed are as follows:

1) Where, within one month before expiry of the time limit, an agent has been appointed for the first time or all the designated agents have been dismissed or changed;
2) Where, within one month before expiry of the time limit, a Notification of Change of Applicant has been submitted;
3) Where, within two months before expiry of the time limit, an examination result has been issued from a foreign Patent Office, and the applicant wishes to reflect the examination result on an Amendment (In this case, a copy of the Notice announcing the examination result and a copy of the claims on which the examination result is based should be submitted when submitting a Request for an Extension of the Time Limit);
4) Where transmission of the Notice of Preliminary Rejection has been delayed for more than one month (In this case, further one month extension is allowed);
5) Where a parent application or divisional application is pending in the Intellectual Property Trial and Appeal Board or Patent Court;
6) Where additional time is required for an experiment and measurement related to grounds of rejection; and
7) Where the examiner considers that a further extension of time is necessary due to a reason beyond the applicant's responsibility and the like.

However, where a Request for Examination has been filed by a third party, a further extension of time is not allowed for the cases 1~5 above.

3. Guidelines on the preliminary review of a draft Amendment

If the applicant submits a draft Amendment prior to one month before the expiry of the time period for filing an Amendment and requests a review of the draft Amendment, the applicant may receive the examiner's preliminary review of the draft Amendment, including the examiner's opinion on whether the rejection grounds could be overcome, through an in-person interview. In this regard, please be advised that a draft Amendment must be submitted in the form of an Amendment or Argument before filing of a Request for Preliminary Review of a Draft Amendment.

10-2016-7015961

발송번호: 9-5-2018-029531397
발송일자: 2018.05.01.
제출기일: 2018.07.01.

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06132

YOUR INVENTION PARTNER

특 허 청

의견제출통지서

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발명자 성명 오기타니 유스케
주소 일본 도쿄도 시나가와쿠 히로마치 1쵸메 2방 58고 다이이찌 산쿄 가부시키가이샤 나이
발명자 성명 마스다 다케시
주소 일본 도쿄도 시나가와쿠 히로마치 1쵸메 2방 58고 다이이찌 산쿄 가부시키가이샤 나이
발명자 성명 나카다 다카시
주소 일본 도쿄도 시나가와쿠 히로마치 1쵸메 2방 58고 다이이찌 산쿄 가부시키가이샤 나이
발명자 성명 요시다 마사오
주소 일본 도쿄도 시나가와쿠 히로마치 1쵸메 2방 58고 다이이찌 산쿄 가부시키가이샤 나이
발명자 성명 아시다 신지
주소 일본 도쿄도 시나가와쿠 히로마치 1쵸메 2방 58고 다이이찌 산쿄 가부시키가이샤 나이
발명자 성명 모리타 고지
주소 일본 도쿄도 시나가와쿠 히로마치 1쵸메 2방 58고 다이이찌 산쿄 가부시키가이샤 나이
발명자 성명 미야자키 히데키
주소 일본 도쿄도 시나가와쿠 히로마치 1쵸메 2방 58고 다이이찌 산쿄 가부시키가이샤 나이
발명자 성명 가스야 유지
주소 일본 도쿄도 시나가와쿠 히로마치 1쵸메 2방 58고 다이이찌 산쿄 가부시키가이샤 나이
발명자 성명 하야카와 이치로

주 소 일본 도쿄도 시나가와쿠 히로마치 1쵸메 2방 58고 다이이찌 산쿄 가부시키가이샤 나이
발 명 자 성 명 아베 유키
주 소 일본 도쿄도 시나가와쿠 히로마치 1쵸메 2방 58고 다이이찌 산쿄 가부시키가이샤 나이

출 원 번 호 10-2016-7015961
출 원 일 자 2016.06.15.
발 명 의 명 칭 항-HER2 항체-약물 접합체

1. 이 출원에 대한 심사결과 다음과 같은 거절이유가 있어 특허법 제63조에 따라 이를 통지하오니 의견이 있거나 보정이 필요할 경우에는 상기 제출기일(2018.07.01.)까지 의견(답변, 소명)서[특허법시행규칙 별지 제24호서식] 또는/및 보정서[특허법시행규칙 별지 제9호서식]를 제출하여 주시기 바랍니다.

2. 상기 제출기일(2018.07.01.)을 연장하려는 경우에는 지정기간연장신청을 통해 그 제출기일을 4개월까지 연장할 수 있습니다. 이 경우 연장신청은 1개월 단위로 해야 하며, 필요 시 4개월을 초과하지 않는 범위에서 2개월 이상을 일괄하여 연장신청할 수 있습니다. 불가피한 사유의 발생(하단의 안내참조)으로 4개월을 초과하여 지정기간을 연장받고자 하는 때에는 그 사유를 기재한 소명서를 추가로 첨부해서 연장신청을 해야 합니다.

[심사결과]

□ 심사 대상 청구항 : 제1, 2, 3, 4, 5, 6, 7, 8, 9, 10항

□ 이 출원의 거절이유가 있는 부분과 관련 법조항

순번	거절이유가 있는 부분	관련 법조항
1	청구항 제1항, 제4항 내지 제10항	특허법 제42조제4항제2호
2	청구항 제1항, 제4항 내지 제10항	특허법 제42조제4항제1호
3	청구항 전항	특허법 제29조제2항

[구체적인 거절이유]

1. 이 출원은 청구범위의 청구항 제1항, 제4항 내지 제10항의 기재가 아래에 지적한 바와 같이 불비하여 특허법 제42조제4항제2호에 따른 요건을 충족하지 못하므로 특허를 받을 수 없습니다.

- 아 래 -

(1) **청구항** 1에는 <항 HER2 항체>라는 그 구조나 성분, 또는 서열 등을 특정하기 곤란한 불명확한 표현이 기재되어 있으며,

(2) **청구항 6, 9**는 ----- 약물 또는 ----- 약학 조성물 등을 청구하면서 그 의약 용도를 구체적으로 특정하지 않고 있는바,

상기항 및 이를 인용하고 있는 항들을 포함하는 **본원발명의 청구항 1, 4-10**은 발명의 보호받고자 하는 사항이 불명확하게 기재된 것입니다.

2. 이 출원은 청구범위의 청구항 제1항, 제4항 내지 제10항의 기재가 아래에 지적한 바와 같이 불비하여 특허법 제42조제4항제1호에 따른 요건을 충족하지 못하므로 특허를 받을 수 없습니다.

- 아 래 -

(1) **청구항 1, 4, 5 발명**은 다음 식으로 나타내는 링커와 약물과, 항 HER2 항체가 결합한 항체-약물 접합체를 청구하고 있으나,

실시예를 비롯한 본원 발명의 상세한 설명을 살펴보면, 단지 항체로써 주로 트라스트주마브에 결합시킨 항체-약물 접합체 및 이의 제조방법만이 기재되어 있어, 상기항은 발명의 상세한 설명에 의해 뒷받침되지 않습니다.

(2) **청구항 6-10 발명**은 제1항 내지 제5항 중 어느 한 항에 따른 항체-약물 접합체 등을 함유하는 약물 또는 약학 조성물 등을 청구하고 있으나,

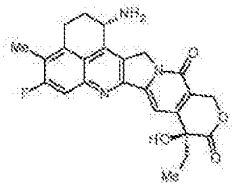
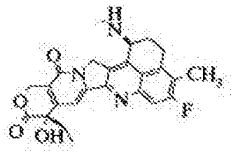
이는 실질적으로 상기 거절이유 2-(1)과 동일한 거절이유를 포함하고 있는 것으로 상기항은 발명의 상세한 설명에 의해 뒷받침되지 않습니다.

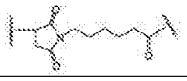
3. 이 출원의 청구범위의 청구항 전항에 기재된 발명은 아래와 같이 그 출원 전에 이 발명이 속하는 기술분야에서 통상의 지식을 가진 사람이 쉽게 발명할 수 있는 것이므로 특허법 제29조제2항에 따라 특허를 받을 수 없습니다.

- 아 래 -

[인용발명 1] 공개특허공보 제10-2001-0052385호 (2001.06.25.)
[인용발명 2] 공개특허공보 제10-2011-0044808호 (2011.04.29.)

(1) **제1항 발명**은 다음 식으로 나타내는 링커 및 약물과, 항 HER2 항체가 결합한 항체-약물 접합체: -(숙신이미드-3-일-N)-$CH_2CH_2CH_2CH_2CH_2$-C(=O)-GGFG-NH-CH_2-O-CH_2-C(=O)-(NH-DX) 에 관한 것이고,
인용발명 1은 고분자-링커-약물 복합체에 관한 것이며, **인용발명 2**는 항체-링커-약물 복합체에 관한 것으로 각 발명을 대비하면 하기와 같습니다.

기술 요지	제1항	인용발명 1	인용발명 2	대비
구성1	링커 ;( )-$CH_2CH_2CH_2CH_2CH_2$ -C(=O)-GGFG-NH-CH_2-O-CH_2 -C(=O)	링커 :GGFG-NH-p-C_6H_4-CH_2-O -CO	링커 ; -아미노산 잔기 (단락 [0165] 등 참조)	용이 도출
구성2	약물 (NH-DX) 	약물 ;DX-8951  (페이지 9 등 참조)	약물 (단락 [0112] 등 참조)	용이 도출
구성3	항 HER2 항체	-	항체	용이 도출
구성4	항 HER2 항체-링커-약물 접합체	고분자-링커-약물 접합체	항체-링커-약물 접합체	

제1항과 인용발명 1을 비교하여 보면, **인용발명 1**은 링커로써 아미노산 잔기(GGFG)-NH-p-C_6H_4-CH_2-O-CO를 포함하고, 상기 링커에 -p-C_6H_4- 를 포함한다는 점 및 항체를 포함하지 않는다는 점에서 제1항과 차이가 있으나, **인용발명 2**에 링커로써  을 포함하고, 상기 링커가 아미노산 잔기를 포함할 수 있다고 기재(단락 [0164], [0165] 등 참조)되어 있어,

통상의 기술자라면 인용발명 1 및 2로부터 각각의 아미노산 잔기를 포함하는 링커를 조합하여 제1항의 링커를 용이하게 도출할 수 있을 것이고, 인용발명 1의 링커에 포함된 -p-C_6H_4- 를 제거하거나, 상기 콘주게이트에 인용발명 2의 항체를 추가하는 것은, 통상의 기술자가 필요에 따라 선택·변경하여 적용해 볼 수 있는 사항이므로, 이에 각별한 구성의 곤란성이 있다고 인정되지 않습니다.

아울러 본원의 상세한 설명 및 실시예를 살펴보아도 링커와 약물 및 이를 항체로써 주로 트라스트주마브에 결합시킨 항체-약물 접합체 및 이의 제조방법만이 기재되어 있어, 효과에 있어서 제1항 발명의 접합체가 통상의 기술자가 인용발명 1 및 2의 결합으로부터 예측할 수 없는 현저한 효과가 있다고 인정되지 않습니다.

따라서 제1항 발명은 통상의 기술자가 인용발명 1 및 2의 결합으로부터 쉽게 발명할 수 있는 것입니다.

(2) **제2항 및 제3항 발명**은 제1항을 인용하면서 항체의 종류 및 상기 항체의 아미노산 배열이 기재되어 있으나, **인용발명 2**에 항-CD22 항체를 포함한다고 기재(청구항 1 등 참조)되어 있고, 상기 항체의 종류는 통상의 기술자가 필요에 따라 선택하여 적용해 볼 수 있는 사항이므로, 이에 각별한 구성의 곤란성이 있다고 인정되지 않고, 상기 아미노산 배열 또한 선택된 항체의 종류에 따라 통상의 기술자가 용이하게 도출할 수 있을 것입니다.

따라서 제2항 및 제3항 발명은 통상의 기술자가 인용발명 1 및 2의 결합으로부터 쉽게 발명할 수 있는 것입니다.

(3) **제4항 및 제5항 발명**은 제1항을 인용하면서 상기 약물-링커 구조의 1 항체 당의 평균 접합수가 기재되어 있으나, 이는 통상의 기술자가 필요에 따라 선택·변경하여 조절해 볼 수 있는 사항이므로 이에 각별한 구성의 곤란성이 있다고 인정되지 않습니다.

따라서 제4항 및 제5항 발명은 통상의 기술자가 인용발명 1 및 2의 결합으로부터 쉽게 발명할 수 있는 것입니다.

(4) **제6항 내지 제10항 발명**은 제1항 내지 제9항 중 어느 한 항에 따른 항체-약물 접합체를 함유하는 의약 등에 관한 것입니다.

그러나 상기 거절이유 3-(1) 내지 3-(3)에서 살핀 바와 같이, 상기의 항체-약물 접합체는 통상의 기술자가 인용발명 1 및 2의 결합으로부터 쉽게 도출할 수 있고,

인용발명 1에 항종양제나 항염증제 등을 함유하는 실질적인 약학 조성물이 기재(요약 등 참조), 항종양제를 약물로 포함한다고 기재(청구항 9 등 참조)되어 있으며,
인용발명 2에 상기 콘주게이트 및 제약상 허용되는 담체를 포함하는 제약 조성물이 기재(단락 [0194] 등 참조)되어 있으며,

아울러 암의 종류가 기재되어 있으나 이는 통상의 기술자가 해당 기술분야에서 널리 알려진 암의 종류를 나열해 본 정도이고, 통상의 기술자라면 상기 항종양제 약물의 종류를 선택하여 치료하고자 하는 암의 종류 또한 선택해 볼 수 있을 것이므로 이에 각별한 구성의 곤란성이 있다고 인정되지 않습니다.

따라서 상기항 발명은 통상의 기술자가 인용발명 1 및 2의 결합으로부터 쉽게 발명할 수 있는 것입니다.

[첨 부]
첨부1 공개특허공보 제10-2001-0052385호(2001.06.25.) 1부.
첨부2 공개특허공보 제10-2011-0044808호(2011.04.29.) 1부. 끝.

10-2016-7015961

2018.05.01.

특허청 특허심사2국

약품화학심사과 심사관 이재정 이재정

<< 안내 >>

1. 특허법 제47조제2항에 따라 특허출원서에 최초로 첨부된 명세서 또는 도면에 기재된 사항의 범위 안에서 명세서 또는 도면을 보정할 수 있음을 알려드립니다.

2. 출원일이 2013년 7월 1일 이후인 출원에 대해서는, 하나의 의견제출통지에 따른 지정기간 내에 보정서를 2회 이상 제출한 경우에는, 마지막 보정 이외의 모든 보정은 취하간주되오니 주의하시기 바랍니다.

3. 보정료 납부안내
 o 명세서 또는 도면을 보정하기 위하여 명세서등 보정서를 전자문서로 제출할 경우 매건 4,000원, 서면으로 제출할 경우 매건 14,000원의 보정료를 납부하여야 합니다.
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ORIGINAL ARTICLE

HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion

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The cancer stem cell hypothesis proposes that cancers arise in stem/progenitor cells through disregulation of self-renewal pathways generating tumors, which are driven by a component of 'tumor-initiating cells' retaining stem cell properties. The *HER2* gene is amplified in 20–30% of human breast cancers and has been implicated in mammary tumorigenesis as well as in mediating aggressive tumor growth and metastasis. We demonstrate that HER2 overexpression drives mammary carcinogenesis, tumor growth and invasion through its effects on normal and malignant mammary stem cells. HER2 overexpression in normal mammary epithelial cells (NMEC) increases the proportion of stem/progenitor cells as demonstrated by *in vitro* mammosphere assays and the expression of stem cell marker aldehyde dehydrogenase (ALDH) as well as by generation of hyperplastic lesions in humanized fat pads of NOD (nucleotide-binding oligomerization domain)/SCID (severe combined immunodeficient) mice. Overexpression of HER2 in a series of breast carcinoma cell lines increases the ALDH-expressing 'cancer stem cell' population which displays increased expression of stem cell regulatory genes, increased invasion *in vitro* and increased tumorigenesis in NOD/SCID mice. The effects of HER2 overexpression on breast cancer stem cells are blocked by trastuzumab in sensitive, but not resistant, cell lines, an effect mediated by the PI3-kinase/Akt pathway. These studies provide support for the cancer stem cell hypothesis by suggesting that the effects of HER2 amplification on carcinogenesis, tumorigenesis and invasion may be due to its effects on normal and malignant mammary stem/progenitor cells. Furthermore, the clinical efficacy of trastuzumab may relate to its ability to target the cancer stem cell population in HER2-amplified tumors.
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Introduction

The *HER2* gene is amplified in 20–30% of human breast cancers and is associated with aggressive metastatic disease (Slamon *et al.*, 1987, 1989). Although a number of signaling pathways including PI3-kinase (PI3-K)/Akt have been shown to be regulated by HER2, the mechanisms by which these pathways contribute to the aggressive characteristics of these tumors remain unclear. The humanized anti-HER2 antibody trastuzumab (Herceptin) has been an important agent used to treat breast cancers displaying HER2 overexpression (Beuzeboc *et al.*, 1999). Despite its benefits, a substantial fraction of patients treated in the adjuvant setting still relapse, one-third of patients with advanced disease fail to respond and the majority of initial responders demonstrate disease progression within 1 year (Seidman *et al.*, 2001; Miller, 2004). The development of effective strategies for treating these trastuzumab-resistant tumors would be facilitated by a clearer understanding of the mechanisms by which HER2 overexpression induces aggressive behavior in breast cancer and the molecular mechanisms of trastuzumab resistance.

There is increasing evidence that a wide variety of malignancies, including breast cancer, may be driven by a small subset of 'tumor-initiating cells' or 'cancer stem cells' (CSCs) that display stem cell properties. Our group and others have described a subpopulation of cells in human mammary carcinomas, with the phenotype CD44+/CD24−/lin− that display 'CSC' properties (Al-Hajj *et al.*, 2003). These cells comprising 1–5% of primary tumors, are able to form tumors in immunocompromised mice as well as to generate the phenotypic heterogeneity of the initial tumor. More recently, we have reported that the stem/progenitor cell population in both the normal mammary gland and in mammary carcinomas may also be identified by virtue of increased expression of the enzyme aldehyde dehydrogenase (ALDH) as assessed by the Aldefluor assay or by immunohistochemistry (Ginestier *et al.*, 2007).

