A Rapid and Sensitive Enzymatic Method for Epidermal Growth Factor Receptor Mutation Screening

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Abstract

Purpose: Mutations in the epidermal growth factor receptor (EGFR) are associated with clinical and radiographic responses to EGFR tyrosine kinase inhibitors gefitinib and erlotinib. Currently available methods of EGFR mutation detection rely on direct DNA sequencing, which requires isolation of DNA from a relatively pure population of tumor cells, cannot be done on small diagnostic specimens, and lack sensitivity. Here we describe the use of a sensitive screening method that overcomes many of these limitations.

Experimental Design: We screened 178 non–small cell lung cancer specimens for mutations in exons 18 to 21 of EGFR using a DNA endonuclease, SURVEYOR, which cleaves mismatched heteroduplexed DNA. Samples were analyzed by high-performance liquid chromatography on the Transgenomic WAVE HS system. Selected specimens that produced digestion products using SURVEYOR were subsequently reanalyzed by size separation or under partially denaturing conditions, followed by fractionation and sequencing. The specimens included DNA isolated from frozen tumor specimens, dissected formalin-fixed, paraffin-embedded tumor specimens undergoing clinical sequencing, and undissected formalin-fixed, paraffin-embedded specimens. One hundred sixty specimens were independently analyzed using direct DNA sequencing in a blinded fashion.

Results: EGFR mutations were detected in 16 of 61 fresh frozen tumor specimens, 24 of 91 dissected formalin-fixed, paraffin-embedded tumor specimens, and 11 of 26 undissected formalin-fixed, paraffin-embedded tumor specimens. Compared with sequencing, the sensitivity and specificity of the present method were 100% and 87%. The positive and negative predictive values were 74% and 100%, respectively. SURVEYOR analysis detected 7 (4%) mutations that were not previously detected by direct sequencing.

Conclusions: SURVEYOR analysis provides a rapid method for EGFR mutation screening with 100% sensitivity and negative predictive value. This unbiased scanning technique is superior to direct sequencing when used with undissected formalin-fixed, paraffin-embedded specimens.

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Mutations in the epidermal growth factor receptor (EGFR) have recently been described in patients with advanced non–small-cell lung cancer (NSCLC). These mutations are detected in 10% to 15% of all patients with NSCLC and in 80% of patients who clinically respond to EGFR tyrosine kinase inhibitors gefitinib or erlotinib (1–4). These mutations are located in exons 18 to 21 of the EGFR tyrosine kinase domain and, to date, ~35 different variants have been described in NSCLC, head and neck, colon, and breast carcinomas (1, 4–10). EGFR mutation detection is being offered to aid in clinical decision-making about the use of EGFR tyrosine kinase inhibitors for patients with advanced NSCLC. At the present time, the most common method of mutation detection is by direct sequencing of EGFR exons 18 to 21 from DNA isolated from tumor cells. This method has limitations for most patients with NSCLC. It requires large-sized specimen, typically at least a core needle biopsy or surgical specimen, subsequent dissection by a pathologist of tumor cells from normal lung and inflammatory cells, and preparation of DNA from these specimens and sequencing. This process can be time-consuming and thus limit its potential as a method for choosing patients for specific therapeutic treatments.

The shortcomings of direct DNA sequencing have led to alternative methods for rapid EGFR mutation detection. Sasaki et al. (11) examined 118 fresh frozen tumor specimens using an allele-specific real-time reverse transcription-PCR assay system. Pan et al. (12) have also used a PCR-based method but only examine mutations in exons 19 and 21. Although these methods may be faster than direct sequencing, allele-specific PCR relies on a priori knowledge of potential mutations, a limitation when scanning for new mutations. New EGFR mutations continue to be reported, including those found in patients who develop acquired resistance to EGFR tyrosine kinase inhibitors (13, 14). To detect such mutations, the method should be unbiased towards any particular EGFR mutation. Marchetti et al. (15) compared direct sequencing and PCR single-strand conformation polymorphism for detecting EGFR mutations in DNA prepared from frozen NSCLC specimens. No false-positive or false-negative results were observed with single-strand conformation polymorphism and eight additional mutations (all point mutations; seven in exon 21 and one in exon 18) not detected by direct sequencing were identified. A significant majority of patients with advanced NSCLC have small diagnostic specimens that are formalin fixed and paraffin embedded. Thus, if EGFR mutation detection is going to be used in clinical decision-making, the development of a method that allows accurate testing of formalin-fixed, paraffin-embedded specimens will be necessary.

