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A Platform for Rapid Detection of Multiple Oncogenic Mutations With Relevance to Targeted Therapy in Non–Small-Cell Lung Cancer

Zengliu Su,* Dora Dias-Santagata,† MarKeesa Duke,* Katherine Hutchinson,* Ya-Lun Lin,* Darrell R. Borger,† Christine H. Chung,* Pierre P. Massion,‡ Cindy L. Vnencak-Jones,§ A. John Iafrate,† and William Pao*

From the Vanderbilt-Ingram Cancer Center, the Department of Medicine/Division of Hematology-Oncology, Vanderbilt University School of Medicine, Nashville, Tennessee; the Translational Research Laboratory,† Massachusetts General Hospital, Boston, Massachusetts; the Division of Allergy, Pulmonary and Critical Care Medicine,‡ Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee; and the Departments of Pathology and Pediatrics,§ Vanderbilt University Medical Center, Nashville, Tennessee*

The identification of somatically acquired tumor mutations is increasingly important in the clinical management of cancer because the sensitivity of targeted drugs is related to the genetic makeup of individual tumors. Thus, mutational profiles of tumors can help prioritize anticancer therapy. We report herein the development and validation of two multiplexed assays designed to detect in DNA from FFPE tissue more than 40 recurrent mutations in nine genes relevant to existing and emerging targeted therapies in lung cancer. The platform involves two methods: a screen (SNaPshot) based on multiplex PCR, primer extension, and capillary electrophoresis that was designed to assess for 38 somatic mutations in eight genes (*AKT1, BRAF, EGFR, KRAS, MEK1, NRAS, PIK3CA*, and *PTEN*) and a PCR-based sizing assay that assesses for *EGFR* exon 19 deletions, *EGFR* exon 20 insertions, and *HER2* exon 20 insertions. Both the SNaPshot and sizing assays can be performed rapidly, with minimal amounts of genetic material. Compared with direct sequencing, in which mutant DNA needs to compose 25% or more of the total DNA to easily detect a mutation, the SNaPshot and sizing assays can detect mutations in samples in which mutant DNA composes 1.56% to 12.5% and 1.56% to 6.25% of the total DNA, respectively. These robust, reliable, and relatively inexpensive assays should help accelerate adoption of a genotype-driven approach in the treatment of lung cancer. ***(J Mol Diagn 2011, 13:74–84; DOI: 10.1016/j.jmoldx.2010.11.010)***

In 2009, nearly 160,000 patients in the United States died of lung cancer, the country's leading cause of cancer-related death.[1] Most patients with advanced lung cancer were treated empirically, based on clinical factors and appearance of tumor histological features. Although multiple genetic variants that "drive" lung tumorigenesis have been shown to influence treatment outcomes, mutation analysis of lung tumors has not yet become a part of standard clinical algorithms.

Most genetic alterations involving "driver mutations" have been uncovered in lung adenocarcinoma, a histological subtype of non–small-cell lung cancer (NSCLC). Driver mutations occur in genes that encode signaling proteins critical for cellular proliferation and survival. Approximately 50% of lung adenocarcinomas harbor such recurrent somatic oncogenic mutations in *EGFR*, *HER2*, *KRAS*, *PIK3CA*, *BRAF*, *MEK1*, and *ALK*. With the exception of *PIK3CA* mutations, a tumor with an alteration in one of these genes rarely has a mutation in one of the other genes. More importantly, mutations in *EGFR*, *HER2*, *PIK3CA*, *BRAF*, *MEK1*, and *ALK* have already been associated with increased sensitivity to specific kinase inhibitors.[2–9] Other mutations, such as those involving *AKT1* and

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Current address of C.H.C.: Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland.

Address reprint requests to William Pao, M.D., Ph.D., Vanderbilt-Ingram Cancer Center, 2220 Pierce Ave, 777 Preston Research Bldg, Nashville, TN 37232. E-mail: william.pao@vanderbilt.edu.

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PTEN, may influence responses to inhibitors of the *EGFR/PI3K/AKT* pathway.[10,11] The *EGFR* mutations represent the best example of the benefits of a genotype-driven approach; treatment-naïve patients with metastatic *EGFR*-mutant tumors experience longer progression-free survival with an *EGFR* tyrosine kinase inhibitor, whereas patients whose tumors harbor wild-type *EGFR* have longer progression-free survival with chemotherapy.[5]

Conventionally, most molecular diagnostic laboratories have tested for mutations in tumor DNA derived from FFPE tissues one gene at a time. A major clinical challenge is prospectively determining the status of multiple clinically relevant genes in FFPE-derived tumor DNA before starting therapy. Further complicating matters, not all types of mutations are readily detected by the same method. For example, missense mutations can be found by one type of assay, but insertions/deletions that add/eliminate nucleotides in specific "hot spots" could be missed. Likewise, protocols to detect insertions/deletions are not optimal for point mutation detection.

We report herein the development and validation of two multiplexed assays designed to detect more than 40 recurrent mutations in nine genes relevant to targeted therapy in lung cancer. The first assay was devised to detect 38 different recurrent point mutations concurrently in eight genes (*EGFR*, *KRAS*, *BRAF*, *NRAS*, *PIK3CA*, *MEK1*, *AKT1*, and *PTEN*) and was adapted from a previously implemented genotyping platform designed for targeted mutational analysis of a broader set of tumor types.[12] This assay uses technology (SNaPshot; Life Technologies/Applied Biosystem, Foster, CA) that involves multiplexed amplification of DNA targets by PCR with unlabeled oligonucleotide primers, multiplexed single-base primer extension with fluorescently labeled dideoxynucleotide triphosphate (ddNTP), and analysis of labeled primer-extension products by capillary electrophoresis.[12] The second assay is a separate PCR-based sizing technique that simultaneously assesses tumors for recurrent insertions in *EGFR* and *HER2* and deletions in *EGFR* that would not be comprehensively detected by the SNaPshot technique. Compared with direct sequencing, these assays offer higher analytical sensitivity and reduced complexity. They also provide a robust and accessible approach for the rapid identification of important mutations in lung cancer.