In addition to driving carcinogenesis, CSCs may contribute to therapeutic resistance. Indeed, it has recently been reported that CD44+/CD24− mammary stem cells are relatively resistant to radiation and chemotherapy *in vitro* and in mouse models (Phillips *et al.*, 2006). The clinical relevance of these observations was confirmed by the demonstration of an increase in the CSC population following neoadjuvant

chemotherapy in patients with locally advanced breast cancer (Li *et al*., 2008).

We have recently reported a significant correlation between HER2 overexpression and expression of the stem cell marker ALDH-1 in a series of 491 breast cancer patients (Ginestier *et al*., 2007). Since tumorigenesis, invasion and metastasis may be mediated by the tumor stem cell component we reasoned that the effects of HER2 overexpression on tumorigenesis might be due to the effects of this signaling pathway on regulating the mammary stem cell population. We report that HER2 overexpression increases the stem/progenitor cell population of both normal and malignant mammary cells. Furthermore, the effects of HER2 overexpression on mammary tumorigenesis and invasion are due to its effects on the CSC population. These effects correlate with increased HER2 and Akt phosphorylation and are inhibited by trastuzumab in sensitive but not in resistant cells. These studies indicate that HER2 signaling regulates the mammary stem/progenitor cell population driving carcinogenesis and tumor invasion.

Results

Effects of HER2 overexpression on normal mammary stem/progenitor cells

We have previously demonstrated that normal human mammary cells generate nonadherent spherical colonies called mammospheres (Dontu *et al*., 2003). These mammospheres are composed of a small number of self-renewing cells capable of mammosphere initiation upon serial passage, as well as a larger number of progenitor cells capable of multilineage differentiation (Dontu *et al*., 2003). To examine the role of HER2 in mammary stem cell self-renewal, we infected normal mammary epithelial cells (NMEC) with lentiviruses expressing either DsRed-HER2 or DsRed. Elevated HER2 protein levels in the HER2 lentivirus-infected compared to DsRed-infected cells was confirmed by western blotting (Figure 1a). HER2 lentivirus-infected cells showed over 90% DsRed-positive cells when analysed by flow cytometry (Supplementary Figure 1A). We have also analysed HER2 surface expression in both noninfected and HER2-infected NMECs by flow cytometry. Over 90% of HER2-infected cells displayed HER2 surface expression as compared to less than 4% of noninfected NMECs (Supplementary Figure 1B).

When DsRed or HER2-infected cells were cultured for 7 days in suspension, both the number and size of mammospheres significantly increased in HER2-expressing NMEC as compared to the DsRed controls (Figure 1a). We have previously demonstrated that the number of mammospheres generated upon serial passage provides an indirect measure of mammary stem cell self-renewal, whereas mammosphere size reflects progenitor cell proliferation. We therefore examined the ability of primary mammospheres from both DsRed and HER2-expressing cells to form secondary and tertiary mammospheres. The number of secondary and tertiary mammospheres generated from the HER2-expressing cells was significantly higher than that of the control cells (Figure 1b).

HER2 overexpression expands the Aldefluor-positive cell population of normal mammary epithelial cells in vitro and increases outgrowths in NOD/SCID mice

We previously demonstrated that NMECs that express ALDH as determined by the Aldefluor assay display characteristics of stem/progenitor cells (Ginestier *et al*., 2007). Figure 1c shows overexpression of HER2 in NMECs increased the Aldefluor-positive population more than twofold compared to DsRed-infected cells. The effects of HER2 overexpression on mammosphere formation and ALDH activity may reflect direct effects of HER2 signaling on these properties. Alternatively, HER2 overexpression may affect stem cell self-renewal producing an increase in the stem cell population. In order to distinguish between these possibilities, we examined the mammosphere-forming capacity of the Aldefluor-positive and Aldefluor-negative cells overexpressing HER2. Only the Aldefluor-positive population was capable of mammosphere formation in HER2-expressing cells (Figure 2a). We also demonstrated that only a small fraction of NMECs express HER2 surface protein (Supplementary Figure 1B) Furthermore, we observed similar HER2 surface expression in Aldefluor-positive and Aldefluor-negative NMECs (Supplementary Figure 1C). This suggests that the effects of HER2 overexpression are due to the generation of increased proportion of primitive mammary cells capable of mammosphere formation.

To determine whether HER2 overexpression increased the mammosphere-forming capacity of the Aldefluor-positive population, we sorted single Aldefluor-positive cells expressing HER2 or DsRed in low-attachment plates and examined the frequency of mammosphere formation. Figure 2b demonstrates a threefold increase in the frequency of single HER2-expressing cells able to form mammospheres as compared to single DsRed cells. Together, these experiments suggest that HER2 overexpression increases the mammary stem cell population and also increases the capacity for sphere formation in this population.

We previously demonstrated that Aldefluor-positive normal human mammary cells have the capacity to generate outgrowths in the humanized mammary fat pads of NOD (nucleotide-binding oligomerization domain)/SCID (severe combined immunodeficient) mice (Ginestier *et al*., 2007). We determined the effect of HER2 overexpression on mammary outgrowths in this model. Outgrowths generated from both control and HER2-overexpressing cells were composed of luminal and myoepithelial cells (Figure 2c). The myoepithelial cell layer was demonstrated by smooth muscle actin staining (Figure 2c). In addition, there were areas of hyperplasia characterized by greater than an eightfold increase in expression of the proliferation marker KI-67 in outgrowths generated from HER2-infected compared

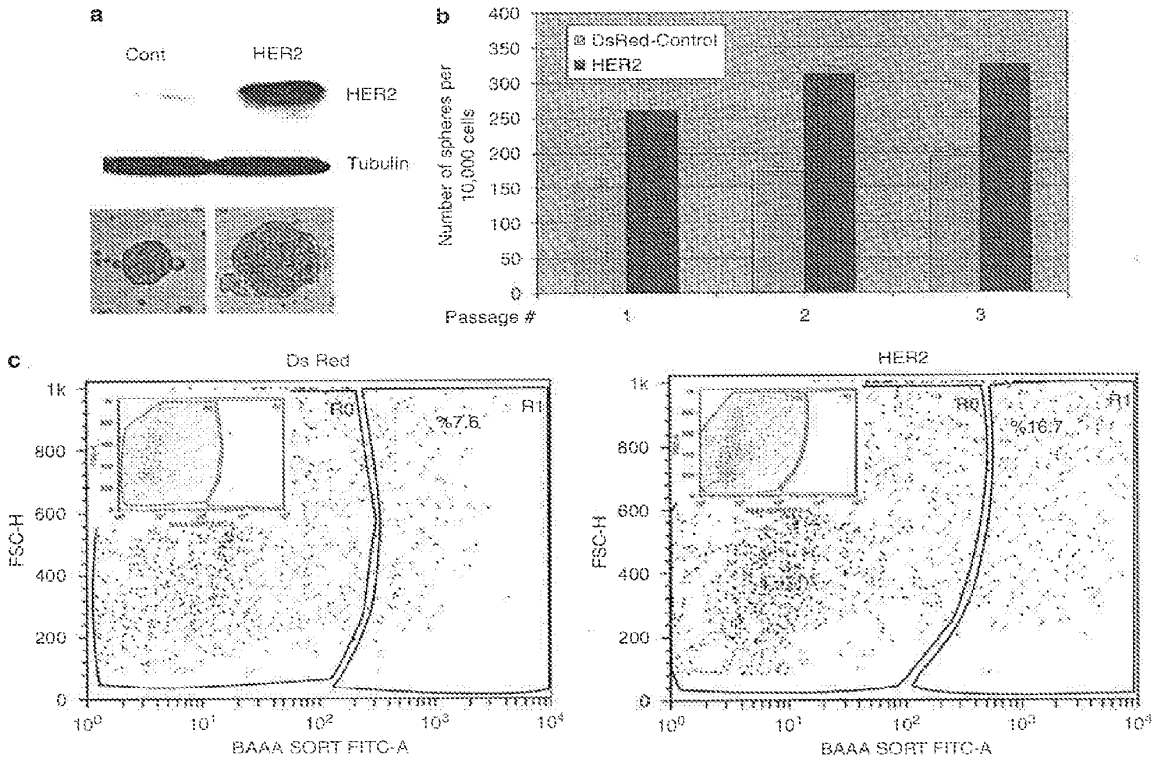

Figure 1 HER2 expression increases mammosphere formation and Aldefluor-positive cell population (a) HER2 expression in normal mammary epithelial cells infected with HER2 or DsRed lentivirus. Increased size of mammospheres in HER2-expressing cells as compared to DsRed control. (b) Serial passage of HER2 and DsRed control mammospheres. HER2 expression increases mammosphere-forming capacity upon serial passage. *P*-values for each passage are <0.01. (c) HER2 or DsRed cells were assessed for aldehyde dehydrogenase (ALDH) activity utilizing the Aldefluor assay. HER2-expressing cells demonstrate greater than a twofold increase in the Aldefluor-positive cell population compared to DsRed control cells. Insert shows diethylaminobenzaldehyde (DEAB)-inhibited flow results for compensation. Data represented here are the mean of five independent experiments performed in triplicates.

to DsRed cells (Figure 2c). We also tested the ability of Aldefluor-positive and Aldefluor-negative cells overexpressing HER2 or DsRed to form mammary outgrowths. Consistent with our previous report, only Aldefluor-positive cells produced outgrowths in this model (Table 1). Furthermore, HER2-overexpressing Aldefluor-positive cells produced outgrowths with as few as 250 cells (Table 1). These results are consistent with the *in vitro* experiments demonstrating that HER2 overexpression increases the stem cell pool as well as driving proliferation of this pool *in vivo*.

HER2 overexpression increases tumorigenicity of mammary carcinomas by modulating the stem/progenitor cell population

In order to determine the effect of HER2 overexpression on the CSC population, we stably infected three mammary carcinoma cell lines: MCF7, SUM149 and SUM159 with HER2 or DsRed lentiviruses. The efficiency of infection was demonstrated by western blotting (Supplementary Figure 2A, B and C). HER2 overexpression resulted in a four to fivefold increase in the Aldefluor-positive population in each of these three mammary carcinoma cell lines (Figure 3a; Supplementary Figure 2A, B and C).

To further examine whether continued HER2 expression was required for maintenance of the stem cell phenotype, we tested the effect of knockdown of HER2 by using a HER2 shRNA in HCC1954 cells, a cell line which displays HER2 amplification (Finn *et al*., 2007). We achieved approximately an 80% reduction in HER2 expression as assessed by western blot utilizing this approach (Supplementary Figure 2D). Control shRNA-transfected cells contained an Aldefluor-positive population of 6–10%, whereas knockdown of HER2 expression reduced the Aldefluor-positive population to less than 2% (Figure 3a). These results complement the gain

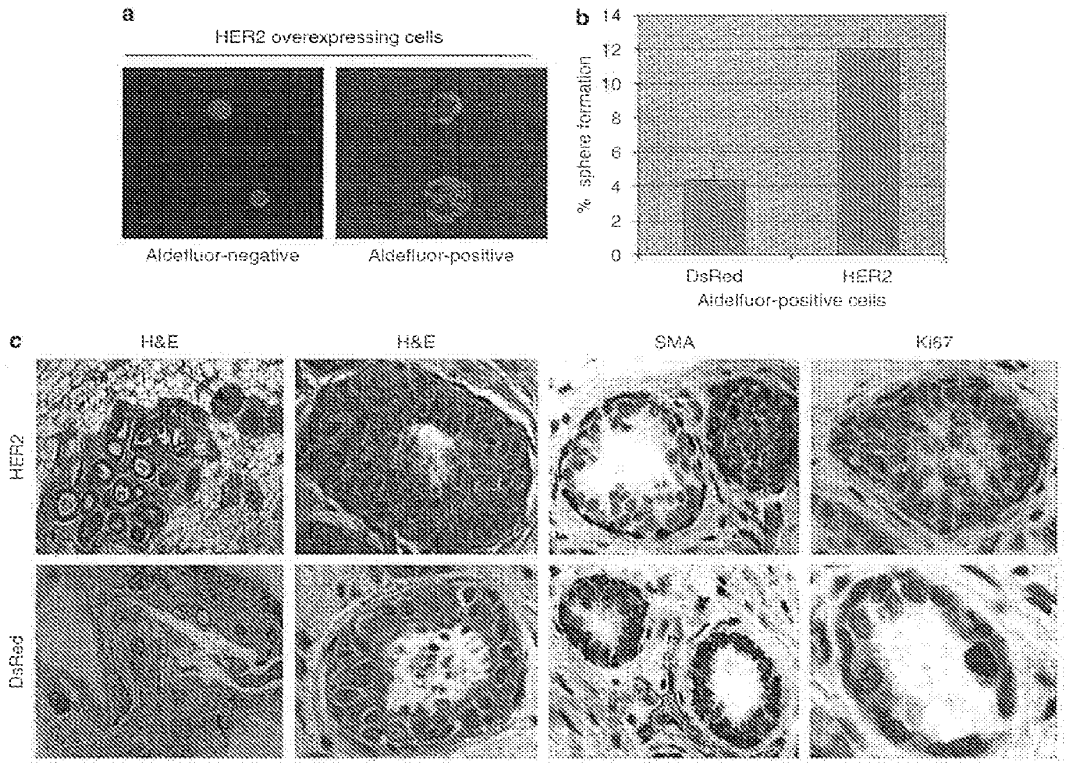

Figure 2 HER2 expression increases the frequency of mammosphere formation and of ductal structures in NOD (nucleotide-binding oligomerization domain)/SCID (severe combined immunodeficient) mice. (a) When plated at clonal density, only the Aldefluor-positive cells are capable of mammosphere formation. (b) A threefold increase in mammosphere-initiating single cells in the HER2 Aldefluor-positive cell population compared to the DsRed Aldefluor-positive population. (c) Serial dilutions of HER2 or DsRed-expressing normal mammary epithelial cells (NMECs) were injected into the mammary fat pads of humanized NOD/SCID mice. HER2-overexpressing cells generated an increased number of mammary outgrowths which displayed hyperplasia (arrows) characterized by an eightfold increase in KI-67 staining. Myoepithelial cells were demonstrated by smooth muscle actin (SMA) staining.

Table 1 Ductal outgrowths generated from HER2 or DsRed-expressing cells

Cell no.	*Unsorted*		*Aldefluor+*			*Aldefluor−*	
	10K	*5K*	*5K*	*500*	*250*	*5K*	*250*
DsRed	8	2	4	0	0	0	0
HER2	23	11	53	5	3	0	0

NMECs were infected with HER2 or DsRed lentiviruses were introduced into the humanized mammary fat pads of NOD-SCID mice. We observed a threefold increase in the number of outgrowths generated by HER2-overexpressing cells compared to DsRed controls. As few as 250 Aldefluor-positive HER2-expressing cells formed outgrowths whereas Aldefluor-negative HER2 or DsRed-expressing cells failed to form outgrowths. The results represent mean of three independent experiments.

of function studies and suggest that in HER2-amplified cells continuous HER2 expression is required for maintenance of the stem cell population.

To determine if the effects of HER2 overexpression on tumorigenicity occurred in all transfected cells or was limited to stem/progenitor cells, we examined the ability of Aldefluor-positive and Aldefluor-negative populations to form tumors in the humanized mammary fat pads of NOD/SCID mice. We demonstrated equal HER2 total and surface expression in Aldefluor-positive and Aldefluor-negative cell populations (Figure 3b and Supplementary Figure 3A). Despite equal levels of HER2 expression in Aldefluor-positive and Aldefluor-negative SUM159-HER2 cells, only the Aldefluor-positive cells were tumorigenic in this model (Figures 3b and c). Figure 3c illustrates the ability of serial dilutions of Aldefluor-positive and Aldefluor-negative populations of HER2-overexpressing cells to form tumors. As few as 500 Aldefluor-positive cells reproducibly formed tumors, whereas only limited tumor growth was seen when up to 50 000 Aldefluor-negative cells were injected. As was the case for MCF7-HER2 cells, we found that only the Aldefluor-positive

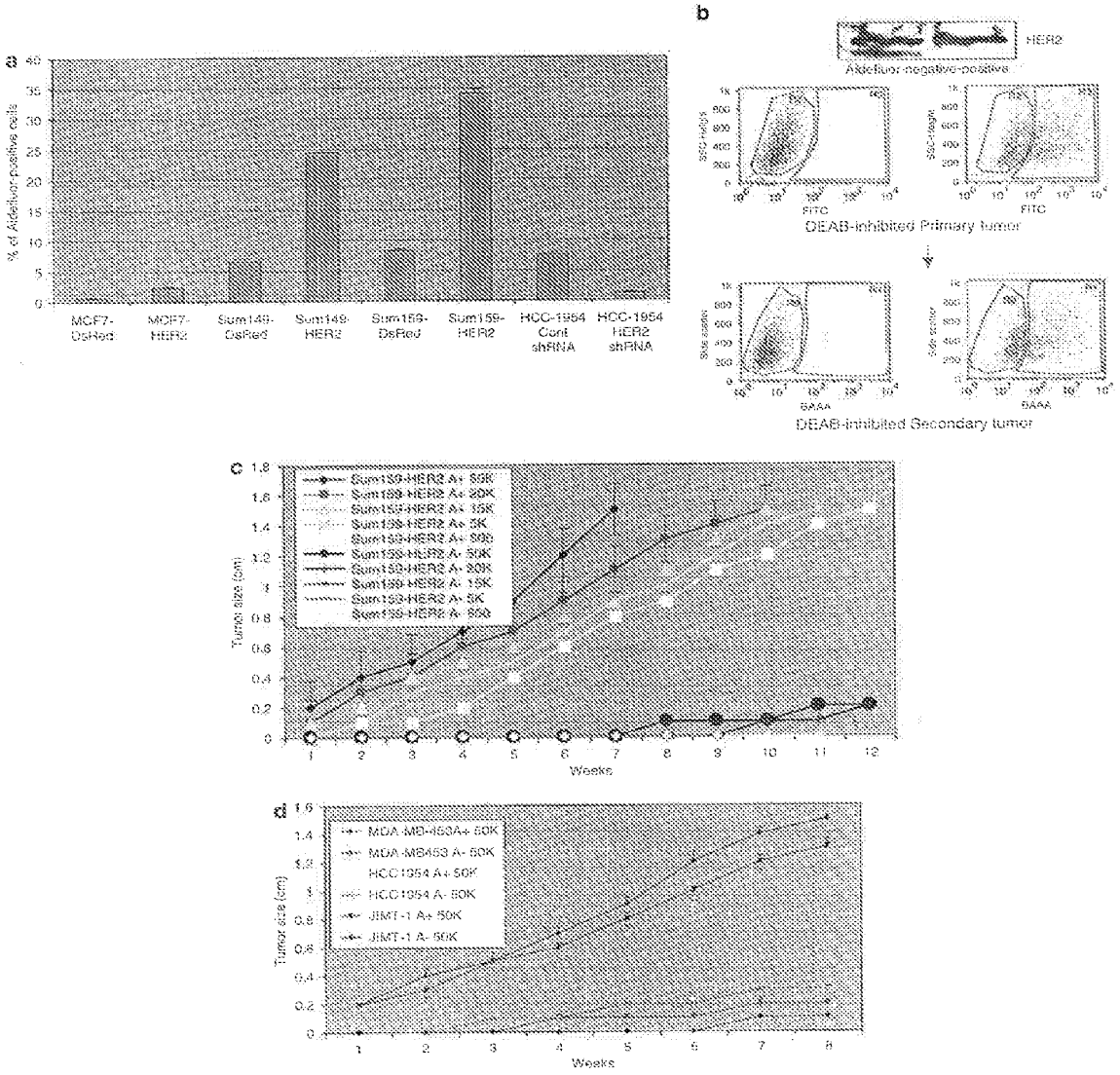

Figure 3 Effects of HER2 overexpression on Aldefluor positivity and tumorigenicity of human mammary carcinoma cell lines. (a) Overexpression of HER2 increased the Aldefluor-positive populations three to fivefold compared to control DsRed cells in indicated breast cancer cell lines. Knockdown of HER2 in HCC1954 cells using HER2 siRNA decreased the Aldefluor-positive cell population. (b) Aldefluor-positive but not Aldefluor-negative cells display properties of tumor stem cells despite equivalent HER2 expression in both populations as shown by western blotting. Tumors generated from the Aldefluor-positive population are composed of both Aldefluor-positive and Aldefluor-negative populations recapitulating the initial tumor phenotype. (c) Growth curves of serial dilutions of Aldefluor-positive and Aldefluor-negative SUM159-HER2 cells. Aldefluor-positive cells form tumors at all cell concentrations whereas the Aldefluor-negative cells fail to do so. (d) In HER2-amplified cell lines MDA-MB-453, HCC1954 and JIMT-1, only Aldefluor-positive cells were tumorigenic.