CEL I nuclease, a DNA endonuclease isolated from celery, has a demonstrated preference for cutting 3′ to mismatched nucleotides on both strands of dsDNA. CEL I recognizes missense, insertion, and deletion mutations in a manner independent of sequence context and position of the mismatch within the DNA fragment (16, 17). A commercial preparation of CEL I endonuclease family (SURVEYOR) has recently been described for mutation detection (18). Briefly, PCR amplification of heterozygous alleles followed by heat denaturation and slow reannealing generates a population of dsDNA-containing mismatches (heteroduplexes). Enzymatic digestion of these heteroduplex PCR products by SURVEYOR yields cleaved fragments indicative of a mutation or mismatch.

In this study, we examine the utility of SURVEYOR digestion of DNA heteroduplexes analyzed on the Transgenomic WAVE Nucleic Acid High Sensitivity Fragment Analysis System (WAVE HS; Transgenomic, Inc., Cambridge, MA) as a method for EGFR mutation screening. We examined 178 specimens using DNA prepared from both frozen and formalin-fixed, paraffin-embedded tumor specimens. We were able to analyze formalin-fixed, paraffin-embedded tumor samples without micro or gross dissection. Furthermore, a comparison of our results with direct sequencing proved that this method was more sensitive, gave no false negatives, and can be adapted to a high throughput format. This method provides a quick and reliable way to scan for clinically relevant mutations and is superior to direct sequencing in its ability to detect mutations in small diagnostic specimens. This greater sensitivity increases the number of useable specimens available in clinical trials.

Materials and Methods

Cell lines and DNA samples. NSCLC cell lines A549, H1975, H3255, and DFCILU-011 were purchased from American Type Culture Collection (Manassas, VA) or established as previously described (1, 19). A549 is wild-type for EGFR. H1975 contains both an L858R and T790M mutation. H3255 and DFCILU-011 contain exon 21 L858R and exon 19 L747_E749 deletion mutations, respectively. DNA from cell lines was extracted using the DNeasy Tissue Kit from Qiagen, Inc. (Valencia, CA). For the dilution curves, wild-type and mutant cell lines were mixed at the desired percentage raging from 0% to 100% and then the DNA was extracted using the DNeasy Tissue Kit from Qiagen. DNA samples were prepared from frozen lung cancer tumor specimens as previously described (1). They were analyzed without knowledge of whether they had mutations and the specific nature of the mutations. Before analyses, the original DNA underwent whole genome amplification using multiple strand displacement with the Phi29 polymerase according to previously described methods (20). For specimens from patients whose tumors underwent sequencing at the clinical EGFR sequencing laboratory, DNA was provided in a blinded fashion by the Laboratory for Molecular Medicine, Harvard Medical School/Partners Healthcare Center for Genetics and Genomics. DNA was prepared from formalin-fixed, paraffin-embedded sections following dissection of tumor material to obtain at least 50% tumor using QIAamp (Qiagen) DNA extraction kit.

Gefitinib-treated patients. Patients with advanced NSCLC treated with gefitinib were identified from the clinical practices of the Dana-Farber Cancer Institute and the Beth Israel Deaconess Medical Center. Their clinical responses were assessed using the WHO criteria (21). Patients were followed for their radiographic and clinical response to gefitinib. All patients provided informed consent for EGFR mutation analyses and the study was approved by the Institutional Review Board at the Dana-Farber Cancer Institute.