Materials and Methods

Cell Lines and Tumor Samples

Genomic DNA was derived from 35 lung cancer cell lines, 73 lung adenocarcinomas, and an additional 34 head and neck cancer cell lines. For cell lines, DNA was isolated using a kit (DNeasy; Qiagen Inc, Valencia, CA). For lung cancers, DNA from 24 specimens with greater than 70% tumor content was obtained from frozen samples using a genomic DNA purification kit (Wizard; Promega Corporation, Madison, WI); and DNA from 49 specimens with 35% to 85% tumor content was extracted from FFPE tissues using a kit (Qiaquick PCR Purification Kit; Qiagen Inc). All lung cancers were analyzed anonymously with informed consent and approval from the local institutional review board. Human male genomic DNA (Promega Corporation) was used as a wild-type control.

Table 1. List of PCR Primers for the SNaPshot Screen

Name	Sequence
AKT1_ex2_a1	5′-GAGGGTCTGACGGGTAGAGT-3′
AKT1_ex2_a2	5′-TCTTGAGGAGGAAGTAGCGT-3′
BRAF_ex11_a1*	5′-TCTGTTTGGCTTGACTTGACTT-3′
BRAF_ex11_a2*	5′-TCACCACATTACATACTTACCATGC-3′
BRAF_ex15_a1*	5′-TGCTTGCTCTGATAGGAAAATG-3′
BRAF_ex15_a2*	5′-CTGATGGGACCCACTCCAT-3′
EGFR_ex18_a1*	5′-CCAACCAAGCTCTCTTGAGG-3′
EGFR_ex18_a2*	5′-CCTTATACACCGTGCCGAAC-3′
EGFR_ex20_a1*	5′-TGTTCCCGGACATAGTCCAG-3′
EGFR_ex20_a2*	5′-ATCTGCCTCACCTCCACCGT-3′
EGFR_ex21_a1*	5′-CCTCCTTCTGCATGGTATTC-3′
EGFR_ex21_a2*	5′-GCAGCATGTCAAGATCACAG-3′
KRAS_ex2_a1*	5′-TCATTATTTTTATTATAAGGCCTGCTG-3′
KRAS_ex2_a2*	5′-AGAATGGTCCTGCACCAGTAA-3′
KRAS_ex3_a1*	5′-AATTGATGGAGAAACCTGTCTCTTG-3′
KRAS_ex3_a2*	5′-TGGTCCCTCATTGCACTGTA-3′
MEK1_ex2_a1	5′-AGCGAAAGCGCCTTGAGGCCTT-3′
MEK1_ex2_a2	5′-AACACCACACCGCCATTGCCAG-3′
NRAS_ex3_a1	5′-ATAGATGGTGAAACCTGTTTGTTGG-3′
NRAS_ex3_a2	5′-TGTATTGGTCTCTCATGGCACT-3′
PIK3CA_ex9_a1*	5′-GACAAAGAACAGCTCAAAGCAA-3′
PIK3CA_ex9_a2*	5′-TTTAGCACTTACCTGTGACTCCA-3′
PIK3CA_ex20_a1*	5′-GAGCAAGAGGCTTTGGAGTA-3′
PIK3CA_ex20_a2*	5′-ATCCAATCCATTTTTGTTGTCC-3′
PTEN_ex7_a1*	5′-GGTGAAGATATATTCCTCCAATTCA-3′
PTEN_ex7_a2*	5′-TTCTCCCAATGAAAGTAAAGTACAAA-3′

*Published previously.[12]

SNaPShot Assay

The SNaPShot technique involves multiplexed PCR, multiplexed single-base primer extension, and capillary electrophoresis. The PCR primers are listed in Table 1. An online primer design tool, "primer-BLAST" (National Center for Biotechnology Information), was used to construct primers with minimal chances of cross-reacting with homologous genes or pseudogenes. A search for known polymorphisms was performed for each sequence and accounted for in the design of all primers. Because DNA from FFPE samples is often degraded, PCR amplicons were designed to be approximately 100 bp. Single-base extension primers are listed in Table 2. Extension primers contain approximately 16 to 20 bp, with melting temperatures of higher than 50°C; and ddNTPs were used for primer extension. Random nucleotides ("GACT") were added to extension primers to adjust their product sizes. Additional details of the rationale and methods used in primer design have been described.[12] SNaPshot analysis was performed as previously described.[12] Briefly, PCR primers were pooled together to amplify DNA using polymerase (platinum TaqDNA; Invitrogen Corporation, Carlsbad, CA) and the following conditions: 95°C for 8 minutes, followed by 95°C for 20 seconds, 58°C for 30 seconds, and 72°C for 1 minute (40 cycles), with a final

Table 2. List of Extension Primers for the SNaPshot Screen*

Name	Sequence†
AKT1.49_extR	5′-*ACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACT*CGCCAGGTCTTGATGTACT-3′
BRAF1397_extF	5′-*TGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGAC*GGGACAAAGAATTGGATCTG-3′
BRAF1406_extF	5′-*AGACTGACTGACTGACTGACTGACTGACTGACTG*GAATTGGATCTGGATCATTTG-3′
BRAF1789_extF	5′-*ACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACT*CAGTAAAAATAGGTGATTTTGGT-3′
BRAF1799_extF‡	5′-*GACTGACTGACTGACTGACTGACT*GTGATTTTGGTCTAGCTACAG-3′
EGFR2155_extF‡	5′-*GACTGACTGACTGACTGACTGACTG*TCAAAAAGATCAAAGTGCTG-3′
EGFR2156_extF	5′-*CTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACT*CAAAAAGATCAAAGTGCTGG-3′
EGFR2369_extR‡	5′-*CTGACTGACTGACTGACTGACTGACTGACT*AAGGGCATGAGCTGC-3′
EGFR2573_extF‡	*GACTGACTGACTGACTGACTGACTGACTGAC*AGATCACAGATTTTGGGC-3′
EGFR2582_extR	5′-TTCTCTTCCGCACCCAGC-3′
KRAS34_extR‡	5′-*GACTGACTG*CTCTTGCCTACGCCAC-3′
KRAS35_extF‡	5′-*CTGACT*CTTGTGGTAGTTGGAGCTG-3′
KRAS37_extF‡	5′-*TGACTGACTGA*TGGTAGTTGGAGCTGGT-3′
KRAS38_extF‡	5′-*GACTGACTGAC*GGTAGTTGGAGCTGGTG-3′
KRAS181_extF	5′-*CTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACT*ATTCTCGACACAGCAGGT-3′
KRAS182_extF	5′-*GACTGACTGACTGACTGACT*ATTCTCGACACAGCAGGTC-3′
KRAS183_extR	5′-*ACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGAC*CTCATTGCACTGTACTCCTC-3′
MEK1.167_extF	5′-*GACTGACTGACTGACT*CTTGAGGCCTTTCTTACCC-3′
MEK1.171_extR	5′-CAGTTCTCCCACCTTCTG-3′
MEK1.199_extR	5′-*GACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACT*CTCACTGATCTTCTCAAAGT-3′
NRAS181_extF‡	5′-*GACTGACTGACTGACTGACTGACTGACTGACTGAC*ACATACTGGATACAGCTGGA-3′
NRAS182_extF‡	5′-*CTGACTGACTGACTGACTGACTGACTGACTGACTG*CATACTGGATACAGCTGGAC-3′
PIK3CA1624_extR‡	5′-*TGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGAC*TTCTCCTGCTCAGTGATTT-3′
PIK3CA1633_extF‡	5′-*GACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACT*GATCCTCTCTCTGAAATCACT-3′
PIK3CA3140_extR‡	5′-GTCCAGCCACCATGA-3′
PTEN697_extR‡	5′-*ACTGACTGACTGACTGACTGACTGACTGACTGACTGACTG*TGAACTTGTCTTCCCGTC-3′