population of the MCF7-HER2 cells was tumorigenic (Supplementary Figure 4A). The ability of Aldefluor-positive but not Aldefluor-negative cells to form tumors was also demonstrated in three HER2-amplified breast cancer cell lines (Figure 3d). Similar level of HER2 surface expression in Aldefluor-positive and Aldefluor-negative cell populations were demonstrated by flow cytometry and immunohistochemistry (Supplementary Figure 3A, B and C). The Aldefluor-positive population was not only capable of self-renewal as demonstrated by

serial transplantation, but was also able to regenerate the phenotypic heterogeneity of Aldefluor-positive and Aldefluor-negative populations found in the initial tumor (Figure 3b). Thus, the Aldefluor-positive-HER2-positive cell population meets the functional definition of 'CSCs' in its ability to both self-renew and to differentiate regenerating the cellular heterogeneity present in the original tumor.

To determine whether HER2 overexpression increased the tumorigenicity of the CSC component, we examined the tumorigenicity of the Aldefluor-positive populations of SUM159-DsRed or SUM159-HER2 cells. HER2 overexpression increased the rate of tumor growth in Aldefluor-positive cells approximately two-fold compared to control Aldefluor-positive cells (Supplementary 4B). Together, these results demonstrate that the effects of HER2 on tumorigenicity are due to its effects on the Aldefluor-positive CSC component. HER2 overexpression expands this population as well as increasing its tumorigenicity.

HER2 overexpression increases invasiveness of the stem/progenitor cell population

Previous studies have demonstrated that HER2 overexpression leads to increased invasion in *in vitro* matrigel assays. It has been suggested that tumor invasion and metastasis may be mediated by the CSC population (Sheridan *et al*., 2006; Wicha *et al*., 2006). To determine whether the effect of HER2 overexpression was due to a direct effect on tumor invasion or alternatively was due to an increase in CSCs, we tested the ability of Aldefluor-positive and Aldefluor-negative populations of HER2 or DsRed-infected MCF7 and Sum159 cells to invade matrigel. The Aldefluor-positive subpopulations were considerably more invasive than Aldefluor-negative populations (Figures 4a and b). Furthermore, overexpression of HER2 resulted in a three to sixfold increase in invasiveness in the Aldefluor-positive populations. These results suggest that HER2 overexpression not only increases the Aldefluor-positive stem cell component but also increases the invasiveness of this population.

HER2 overexpression induces the expression of stem cell-related genes and activates the PI3-K/Akt pathway

A number of signaling pathways have been implicated in stem cell self-renewal (12). To determine whether HER2 affected the expression of genes involved in stem cell behavior, we examined the expression of *Oct3/4*, *Notch1*, *Notch2*, *Jag1* and *Gli1* in Aldefluor-positive and Aldefluor-negative populations from SUM159-HER2 or DsRed cells. Expression of these stem cell-related genes was significantly increased in Aldefluor-positive compared to Aldefluor-negative cell populations (Figure 5a). Furthermore, there was a significant increase in the expression of these genes in SUM159-HER2-Aldefluor-positive cells compared to Aldefluor-positive DsRed cells. This suggests that HER2 overexpression not only increases the stem cell pool, but also increases the

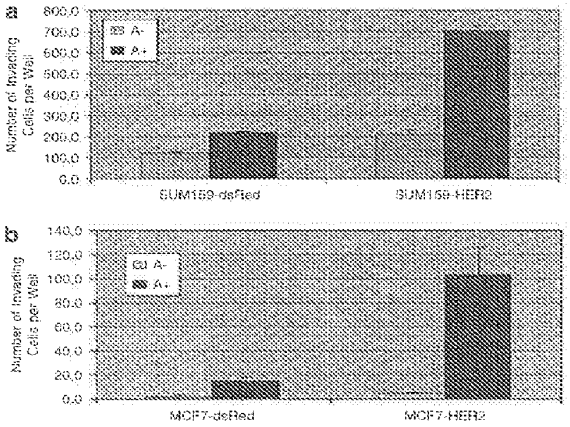

Figure 4 Effects of HER2 overexpression on invasive properties of Aldefluor-positive and Aldefluor-negative populations of breast cancer cell lines. Aldefluor-positive populations from Sum159-DsRed, SUM159-HER2 (a), MCF7-DsRed or MCF7-HER2 cells (b) showed significantly higher invasiveness compared to Aldefluor-negative populations. Furthermore, HER2 overexpression resulted in a five to sixfold increase in the invasive potential of Aldefluor-positive cells.

expression of stem cell-related genes within this cell population.

HER2 has been shown to signal through the PI3-K/Akt pathway. In order to determine the relationship between activation of this pathway and the stem cell phenotype, we examined the levels of Akt phosphorylation by western blot in Aldefluor-positive and Aldefluor-negative cell populations isolated from control or HER2-overexpressing cell lines. Aldefluor-positive cells showed significantly higher Akt phosphorylation compared to Aldefluor-negative cells isolated from the same cell line (Figure 5b). Furthermore, the Aldefluor-positive population from SUM159-HER2 showed increased levels of phospho-Akt compared to the Aldefluor-positive population from SUM159-DsRed cells. These results were further confirmed by phospho-Akt immunoflourescence staining of Aldefluor-positive and Aldefluor-negative SUM159-DsRed and SUM159-HER2 cells (Figure 5c).

Trastuzumab decreases the Aldefluor-positive population in sensitive but not in resistant breast cancer cells through inhibition of PI3-K/Akt signaling

We examined the effect of trastuzumab on the Aldefluor-positive populations in several breast cancer cell lines. Figure 6a (and Supplementary 5C) demonstrates that trastuzumab treatment resulted in a greater than 80% reduction in the population of Aldefluor-positive SUM159-HER2 cells. Interestingly, treatment of SUM159-HER2 cells decreased the Aldefluor-positive cell population to the same level found in DsRed control cells suggesting that this antibody inhibited

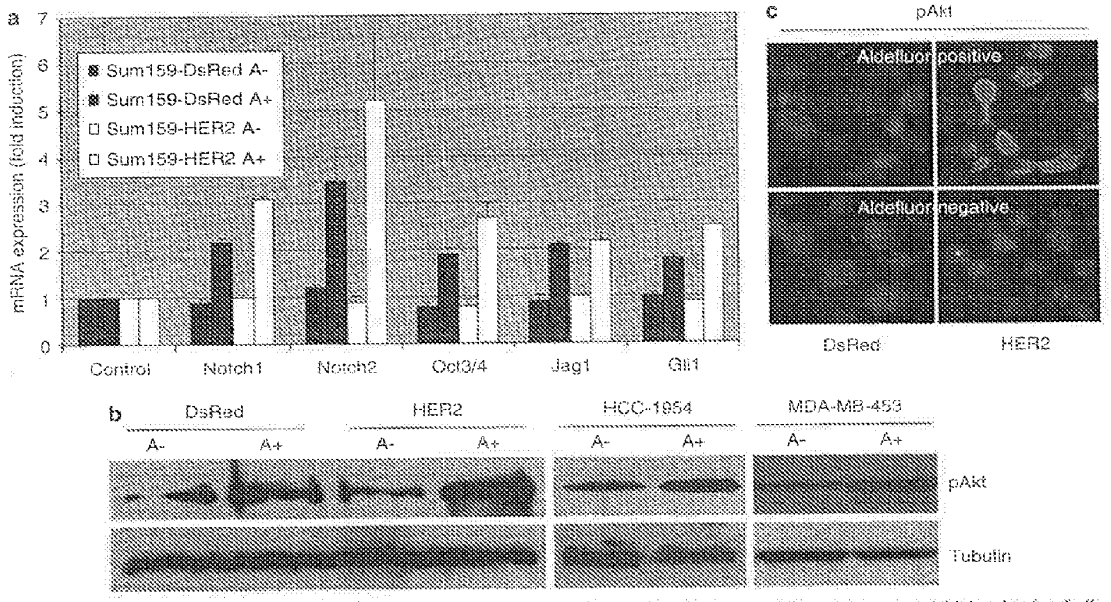

Figure 5 Expression of stem cell-related genes and Akt phosphorylation. (a) Aldefluor-positive cells expressed higher levels of all indicated stem cell-related genes than Aldefluor-negative cells. The expression of these genes was further elevated by HER2 overexpression. (b) Aldefluor-positive populations of indicated breast cancer cell lines showed higher Akt-phosphorylation when compared to Aldefluor-negative populations. (c) Akt-phosphorylation as assessed by immunofluorescence in Aldefluor-positive and Aldefluor-negative SUM159-DsRed and SUM159-HER2 cells. Increased phospho-Akt is detected in Aldefluor-positive and HER2-overexpressing cells.

HER2 signaling to baseline levels. The ability of trastuzumab to reduce the percent of stem cells was also confirmed by immunofluorescent staining utilizing an ALDH-1 monoclonal antibody (Supplementary Figure 5D). We found approximately 50% reduction in HER2 surface expression following trastuzumab treatment of SUM159-HER2 cells (Supplementary Figure 3A). When we analysed whether HER2 surface expression was equally reduced in both Aldefluor-positive and Aldefluor-negative populations, we found no significant difference in HER2 surface expression in both populations (Supplementary Figure 3A). In order to examine trastuzumab-induced cell death, we analysed apoptosis following trastuzumab treatment using Annexin V staining. Trastuzumab did not induce apoptosis in these cells as assessed by flow cytometry (Supplementary Figure 6A).

To determine whether there was a correlation between trastuzumab's effects on the stem cell phenotype and cellular sensitivity to this antibody, we examined the effect of trastuzumab on the Aldefluor-positive populations of HER2-amplified breast cancer cell lines, one HCC1954, which is trastuzumab sensitive, and MDA-MB-453 and JIMT-1, which are trastuzumab resistant. Trastuzumab reduced the Aldefluor-positive population more than 50% in trastuzumab-sensitive HER2-amplified HCC1954 cells (Figure 6a). In contrast, the antibody had no effect on the percent of Aldefluor-positive cells in the trastuzumab-resistant cell lines MDA-MB-453 and JIMT-1 (Figure 6a; Supplementary Figure 5A and B).

In order to determine whether trastuzumab resistance was associated with Akt phosphorylation, we examined the effects of trastuzumab on Akt phosphorylation in trastuzumab-sensitive and -resistant HER2-overexpressing cells. As shown in Figure 6b, trastuzumab reduced Akt phosphorylation in the trastuzumab-sensitive SUM159-HER2 cell line, but had no effect on Akt phosphorylation in the trastuzumab-resistant MDA-MB-453 cell line, which displays constitutive Akt activation (Figure 6b).

As HER2 activates the PI3-K/Akt pathway, we examined the effect of the PI3-K inhibitor LY294002 on the Aldefluor-positive population in these cell lines. Treatment of trastuzumab-sensitive and -resistant cell lines with LY294002 resulted in a significant decrease in the Aldefluor-positive populations (Figure 6a). Furthermore PI3-K inhibitor LY294002 induced apoptosis in 50% of cells as assessed by Annexin V staining (Supplementary Figure 6B).

Discussion

The 'CSC hypothesis' proposes that cancers originate in tissue stem or progenitor cells through disregulation of

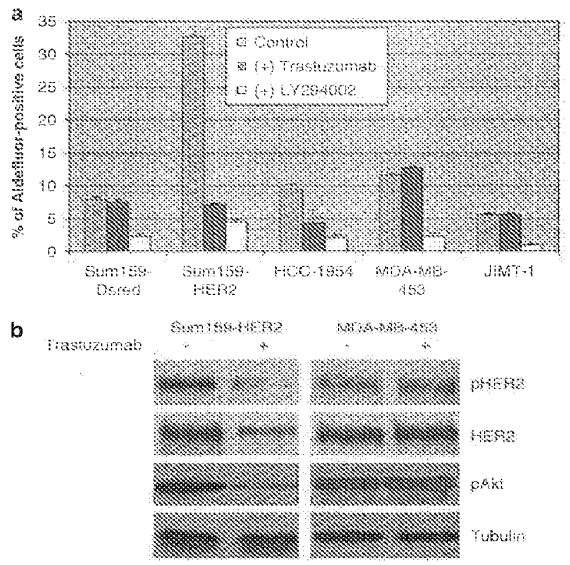

Figure 6 Effects of trastuzumab and LY294002 on the Aldefluor-positive population in breast cancer cell lines. (*a*) Trastuzumab reduced the proportion of Aldefluor-positive cells by as much as 80% in SUM159-HER2 and HCC1954 cells. Trastuzumab had no effect on the Aldefluor-positive population in MDA-MB-453 and JIMT-1 trastuzumab-resistant cell lines. In contrast, LY294002 reduced the proportion of Aldefluor-positive cells in all cell lines. (b) Trastuzumab reduced phospho-HER2 and phospho-AKT expression in Sum159-HER2 cells but has no effect on expression of these proteins in trastuzumab-resistant MDA-MB-453 cells.

the normally tightly regulated process of self-renewal. As a consequence cancers are composed of a cellular hierarchy with a limited number of 'CSCs' capable of self-renewal driving the malignant phenotype and differentiation generating tumor heterogeneity (Korkaya and Wicha, 2007). A frequent genetic alteration in human breast cancer is the amplification of the *HER2* gene (Slamon *et al.*, 1987). Although HER2 overexpression has been demonstrated to affect tumor growth, invasion and metastasis, the mechanisms involved remain undefined. We provide evidence that the effects of HER2 on mammary carcinogenesis, tumorigenicity and invasion result from effects of this signaling pathway on the CSC population. Overexpression of HER2 in NMECs increases the stem/progenitor cell population as demonstrated by increased ALDH activity and mammosphere formation *in vitro* and generation of hyperplastic lesions in NOD/SCID mice. In contrast, Aldefluor-negative HER2-expressing cells neither formed mammospheres nor produced outgrowths in humanized NOD/SCID mice.

In women, HER2 overexpression frequently occurs in premalignant lesions including atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS), suggesting that dysregulation of HER2 signaling is important in tumor initiation. Our findings suggest that HER2 amplification may lead to an expansion of mammary stem cells, providing targets for further carcinogenic events. This model could account for the demonstrated clonality of ADH and DCIS (Allred *et al.*, 1994; Xu *et al.*, 2002; Park *et al.*, 2006).

HER2 overexpression may also affect the behavior of mammary carcinomas by expanding their 'CSC' populations. We show that overexpression of HER2 in a series of human breast cancer cell lines increases the percentage of cells with ALDH activity. The functional significance of this was demonstrated by the ability of Aldefluor-positive/HER2 cells to form tumors, which could be serially passaged in NOD/SCID mice. Despite equivalent levels of HER2 expression in Aldefluor-positive and Aldefluor-negative cell populations, Aldefluor-negative cells failed to form tumors even with 100-fold more cells. HER2 overexpression also increased the tumorigenicity within the Aldefluor-positive population. These experiments suggest that HER2 increases the CSC population as well as increasing tumorigenicity within this population. In contrast, HER2 overexpression does not affect the phenotype of more differentiated Aldefluor-negative cells.

A number of genes and signaling pathways have been identified which regulate the self-renewal of stem cells in a variety of developmental systems. These include Oct3/4 and genes involved in the Notch and Hedgehog signaling pathways (Clarke *et al.*, 2003; Dontu *et al.*, 2004; Liu *et al.*, 2006). We demonstrate that these genes show increased expression in Aldefluor-positive as compared to Aldefluor-negative Sum159 cells. Furthermore, Aldefluor-positive-HER2-positive cells showed increased expression of these stem cell genes compared to Aldefluor-positive DsRed control cells. This suggests that in addition to increasing the Aldefluor-positive population, HER2 overexpression may also drive expression of stem cell regulatory genes within this population. HER2 has been shown to signal through the PI3-K/Akt pathway generating a multidrug resistance phenotype (Knuefermann *et al.*, 2003). We found that the Aldefluor-positive cell population displayed significantly higher Akt phosphorylation than Aldefluor-negative cells. Akt phosphorylation was further increased by HER2 overexpression in this cell population.