DNA extraction from gefitinib-treated patients. For gefitinib-treated patients, formalin-fixed, paraffin-embedded tissues were obtained either as a set of ten 5-μm slides or as uncut tissue blocks. For the slides, excess paraffin was scraped off first and then tissue was scraped into a microcentrifuge tube. For the blocks, 4 × 40 μm sections were cut and excess paraffin was removed before DNA extraction. To extract DNA from tissues, Qiagen DNeasy Tissue Kit was used with the previously described modifications (22). The yield of DNA from 23 tissue samples was between 3.15 and 30.71 μg with a mean of 8.93 μg.

PCR primers and cycling conditions. The PCR primers for amplification of the EGFR exons 18 to 21 were designed to hybridize to intron sequences flanking the corresponding exon so that all coding sequence mutations or polymorphisms could be detected. The EGFR amplicons generated were 264 bp for exon 18, 203 bp for exon 19, 337 bp for exon 20, and 286 bp for exon 21. The specific PCR primer sequences are available on request. PCR reactions were done using the JumpStart Taq from Sigma and following the recommendations of the manufacturer. The specific PCR conditions are available on request. Following PCR amplification, all samples were then denatured and slowly renatured to form heteroduplexes using the following conditions: 95°C for 2 minutes, followed by a step decrease in temperature of 0.5°C for 15 seconds per step, until the temperature reached 45°C.

To generate mutation positive controls when the cell lines with relevant mutations were not available, we used a modified PCR mutagenesis method in which a third oligo was added to the PCR reaction. We generated positive controls for exon 18 (G719S) and exon 20 (insertion). The specific *in vitro* mutagenesis conditions and primers are available on request.

SURVEYOR digestion, RFLP assays, and dsDNA sizing. All EGFR exons were analyzed by SURVEYOR digestion. In addition, exons 19 and 20, which are known to harbor deletions and insertions, were analyzed without digestion. RFLP assays were used on exons 20 and 21. For the SURVEYOR digestion, 12 μL of the PCR product were used in a reaction containing 1 μL Enhancer and 1 μL SURVEYOR. The reaction was incubated at 42°C for 20 minutes and stopped by adding 1.5 μL of stop buffer. All reagents were provided with the SURVEYOR kit from Transgenomic. Digestions with the *Nla*III or *Bst*UI restriction enzyme were as follows: 12 μL of the PCR product, 1 μL restriction enzyme (New England Biolabs, Beverly, MA), 2 μL of 10× NEB recommended

buffer, and water up to 20 μL. The reaction was incubated at the recommended temperature for 1 hour, followed by purification of the DNA using the QIAquick PCR purification kit (Qiagen). All analyses were carried out by high-performance liquid chromatography on the Transgenomic WAVE Nucleic Acid High Sensitivity Fragment Analysis System (WAVE HS system; Transgenomic, Omaha, NE).

Samples were loaded on a DNASep cartridge (Transgenomic) at 50°C and eluted with a linear acetonitrile gradient in a 0.1 mol/L triethylammonium acetate buffer (pH 7) at a constant flow rate of 0.9 mL/min. Eluted DNA fragments were detected by a UV-C detector (Transgenomic). To improve the sensitivity of SURVEYOR analysis, Transgenomic WAVE Optimized HS Staining Solution I (Transgenomic), a DNA-intercalating dye, was mixed with the eluate following UV detection, and fluorescent intensity was measured by fluorescence detector with excitation at 490 nm and emission at 520 nm according to the instructions of the manufacturer.