*Primers were purified by polyacrylamide gel electrophoresis.
†Certain "*GACT*" nucleotides (italicized) were added to the extension primers to adjust their product sizes.
‡Published previously.[12]

extension of 72°C for 3 minutes. Next, corresponding polyacrylamide gel electrophoresis–purified primers were pooled together with a multiplex ready reaction mix (SNaPShot; Applied Biosystems) to perform multiplex extension reactions [96°C for 30 seconds, 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 30 seconds (for 25 cycles)]. Third, extension products were separated by capillary electrophoresis in an analyzer (ABI 3730) using standard conditions with the following reagents and parameters: dye set, Any5Dye; polymer, POP-7; matrix standard, DS-02; size standard, GeneScan-120 LIZ; injection time, 10 seconds; run time, 1200 seconds; and run voltage, 15 kV. Data were interpreted using computer software (ABI GeneMapper, version 4.0). The ability of the assay to detect all potential mutations was validated with cell lines or spiking primers that contained specific mutations. The latter are listed in Supplemental Table S1 at *http://jmd.amjpathol.org*.

Triplex Sizing Assay

An *EGFR* exon 19 deletion sizing assay, established previously,[13] was adapted to allow for further analysis of *EGFR* and *HER2* exon 20 insertions. The *EGFR* exon 19 deletions range from 9 to 24 nucleotides; *EGFR* exon 20 insertions, 3 to 12 nucleotides; and *HER2* exon 20 insertions, 3 to 12 bp. The following primers were used: for *EGFR* exon 19 (wild-type amplicon size, 207 bp), there were EGFR_Ex19_FWD1, 5′-GCACCATCTCACAATTGCCAGTTA-3′; and EGFR_Ex19_REV1, 5′-/6FAM/AAAAGGTGGGCCTGAGGTTCA-3′; and for EGFR exon 20 (wild-type amplicon size, 155 bp), there were EGFR_Ex20ins_FWD, 5′-TCTTCACCTGGAAGGGGTCC-3′; and EGFR_Ex20ins_REV, 5′-/HEX/ACGGTGGAGGTGAGGCAGAT-3′. For *HER2*/*ERBB2* exon 20 (wild-type amplicon size, 245 bp), the following were used: ERBB2_Ex20_FWD, 5′-ACCGTGCCCGGCCTAATCTT-3′; and ERBB2_Ex20_REV, 5′-/HEX/TCAGGCAGATGCCCAGAAGG-3′. A 1-ng template DNA was used to perform PCR with a kit (HotStarTaq Master Mix Kit; Qiagen Inc), as follows: 95°C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds, and a final extension of 72°C for 30 minutes. Fluorescently labeled amplicons were separated with a capillary electrophoresis instrument (ABI 3730), and output data were interpreted using software (GeneMapper, version 4.0).

Direct Dideoxynucleotide-Based Sequencing

All mutations detected by SNaPShot and sizing assays were further confirmed with direct sequencing. Exons with positive mutations were amplified using a kit (HotStarTaq Master Mix Kit; Qiagen Inc) and M13-tagged gene-specific primers (see Supplemental Table S2 at *http://jmd.amjpathol.org*). The following conditions were used: 95°C for 15 minutes, followed by 40 cycles of 95°C

for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds, and a final extension of 72°C for 30 minutes. Excess primers and nucleotides were digested using ExoSAP (USB Corporation, Cleveland, OH). ExoSAP is an enzyme mixture of exonuclease I, which removes leftover primers, and Shrimp Alkaline Phosphatase, which removes leftover dNTPs. Sequencing reactions were performed using chemistry (Version 3.1 Big Dye Terminator; Applied Biosystems) and analyzed on a sequencer (model 3730XL; Applied Biosystems). All sequence chromatograms were read in both forward and reverse directions.

Results

Development of a SNaPShot Assay to Assess Multiple Somatic Point Mutations in Lung Cancer

After performing a literature search and reviewing the Catalogue of Somatic Mutations in Cancer database, we decided to include in our SNaPshot screen (v1.0) 38 somatic point mutations occurring at 26 different loci in eight genes potentially relevant to targeted therapy in lung cancer (Table 3). The following criteria were used for selection of mutations: i) they were found in NSCLCs, ii) they occurred at a frequency of 1% or greater, and iii) they could be used as a predictor for targeted therapy. In this study, we included 14 SNaPshot assays derived from a 58-mutation genotyping panel that is being used for clinical testing of FFPE-derived tumor samples[12] and designed 12 additional assays (Tables 1 and 2). All mutations were incorporated into five multiplexed panels, each capable of detecting mutations at four (panels 3 and 5) to six (panels 1, 2, and 4) loci. We optimized the concentration of PCR and extension primers in each panel so that all fluorescently labeled fragments displayed roughly the same peak height after capillary electrophoresis (Figure 1A). Each peak was validated with DNA from cell lines containing known mutations or "spiking primers" (ie, oligonucleotides containing mutations of interest; see Supplemental Figure S1 at *http://jmd.amjpathol.org*). We also developed a "pan-positive" control for the whole screen, using pools of spiking primers (Figure 1B). Spiking primers were mixed with the appropriate PCR products before primer extension reactions. By using normal genomic DNA, we performed the entire SNaPshot screen with all five panels reliably with as little as 2 ng per panel.