A recent report suggested that the subpopulation of breast cancer cells, previously characterized by the stem cell phenotype CD44+/CD24− (Al-Hajj *et al.*, 2003) show higher levels of proinvasive genes and exhibit increased invasive properties (Sheridan *et al.*, 2006). We demonstrate here that HER2 overexpression increases the invasiveness of both MCF7 and SUM159 cells. Furthermore, the Aldefluor-positive populations are significantly more invasive than the Aldefluor-negative populations. These results suggest that HER2 overexpression may have a direct effect on tumor invasion as well as an indirect effect resulting from increases in the invasive CSC population.

In the metastatic setting, trastuzumab increases the effectiveness of chemotherapy resulting in an increased response rate and duration of response (Burstein *et al.*,

2001). Recent evidence suggests that CD44+/CD24− breast CSCs may be relatively resistant to both chemotherapy and radiation. In contrast to the resistance of CSCs to these conventional treatments, our results suggest that the effectiveness of trastuzumab may be directly related to its effects on the CSC population. A recent report demonstrating that the HER2/EGFR inhibitor, Lapatinib when used in neoadjuvant setting decreases the CSC component supports the clinical relevance of our findings (Li *et al.*, 2008).

Despite the significant clinical benefits of trastuzumab, many patients treated in the adjuvant setting still relapse and most patients with advanced disease become trastuzumab-resistant within 1 year (Slamon *et al.*, 1987; Park *et al.*, 1990). The development of secondary mutations such as deletion of the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) may contribute to trastuzumab resistance (Nagata *et al.*, 2004; Saal *et al.*, 2007). We demonstrate that treatment of SUM159-HER2 cells with trastuzumab results in an 80% reduction of the Aldefluor-positive cell population. The effect of trastuzumab on Sum159-HER2 cells was associated with decreased expression of phospho-HER2 and phospho-Akt. In contrast to its effect on SUM159-HER2 cells, trastuzumab had no measurable effect on phospho-HER2, phopho-Akt, or on the proportion of the Aldefluor positivity in two trastuzumab-resistant cell lines. However, the PI3-K inhibitor LY294002 reduced the Aldefluor-positive population in both trastuzumab-sensitive and -resistant cell lines. These findings are consistent with previous reports showing increased Akt activity in trastuzumab-resistant cell lines (Tanner *et al.*, 2004; Kucab *et al.*, 2005) and suggest that the PI3-K/Akt pathway is important in mediating the effects of HER2 signaling. It is also consistent with recent reports suggesting a role for Akt signaling in stem cell self-renewal (Welham *et al.*, 2007) and in mediating treatment resistance (Ma *et al.*, 2007; Saal *et al.*, 2007). Most recently, Berns *et al.* (2007) identified the PI3K pathway as a major determinant of trastuzumab resistance in human breast tumors using a functional genomic approach.

In conclusion, our results suggest that the effects of HER2 amplification on mammary carcinogenesis, tumorigenicity and invasion result from effects of this signaling pathway on mammary CSCs. These effects are due to the ability of HER2 to expand the stem/progenitor cell population as well as increasing the tumorigenicity and invasiveness of this population. Carcinogenesis may be initiated by expansion of stem cell pools which provide targets for further carcinogenic events. Increased HER2 expression in breast cancers, in turn, may increase the CSC component driving tumorigenesis, invasion and metastasis. An elucidation of the molecular mechanisms by which HER2 signaling drives the stem cell pool may lead to further insights into the behavior of normal and malignant mammary stem cells. In addition, identification of components of these pathways may lead to new targets for cancer prevention and therapy.

Materials and methods

Dissociation of normal mammary tissue and mammosphere assay

Mammary tissues from reduction mammoplasties were dissociated as previously described (Stingl *et al.*, 1998). For mammosphere experiments, single cell suspensions of NMEC were plated on 1% agarose-coated plates at a density of 1×10^5 and grown for 7–10 days. Subsequent cultures after dissociation of primary spheres were plated on ultralow attachment plates at a density of 5×10^4 to 1×10^5. Mammosphere cultures were grown in a serum-free mammary epithelium basal medium as previously described (Dontu *et al.*, 2003).

Lentiviral construction and cell infections

All lentiviral constructs were prepared by the University of Michigan Vector Core facility. The pLentiLox RSV-GFP-HER2 construct was generated by ligating the *Nhe*I and *Xba*I HER2 fragment from pCI-HER2 (generous gift from K. Ignatoski, Urology Medical School, University of Michigan, Ann Arbor, MI, USA) into *Nhe*I site of pLentiLox RSV lentiviral-GFP vector. After confirmation by restriction digestion and DNA sequencing, large-scale pLentiLox-HER2 virus was produced and used for infecting NMECs and breast cancer cell lines. pLentiLox RSV-dsRed-HER2 vector was generated by replacing the green fluorescent protein (GFP) with a DsRed insert.

Implantation of cells in NOD/SCID mice

Fat pads of 3 weeks old NOD/SCID mice were cleared and replaced with 1:1 mixture of irradiated and nonirradiated human fibroblasts. Within 2–3 weeks, human fibroblasts generated a humanized mammary fat pad in mice. These resulting fat pads were injected with NMECs infected with DsRed or HER2 lentivirus. An estrogen pellet was also implanted subcutaneously in each mouse. The mice were killed after 4–8 weeks and the fat pads were analysed for outgrowths.

Breast cancer cells at serial dilutions were directly injected into the fat pads of 5 weeks old NOD/SCID mice with an estrogen pellet implantation. The kinetics of tumor growth in these mice were analysed at indicated time points.

Aldefluor assay and flow cytometry

To measure and isolate cells with high ALDH activity, the Aldefluor assay was performed according to manufacturer's (Stemcell Technologies, Durham, NC, USA) guidelines. Dissociated single cells were suspended in Aldefluor assay buffer containing the ALDH substrate, Bodipy-aminoacetaldehyde (BAAA) at 1.5 μM and incubated for 40 min at 37 °C. To distinguish between ALDH-positive and ALDH-negative cells, a fraction of cells was incubated under identical condition in the presence of a 10-fold molar excess of the ALDH inhibitor, diethylaminobenzaldehyde (DEAB). This results in a significant decrease in the fluorescence intensity of ALDH-positive cells and was used to compensate the flow cytometer.

Cell lines and HER2, DsRed lentiviral infection

MDA-MB-453 and HCC1954 cells were maintained in Roswell Park Memorial Institute (RPMI) supplemented with 10% fetal bovine serum and antibiotic/antimycotic (10 000 units per ml penicillin G sodium, 10 000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B) and the HMT-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% serum and antibiotic/antimycotic. SUM159, SUM149 and MCF7 cells were infected with the lentivirus-

expressing human HER2 or control lentiviruses expressing either GFP or DsRed. Following 12–16 h of incubation, the viruses were removed and replaced with fresh medium.

Immunoblotting

Cells were lysed in a laemmli buffer, boiled and loaded onto SDS–polyacrylamide gels. Following electrophoresis, proteins were transferred onto Nitrocellulose Membranes (Pierce, Rockford, IL, USA) using semidry Trans-Blot (BioRad Laboratories, Hercules, CA, USA). Blots were first incubated in Tris-buffered saline (TBS) blocking buffer containing either 2% milk or 2% BSA (bovine serum albumin, for phospho-specific antbodies) for 1–2 h at room temperature and then with the respective primary antibodies diluted in TBST (Tris-buffered saline Tween-20, containing 0.1% Tween-20 and 2% BSA) overnight at 4 °C. Subsequently, blots were washed and incubated with appropriate secondary antibodies (GE Healthcare, Little Chalfont, UK) in TBST and detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Antibodies to human HER2, c-erbB-2/HER-2/neu Ab-17 (Clone e2-4001 + 3B5) mouse monoclonal antibody and anti-phospho-erbB-2/HER-2 (Tyr1248) antibody were from Upstate (Temecula, CA, USA), and the phospho-Akt1 (p-Akt1/2/3 (Ser 473)) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunohistochemisty and immunofluorescent staining were carried out as described previously (6).

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References

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* **100**: 3983–3988.

Allred DC, O'Connell P, Fuqua SA, Osborne CK. (1994). Immunohistochemical studies of early breast cancer evolution. *Breast Cancer Res Treat* **32**: 13–18.

Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K *et al*. (2007). A functional genetic approach identifies the PI3K pathway as a major determinant of Trastuzumab resistance in breast cancer. *Cancer Cell* **12**: 395–402.

Beuzeboc P, Scholl S, Garau XS, Vincent-Salomon A, Cremoux PD, Couturier J *et al*. (1999). Herceptin, a monoclonal humanized antibody anti-HER2: a major therapeutic progress in breast cancers overexpressing this oncogene? *Bull Cancer* **86**: 544–549.

Burstein HJ, Kuter I, Campos SM, Gelman RS, Tribou L, Parker LM *et al*. (2001). Clinical activity of trastuzumab and vinorelbine in women with HER2-overexpressing metastatic breast cancer. *J Clin Oncol* **19**: 2722–2730.

Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ *et al*. (2003). *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* **17**: 1253–1270.

Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, Wicha MS. (2004). Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res* **6**: R605–R615.

Finn RS, Dering J, Ginther C, Wilson CA, Glaspy P, Tchekmedyian N *et al*. (2007). Dasatinib, an orally active small molecule inhibitor of both the src and abl kinases, selectively inhibits growth of basal-type/'triple-negative' breast cancer cell lines growing *in vitro*. *Breast Cancer Res Treat* **105**: 319–326.

Ginestier C, Hur M, Charafe-Jauffret E, Monville F, Dutcher J, Brown M *et al*. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* **1**: 555–567.

Knuefermann C, Lu Y, Liu B, Jin W, Liang K, Wu L *et al*. (2003). HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. *Oncogene* **22**: 3205–3212.

Korkaya H, Wicha MS. (2007). Selective targeting of cancer stem cells: a new concept in cancer therapeutics. *BioDrugs* **21**: 299–310.

Kucab JE, Lee C, Chen CS, Zhu J, Gilks CB, Cheang M *et al*. (2005). Celecoxib analogues disrupt Akt signaling, which is commonly activated in primary breast tumours. *Breast Cancer Res* **7**: R796–R807.

Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu M-F *et al*. (2008). Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst* **100**: 672–679.

Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW *et al*. (2006). Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res* **66**: 6063–6071.

Ma S, Lee TK, Zheng BJ, Chan KW, Guan XY. (2007). CD133(+) HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. *Oncogene* **27**: 1749–1758.

Miller KD. (2004). The role of ErbB inhibitors in trastuzumab resistance. *Oncologist* **9**(Suppl 3): 16–19.

Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA *et al*. (2004). PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* **6**: 117–127.

Paik S, Hazan R, Fisher ER, Sass RE, Fisher B, Redmond C *et al*. (1990). Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: prognostic significance of erbB-2 protein overexpression in primary breast cancer. *J Clin Oncol* **8**: 103–112.

Park K, Han S, Kim HJ, Kim J, Shin E. (2006). HER2 status in pure ductal carcinoma *in situ* and in the intraductal and invasive components of invasive ductal carcinoma determined by fluorescence *in situ* hybridization and immunohistochemistry. *Histopathology* **48**: 702–707.

Phillips TM, McBride WH, Pajonk F. (2006). The response of CD24(-/low)/CD44 + breast cancer-initiating cells to radiation. *J Natl Cancer Inst* **98**: 1777–1785.

Saal LH, Johansson P, Holm K, Gruvberger-Saal SK, She QB, Maurer M *et al*. (2007). Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. *Proc Natl Acad Sci USA* **104**: 7564–7569.

Seidman AD, Fornier MN, Esteva FJ, Tan L, Kaptain S, Bach A *et al*. (2001). Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by HER2 immunophenotype and gene amplification. *J Clin Oncol* **19**: 2587–2595.

Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH *et al*. (2006). CD44 + /CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res* **8**: R59.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**: 177–182.

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE *et al.* (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**: 707–712.

Stingl J, Eaves CJ, Kuusk U, Emerman JT. (1998). Phenotypic and functional characterization *in vitro* of a multipotent epithelial cell present in the normal adult human breast. *Differentiation* **63**: 201–213.

Tanner M, Kapanen AI, Junttila T, Raheem O, Grenman S, Elo J *et al.* (2004). Characterization of a novel cell line established from a patient with Herceptin-resistant breast cancer. *Mol Cancer Ther* **3**: 1585–1592.

Tokuzawa Y, Kaiho E, Maruyama M, Takahashi K, Mitsui K, Maeda M *et al.* (2003). Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. *Mol Cell Biol* **23**: 2699–2708.

Welham MJ, Storm MP, Kingham E, Bone HK. (2007). Phosphoinositide 3-kinases and regulation of embryonic stem cell fate. *Biochem Soc Trans* **35**: 225–228.

Wicha MS, Liu S, Dontu G. (2006). Cancer stem cells: an old idea—a paradigm shift. *Cancer Res* **66**: 1883–1890; discussion 1895–6.

Xu R, Perle MA, Inghirami G, Chan W, Delgado Y, Feiner H. (2002). Amplification of Her-2/neu gene in *Her-2/neu*-overexpressing and -nonexpressing breast carcinomas and their synchronous benign, premalignant, and metastatic lesions detected by FISH in archival material. *Mod Pathol* **15**: 116–124.

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Biochemistry

Demonstration of ligand-dependent signaling by the *erbB-3* tyrosine kinase and its constitutive activation in human breast tumor cells

(gp180^{erbB-3}/receptor tyrosine kinase/tyrosine phosphorylation/mitogenic signaling/neoplasia)

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ABSTRACT **The predicted human *erbB-3* gene product is closely related to epidermal growth factor receptor (EGFR) and *erbB-2*, which have been implicated as oncogenes in model systems and human neoplasia. We expressed the *erbB-3* coding sequence in NIH 3T3 fibroblasts and identified its product as a 180-kDa glycoprotein, gp180^{erbB-3}. Tunicamycin and pulse–chase experiments revealed that the mature protein was processed by N-linked glycosylation of a 145-kDa *erbB-3* core polypeptide. The intrinsic catalytic function of gp180^{erbB-3} was shown by its ability to autophosphorylate *in vitro*. Ligand-dependent signaling of its cytoplasmic domain was established employing transfectants that express a chimeric EGFR/*erbB-3* protein, gp180$^{EGFR/erbB-3}$. EGF induced tyrosine phosphorylation of the chimera and promoted soft agar colony formation of such transfectants. These findings combined with the detection of constitutive tyrosine phosphorylation of gp180^{erbB-3} in 4 of 12 human mammary tumor cell lines implicate the activated *erbB-3* product in the pathogenesis of some human malignancies.**

A small family of growth factor receptors with the *erbB*/epidermal growth factor receptor (EGFR) as a prototype has been frequently implicated in malignancy. The EGFR and the related *erbB-2* gene are frequently overexpressed in the presence or absence of gene amplification in various human tumors (1–3). Moreover, they can become activated by mutations that constitutively enhance the catalytic function of their encoded proteins (4, 5). When such abnormalities are reproduced in model systems, these receptors have been shown capable of conferring the malignant phenotype (6–9), supporting their critical role in the development of human neoplasia.

Recently, we have identified a third family member, designated *erbB-3* (10). The *erbB-3* coding sequence predicts a receptor-like tyrosine kinase with close similarity to the EGFR and *erbB-2* proteins. Though normally expressed in cells of epithelial and neuroectodermal origin, increased *erbB-3* transcript levels were detected in some human breast tumor cell lines. These findings raised the possibility that overexpression of this gene plays a role in some human epithelial malignancies (10). Our present studies were undertaken in an effort to identify and characterize the normal *erbB-3* gene product. By analysis of an EGFR/*erbB-3* chimeric receptor, we demonstrate that EGF-dependent activation of the *erbB-3* catalytic domain results in a proliferative response in transfected NIH 3T3 cells. Finally, we show that some human mammary tumor cell lines exhibit a dramatic elevation of steady-state *erbB-3* tyrosine phosphorylation implying functional *erbB-3* activation in these tumor cells.

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MATERIALS AND METHODS

Engineering of LTR-*erbB-3* Expression Vector and DNA Transfection. The pZIPneo expression vector (11) was modified by introduction of a unique *Sal* I cloning site. Following deletion of the *Sal* I site in the tetracycline-resistance gene, the synthetic oligonucleotides 5′-GATCTCGAGTCGAC-3′ and 5′-GATCGTCGACTCGA-3′ were annealed and ligated into the single *Bam*HI site to generate pZIPneo$_{Sal}$. The *erbB-3* open reading frame (10) including 7 nucleotides upstream of the initiation codon and the termination codon (nucleotides 93–4128) was linked with *Sal* I ends employing the polymerase chain reaction (PCR) and cloned into pZIPneo$_{Sal}$ (LTR-*erbB-3*). Sense orientation and integrity of the open reading frame were confirmed by restriction analysis as well as nucleotide sequence analysis of cloning boundaries and PCR-amplified regions. The chimeric EGFR/*erbB-3* receptor was engineered by joining extracellular and transmembrane domains of the EGFR (amino acids 1–682; ref. 12) with the intracellular portion of *erbB-3* (amino acids 681–1342). Its construction will be described in detail elsewhere. Linearized expression constructs (0.01–10 μg per plate) were transfected into NIH 3T3 cells by calcium phosphate precipitation using 40 μg of calf thymus DNA as carrier. Mass cultures expressing the recombinant proteins were obtained by selection with 750 μg of G418 per ml.

Soft Agar Assays and Human Tumor Cell Lines. To test anchorage-independent growth, cell suspensions were seeded at 10-fold serial dilutions in semisolid agarose medium containing growth medium and 0.45% seaplaque agarose (FMC). Visible colonies comprising >100 cells were scored at 14 days. EGF was added at a concentration of 20 ng/ml as indicated. Human mammary tumor cell lines were obtained from the American Type Culture Collection and propagated in Dulbecco's modified Eagle medium containing 10% fetal calf serum.

Generation of *erbB-3* Monoclonal Antibody and Peptide Antisera. BALB/c mice were immunized with live LTR-*erbB-3* cells. Somatic cell hybrids were prepared by fusion of immune splenocytes with murine nonsecreting NS-1 myeloma cells (13, 14). Hybridoma supernatants were screened for differential immunoreactivity with LTR-*erbB-3* but not LTR-neo transfectants by enzyme-linked immunosorbent assay (ELISA) using live cells or cell extracts as antigen source. Positive hybridoma cell lines were cloned twice by limiting dilution and further characterized by immunoprecipitation and immunofluorescence analysis. One monoclonal antibody, MAb E3-1 (IgG2a isotype), specifically recognized the extracellular domain of the human *erbB-3* protein and did not show any cross-reactivity with the related EGFR or *erbB-2* proteins overexpressed in an NIH 3T3 cell background. Polyclonal antisera were raised in rabbits against two

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor.

synthetic peptides in the carboxyl-terminal *erbB-3* coding sequence (10): MK4 (1191–1205), EDEDEEYEYMNRRRR; MK5 (1254–1268), TTPDEDYEYMNRQRD. For immunization, peptides were coupled to thyroglobulin using glutaraldehyde.