Fractionation and sequencing. Amplicons with new digestion products following SURVEYOR analysis were fractionated by denaturing high-performance liquid chromatography under partially denaturing conditions designed to enrich for a fraction containing heteroduplexes. Running temperatures were determined using Navigator Software (Transgenomic) based Tm of each amplicon. Samples harboring a deletion or insertion were size fractionated under nondenaturing conditions. Fractions were collected and peaks of interest were amplified using Expand High Fidelity PCR System (5 μL fraction per 50 μL PCR reaction). The amplified PCR products were purified by using the QIAquick kit from Qiagen and sequenced in the forward and reverse directions by the Molecular Biology Core Facilities at Dana-Farber Cancer Institute. The sequencing primers were the same used for the PCR amplifications.

Results

Sensitivity of EGFR mutation detection using DNA from EGFR mutant NSCLC cell lines. The sensitivity of heteroduplex detection by Surveyor analysis on the WAVE HS system was tested by making dilution curves. H1975 containing the heterozygous EGFR mutation L858R or DFCILU-011 containing the heterozygous EGFR deletion L747_E749del were diluted with EGFR wild-type cells A549. Neither cell line contains a significant amplification of the EGFR locus (23). Genomic DNA was isolated from cell mixtures containing 100%, 50%, 20%, 5%, 1%, and 0% mutant cells. Exon 19 of EGFR (Fig. 1) or exon 21 (Fig. 2) was amplified and digested with Surveyor. The digested PCR products were size fractionated on the WAVE HS system using the high-sensitivity fluorescence detector with WAVE Optimized Staining Solution I. As can be seen in Fig. 1, the presence of a mutant allele is easily detected by the appearance of two new fragments following SURVEYOR digestion. The SURVEYOR digestion products could be detected in genomic DNA from mixtures containing as little as 1% mutant cells. Because the DFCILU-011 cell line contains a 9-bp deletion in EGFR, we were also able to use the WAVE HS system to separate intact heteroduplexes, mutant homoduplexes, and wild-type homoduplexes. We were able to detect heteroduplexes in dilutions with as little as 1% of the mutant cell line (Fig. 1). Sequencing of each of these three peaks has confirmed that the heteroduplexes elute first, followed by the mutant homoduplexes, and, lastly, the wild-type homoduplexes (data not shown). Parallel direct sequencing and analysis by the Mutation Surveyor (Softgenetics) software were unable to detect the presence of the mutant allele in dilutions containing ≤20% DFCILU-011 (data not shown). Similar findings were observed for the H1975 cell line dilutions containing the L858R mutation (Fig. 2). Using SURVEYOR analysis, mutations were detected in dilutions containing as little as 1% of H1975 cells whereas direct sequencing and analysis by the Mutation Surveyor software were only able to detect a mutation in >20% H1975 cells (data not shown). To confirm that the mutant allele could be identified in these samples, we fractionated these samples using partially denaturing high-performance liquid chromatography. Under these conditions, heteroduplexes were eluted off the column faster than the homoduplexes. To enrich for the heteroduplexes, we collected and sequenced the first few fractions from the first peak in the chromatogram (24). As can be seen in Fig. 2, sequencing of the fractions isolated from the 10% and 5% dilutions of H1975 clearly shows the presence of the mutation. Using this strategy of fractionation, reamplification, and sequencing, we were able to enrich for the mutant-containing heteroduplex PCR product by at least 4-fold when compared with direct sequencing. Thus, using SURVEYOR analysis coupled with sequencing of the enriched fractions, we were able to detect and characterize mutations present in >1% of the cell population.

EGFR mutation detection from frozen NSCLC specimens. We next examined the utility of EGFR mutation detection with SURVEYOR digestion using DNA isolated from frozen NSCLC specimens. We chose to examine these 66 tumor specimens because they had all previously undergone direct sequencing (1). Of these samples, five specimens were excluded from the analysis due to either poor or inconsistent PCR amplifications. Sixty-one specimens were analyzed using SURVEYOR in a blinded fashion. EGFR exons 18 to 21 were amplified by PCR, subjected to SURVEYOR digestion, and the products separated using the WAVE HS system. We used dsDNA sizing to corroborate deletions and insertions in exons 19 and 20. Additionally, exon 20 frequently harbors a SNP that results in a similar SURVEYOR digestion pattern to a previously reported rare T790M mutation associated with resistance to gefitinib