We subsequently validated our SNaPshot screen against a panel of 35 NSCLC cell lines with known mutation status.[14–16] All cell lines were shown by us or others previously to harbor specific missense changes. We obtained 100% concordance with published results (Table 4 and Supplemental Table S3 at *http://jmd.amjpathol.org*). No false-positive or false-negative cases were observed.

Finally, we measured the sensitivity of the SNaPShot assays in serial dilution experiments, using the empirically established method described in detail previously.[12] One representative mutation in each of the five panels was studied, using mixtures of DNA from the male human genomic control and positive control cell lines with known mutations. Briefly, for any given locus, a mutation was called confidently if its peak height exceeded 10% of the corresponding heterozygous wild-type peak in the same sample (Figure 1C). If the height of a potential mutation peak was less than 10% of the corresponding wild-type peak, then a mutation

Table 3. SNaPshot Screen Designed to Detect 38 Somatic Point Mutations in Eight Genes Relevant to Targeted Therapy in the NSCLC*

Position	AA mutant	Nucleotide mutant
EGFR		
G719	p.G719C†	c.2155G>T
	p.G719S†	c.2155G>A
	p.G719A	c.2156G>C
T790M	p.T790M	c.2369C>T
L858	p.L858R†	c.2573T>G
L861	p.L861Q	c.2582T>A
KRAS		
G12	p.G12C†	c.34G>T
	p.G12S†	c.34G>A
	p.G12R†	c.34G>C
	p.G12V†	c.35G>T
	p.G12A†	c.35G>C
	p.G12D†	c.35G>A
G13	p.G13C†	c.37G>T
	p.G13S†	c.37G>A
	p.G13R†	c.37G>C
	p.G13D†	c.38G>A
	p.G13A†	c.38G>C
Q61	p.Q61K	c.181C>A
	p.Q61R	c.182A>G
	p.Q61L	c.182A>T
	p.Q61H	c.183A>T
	p.Q61H	c.183A>C
BRAF		
G466	p.G466V	c.1397G>T
G469	p.G469A	c.1406G>C
L597	p.L597V	c.1789C>G
V600	p.V600E†	c.1799T>A
PIK3CA		
H1047	p.H1047R†	c.3140A>G
E542	p.E542K†	c.1624G>A
E545	p.E545K†	c.1633G>A
	p.E545Q†	c.1633G>C
NRAS		
Q61	p.Q61K†	c.181C>A
	p.Q61L†	c.182A>T
	p.Q61R†	c.182A>G
MEK1 (MAP2K1)		
Q56	p.Q56P	c.167A>C
K57	p.K57N	c.171G>T
D67	p.D67N	c.199G>A
AKT1		
E17	p.E17K	c.49G>A
PTEN		
R233	p.R233X†‡	c.697C>T

*The *EGFR* exon 19 deletions, *EGFR* exon 20 insertions, and *HER2* exon 20 insertions are detected by a separate sizing assay. The *PIK3CA* H1047L mutation detected in 1 FFPE sample (see text) was not listed because that mutation was not reported in COSMIC (Catalogue of Somatic Mutations in Cancer) in NSCLC when the assay was designed.
†Previously published SNaPshot assays.[12]
‡Truncation.