Protein Analysis. Protein lysates were prepared in StaphA buffer (15) containing the protease inhibitors phenylmethylsulfonyl fluoride (1 mM) and aprotinin (10 μg/ml; Boehringer Mannheim). For the analysis of phosphotyrosine (*P*-Tyr) proteins, the phosphatase inhibitors sodium orthovanadate (2 mM) and sodium pyrophosphate (10 mM) were added. Immunoblot analysis using peptide antisera was essentially conducted as reported (15). An EGFR peptide antiserum was generated against a carboxyl-terminal epitope of the receptor (E7) as described (16). For the detection of *P*-Tyr proteins, membranes were blocked in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) and immunostained with a mixture of monoclonal anti-*P*-Tyr antibodies (PY20 and PY69; ICN) diluted 1:500 in PBS containing 1% BSA. Filters were washed with PBS containing 0.05% Tween 20. Immunoprecipitation was conducted as described (17) using GammaBind G agarose (Pharmacia) to collect the immunocomplexes. The beads were coupled with goat anti-mouse IgG second antibody (Boehringer Mannheim) in immunoprecipitations using *erbB-3* or EGFR monoclonal antibodies. A commercially available monoclonal antibody, AB-1 (Oncogene Sciences, Mineola, NY), reactive with the EGFR extracellular domain, was employed for immunoprecipitation of EGFR and EGFR/*erbB-3* proteins. For *in vitro* kinase assays, 4 mg total lysates were precleared with GammaBind G agarose. Following immunoprecipitation, washed immunocomplexes were equilibrated in autokinase buffer containing 40 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 3 mM $MnCl_2$, and 0.05% Triton. The immunocomplexes were subsequently divided for immunoblot analysis or immunocomplex kinase assay. Autokinase reactions were carried out in 40 μl of autokinase buffer containing 20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) at 25°C for 10 min and terminated by addition of SDS-containing sample buffer.

RESULTS

Recombinant Expression of the *erbB-3* Protein in NIH 3T3 Fibroblasts. For structural and functional characterization of the *erbB-3* gene product, we inserted the complete *erbB-3* open reading frame (10) into a modified ZIPneo vector (11), placing the cDNA under the transcriptional control of the Moloney murine leukemia virus long-terminal-repeat sequence (LTR-*erbB-3*). NIH 3T3 fibroblasts were transfected with LTR-*erbB-3* or LTR-neo control DNA and cultured in the presence or absence of the selective drug G418. Under conditions in which efficient drug resistance (6×10^3 colonies per pmol) was conferred by LTR-*erbB-3*, no transformed foci were detectable. In contrast, LTR-*erbB-2* or EGF-triggered LTR-EGFR induced morphological transformation of NIH 3T3 cells with efficiencies of around 1.2×10^4 per pmol and 2.3×10^2 per pmol, respectively, as described (6, 8).

To test for expression of the *erbB-3* protein, we developed two polyclonal rabbit antisera against synthetic peptides encompassing residues 1191–1205 (MK4) or 1254–1268 (MK5) within the carboxyl terminus of the predicted *erbB-3* product (10). Immunoblot analysis of lysates from marker-selected LTR-neo and LTR-*erbB-3* transfectants revealed a major 180-kDa band only in LTR-*erbB-3* cells. This band was independently recognized by both antisera (Fig. 1*A*). There was no cross-reactivity of either antiserum with the related EGFR or *erbB-2* proteins overexpressed in NIH 3T3 cells. Immunoreactivity of either antiserum with the 180-kDa band in LTR-*erbB-3* transfectants was competitively inhibited by the antigenic peptide, whereas MK4 reactivity with a faint

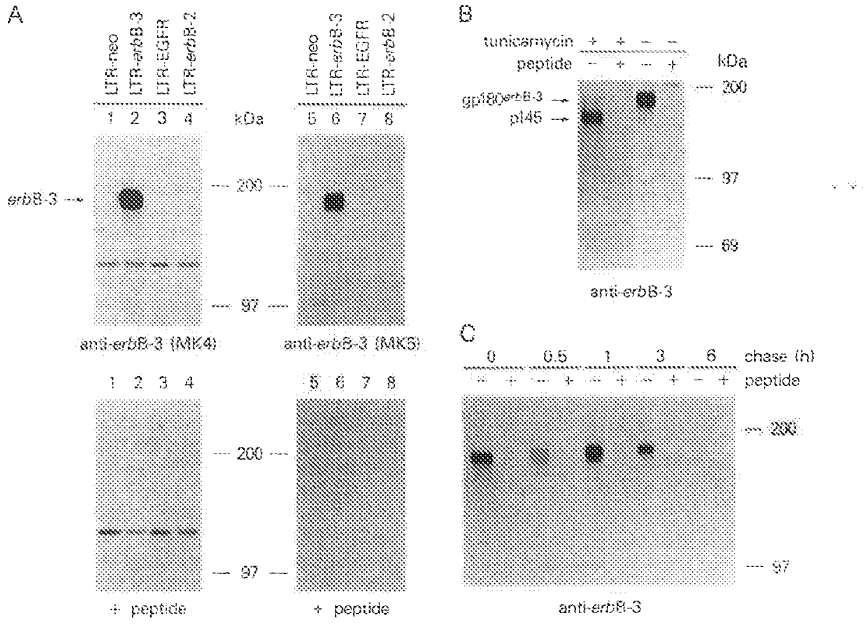

FIG. 1. Characterization of gp180^{erbB-3} recombinantly expressed in NIH 3T3 cells. (*A*) Immunoblot analysis of transfectants with *erbB-3* peptide antisera MK4 (left) and MK5 (right) and peptide competition. (*B*) Immunoprecipitation with MK5 antiserum of LTR-*erbB-3* transfectants metabolically labeled for 2 h in the presence or absence of glycosylation inhibitor tunicamycin (1 μg/ml). (*C*) Pulse–chase analysis. LTR-*erbB-3* transfectants were pulse-labeled for 15 min with 0.5 mCi each of [^{35}S]methionine and [^{35}S]cysteine and immediately lysed (0) or chased with 100 μg/ml each of unlabeled methionine and cysteine for the indicated time periods. Trichloroacetic acid-precipitable counts (1×10^7) were immunoprecipitated from total lysates using MK5 antiserum.

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125-kDa band was not affected by peptide competition (Fig. 1*A*). These results established specificity of *erbB-3* protein detection by both polyclonal antisera. By comparison, the 180-kDa *erbB-3* protein migrated distinctly slower than the 170-kDa EGFR and slightly faster than the 185-kDa *erbB-2* protein (data not shown).

The Mature *erbB-3* Product, gp180^{erbB-3}, Represents a Catalytically Active Glycoprotein Associated with the Cell Membrane. To characterize processing of the *erbB-3* protein, we performed immunoprecipitation experiments with MK5 antiserum. Metabolic labeling of LTR-*erbB-3* transfectants in the presence or absence of tunicamycin demonstrated that the 145-kDa *erbB-3* core polypeptide is modified by N-linked glycosylation (Fig. 1*B*). Pulse–chase analysis further indicated cotranslational processing, resulting in a predominant 170-kDa precursor protein in addition to faint *erbB-3*-specific bands of 150 kDa and 160 kDa, following 15 min of pulse-labeling (Fig. 1*C*). The mature 180-kDa *erbB-3* protein appeared after 0.5 h of chase, and the majority was converted into gp180^{erbB-3} by 1 h. By analysis of further time points, we estimate an approximate half-life of 2–3 h (Fig. 1*C*). Thus, in NIH 3T3 cells, gp180^{erbB-3} exhibits an apparently faster turnover than the EGFR, for which a biosynthesis time of 3 h and approximate half-life of 3–6 h have been reported (18).

For immunolocalization of the *erbB-3* protein, an *erbB-3*-specific monoclonal antibody, MAb E3-1, was raised against the recombinantly expressed protein. MAb E3-1 specifically immunoprecipitated gp180^{erbB-3} from LTR-*erbB-3* transfectants (Fig. 2*A*) and did not exhibit cross-reactivity with the EGFR or *erbB-2* proteins (data not shown). Immunofluorescence analysis revealed heterogeneous membrane immunostaining of formalin-fixed LTR-*erbB-3* cells using MAb E3-1 but not with a nonspecific mouse immunoglobulin of matching isotype (Fig. 2*B*). MAb E3-1-specific membrane fluorescence of native LTR-*erbB-3* cells (Fig. 2*B*) indicated that gp180^{erbB-3} was expressed at the cell surface, as expected for a membrane-anchored protein.

To investigate its function, we next analyzed the *erbB-3* protein for *in vitro* kinase activity. LTR-*erbB-3* and control LTR-neo cell lysates were first immunoprecipitated with E3-1 followed by immunoblot analysis with MK4 antiserum (Fig. 3*A*). When the same immunoprecipitates were incubated in autokinase buffer containing [γ-^{32}P]ATP, a predominant 180-kDa phosphoprotein was labeled only in immunoprecipitates containing the *erbB-3* protein (Fig. 3*B*). These

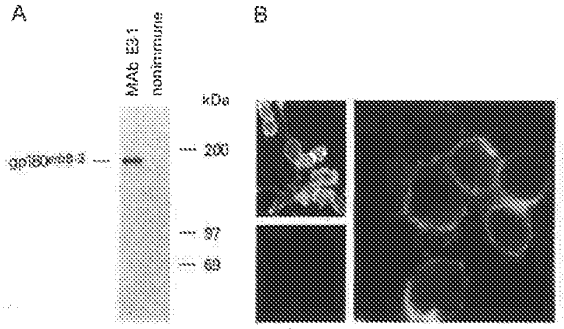

FIG. 2. Immunolocalization of gp180^{erbB-3} on the surface of LTR-*erbB-3* cells. (*A*) Immunoprecipitation analysis of metabolically labeled LTR-*erbB-3* transfectants with monoclonal antibody E3-1 and a nonimmune control. (*B*) Indirect immunofluorescence. Formalin-fixed LTR-*erbB-3* transfectants were incubated with MAb E3-1 (*Upper Left*) or nonimmune IgG (*Lower Left*) and stained with a fluorescein-conjugated secondary antibody. (×50.) (*Right*) Indirect immunofluorescence with MAb E3-1 of native LTR-*erbB-3* cells. (×500.)

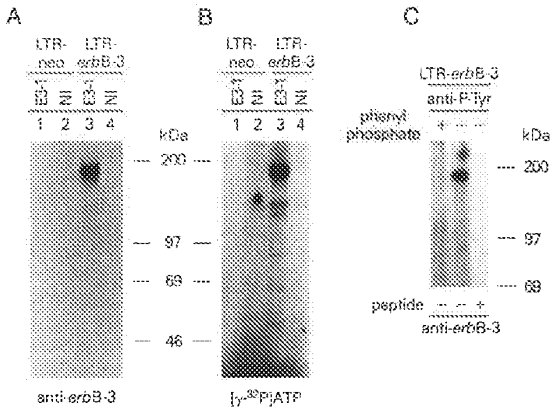

FIG. 3. Autophosphorylation *in vitro* and chronic tyrosine phosphorylation *in vivo* of gp180^{erbB-3}. LTR-*erbB-3* or control lysates were immunoprecipitated with *erbB-3* monoclonal antibody (E3-1) or nonimmune IgG (NI). Parallel immunoprecipitates were subjected either to immunoblot analysis with MK4 antiserum (*A*) or to an immunocomplex kinase assay in the presence of [γ-^{32}P]ATP (*B*). (*C*) Tyrosine phosphorylation *in vivo* was assayed by immunoprecipitation with monoclonal anti-*P*-Tyr antibodies followed by immunoblotting with MK4 antiserum.

findings indicated that gp180^{erbB-3} possessed intrinsic protein kinase activity. To assess its enzymatic activity *in vivo*, LTR-*erbB-3* lysates were subjected to immunoprecipitation with *P*-Tyr-specific monoclonal antibodies (anti-*P*-Tyr) followed by immunoblotting with MK4 antiserum. As shown in Fig. 3*C*, the *erbB-3* protein was recovered from anti-*P*-Tyr immunoprecipitates, and immunodetection was competitively inhibited either by phenyl phosphate in the immunoprecipitation or the *erbB-3* peptide in Western blot analysis. These findings indicated that recombinant gp180^{erbB-3} expressed in NIH 3T3 cells was chronically phosphorylated on tyrosine residues.

EGF-Dependent Signaling by an EGFR/*erbB-3* Chimeric Receptor. To explore *erbB-3* signaling, we utilized a chimeric receptor, LTR-EGFR/*erbB-3* containing the ligand-binding domain of the closely related EGF receptor (amino acids 1–682) and the intracellular portion of *erbB-3* (amino acids 681–1342; unpublished data). Transfection of NIH 3T3 cells with this construct did not result in detectable focus formation either in the presence or absence of EGF. To investigate ligand dependence of *erbB-3* enzymatic activity, we compared *in vivo* tyrosine phosphorylation of the chimeric receptor in the presence or absence of EGF. This protein, EGFR (8), and *erbB-3* proteins from independent transfectants were enriched by immunoprecipitation and subjected to immunoblot analysis with either anti-*P*-Tyr or the appropriate specific antiserum.

As shown in Fig. 4, the steady-state level of tyrosine phosphorylated gp180^{erbB-3} in NIH 3T3 cells was not altered upon EGF exposure (lanes 1 and 2). The chimeric EGFR/*erbB-3* receptor that was expressed as a 180-kDa protein, gp180$^{EGFR/erbB-3}$, displayed a low but detectable level of tyrosine phosphorylation in serum-free medium (lane 3). However, EGF triggering of the chimera resulted in a substantial increase in tyrosine phosphorylation, demonstrating EGF-dependent activation of *erbB-3* catalytic function (lane 4). The wild-type EGFR showed a somewhat higher level of EGF-dependent tyrosine phosphorylation under the same conditions (lane 6). Of note, the relative level of gp180^{erbB-3} tyrosine phosphorylation was comparable to that of the EGF-activated chimeric receptor expressed at a similar pro-

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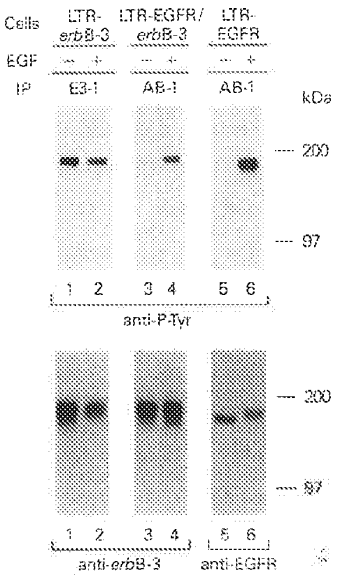

FIG. 4. EGF-dependent tyrosine phosphorylation of an EGFR/*erbB-3* chimeric receptor, $gp180^{EGFR/erbB-3}$. Serum-starved LTR-*erbB-3*, LTR-EGFR/*erbB-3*, and LTR-EGFR transfectants were triggered with 100 ng of EGF per ml. Similar amounts of $gp180^{erbB-3}$, $gp180^{EGFR/erbB-3}$, and EGFR were immunoprecipitated (IP) with *erbB-3* (E3-1) or EGFR (AB-1) monoclonal antibodies followed by immunoblot analysis with anti-*P*-Tyr antibodies or anti-*erbB-3* (MK4) and anti-EGFR (E7) peptide antisera, respectively.

tein level, implying constitutive activation of *erbB-3* catalytic properties in LTR-*erbB-3* transfectants.

We next assessed the biological effects of activated *erbB-3* catalytic function by testing the transfectants for anchorage-independent growth (Table 1). LTR-neo controls (3×10^5 EGF binding sites per cell) failed to exhibit significant soft agar growth in the presence or absence of EGF. In contrast, EGF induced LTR-EGFR/*erbB-3* (1×10^6 binding sites) and LTR-EGFR transfectants (3×10^5 binding sites) to form colonies, although the latter showed greater numbers (Table 1) as well as increased colony size (data not shown). Conversely, the LTR-*erbB-3* transfectant, which expressed 0.6–1 $\times 10^6$ *erbB-3* molecules per cell as compared by immunoblotting with the chimeric receptor transfectant, displayed EGF-independent colony formation with an efficiency similar to that of EGF-activated LTR-EGFR/*erbB-3* cells (Table 1). All of these findings establish that ligand activation of a chimeric EGFR/*erbB-3* receptor causes mitogenic signaling in NIH 3T3 cells. Furthermore, chronic tyrosine phosphorylation of *erbB-3* in LTR-*erbB-3* transfectants is associated with its constitutive signaling in these cells.

Evidence for Activated *erbB-3* Signaling Function in Human Breast Tumor Cells. The availability of *erbB-3*-specific antibodies made it possible to explore expression and activity of $gp180^{erbB-3}$ in human tumor cells. Based upon our previous evidence of *erbB-3* mRNA overexpression in certain breast cancer cell lines (10), we measured *erbB-3* protein levels and tyrosine phosphorylation in such tumor lines. Following immunoprecipitation with MAb E3-1, immunoblot analysis with MK4 antiserum revealed the natural human *erbB-3* product as a 180-kDa protein. The levels of *erbB-3* protein varied markedly among the tumor lines analyzed with highest expression in BT483, MDA-MB453, MDA-MB134, MDA-MB361, SK-BR-3, and MDA-MB468 (Fig. 5). The lowest levels were observed in BT20 and MDA-MB175 cell lines (Fig. 5), comparable with that expressed by nonmalignant 184B5 mammary epithelial cells (data not shown). Thus, *erbB-3* protein expression varied by at least 20- to 30-fold among the lines tested.

Table 1. Anchorage-independent growth of NIH 3T3 transfectants

NIH 3T3 transfectant	Colonies, no. per 10^4 cells	
	Without EGF	With EGF
LTR-neo	1 ± 1	4 ± 2
LTR-EGFR	2 ± 2	206 ± 49
LTR-EGFR/*erbB-3*	7 ± 3	88 ± 16
LTR-*erbB-3*	97 ± 20	94 ± 29

Data are expressed as mean ± standard error of three independent assays.