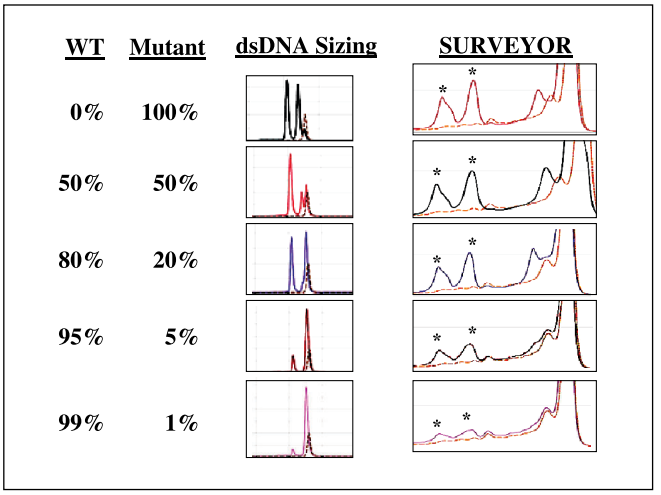

Fig. 1. Dilution curve of DFCILU-011 cells containing an EGFR exon 19 deletion. DFCILU-011 cells (EGFR L747.E749del) were diluted with A549 (EGFR wild-type) cells. The percentages of mutant cells were 100%, 50%, 20%, 5%, and 1%. Genomic DNA was extracted, PCR amplified for exon 19, and analyzed using the WAVE HS system (*solid lines*). Dashed line, EGFR wild-type control (100% A549). Double-stranded DNA size separation of amplified exon 19 shows three distinct peaks corresponding to the heteroduplex, deletion mutant homoduplex, and the wild-type homoduplex. *, two new fragments generated by SURVEYOR digestion of these samples; wild-type control is uncut.

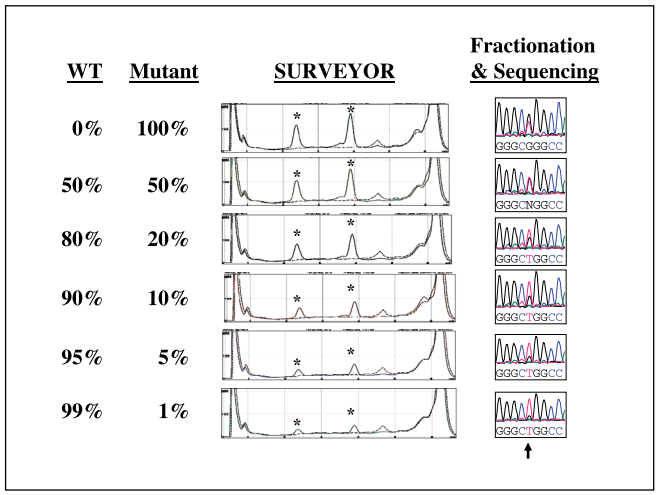

Fig. 2. Dilution curve of H1975 cells containing a EGFR exon 21 missense mutation. H1975 cells (EGFR L858R) were diluted with A549 cells (EGFR wild-type). The percentages of mutant cells were 100%, 50%, 20%, 10%, 5%, and 1%. Genomic DNA was extracted, PCR amplified for exon 21, and analyzed using the WAVE HS system (*solid lines*). Dashed line, wild-type control (100% A549). *, two fragments generated by SURVEYOR digestion of these samples; the wild-type control is uncut. The samples were rerun under partially denaturing conditions; fractions were collected, reamplified, and sequenced (as described in Materials and Methods). The T→G alteration resulting in the L858R is visualized at mixtures of ›5% mutant cells (*arrow*).