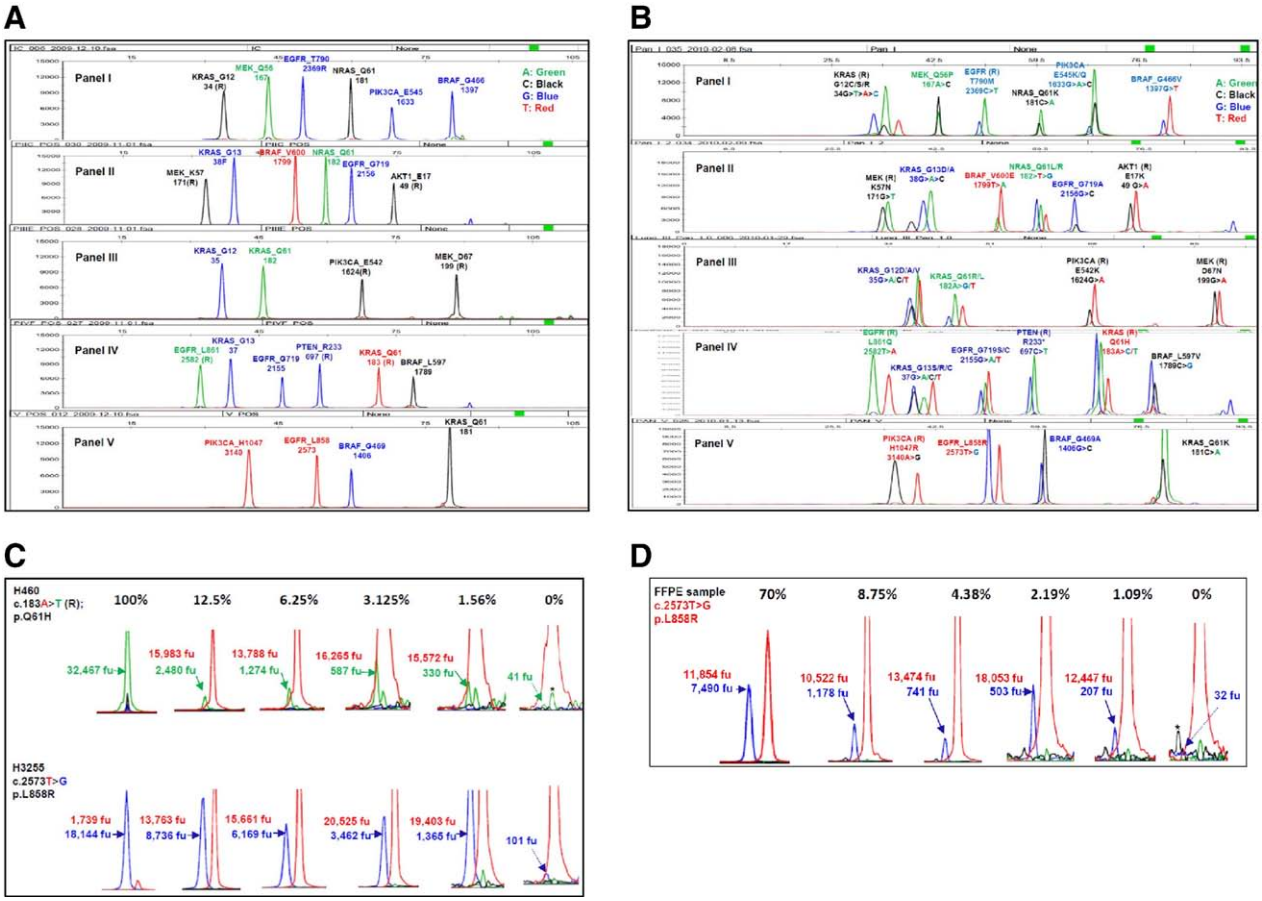

Figure 1. Lung cancer SNaPShot screen (v1.0). **A:** Human genomic DNA was used as a wild-type control for the multiplex SNaPShot screen, which consists of five panels. Each peak represents a locus where a driver mutation may occur. The name of each gene and the name and position of the amino acid are labeled on the top of each peak. The number under the gene name is the nucleotide position. **R** designates use of an extension primer encoding the reverse (complementary) strand. **B:** Pan-positive controls for the SNaPshot screen. Spiking primers were used to display all positive peaks in each locus. **C:** Sensitivity measurement with cell lines. DNAs from cell lines carrying the known mutations were diluted with human genomic DNA in ratios of 100%, 25% (data not shown), 12.5%, 6.25%, 3.125%, 1.56%, and 0% (wild-type control). Mixtures were then used to perform the SNaPShot screen. Numbers indicate the arbitrary fluorescence units (fu) of wild-type (above) and mutant (underneath) peaks separately. **Solid arrows** show mutant peaks; and **dotted arrows**, background peaks. The *y* axis was adjusted to the appropriate scale to visualize various peaks. Based on previously established criteria,[12] the following rules were used to call a mutation: i) A mutation is called confidently if the mutant peak height is 10% or greater of the corresponding wild-type peak [eg, 12.5% dilution of the H460 cell line, as follows: (2480/15,983)×100 = 15.5%]. ii) If the potential mutant peak is less than 10%, the cutoff value [eg, 1.56% dilution of the H460 cell line, as follows: (330/15,572)×100 = 2.1%], a background peak of the same color and size (**dotted arrow**) in a separate wild-type DNA control (0%) is used as a reference. If the potential mutant peak height is three times or more than the background peak (330/41 = 8.0, >3), a mutation is called positive (see text for further details). *A background peak of the same color but not the same size as a mutant peak. **D:** Sensitivity measurements with FFPE-derived DNAs. The FFPE-derived DNA from a patient sample containing approximately 70% tumor cells was diluted with FFPE-derived DNA from a patient's normal tissue [ie, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 corresponded to samples with 70% tumor cells, 35% (data not shown), 17.5% (data not shown), 8.75%, 4.38%, 2.19%, and 1.09%, respectively]; 0% tumor cells (with 100% normal cells) were also analyzed. Numbers indicate the arbitrary fluorescence units (fu) of wild-type (above) and mutant (underneath) peaks separately. **Solid arrows** show mutant peaks; and **dotted arrow**, the background peak. The *y* axis was adjusted to the appropriate scale to visualize various peaks. **Asterisks** mark background peaks of the same size but not the same color as a mutant peak.

was called if the potential mutant peak was three times higher than any background peaks of the same color and size in separate analyses of wild-type DNA controls (Figure 1C). In this way, the calculated sensitivity of our SNaPshot assays ranged from 1.56% to 12.5% (Figure 1C), as previously published.[12] Assay sensitivity was further validated on FFPE-derived DNA harboring an *EGFR* L858R mutation. This sample contained approximately 70% tumor cells and was serially diluted with DNA extracted from FFPE-derived normal tissue. The L858R mutation could be detected in dilutions as low as 1.09% (Figure 1D). Notably, the sensitivity of a specific allele can vary, depending on the quality of the DNA, the level of background noise, and the size (position) of the peak in a panel. Furthermore, because of ploidy differences between cancer cell lines and nonneoplastic cells, sensitivities based on dilutions of DNA should be viewed only as approximations of absolute sensitivity. In both cell lines and FFPE samples, we observed a linear correlation between relative mutant peak intensity and tumor cell content (see Supplemental Figure S2 at *http://jmd.amjpathol.org*).

Triplex Sizing Assay

Concurrently, we developed a triplex sizing assay to detect recurrent insertions and deletions occurring in *EGFR* exons 19 and 20 and in *HER2* exon 20 (Figure 2A). This

Table 4. Summary of Mutations Detected in Various Lung Cancer Samples

Genes	Amino acids	Nucleotides	No. (%) of mutations
Cell lines*			
EGFR	T790M	2368 C>T	2 (5.7)
	L858R	2573 T>G	3 (8.6)
	Exon 19 del (12 bp)	NA	1 (2.9)
	Exon 19 del (15 bp)	NA	2 (5.7)
KRAS	G12A	35 G>C	1 (2.9)
	G12C	34 G>T	3 (8.6)
	G12R	34 G>C	1 (2.9)
	G12S	34 G>A	1 (2.9)
	G12V	35 G>T	1 (2.9)
	Q61H	183 A>T	1 (2.9)
BRAF	G466V	1397 T>C	1 (2.9)
NRAS	Q61L	182 A>T	1 (2.9)
	Q61K	181 C>A	1 (2.9)
PIK3CA	E545K	1633 G>A	1 (2.9)
MEK1	Q56P	167 A>C	1 (2.9)
HER2	Exon 20 ins (3 bp)	NA	1 (2.9)
Frozen tissues†			
EGFR	L858R	2573 T>G	1 (4.2)
	Exon 19 del (15 bp)	NA	1 (4.2)
	Exon 20 ins (3 bp)	NA	1 (4.2)
KRAS	G12A	35 G>C	1 (4.2)
	G12V	35 G>T	2 (8.3)
	G12C	34 G>T	1 (4.2)
	G13C	37 G>T	1 (4.2)
	G13D	38 G>A	1 (4.2)
NRAS	Q61K	181 C>A	1 (4.2)
PIK3CA	E545K	1633 G>A	1 (4.2)
FFPE samples‡			
EGFR	G719A	2156 G>C	1 (2.0)
	T790M	2369 C>T	1 (2.0)
	L858R	2573 T>G	2 (4.1)
	Exon 19 del (15 bp)	NA	5 (10.2)
	Exon 19 del (18 bp)	NA	2 (4.1)
	Exon 20 ins (6 bp)	NA	1 (2.0)
KRAS	G12C	34 G>T	6 (12.2)
	G12V	35 G>T	3 (6.1)
	Q61H	183 A>C	1 (2.0)
PIK3CA	H1047L	3140 A>T	1 (2.0)