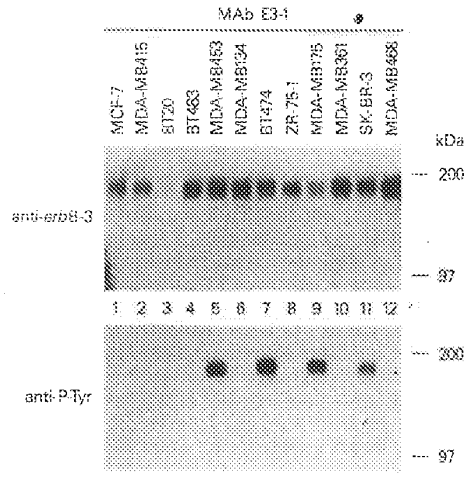

FIG. 5. Activation of $gp180^{erbB-3}$ signaling function in human breast tumor cells. The *erbB-3* protein was immunoprecipitated with MAb E3-1 from 1 mg of total protein lysate and subjected to immunoblot analysis with *erbB-3* peptide antiserum MK4 or phosphotyrosine antibodies as indicated.

Immunoblot analysis of the same immunoprecipitates with anti-*P*-Tyr antibodies revealed that tyrosine phosphorylation of the native *erbB-3* product was undetectable in eight of the tumor cell lines, including MDA-MB134, MDA-MB361, and MDA-MB468, which harbored increased *erbB-3* protein levels. In contrast, the *erbB-3* protein expressed by four cell lines, including MDA-MB453, BT474, MDA-MB175, and SK-BR-3, was chronically phosphorylated on tyrosine (Fig. 5, lanes 5, 7, 9, and 11). In one of these cell lines, MDA-MB175, there was no significant elevation of *erbB-3* protein. These observations provide evidence for constitutive activation of the *erbB-3* signaling function in certain human tumor cell lines.

DISCUSSION

In the present study, we characterized the human *erbB-3* gene product, $gp180^{erbB-3}$, as a transmembrane glycoprotein exhibiting properties characteristic of a receptor-like tyrosine kinase. The recombinant human *erbB-3* protein exhibited electrophoretic mobility indistinguishable from the natural *erbB-3* product expressed by human breast tumor cell lines. Moreover, recombinant and endogenously expressed $gp180^{erbB-3}$ were specifically recognized by different antibodies directed against distinct epitopes. The 145-kDa *erbB-3* polypeptide precursor conformed with the predicted *erbB-3*-encoded protein following cleavage of its signal sequence.

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Finally, our demonstration of *in vitro* kinase activity established that recombinant gp180^{erbB-3} possessed catalytic function. A 160-kDa protein has been reported for *erbB-3* cDNA transiently expressed in COS cells (19). Although functional activity has not been demonstrated for this protein, it remains to be determined whether this lower molecular mass species reflects incomplete processing or some structural difference in its coding sequence.

Although the *erbB-3* tyrosine kinase domain shares >60% amino acid identity with the EGFR and *erbB-2* proteins, there are single amino acid substitutions in highly conserved residues shared among known tyrosine kinases. This has raised the question as to whether *erbB-3* actually harbors intrinsic catalytic activity involved in signal propagation instead of signal attenuation as has been postulated for certain other receptor tyrosine kinase-like molecules (20). Most notably, codon 834 within the tyrosine kinase domain predicts asparagine in *erbB-3*, whereas aspartate is present at this position in essentially all known protein kinases (10, 19, 21). Moreover, substitution of asparagine for aspartate at this position abolishes c-*kit* and v-*fps* tyrosine kinase activity (22, 23). Our characterization of the *erbB-3* cytoplasmic domain demonstrated not only its catalytic function but also the ability to transduce a mitogenic signal. Thus, the *erbB-3* gene encodes a membrane-spanning molecule possessing all properties of a functional growth factor receptor.

Constitutive activation of *erbB-3* catalytic activity was demonstrated in LTR-*erbB-3* transfectants. These results raise the possibility that NIH 3T3 cells may express an *erbB-3* ligand. If so, this putative ligand would unlikely interact with the EGFR, since overexpression of the latter in NIH 3T3 cells is not associated with its tyrosine phosphorylation in the absence of exogenous EGF. We further established that EGF neither enhanced *in vivo* tyrosine phosphorylation of gp180^{erbB-3} nor elicited a mitogenic response in LTR-*erbB-3* cells. Additional ligands of the EGF family including transforming growth factor α (24), amphiregulin (25), and heparin-binding EGF (26) have also failed to stimulate gp180^{erbB-3} tyrosine phosphorylation or DNA synthesis in LTR-*erbB-3* cells (unpublished observations). Though a low-affinity interaction of known EGF-related ligands with gp180^{erbB-3} cannot be excluded, our findings suggest that *erbB-3* and EGFR proteins possess distinct ligand specificities.

EGFR and *erbB-2* genes have been implicated as oncogenes based upon demonstration of their overexpression and constitutive activation in various human tumors (15, 18, 27–31). Our present findings argue strongly that the most recently identified family member, *erbB-3*, is activated in some human breast tumors. Overexpression of the *erbB-3* protein did not invariably correlate with its chronic tyrosine phosphorylation. Hence, *erbB-3* activation may involve autocrine stimulation or subtle genetic alterations. In addition to breast tumors, expression of the *erbB-3* transcript has been observed in a wide range of human carcinomas including colon, lung, kidney, pancreas, and skin (10, 32). This prompts the search for evidence of *erbB-3* activation as an oncogene in these other common human cancers as well.

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1. Ullrich, A. & Schlessinger, J. (1990) *Cell* **61**, 203–212.
2. Aaronson, S. A. (1991) *Science* **254**, 1146–1153.
3. Di Fiore, P. P. & Kraus, M. H. (1991) in *Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer*, eds. Dickson, R. B. & Lippman, M. E. (Kluwer, Boston), Vol. 61, pp. 139–160.
4. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. & Waterfield, M. D. (1984) *Nature (London)* **307**, 521–527.
5. Bargmann, C. I., Hung, M. C. & Weinberg, R. A. (1986) *Cell* **45**, 649–657.
6. Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R. & Aaronson, S. A. (1987) *Science* **237**, 178–182.
7. Hudziak, R. M., Schlessinger, J. & Ullrich, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7159–7163.
8. Di Fiore, P. P., Pierce, J. H., Fleming, T. P., Hazan, R., Ullrich, A., King, C. R., Schlessinger, J. & Aaronson, S. A. (1987) *Cell* **51**, 1063–1070.
9. Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I. & Lowy, D. R. (1987) *Science* **238**, 1408–1410.
10. Kraus, M. H., Issing, W., Miki, T., Popescu, N. C. & Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9193–9197.
11. Cepko, C. L., Roberts, B. E. & Mulligan, R. C. (1984) *Cell* **37**, 1053–1062.
12. Di Fiore, P., Segatto, O., Taylor, W. G., Aaronson, S. A. & Pierce, J. H. (1990) *Science* **248**, 79–83.
13. Gaifré, G. & Milstein, C. (1981) *Methods Enzymol.* **73**, 3–46.
14. Muraro, R., Kuroki, M., Wunderlich, D., Poole, D. J., Colcher, D., Thor, A., Greiner, J. W., Simpson, J. F., Molinolo, A., Noguchi, P. & Schlom, J. (1988) *Cancer Res.* **48**, 4588–4596.
15. Kraus, M. H., Popescu, N. C., Amsbaugh, S. C. & King, C. R. (1987) *EMBO J.* **6**, 605–610.
16. Di Fiore, P., Segatto, O., Lonardo, F., Fazioli, F., Pierce, J. H. & Aaronson, S. A. (1990) *Mol. Cell. Biol.* **10**, 2749–2756.
17. King, C. R., Kraus, M. H., Williams, L. T., Merlino, G. T., Pastan, I. H. & Aaronson, S. A. (1985) *Nucleic Acids Res.* **13**, 8477–8486.
18. Di Marco, E., Pierce, J. H., Fleming, T. P., Kraus, M. H., Molloy, C. J., Aaronson, S. A. & Di Fiore, P. P. (1989) *Oncogene* **4**, 831–838.
19. Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J. & Shoyab, M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4905–4909.
20. Chou, Y. H. & Hayman, M. J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4897–4901.
21. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241**, 42–52.
22. Moran, M. F., Koch, C. A., Sadowski, I. & Pawson, T. (1988) *Oncogene* **3**, 665–672.
23. Tan, J. C., Nocka, K., Ray, P., Traktman, P. & Besmer, P. (1990) *Science* **247**, 209–212.
24. Marquardt, H., Hunkapiller, M. W., Hood, L. E., Twardzik, D. R., De, L. J., Stephenson, J. R. & Todaro, G. J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4684–4688.
25. Shoyab, M., Plowman, G. D., McDonald, V. L., Bradley, J. G. & Todaro, G. J. (1989) *Science* **243**, 1074–1076.
26. Higashiyama, S., Abraham, J. A., Miller, J., Fiddes, J. C. & Klagsbrun, M. (1991) *Science* **251**, 936–939.
27. Xu, Y. H., Richert, N., Ito, S., Merlino, G. T. & Pastan, I. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7308–7312.
28. Lin, C. R., Chen, W. S., Kruiger, W., Stolarsky, L. S., Weber, W., Evans, R. M., Verma, I. M., Gill, G. N. & Rosenfeld, M. G. (1984) *Science* **224**, 843–848.
29. Libermann, T. A., Nusbaum, H. R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M. D., Ullrich, A. & Schlessinger, J. (1985) *Nature (London)* **313**, 144–147.
30. vandeVijver, M., vandeBersselaar, R., Devilee, P., Cornelisse, C., Peterse, J. & Nusse, R. (1987) *Mol. Cell. Biol.* **7**, 2019–2023.
31. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. & Press, M. F. (1989) *Science* **244**, 707–712.
32. Ciardiello, F., Kim, N., Saeki, T., Dono, R., Persico, M. G., Plowman, G. D., Garrigues, J., Radke, S., Todaro, G. J. & Salomon, D. S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7792–7796.

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Biochemistry

Isolation and characterization of *ERBB3*, a third member of the *ERBB*/epidermal growth factor receptor family: Evidence for overexpression in a subset of human mammary tumors

(receptor-like kinase/sequence/chromosomal mapping/expression)

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ABSTRACT **A related DNA fragment distinct from the epidermal growth factor receptor and *ERBB2* genes was detected by reduced stringency hybridization of v-*erbB* to normal genomic human DNA. Characterization of the cloned DNA fragment mapped the region of v-*erbB* homology to three exons with closest identity of 64% and 67% to a contiguous region within the tyrosine kinase domains of the epidermal growth factor receptor and ERBB2 proteins, respectively. cDNA cloning revealed a predicted 148-kDa transmembrane polypeptide with structural features identifying it as a member of the *ERBB* gene family, prompting us to designate the gene as *ERBB3*. It was mapped to human chromosome 12q13 and was shown to be expressed as a 6.2-kilobase transcript in a variety of normal tissues of epithelial origin. Markedly elevated ERBB3 mRNA levels were demonstrated in certain human mammary tumor cell lines. These findings suggest that increased *ERBB3* expression, as in the case of epidermal growth factor receptor and *ERBB2*, may play a role in some human malignancies.**

Protooncogenes encoding growth factor receptors constitute several distinct families with close overall structural homology. The highest degree of homology is observed in their catalytic domains, essential for the intrinsic tyrosine kinase activity of these proteins (1). Examples of such families include genes encoding epidermal growth factor receptor (EGF-R) and ERBB2, the colony-stimulating factor 1/platelet-derived growth factor receptors, insulin/insulin-like growth factor I receptors, and EPH/ELK (2–12). Growth factor receptors in several of these families play critical roles in regulation of normal growth and development. Some of these molecules have been implicated in the neoplastic process as well. In particular, both the EGF-R gene and *ERBB2* have been shown to be activated as oncogenes by mechanisms involving overexpression or mutations that constitutively activate the catalytic activity of their encoded proteins (13–16). Thus, we undertook the present studies in an effort to identify and isolate additional members of the *ERBB* protooncogene family.

MATERIALS AND METHODS

Human Cells. Mammary epithelial cells AB589 (17) and immortalized keratinocytes RHEK (18) were provided by M. Stampfer (Lawrence Berkeley Laboratory) and J. Rhim, respectively. Normal human epidermal melanocytes (NHEM) and keratinocytes (NHEK) were obtained from Clonetics (San Diego, CA). Sources for human embryo fibroblasts (19) or mammary tumor cell lines (20) have been described.

DNA and RNA Hybridization. High-stringency hybridization was conducted as described (20). Reduced-stringency hybridization of DNA was carried out in 30% (vol/vol) formamide followed by washes in 0.6× SSC, whereas intermediate stringency was achieved by hybridization in 40% (vol/vol) formamide and washing in 0.25× SSC.

Molecular Cloning. An oligo(dT)-primed human placenta cDNA library was obtained from Clontech. The oligo(dT) primed MCF-7 cDNA library was constructed in ApCEV9 (21). After plaque purification, phage DNA inserts were subcloned into pUC-based plasmid vectors for further characterization.

Nucleotide and Amino Acid Sequence Analysis. The nucleotide sequence was determined for both DNA strands by the dideoxy chain-termination method (22) using supercoiled plasmid DNA as template.[‡] Amino acid sequence comparison was performed with the alignment program by Pearson and Lipman (23). Hydrophobic and hydrophilic regions in the predicted protein were identified according to Kyte and Doolittle (24).

RESULTS

Identification of a Third Member of the *ERBB* Protooncogene Family. In an effort to detect novel *ERBB*-related genes, human genomic DNA was cleaved with a variety of restriction endonucleases and subjected to Southern blot analysis with v-*erbB* as probe. Under reduced stringency hybridization, four *Sac* I restriction fragments were detected. Two were identified as EGF-R gene fragments by their amplification in MDA-MB468 cells (Fig. 1*A*, lanes 1 and 2) known to contain EGF-R gene amplification and one as an *ERBB2*-specific gene fragment due to its increased signal intensity in *ERBB2*-amplified SK-BR-3 cells (Fig. 1*A*, lanes 1 and 3). However, a single 9-kbp *Sac* I fragment exhibited equal signal intensities in normal human thymus, A431, and SK-BR-3 DNA (Fig. 1*A*). When the hybridization stringency was raised by 7°C, this fragment did not hybridize, whereas EGF-R and *ERBB2*-specific restriction fragments were still detected with v-*erbB* as a probe (Fig. 1*B*). Taken together, these findings suggested the specific detection of another v-*erbB*-related DNA sequence within the 9-kbp *Sac* I fragment.

For further characterization we prepared a normal human genomic library from *Sac* I-cleaved thymus DNA enriched for 8- to 12-kbp fragments. Ten recombinant clones detected by v-*erbB* under reduced stringency conditions did not hybridize with human EGF-R or ERBB2 cDNA probes at high stringency. As shown in the restriction map of a repre-

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Abbreviation: EGF, epidermal growth factor.

‡The *ERBB3* nucleotide sequence has been deposited in the GenBank data base (accession no. M29366).

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sentative clone with a 9-kbp insert, the region of v-*erbB* homology was localized by hybridization analysis to a 1.5-kbp segment spanning from the *Eco*RI to the downstream *Pst* I site. Nucleotide sequence analysis revealed that this region contained three open reading frames bordered by splice junction consensus sequences (Fig. 2). The predicted amino acid sequence of these three open reading frames revealed the highest identity scores of 64–67% to three regions that are continuous in the tyrosine kinase domains of v-*erbB*, as well as human EGF-R and ERBB2 proteins. Furthermore, all splice junctions of the three characterized exons in the gene were conserved with *ERBB2*. Amino acid sequence homology to other known tyrosine kinases was significantly lower, ranging from 39–46%.

A single 6.2-kb-specific mRNA was identified by Northern (RNA) blot analysis of human epithelial cells by using the 150-base pair (bp) *Spe* I–*Acc* I exon-containing fragment as probe (Fig. 2). Under the stringent hybridization conditions used, this probe detected neither the 5-kb ERBB2 mRNA nor the 6- and 10-kb EGF-R mRNAs (data not shown). All of these findings suggested that we had identified an additional functional member of the *ERBB* protooncogene family, which we tentatively designated as *ERBB3*.

Close Structural Similarity of the Predicted ERBB3 Protein with Other *ERBB* Family Members. In an effort to characterize the entire *ERBB3* coding sequence, overlapping cDNA clones were isolated from oligo(dT)-primed cDNA libraries from sources with known *ERBB3* expression, utilizing gene-specific genomic exons or cDNA fragments as probes. The clones were initially characterized by restriction analysis and hybridization to the mRNA and were subsequently subjected to nucleotide sequence analysis. The clones pE3-8, pE3-9, pE3-11, and pE3-16 contained identical 3′ ends terminating in a poly(A) stretch (Fig. 2).

The complete coding sequence of *ERBB3* was contained within a single long open reading frame of 4080 nucleotides extending from position 46 to an in-frame termination codon at position 4126. The most upstream ATG codon at position

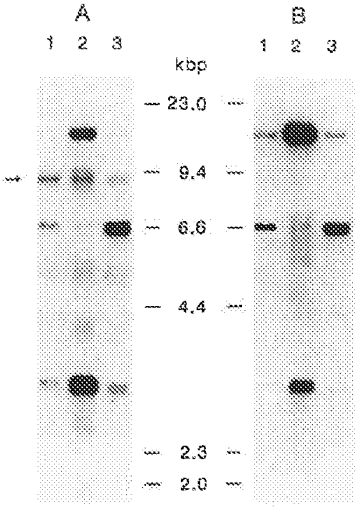

FIG. 1. Detection of v-*erbB*-related gene fragments in normal human thymus (lane 1), MDA-MB468 (lane 2), SK-BR-3 (lane 3). DNAs were restricted with *Sac* I and hybridized with a v-*erbB*-specific probe spanning from the upstream *Bam*HI to the *Eco*RI site in avian erythroblastosis proviral DNA (25). Hybridization was conducted at reduced (*A*) or intermediate (*B*) stringency conditions. The arrow denotes a 9-kbp *ERBB*-related restriction fragment distinct from those of EGF-R and *ERBB2*.