(13, 14). The high sensitivity of the WAVE HS system allows the use of RFLP to distinguish between mutations and SNPs. To characterize 14 specimens that showed positive exon 20 patterns using SURVEYOR, we used the *Nla*III restriction enzyme which generates an additional exon 20 fragment in T790M samples (14). We used H1975 as a positive control as it is known to contain the T790M mutation (data not shown). This mutation was not found in any of the patients analyzed.

We were able to detect all EGFR mutations previously reported by direct sequencing from this sample group of 61 specimens. Direct sequencing had previously detected 15 mutations (10 exon 19 deletions, 1 exon 20 insertion, 1 exon 18 G719S, and 3 exon 21 L858R mutations) in the 60 tumor specimens. However, our analysis identified a second mutation (E709A) in the specimen containing the G719S mutation. The E709A mutation has previously been described (6). Of note, the E709A mutation was also detected in this same specimen using the 454 technology.[12] We also found an EGFR exon 18 mutation in one specimen that was classified as wild-type by direct sequencing. Fractionation and sequencing found this mutation to be G719A. Seventeen specimens were found to have the exon 20 (Q787Q) SNP and, of those, 12 contained the exon 20 SNP as their only variant. Based on these findings, the sensitivity of SURVEYOR combined with denaturing high-performance liquid chromatography for EGFR mutation detection using DNA from frozen tumor specimens is 100% and the specificity is 97.8% in this sample set.

EGFR mutation detection from gross dissected formalin-fixed, paraffin-embedded tumor specimens undergoing clinical sequencing. The utility of this mutation detection method using DNA from frozen tumor specimens was initially studied to develop the methods for using SURVEYOR analysis. DNA isolated from paraffin-embedded tumor specimens was studied because this is the most common source of material for mutation detection in tumors. For these studies, we used DNA isolated from tumor specimens undergoing clinical EGFR sequencing in a Clinical Laboratory Improvement Amendment–certified laboratory (Laboratory for Molecular Medicine, Harvard-Partners Center for Genetics and Genomics, Cambridge, MA). We examined 96 specimens using SURVEYOR in a blinded fashion. Specimens were either derived from formalin-fixed, paraffin-embedded (78) or from frozen tumors (18), had undergone gross dissection to enrich for tumor cells, followed by DNA isolation and sequencing of EGFR exons 18 to 24. Eighty-five of the 96 specimens were analyzed by direct sequencing. Eleven had either failed PCR (n = 5) or were not tested (n = 6). Of these, five specimens could not be analyzed due to poor PCR amplifications for some or all EGFR exons. Of the five failed samples, four also failed independent PCR when used in the present analysis. By direct sequencing, the 85 specimens were found to contain 27 mutations in 24 patients: G719A (1), exon 19 deletions (12), exon 20 insertions (2), T790M (1), R776H (1), R831C (1), L858R (6), L861Q (1), and P848L (2). Three patients had double mutations: one patient with L858R and R831C, one patient with L858R and T790M, and one patient with G719A and R776H. Our SURVEYOR analysis identified all of these mutations. SNPs in exons 21 (R836R) and 20 (Q787Q) were found in 1 and 47 specimens, respectively. Twenty-nine specimens had the exon 20 SNP as the only variant. In addition, 15 tumor specimens had 20 unique SURVEYOR patterns and were fractionated and sequenced. Of the 20 unique SURVEYOR patterns, there were 10 variants, 4 SNPs, and 6 mutations in seven patients not detected by direct sequencing (Table 1). The remaining 10 unique SURVEYOR patterns in eight patients were all classified as false positives or below the limit of detection as independent PCR reactions and SURVEYOR analyses could not reproduce the initial observations. Furthermore, fractionation and sequencing were unable to identify any sequence variants. The six additional mutations we found included five in exon 20. Four of these (T790M, V769M, G779F, and E866K) have been previously characterized (6, 13, 14, 25, 26). Mutations in T783 (T783A) have also been previously described although we detected a different missense mutation (T783I; TT55 in Table 1; ref. 27). The remaining mutation (I789_L792 del) was novel. The T790M mutation has been associated with resistance to EGFR tyrosine kinase inhibitors whereas T783A has been observed in a patient who developed a partial response to gefitinib (13, 14, 27). Of note, two of these samples (TT53 and TT90) were found to have <50% tumor content on review of the pathology reports. In addition, we were able to detect an exon 21 mutation in a sample that had failed direct sequencing (TT60; Table 1). No data on treatment with gefitinib or erlotinib and the subsequent outcome are available for these patients. These results show that the sensitivity of the SURVEYOR analysis combined with denaturing high-performance liquid chromatography is 100% whereas the specificity is 78% in this sample set.