NA, not available.
*Among 35 samples, 23 mutations were found in 20 of them.
†Among 24 samples, 11 mutations were found in 10 of them.
‡Among 49 samples, 23 mutations were found in 21 of them.

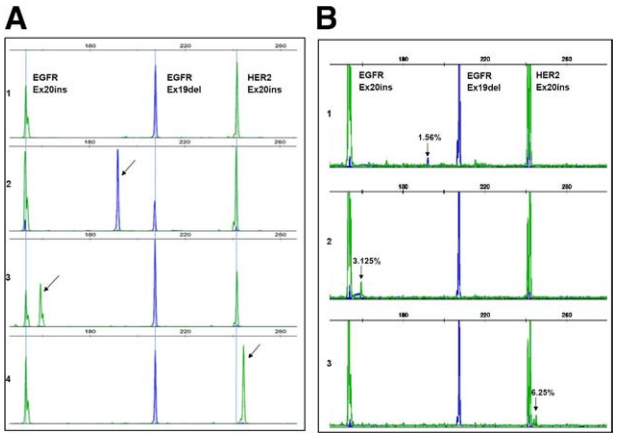

Figure 2. Lung cancer triplex sizing assay. The triplex sizing assay was established to detect simultaneously *EGFR* exon 19 deletions, *EGFR* exon 20 insertions, and *HER2* 20 insertions. **A:** Examples of results with known positive controls. **1**, human genomic DNA was used as a wild-type control (peaks are indicated by dashed lines); **2**, H1650 cell line DNA showed a 15-nucleotide deletion in *EGFR* exon 19 (**arrow**); **3**, DNA from a previously characterized lung adenocarcinoma sample showed a three-nucleotide insertion in *EGFR* exon 20 (**arrow**); **4**, H1781 cell line DNA showed a homozygous three-nucleotide insertion in *HER2* exon 20 (**arrow**). **B:** Sensitivity assays. Samples carrying the known mutations were diluted with human genomic DNA in ratios of 100%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, and 0%. Mixtures were then used to perform the sizing assay. The **arrows** indicate the mutation peaks at the lowest dilution rate.

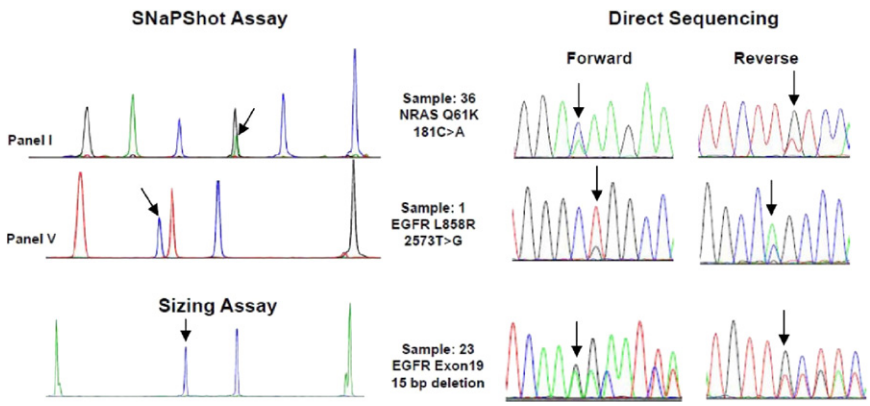

Figure 3. The SNaPshot and sizing assays results confirmed by forward and reverse direct sequencing. **Arrows** show the positions of mutations. The *y* axis of the SNaPshot assay involves arbitrary fluorescence units and was adjusted to an appropriate scale for observation of mutant peaks in each panel. Only representative examples are shown; remaining data are not shown.

assay is based on length analysis of fluorescently labeled PCR products. By using normal genomic DNA, we performed the assay reliably with as little as 1 ng. In validation studies with 35 NSCLC cell lines with known mutation status, we obtained 100% concordance with published results (Table 4 and Supplemental Table S3 at *http://jmd.amjpathol.org*). In serial dilution assays using wild-type genomic DNA and positive controls (H1650 for *EGFR* exon 19 deletion, H1781 for *HER2* exon 20 insertion, and a lung tumor sample for *EGFR* exon 20 insertion), all three mutations could be detected when the starting template was composed of 1.56% to 6.25% mutant DNA (Figure 2B).

Application of Multiplex SNaPShot and Sizing Assays

We used both assays to analyze DNA from 24 frozen lung adenocarcinoma samples of previously unknown mutation status. We detected 11 mutations (46%): three (13%) in *EGFR*, six (25%) in *KRAS*, one (4.2%) in *PIK3CA*, and one (4.2%) in *NRAS*. Consistent with the literature, *EGFR* and *KRAS* mutations were mutually exclusive (Table 4 and Supplemental Table S4 at *http://jmd.amjpathol.org*). The results in 9 of 11 positive cases were confirmed by direct sequencing (Figure 3, remaining data not shown). In the remaining two cases, direct sequencing showed only equivocally positive results, consistent with the notion that SNaPshot assays are more sensitive than direct sequencing (Figure 4).

We next used the SNaPshot and sizing assays to study DNAs from 49 FFPE-derived lung tumor samples, some of which were previously analyzed. Twenty-three changes were detected (Table 4 and Supplemental Table S5 at *http://jmd.amjpathol.org*). Three *KRAS* mutations (two *KRAS* G12C and one *KRAS* G12V), five *EGFR* exon 19 deletions, and one *EGFR* exon 20 insertion matched perfectly with previous results (data not shown). The other 14 mutations detected by SNaPshot and sizing assays (four *KRAS* G12C, two *KRAS* G12V, one *KRAS* Q61H, two *EGFR* exon 19 deletions, one *EGFR* G719A, one *EGFR* T790M, two *EGFR* L858R, and one *PIK3CA* H1047L) were further confirmed by direct sequencing (Figure 3, remaining data not shown). Notably, when we designed the assay, the *PI3KCA* H1047L variant had not yet been reported specifically in NSCLCs; it was detected as part of the screen for the previously reported H1047R mutation.