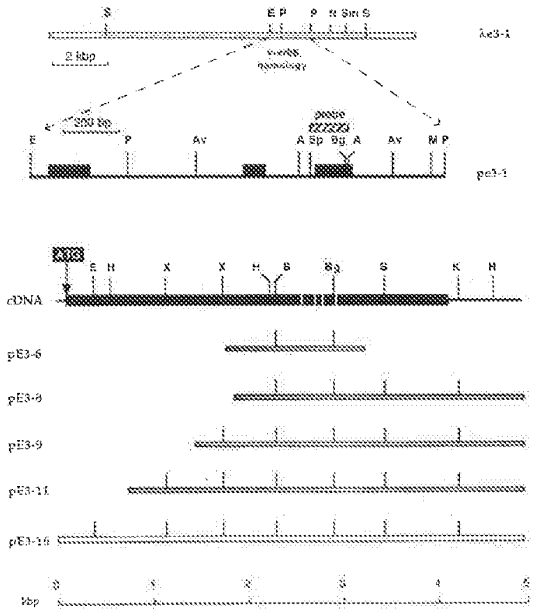

FIG. 2. Genomic and cDNA cloning of *ERBB3*. The region of v-*erbB* homology within the genomic 9-kbp *Sac* I insert of λe3-1 was subcloned into pUC (pe3-1) and subjected to nucleotide sequence analysis. The three predicted exons are depicted as solid boxes. ERBB3 cDNA clones were isolated from normal human placenta (shaded bars) and MCF-7 (open bar) oligo(dT)-primed libraries. The entire nucleotide sequence was determined for both strands on ERBB3 cDNA from normal human placenta and upstream of the 5′ *Xho* I site on pE3-16. The coding sequence is shown as a solid bar, and splice junctions of the three characterized genomic exons are indicated by vertical white lines. Solid lines in the cDNA map represent untranslated sequences. Restriction sites: A, *Acc* I; Av, *Ava* I; B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; *H*, *Hin*dIII; K, *Kpn* I; M, *Mst* II; P, *Pst* I; S, *Sac* I; Sm, *Sma* I; and Sp, *Spe* I.

100 was the likely initiation codon, as it was preceded by an in-frame stop codon at nucleotide position 43 and fulfilled Kozak's criteria (26) for an authentic initiation codon. The open reading frame comprised 1342 codons, predicting a 148-kDa polypeptide. As shown in Fig. 3, the deduced amino acid sequence of the ERBB3 polypeptide predicted a transmembrane receptor tyrosine kinase most closely related to EGF-R and ERBB2. A hydrophobic signal sequence of ERBB3 was predicted to comprise the 19 amino-terminal amino acid residues. Cleavage of this signal sequence between Gly-19 and Ser-20 would generate a processed polypeptide of 1323 amino acids with an estimated molecular mass of 145 kDa. A single hydrophobic membrane-spanning domain encompassing 21 amino acids was identified (Fig. 3).

The putative ERBB3 ligand-binding domain was 43% and 45% identical in amino acid residues with the predicted ERBB2 and EGF-R protein, respectively. Within the extracellular domain, all 50 cysteine residues of the processed ERBB3 polypeptide were conserved and similarly spaced when compared with the EGF-R and ERBB2. Forty-seven cysteine residues were organized in two clusters containing 22 and 25 cysteines, respectively, a structural hallmark of this tyrosine kinase receptor subfamily (2–4). Ten potential N-linked glycosylation sites were localized within the ERBB3 extracellular domain. In comparison with the EGF-R and ERBB2 proteins, five and two of these glycosylation sites were conserved, respectively. Among these, the site proximal to the transmembrane domain was conserved among all three proteins (Fig. 3).

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Within the cytoplasmic domain, a core of 277 amino acids from position 702–978 revealed the most extensive homology with the tyrosine kinase domains of EGF-R and ERBB2. In this region 60 or 62% of amino acid residues were identical and 90 or 89% were conserved, respectively. This stretch of amino acid homology coincides with the minimal catalytic domain of tyrosine kinases (1). There was significantly lower homology with other tyrosine kinases (Fig. 3). The consensus sequence for an ATP-binding site Gly-Xaa-Gly-Xaa-Xaa-Gly (1) at amino acid position 716–721 as well as a lysine residue located 21 amino acid residues farther carboxyl-terminal were conserved between the three ERBB-related receptors. Taken together, these findings defined the region between amino acid position 702 and 978 as the putative catalytic domain of the ERBB3 protein (Fig. 3).

The most divergent region of ERBB3 compared with either EGF-R or ERBB2 was its carboxyl terminus, comprising 364 amino acids. Tyrosine residues at positions 1197, 1199, and 1262 matched closest with the consensus sequence for putative phosphorylation sites (28). The peptide one-letter sequence YEYMN, encompassing Tyr-1197 and Tyr-1199, was repeated at positions 1260–1264 and was at both locations surrounded by charged residues. These observations render Tyr-1197, Tyr-1199, and Tyr-1262 likely candidates for autophosphorylation sites of the ERBB3 protein.

Chromosomal Mapping of Human *ERBB3*. We determined the chromosomal location of the *ERBB3* gene by *in situ* hybridization (29) with an ^{3}H-labeled plasmid containing the *ERBB3* amino-terminal coding sequence. A total of 110 human chromosome spreads were examined prior and subsequent to G banding for identification of individual chromosomes. One hundred forty-two grains were localized on a 400-band ideogram. We observed specific labeling of chromosome 12, where 38 out of 51 grains were localized to band q13 (data not shown). Thus, the genomic locus of *ERBB3* was assigned to 12q13. In this region of chromosome 12, several genes have previously been mapped including the melanoma-associated antigen ME491 (30), histone genes (31) and the gene for lactalbumin (32). In addition, two protooncogenes, *INT1* (33) and *GLI* (34), are located in close proximity to *ERBB3*.

***ERBB3* Expression in Normal and Malignant Human Cells.** To investigate its pattern of expression, we surveyed a number of human tissues for the ERBB3 transcript. The 6.2-kb ERBB3-specific mRNA was observed in term placenta, postnatal skin, stomach, lung, kidney, and brain, but it was not detectable in skin fibroblasts, skeletal muscle, or lymphoid cells (data not shown). Among the fetal tissues analyzed, the ERBB3 transcript was expressed in liver, kidney, and brain but not in fetal heart or embryonic lung fibroblasts. These

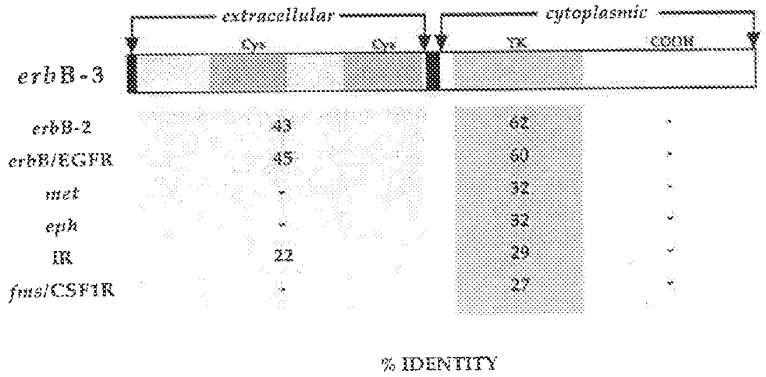

FIG. 3. (*Upper*) Predicted amino acid sequence of the ERBB3 polypeptide (as deduced from the GenBank sequence M29366) and comparison with other receptor-like tyrosine kinases. The amino acid sequence is shown in single-letter code and is numbered at left. The putative extracellular domain (light shading) extends between the predicted signal sequence (solid box) at the amino terminus and a single hydrophobic transmembrane region (solid box) within the polypeptide. The putative ATP-binding site at the amino terminus of the TK domain is circled. Potential autophosphorylation sites within the carboxyl-terminal domain (COOH) are indicated by asterisks. Potential N-linked glycosylation sites (⟜) are marked above the amino acid sequence. (*Lower*) The two cysteine clusters (Cys) in the extracellular domain and the predicted tyrosine kinase domain (TK) within the cytoplasmic portion of the polypeptide are outlined by dark shading. The percentage of amino acid homology of ERBB3 in individual domains with ERBB2 (4), EGF-R (2), MET (27), EPH (11), insulin receptor [IR (9)], and FMS (5) is listed below. Less than 16% identity is denoted by -.

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observations indicated the preferential expression of the ERBB3 transcript in epithelial tissues and brain.

We also investigated *ERBB3* expression in individual cell populations in comparison to EGF-R and ERBB2 transcripts. As shown in Table 1, mRNA levels of each were relatively high in keratinocytes and low but similar in cells derived from glandular epithelium. These findings are consistent with growth regulatory roles of all three receptor-like molecules in squamous and glandular epithelium. Whereas ERBB2 and EGF-R transcripts were also readily seen in normal fibroblasts, the same cells lacked detectable ERBB3 mRNA. In contrast, normal human melanocytes, which expressed both ERBB3 and ERBB2 at levels comparable with human keratinocytes, lacked detectable EGF-R transcripts. Thus, the expression patterns of these receptor-like molecules were different in specialized cell populations derived from epidermal tissues.

The ERBB3 transcript was detected in 36 of 38 carcinomas and 2 of 12 sarcomas, whereas 7 tumor cell lines of hematopoetic origin lacked measurable ERBB3 mRNA. Markedly elevated levels of a normal-sized transcript were observed in 6 of 17 tumor cell lines derived from human mammary carcinomas. By Southern blot analysis, neither gross gene rearrangement nor amplification was detected in the cell lines (data not shown). Fig. 4*A* shows the results of Northern blot analysis with control AB589 nonmalignant human mammary epithelial cells (lane 1) and two representative human mammary tumor lines, MDA-MB415 (lane 2) and MDA-MB453 (lane 3). Hybridization of the same filter with a human β actin probe (Fig. 4*B*) verified levels of mRNA in each lane. Densitometric scanning indicated that the ERBB3 transcript in each tumor cell line was elevated more than 100-fold above that of the control cell line. Thus, overexpression of this third member of the *ERBB* family, as for the EGF-R and *ERBB2* genes, may play an important role in some human malignancies.

DISCUSSION

In the present report, we describe the identification of a third member of the *ERBB*/EGF receptor family of membrane-spanning tyrosine kinases and the cloning of its full-length coding sequence. This gene, designated *ERBB3*, encodes a predicted protein with striking structural similarities to other members of this family. These features include overall size, extracellular domain with two signature cysteine clusters, and its uninterrupted tyrosine kinase domain, exhibiting with 81% and 83% significantly greater overall similarities to EGF-R and ERBB2 products than to any other tyrosine kinase. The structural relatedness of its extracellular domain with that of the EGF-R raises the possibility that one or more of an increasing number of EGF-like ligands (36) may interact with the ERBB3 product.

Distinct regions within the predicted *ERBB3* coding sequence revealed relatively higher degrees of divergence. For example, its carboxyl-terminal domain failed to exhibit significant colinear identity scores with either ERBB2 or EGF-R. Within the tyrosine kinase domain, which represents the most conserved region of the predicted ERBB3 protein, a short stretch of 29 amino acids carboxyl-terminal to the ATP-binding site differed from regions of the predicted ERBB2 and EGF-R coding sequence in 28 and 25 positions, respectively. Such regions of higher divergence in their cytoplasmic domains may confer functional specificity to these closely related receptor-like molecules.

Chromosomal mapping localized *ERBB3* to human chromosome 12q11-13, whereas the related EGF-R and *ERBB2* genes are located on chromosomes 7p12-13 (37) and 17p12-21.3 (3, 29), respectively. Thus, each appears to localize to regions containing different respective homeobox (38, 39) and collagen gene (40) loci. Keratin type I and type II genes also map to regions of 12 and 17 (41, 42), consistent with localization of *ERBB3* and *ERBB2*, respectively.

Table 1. Normal expression pattern of human *ERBB* gene family members

Source	Relative transcript levels		
	ERBB3	ERBB2	EGF-R
Embryonic fibroblast (M426)	−	+	+
Skin fibroblast (501T)	−	+	+
Immortal keratinocyte (RHEK)	++	++	++
Primary keratinocyte (NHEK)	+	+	++
Glandular epithelium (AB589)	(+)	(+)	(+)
Melanocyte (NHEM)	++	++	−

Replicate Northern blots were hybridized with equal probe counts of similar specific activity for ERBB3, ERBB2, and EGF receptor, respectively. Relative signal intensities were semiquantitatively estimated: −, not detectable; (+), weakly positive; +, positive; ++, strongly positive.

Recent studies in *Drosophila* have emphasized how critical and multifunctional are developmental processes mediated by ligand–receptor interactions. An increasing number of *Drosophila* mutants with often varying phenotypes have now been identified as being due to lesions in genes encoding such proteins (43, 44) including the *Drosophila* EGF-R homologue, designated DER. It is not yet known whether DER is the Drosophila counterpart of all three mammalian *ERBB* genes. If so, functions assigned to DER may eventually be associated with one or more of the divergent mammalian *ERBB* genes as well as other functions that have evolved in more complex mammalian organisms.

There is evidence for autocrine (45, 46) as well as paracrine (19, 47) effectors of normal cell proliferation. However, the inherent transforming potential of autocrine growth factors (48, 49) suggests that growth factors most commonly act on their target cell populations by a paracrine route. Our survey of *ERBB3* gene expression indicated its normal expression in cells of epithelial and neuroectodermal derivation. Comparative analysis of the three *ERBB* receptor-like genes in different cell types of epidermal tissue revealed that keratinocytes expressed all three genes. In contrast, melanocytes and stromal fibroblasts specifically lacked EGF-R and

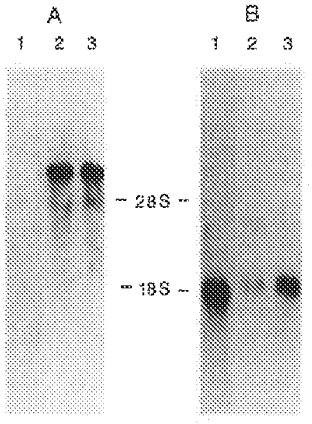

FIG. 4. Elevated ERBB3 transcript levels in human mammary tumor cell lines. A Northern blot containing 10 μg of total cellular RNA from AB589 mammary epithelial cells (lane 1), as well as MDA-MB415 (lane 2) and MDA-MB453 (lane 3) mammary tumor cell lines was hybridized with an ERBB3 cDNA probe (*A*). After signal decay the same blot was rehybridized with a human β actin cDNA probe (35).

ERBB3 transcripts, respectively. Thus, melanocytes and stromal fibroblasts may be sources of paracrine growth factors for EGF-R and ERBB3 products, respectively, that are expressed by the other cell types residing in close proximity in epidermal tissues.

To date, both *ERBB*/EGFR and *ERBB2* have been causally implicated in human malignancy. EGF-R gene amplification and/or overexpression in tumors has been demonstrated in squamous cell carcinomas and glioblastomas (50). *ERBB2* amplification and/or overexpression has been observed in human breast and ovarian carcinomas (51, 52), and *ERBB2* overexpression has been reported to be an important prognostic indicator of particularly aggressive tumors (52). Thus, our present findings that the ERBB3 transcript is overexpressed in a significant fraction of human mammary tumor cell lines raises the possibility that this new member of the ERBB/EGF receptor family may also play an important role in some human malignancies.