To evaluate the reproducibility of the lung tests, 19 cell lines and 11 FFPE-derived DNA samples were assayed independently by another operator in the laboratory. We obtained the exact same results (see Supplemental Tables S3 and S5 at *http://jmd.amjpathol.org*).

Finally, to demonstrate further the specificity of both the SNaPshot and sizing assays, we examined a cohort of 34 head and neck squamous cell cancer cell lines of unknown mutation status. Only three mutations (9% of 34) were found (see Supplemental Table S6 at *http://jmd.amjpathol.org*), all in *PIK3CA*. Results were confirmed by direct sequencing (data not shown). The lack of mutations in these samples is consistent with published results[17] and demonstrates a low likelihood of detecting false positives using these molecular tests.

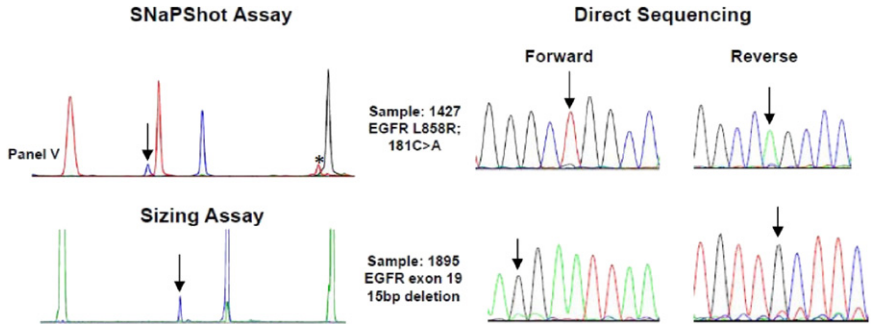

Figure 4. SNaPshot and sizing assays are more sensitive than direct sequencing. Mutations were detected in two samples by SNaPshot and sizing assays, but the calls were only equivocally positive by direct sequencing, consistent with the notion that SNaPshot assays are more sensitive than direct sequencing. An **asterisk** marks a background peak; no mutant allele exists that will show this position and color (panel V of Figures 1B and Supplemental Figure S1E at *http://jmd.amjpathol.org*). **Arrows** indicate mutant peaks.

Table 5. Frequencies of Mutations Detected by the SNaPshot and Sizing Assays for Lung Cancer*

Gene	Mutation	Frequency	Method used	Reference	Prediction	Reference
EGFR	G719C/S/A	a, 0.5% (AD); b, 0.5% (NSCLC)	a and b, direct sequencing	a, Ding et al[19]; b, Shigematsu et al[20]	Increased sensitivity to EGFR TKIs	Lynch et al[29] and Paez et al[30]
	Exon 19 del	a, 13% (AD); b, 10% (NSCLC)	a, sequenom; b, direct sequencing;	a, Chitale et al[21]; b, Shigematsu et al[20]	Increased sensitivity to EGFR TKIs	Lynch et al,[29] Paez et al,[30] and Pao et al[31]
	L858R	a, 9% (AD); b, 8% (NSCLC)	a, sequenom; b, direct sequencing	a, Chitale et al[21]; b, Shigematsu et al[20]	Increased sensitivity to EGFR TKIs	Lynch et al,[29] Paez et al,[30] and Pao et al[31]
	T790M	a, 0.5% (AD); b, 4% (AD); c, 0.7% (NSCLC)	a, sequenom; b, mutant-enriched PCR; c, NA	a, Chitale et al[21]; b, Inukai et al[22]; c, COSMIC	Decreased sensitivity to EGFR TKIs	Pao et al[32] and Kobayashi et al[33]
	Exon 20 ins	a, 3% (AD); b, 2% (NSCLC)	a and b, direct sequencing	a, Ding et al[19]; b, Shigematsu et al[20]	Decreased sensitivity to EGFR TKIs	Wu et al[34]
	L861Q	a, 0.5% (AD); b, 0.3% (NSCLC)	a, direct sequencing; b, NA	a, Ding et al[19]; b, COSMIC	Increased sensitivity to EGFR TKIs	Lynch et al[29] and Paez et al[30]
HER2	Exon 20 ins	a, 2.6% (AD); b, 2% (NSCLC)	a, PCR-SSCP; b, direct sequencing	a, Sonobe et al[23] ; b, Shigematsu et al[20]	Increased sensitivity to HER2 TKIs	De Greve et al[7]
KRAS	G12C/S/R/D/A/V and G13C/S/R/D/A	a, 20% (AD); b, 26% (NSCLC)	a and b, direct sequencing	a, Riely et al[24]; b, Tsao et al[25]	Decreased sensitivity to EGFR TKIs	Pao et al[35]
	Q61H	a, 0.5% (AD); b, 0.2% (NSCLC)	a, direct sequencing; b, NA	a, Ding et al[19]; b, COSMIC	NA	NA
PIK3CA	E542K, E545K/Q, and H1047R	a, 2% (AD); b, 1.2% (NSCLC)	a, NA; b, direct sequencing	a, COSMIC; b, Yamamoto et al[26]	Increased sensitivity to PI3K inhibitors	Shapiro et al[8]
BRAF	V600E	a, 2% (AD); b, 1% (NSCLC)	a, sequenom; b, sequenom	a, Chitale et al[21]; b, Pratilas et al[27]	Increased sensitivity to BRAF V600E inhibitors	Flaherty et al[6]
	Non-V600E	a, 0.5% (AD); b, 0.7% (NSCLC)	a, sequenom; b, sequenom	a, Chitale et al[21]; b, Pratilas et al[27]	NA	NA
MEK1	Q56P, K57N, and D67N	1% (AD)	Direct sequencing	Marks et al[3]	Increased sensitivity to MEK inhibitors	Marks et al[3]
AKT1	E17K	a, 0.5% (AD); b, 0.5% (NSCLC)	a, direct sequencing; b, NA	a, Ding et al[19]; b, COSMIC	NA	NA
NRAS	Q61L/R	a, 1.6% (AD); b, 0.8% (NSCLC)	a and b, direct sequencing	a, Ding et al[19]; b, Brose et al[28]	NA	NA
PTEN	R233†	a, 1% (AD); b, 0.9% (NSCLC)	a and b, NA	a and b, COSMIC	Decreased sensitivity to EGFR TKIs	Sos et al[11]

AD, adenocarcinoma; TKI, tyrosine kinase inhibitor; Sequenom, a method based on Sequenom's MassARRAY system, involving multiplexed PCR, multiplexed single-base primer extension, and analysis of primer extension products using mass spectrometry; NA, not available; COSMIC, Catalogue of Somatic Mutations in Cancer; SSCP, single-strand conformational polymorphism; PI3K, phosphatidylinositol 3-kinase.

**ALK* fusions predict for sensitivity to *ALK* inhibitors; however, a different assay (fluorescence in situ hybridization) is used to detect ALK fusions. Version 46 of COSMIC was used as a reference for this table.

†Truncation.

Discussion

Historically, cancer treatment decisions have been based on stage and histological classification of tumors, with the choice of chemotherapies guided mostly by empirical data. However, basic and translational research has uncovered molecular abnormalities that drive and sustain cancers. Clinical research[18] has shown that patients' tumors respond differently to therapies targeted against these molecular abnormalities. For example, increased sensitivity of tumors harboring *EGFR*, *HER2*, *PIK3CA*, *BRAF*, *MEK1*, and *ALK* alterations to specific kinase inhibitors has already been established (Table 5).[2–9] Thus, if physicians can identify genetic abnormalities required for tumor growth before a patient's therapy is chosen, they can begin to administer more appropriate specific agents to destroy tumors in a targeted fashion. Prospective incorporation of molecular tumor testing into everyday practice will prioritize for patients and physicians the treatments with the highest probability of positive outcomes. Unfortunately, clinical implementation of large-scale molecular diagnostics to cancer treatment remains in its infancy. Many hurdles remain, including the need to develop rapid, inexpensive, multiplexed genotyping tests for clinical use.

For multiplexed analysis of point mutations in DNA from FFPE tissue samples, at least two major platforms are in use. The first relies on Sequenom's MassARRAY system, based on multiplexed PCR, multiplexed single-base primer extension, and analysis of primer extension products using mass spectrometry (ie, matrix-assisted laser desorption/ionization time-of-flight analysis).[36] The second is based on a system (SNaPshot; Life Technologies/Applied Biosystems) that depends on the analysis of fluorescently labeled primer-extension products by conventional capillary electrophoresis. In both assays, the identity of the incorporated nucleotide indicates the presence or absence of a mutation. Recently, some of us (D.D.-S., D.R.B., and A.J.I.) developed a fully operational SNaPshot assay that has been used as a clinical test for longer than 1 year to profile FFPE-derived tumor samples; it detects mutations in 58 different loci from 13 cancer genes (*APC*, *BRAF*, *CTNNB1*, *EGFR*, *FLT3*, *JAK2*, *KIT*, *KRAS*, *NOTCH1*, *NRAS*, *PIK3CA*, *PTEN*, and *TP53*) in eight multiplexed reactions.[12] Similarly, others[37] have used the SNaPshot technique to simultaneously screen colorectal carcinomas for 22 distinct mutations in four genes (*KRAS*, *NRAS*, *BRAF*, and *PIK3CA*).

Herein, we extended the SNaPshot technique to develop a multiplexed screen that was designed to assess DNA samples simultaneously for 38 somatic recurrent point mutations in eight genes with relevance to targeted therapy specifically in lung cancer. We chose to adopt the SNaPshot platform for the following reasons: i) familiarity with capillary electrophoresis, ii) ease of data interpretation, and iii) local availability of all necessary equipment. We also developed a triplex sizing assay, based on differential fragment lengths of fluorescently labeled products, to simultaneously assess samples for *EGFR* exon 19 deletions and *EGFR* exon 20 and *HER2* exon 20 insertions. Such alterations are not amenable to comprehensive detection by the SNaPshot method. Both SNaPshot and sizing assays can be performed rapidly with minimal amounts of starting FFPE-derived DNA material and high sensitivity. Compared with direct sequencing, in which mutant DNA needs to compose 25% or more of the total DNA to easily detect a mutation, the SNaPshot and sizing assays can detect mutations in samples in which mutant DNA composes 1.56% to 12.5% and 1.56% to 6.25% of the total DNA, respectively. More important, the assays are robust, rapid, reliable, and relatively inexpensive. Compared with direct sequencing of all exons involved, it has been estimated that SNaPshot assays cost 80% less.[37]

The SNaPshot assay does have some limitations. The technique involves multiple primer sets for both PCR amplification and primer extension. The addition of new mutations to existing panels is straightforward but still requires effort (ie, for each additional mutation, the concentrations of PCR and extension primers in a panel may need to be reevaluated because of competition between different primers). Moreover, SNaPshot detects point mutations at specific sites. It is not designed to detect amplifications, insertions, or deletions; it is also not the optimal method if mutations occur at multiple spots across coding exons (eg, in tumor suppressor genes) or for mutations involving fusions (eg, *EML4-ALK* translocations). For lung cancer specifically, novel platforms are needed that can simultaneously detect gene fusions, point mutations, insertions, deletions, and amplifications.

In its present form, the SNaPshot and sizing assays are designed to detect more than 40 types of recurrent genetic alterations in lung cancer. Together, a mutation in one of these genes can be found in approximately 50% of lung adenocarcinomas. As additional novel mutations are identified through efforts such as The Cancer Genome Atlas,[38] we plan to incorporate the clinically relevant ones into our screens. In the meantime, the current screens will be used to assess samples for multiple mutations and, in particular, as part of a 13-center US consortium (*http://clinicaltrials.gov/ct2/show/NCT01014286;* date of accession, August 18, 2010) among academic cancer centers that seek to genotype 1000 lung cancers nationwide. Mutation results will be used to prioritize therapy for patients, either as part of the standard of care or for clinical trials directed at specific mutation types. Efforts such as these will accelerate the use of genotypic information into standard treatment algorithms for lung cancer.

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