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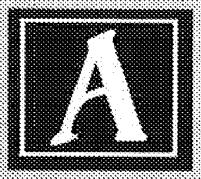
1. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241**, 42–52.
2. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D. & Seeburg, P. H. (1984) *Nature (London)* **309**, 418–425.
3. Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., Francke, U., Levinson, A. & Ullrich, A. (1985) *Science* **230**, 1132–1139.
4. Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T. & Toyoshima, K. (1986) *Nature (London)* **319**, 230–234.
5. Coussens, L., van Beveren, C., Smith, D., Chen, E., Mitchell, R. L., Isacke, C. M., Verma, I. M. & Ullrich, A. (1986) *Nature (London)* **320**, 277–280.
6. Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T. & Stanley, E. R. (1985) *Cell* **41**, 665–676.
7. Yarden, Y., Escobedo, J. A., Kuang, W.-J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Francke, U., Fried, V. A., Ullrich, A. & Williams, L. T. (1986) *Nature (London)* **323**, 226–232.
8. Matsui, T., Heidaran, M., Miki, T., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J. & Aaronson, S. (1989) *Science* **243**, 800–804.
9. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M. & Ramachandran, J. (1985) *Nature (London)* **313**, 756–761.
10. Ullrich, A., Gray, A., Tam, W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J. & Fujita-Yamaguchi, Y. (1986) *EMBO J.* **5**, 2503–2512.
11. Hirai, H., Maru, Y., Hagiwara, K., Nishida, J. & Takaku, F. (1987) *Science* **238**, 1717–1720.
12. Letwin, K., Yee, S.-P. & Pawson, T. (1988) *Oncogene* **3**, 621–627.
13. Bargmann, C. I., Hung, M. C. & Weinberg, R. A. (1986) *Cell* **45**, 649–657.
14. Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R. & Aaronson, S. A. (1987) *Science* **237**, 178–182.
15. Di Fiore, P. P., Pierce, J. H., Fleming, T. P., Hazan, R., Ullrich, A., King, C. R., Schlessinger, J. & Aaronson, S. A. (1987) *Cell* **51**, 1063–1070.
16. Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I. & Lowy, D. R. (1987) *Science* **238**, 1408–1410.
17. Walen, K. H. & Stampfer, M. R. (1989) *Cancer Genet. Cytogenet.* **37**, 249–261.
18. Rhim, J. S., Jay, G., Arnstein, P., Price, F. M., Sanford, K. K. & Aaronson, S. A. (1985) *Science* **227**, 1250–1252.
19. Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G. & Rudikoff, S. & Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 802–806.
20. Kraus, M. H., Popescu, N. C., Amsbaugh, S. C. & King, C. R. (1987) *EMBO J.* **6**, 605–610.
21. Miki, T., Matsui, T., Heidaran, M. A. & Aaronson, S. A. (1989) *Gene*, in press.
22. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
23. Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2444–2448.
24. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
25. Vennstrom, B., Fanshier, L., Moscovici, C. & Bishop, J. M. (1980) *J. Virol.* **36**, 575–585.
26. Kozak, M. (1987) *Nucleic Acids Res.* **15**, 8125–8148.
27. Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. A. & van de Woude, G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6379–6383.
28. Smart, J. E., Oppermann, H., Czernilofsky, A. P., Purchio, A. F., Erikson, R. L. & Bishop, J. M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6013–6017.
29. Popescu, N. C., King, C. R. & Kraus, M. H. (1989) *Genomics* **4**, 362–366.
30. Hotta, H., Ross, A. H., Huebner, K., Isobe, M., Wendeborn, S., Chao, M. V., Ricciardi, R. P., Tsujimoto, Y., Croce, C. M. & Koprowski, H. (1988) *Cancer Res.* **48**, 2955–2962.
31. Trippoti, P., Emanuel, B. S., Croce, C. M., Green, L. G., Stein, G. S. & Stein, J. L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3185–3188.
32. Davies, M. S., West, L. F., Davies, M. B., Povey, S. & Craig, R. K. (1987) *Ann. Hum. Genet.* **51**, 183–188.
33. Turc-Carel, C., Pietrzak, E., Kakati, S., Kinniburgh, A. J. & Sandberg, A. A. (1987) *Oncogene Res.* **1**, 397–405.
34. Kinsler, K. W., Bigner, S. H., Bigner, D. D., Trent, J. M., Law, M. L., O'Brien, S. J., Wong, A. J. & Vogelstein, B. (1987) *Science* **236**, 70–73.
35. Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H. & Kedes, L. (1983) *Mol. Cell. Biol.* **3**, 787–795.
36. Shoyab, M., Plowman, G. D., McDonald, V. L., Bradley, J. G. & Todaro, G. J. (1989) *Science* **243**, 1074–1076.
37. Spurr, N. K., Solomon, E., Jansson, M., Sheer, D., Goodfellow, P. N., Bodmer, W. F. & Vennstrom, B. (1984) *EMBO J.* **3**, 159–163.
38. Rabin, M., Ferguson-Smith, A., Hart, C. P. & Ruddle, F. H. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9104–9108.
39. Cannizzaro, L. A., Huebner, K., Griffen, C. A., Simeone, A., Boncinelli, E. & Croce, C. M. (1987) *Am. J. Hum. Genet.* **41**, 1–15.
40. Retief, E., Parker, M. I. & Retief, A. E. (1985) *Hum. Genet.* **69**, 304–308.
41. Rosenberg, M., RayChaudury, A., Shows, T. B., Le Beau, M. M. & Fuchs, E. (1988) *Mol. Cell. Biol.* **8**, 722–736.
42. Popescu, N. C., Bowden, P. & DiPaolo, J. A. (1989) *Hum. Genet.* **82**, 109–112.
43. Schejter, E. D. & Shilo, B. Z. (1989) *Cell* **56**, 1093–1104.
44. Price, J. V., Clifford, R. J. & Schupbach, T. (1989) *Cell* **56**, 1085–1092.
45. Sporn, M. B. & Todaro, G. J. (1980) *N. Engl. J. Med.* **303**, 878–880.
46. Kaplan, P. L., Anderson, M. & Ozanne, B. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 485–489.
47. Weiner, H. L. & Swain, J. L. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2683–2687.
48. Doolittle, R. F., Hunkapiller, M. W., Hood, L. E., Devare, S. G., Robbins, K. C., Aaronson, S. A. & Antoniades, H. N. (1983) *Science* **221**, 275–277.
49. Waterfield, M. D., Scrace, G. T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C. H., Huang, J. S. & Deuel, T. F. (1983) *Nature (London)* **304**, 35–39.
50. Libermann, T. A., Nusbaum, H. R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M. D., Ullrich, A. & Schlessinger, J. (1985) *Nature (London)* **313**, 144–147.
51. King, C. R., Kraus, M. H. & Aaronson, S. A. (1985) *Science* **229**, 974–976.
52. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. & Press, M. F. (1989) *Science* **244**, 707–712.



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Update

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Antitumour activity of DX-8951f: a new camptothecin derivative

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DX-8951f is a water-soluble camptothecin analogue with a unique hexacyclic structure. Compared to other current camptothecin derivatives, DX-8951f is the most effective topoisomerase I (topo I) inhibitor and has the most potent cytotoxic activity against various tumour cell lines *in vitro*. Of particular interest is DX-8951f's significant effect on certain tumour cell lines resistant to other camptothecin derivatives, as well as on multi-drug resistant variants that overexpress P-glycoprotein. In addition, in *in vivo* xenograft systems using nude mice, DX-8951f strongly inhibits the growth of human solid tumours, including resistant tumours. Its antitumour effects and resulting life prolongation in tumour-bearing mice have also been confirmed in several metastasis models. DX-8951f provides greater therapeutic efficacy and broader effective dose ranges using multiple injections than with a bolus injection and simple intermittent applications. The *in vivo* effects of the compound are superior to those of CPT-11 and SK&F104864, suggesting that DX-8951f is a promising therapeutic agent for the treatment of cancer patients. Phase I clinical trials are ongoing in Europe, the USA and Japan.

Keywords: *antitumour activity, camptothecin derivative, DX-8951f, preclinical studies*

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1. Introduction

Camptothecin, isolated from the plant *Camptotheca acuminata*, demonstrates antitumour activity by the inhibition of DNA topoisomerase I (topo I) [1,2]. In spite of its activity against murine tumours, this compound has proven disappointing due both to its low response rate in clinical trials and its significant and unpredictable toxicity [3,4]. Following extensive research into new camptothecin derivatives, CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin hydrochloride trihydrate, **Figure 1**) and SK&F104864 (9-[(dimethylamino)methyl]-10-hydroxy-(20*S*)-camptothecin, topotecan, **Figure 1**), semi-synthetic water soluble derivatives were found to exhibit higher antitumour activity with less toxicity than camptothecin, both *in vitro* and *in vivo* [5-9]. Further clinical evaluation revealed that CPT-11 is a promising agent for human cancers [10]. However, CPT-11 has been reported to characteristically induce severe watery diarrhoea that is occasionally life-threatening [11]. In addition, interpatient variations in both its effects and toxicity have been observed due to the fact that CPT-11 is a pro-drug and is converted *in vivo* to the active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38, **Figure 1**) [7,12]. The clinical activity and toxicity of CPT-11 strongly depend on its conversion to SN-38. SK&F104864

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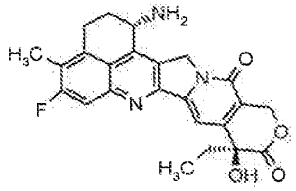
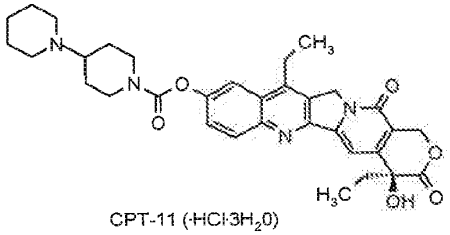
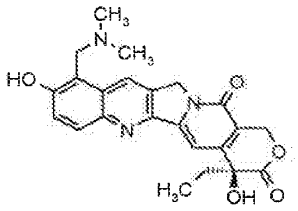
Figure 1: Chemical structures of camptothecin analogues. Empirical formula of the salt hydrate of each compound is indicated in parentheses.



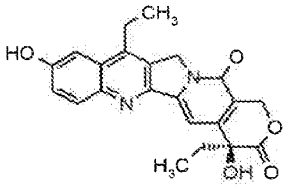
DX-8951f ($\cdot CH_3SO_3H \cdot 2H_2O$)



CPT-11 ($\cdot HCl \cdot 3H_2O$)



SK&F104864 ($\cdot HCl \cdot H_2O$)



SN-38 ($\cdot H_2O$)

has also been administered by several investigators using a variety of regimens, with antitumour activity reported in certain cancers [13,14,15].

In order to obtain a more effective analogue with lower toxicity than the older camptothecin derivatives, Daiichi synthesised many camptothecin derivatives and finally obtained a novel, non-prodrug water-soluble analogue, DX-8951f, (1*S*,9*S*)-1-amino--9ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1*H*,12*H*-benzo[*de*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione monomethanesulfonate dihydrate (**Figure 1**; [16,101]). This is a unique hexacyclic compound with a fluorine substituent. Camptothecin and other current derivatives, such as CPT-11, SK&F104864, SN-38 and 9-amino-camptothecin (9-AC) are pentacyclic analogues. In this paper, the *in vitro* and *in vivo* antitumour effects of DX-8951f against various human and murine tumours, including certain drug-resistant variants, are discussed.

2. Mode of action

Camptothecin and its derivatives have been shown to inhibit topo I by stabilising the enzyme-DNA cleavable complex [17,18], thereby inducing the lethal impairment of cells through the strong inhibition of DNA synthesis. Although the mechanisms of their cytotoxicity have not been precisely determined, CPT-11 and SN-38, like other anticancer agents were reported to induce apoptotic cell death [19]. Compared to camptothecin, SN-38 and SK&F104864, DX-8951f is a far more potent topo I inhibitor [20]. This inhibitory activity, as indicated by the IC_{50} value in the DNA relaxation assay, was approximately 3- to 24-times as potent as that of other analogues. The activity of DX-8951f in stabilising the DNA-topo I cleavable complex was approximately 2.5-times and 5-times higher than that of SN-38 and SK&F104864, respectively [21]. DX-8951f, in addition to SN-38, has been shown to induce apoptotic death. DNA fragmentation, a process believed to be an outcome of cell apoptosis, was dose-dependently induced by DX-8951f, and this effect was more potent than that of SN-38 [22]. These results indicate that DX-8951f has the potential to induce apoptosis and that this seems to be the major pathway by which the drug kills cells, following topo I inhibition [22].

3. Cell-killing kinetics

Cancer chemotherapeutics can be categorised by their cytocidal activity as either concentration-dependent or time-dependent [23-25]. Concentration-dependent agents exhibit strong cytotoxicity when their concentrations reach sufficient levels, even transiently, but are not cytocidal at lower levels, even following long periods of exposure [23]. By contrast, time-dependent-type agents can exhibit significant cytotoxicity following continuous, long-term exposure at certain effective dose levels, but fail to

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show activity with short-term exposure, even at higher concentrations [24]. In general, cycle-phase nonspecific drugs, for example, anticancer antibiotics and alkylating agents, belong to the concentration-dependent category [23], and cycle-phase specific agents, such as antimetabolites and Vinca alkaloids, fall into the time-dependent category [24]. It has been demonstrated that optimal dosing schedules for different types of chemotherapeutic agents vary depending on their cell-killing kinetics [25,26]. Cytocidal patterns (concentration-lethality curves and exposure time-lethality curves) of DX-8951f were similar to those of two typical time-dependent drugs, 5-fluorouracil and cytarabine, and were quite different from those of a typical concentration-dependent agent, nimustine hydrochloride. Two other camptothecin derivatives, SN-38 and SK&F104864, also exhibited time-dependent cytocidal patterns. The cytotoxic activity of DX-8951f in this system, as indicated by MLC_{95} (the minimum concentration causing 95% lethality), was 4 or 20 times greater than that of SN-38 or SK&F104864, respectively [unpublished results]. It has been concluded that DX-8951f shows potent cytocidal activity in a time-dependent manner.

4. *In vitro* cytotoxicity against a series of 32 malignant cell lines

The cytotoxic activity of DX-8951f was tested *in vitro* and compared to that of camptothecin, SN-38 and SK&F104864, using 31 human cancer cell lines, including breast cancer (9 lines), stomach cancer (4 lines), colon cancer (6 lines), lung cancer (9 lines), leukaemia (2 lines) and ovarian cancer (1 line) and a murine leukaemia cell line [20]. The cytotoxic activities of DX-8951f were stronger than those of other camptothecin derivatives examined and showed the smallest GI_{50} values (drug concentrations at which cell proliferation is inhibited by 50%) against all of the 32 cell lines used [20]. Comparing the mean GI_{50} values of the compounds against all cell lines, DX-8951f was approximately 6 times as potent as SN-38 and about 28 times as potent as SK&F104864 [20].

A comparative study of molecular and cellular pharmacology by Tanizawa *et al.* revealed that SN-38 is the most potent compound among the various camptothecin derivatives, including those previously and presently evaluated in clinical trials [18]. These researchers demonstrated that SN-38 is superior to camptothecin, SK&F104864, 9-AC, 10-hydroxy-camptothecin and 10,11-methylenedioxy-camptothecin (MDO-CPT) in the formation and stabilisation of cleavable complexes, inhibition of topo I, DNA-damaging effect and resulting cytotoxic activity [18]. Due to the superiority of DX-8951f in relation to SN-38 in topo I inhibition, apoptosis induction and cytotoxicity, it has been concluded that DX-8951f is by far the most active camptothecin derivative currently available [22].

5. *In vitro* cytotoxicity against drug-resistant variants

The development of resistance to antitumour agents is the main cause of treatment failure in cancer patients; it is therefore important to evaluate the efficacy of drugs on several types of drug-resistant tumour cells. Multi-drug resistance mediated by P-glycoprotein, a plasma membrane protein playing an important role as a drug efflux pump, is known to be one of the typical mechanisms of drug resistance [27-29]. Camptothecin and its derivatives are comparatively less affected by P-glycoprotein, but SK&F104864 is reported to be subject to this type of resistance [30,31]. In addition, several kinds of resistance mechanisms in CPT-11-resistant tumour cells have been reported [12,32-37], including a decrease in the cellular levels and/or in activities of topo I [12,33,34], alteration in topo I gene [35], a low population of S-phase cells [12], a decrease in the activity of intracellular carboxylesterase [12,37] and a decrease in drug accumulation [36].

A human oat cell carcinoma, PC-6, and its variants, PC-6/VCR29-9 and PC-6/SN2-5, were used to evaluate the antitumour effect of DX-8951f on certain drug-resistant cell lines [20,21]. PC-6/VCR29-9 and PC-6/SN2-5 were obtained by a stepwise selection procedure with sequential increases in concentrations of vincristine (VCR), and SN-38, respectively. PC-6/VCR29-9 cells overexpress P-glycoprotein and are approximately a 1000-times more resistant to VCR than parental PC-6 cells. Cross-resistance to adriamycin, methotrexate and etoposide has been confirmed in this variant. The cells also demonstrated a slight resistance to SN-38 and SK&F104864 (degree of resistance was 2- or 3-fold), but no resistance to DX-8951f [20]. On the other hand, PC-6/SN2-5 cells do not express P-glycoprotein and show no cross-

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resistance to non-camptothecin-type chemotherapeutics, except for mitoxantrone [21]. The cells were resistant to SN-38 (32-fold) and SK&F104864 (14-fold), but barely resistant to DX-8951f (2-fold) [21]. Topo I protein levels and topo I activities of parental cells were similar to those of resistant cells. The determination of the cellular drug concentration by either flow-cytometric analysis or HPLC confirmed that the cellular accumulation of SN-38 and SK&F104864 was significantly reduced in PC-6/SN2-5 cells, whereas that of DX-8951f was only slightly reduced [21]. Furthermore, DX-8951f stabilised the cleavable complex formations in intact PC-6/SN2-5 cells, as well as in parental cells, but SN-38 and SK&F104864 had no effect in the resistant cells [21]. These data suggest that PC-6/SN2-5 cells may have acquired resistance to camptothecin analogues by a decrease in intracellular drug accumulation and that DX-8951f may have the potency to overcome such a type of resistance mechanism induced by camptothecin compounds.

Similar results were obtained in another series of experiments using human pancreatic cancer cell lines, SUIT-2, KP-1N and their CPT-11-resistant variants, SUIT-2/CPT-11 and KP-1N/CPT-11 [22]. In these resistant variants, reduced levels of topo I mRNA and protein may contribute to the mechanism of resistance [32]. A one-third decrease in the uptake of CPT-11 was also found in KP-1N/CPT-11 compared with its parental cell line, while no difference in the uptake of CPT-11 was detected between SUIT-2 and SUIT-2/CPT-11 [32]. The acquired-CPT-11 resistant cells also showed marked cross-resistance to SN-38 and SK&F104864, but less resistance to DX-8951f, with the resistance rates to DX-8951f being the lowest among current camptothecin derivatives [22].

The reason why DX-8951f shows sufficient antitumour activity against the above resistant cells is presently unclear, but it is possible that its uptake is not influenced even in resistant cells. This idea seems to be supported by the previously described finding that DX-8951f overcame P-glycoprotein-mediated resistance [20] and that this effect might be attributed to its good membrane permeability [38]. Further detailed analysis is required.

6. Administration regimens and therapeutic ratios

As previously described, it has been shown that optimal dosing schedules for different types of chemotherapeutic agents vary depending on their cell-killing kinetics, and it is quite clear that optimal scheduling is an important key for therapeutic success. CPT-11 has been evaluated in clinical trials in Japan, Europe and the USA with different treatment regimens: daily administration for several consecutive days, every 3 to 4 weeks [11], or one dose every 1 - 3 weeks [39]. The latter has commonly been adopted as the clinical treatment regimen against solid tumours as CPT-11 is converted *in vivo* to SN-38 [7,12] and the plasma SN-38 concentration decreases slowly in mice and humans [40,41]. SK&F104864, a non-prodrug-type derivative, has been studied using daily dosing regimens for 5 consecutive days [42], or low-dose continuous infusions for 24 h - 21 days [43-46]. The results of these studies suggest that the optimal regimen and dosage for camptothecin derivatives differs greatly among the compounds, probably due to differences in their pharmacokinetic profiles.

The therapeutic efficacy of DX-8951f was investigated against the murine fibrosarcoma, Meth A, using various iv. doses. The purpose of this study was to determine the optimal regimen for the administration of this compound. When the total dose was fixed, DX-8951f demonstrated greater antitumour activity with daily or intermittent administration (such as every 2nd day or every 4th day for 4 treatments) than with single dose administration [47]. Shorter intervals between injections resulted in greater efficacy and toxicity (body weight loss and toxic death) [47]. DX-8951f exhibited higher therapeutic efficacy with an infusion-like administration (3 injections per day at intervals of 3 - 4 h) than with a bolus injection [unpublished results]. In addition, rather than a simple intermittent application, such as once daily every 4th day (q4dx4), multiple administrations (such as 4 cycles of 3 injections per day every 4th day: q3hx3/q4dx4, and 4 cycles of daily injections for 2 days every 4 days: qdx2/q4dx4) resulted in greater antitumour effects of DX-8951f, with broader effective dose ranges [unpublished results]. The efficacy of DX-8951f was superior to that of either CPT-11 and SK&F104864 with multiple administration; therapeutic ratios (ratios calculated as maximum tolerated dose/the minimum effective dose) of DX-8951f were 16, while those of CPT-11 and SK&F104864 were 1 or < 1, and 2 - 6, respectively [unpublished results]. Therefore, multiple administrations followed by recovery periods are recommended as the optimal regimen for the clinical application of DX-8951f.

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