EGFR mutation detection from patients treated with gefitinib using undissected formalin-fixed, paraffin-embedded tumor specimens. Because we had established the sensitivity of our mutation detection method using cell line dilutions, frozen tumors, and dissected formalin-fixed, paraffin-embedded tumor specimens, we next wished to determine whether we could

[12] M. Meyerson, unpublished data.

apply this method to undissected formalin-fixed, paraffin-embedded tumor specimens. If successful, it would eliminate a costly and time-consuming dissection step in the EGFR mutation detection process. We examined 26 patients with NSCLC who were treated with gefitinib. For our blinded analyses, DNA was prepared from formalin-fixed, paraffin-embedded tumor specimens using the entire tumor section without gross or microdissection.

All patients have been followed in the clinics of Dana-Farber Cancer Institute or Beth Israel Deaconess Medical Center and their clinical outcome has been characterized. Among the 26 patients, there were 12 who achieved partial response, 8 who had disease progression, and 6 who had stable disease (defined as no evidence of tumor progression >60 days) with gefitinib treatment. We enriched for patients who had developed partial responses to have adequate numbers of patients with different mutations. The definition of a partial response was based on the WHO criteria (21). Patients were chosen for these analyses based on the availability of tumor tissue for analyses. Fourteen (54%) of these patients had also undergone direct sequencing of their tumor specimen following dissection and seven (27%) have been previously reported (1). The tumor content in each slide was estimated by a pathologist (N.L.) and varied from 5% to 90% (Table 2).

Mutation analysis was successful in all of the specimens. EGFR mutations were detected in 11 of 12 (92%) patients with partial responses and in 0 of 14 (0%) patients with either stable or progressive disease. All specimens that gave digestion products by SURVEYOR analysis were rerun under partially denaturing conditions for point mutations or dsDNA size separation for deletions/insertions. Fractions enriched for heteroduplexes were collected, reamplified, and sequenced. Using these techniques, we were able to differentiate among three nearly identical EGFR exon 21 SURVEYOR digestion patterns (Fig. 3A). These samples exhibited different elution profiles under partially denaturing conditions (Fig. 3B) that corresponded to three distinct variants: two mutations, L858R and L861Q, and one SNP, R838R (Fig. 3C). As an alternative approach, we also used an RFLP assay to distinguish between this R838R SNP and the two exon 21 mutations as the SNP eliminates a *Bst*UI restriction enzyme site (data not shown).

Of the 12 patients who responded to gefitinib treatment, six patients underwent direct sequencing of DNA extracted from gross dissected tumor tissue and our results were 100% concordant. Four of 12 specimens were considered inadequate for direct sequencing due to either a low tumor content ($n = 3$) or small specimen size ($n = 1$). The SURVEYOR analysis was successful for all four specimens and mutations were detected in three of them. The remaining two specimens were not sequenced. No mutations were detected in 14 patients with either disease progression ($n = 8$) or stable disease ($n = 6$). Eight of these patients had previously undergone sequencing and, again, our results were 100% concordant. Seven of the 26 patients had a positive SURVEYOR digestion pattern in exon 20. Using denaturing high-performance liquid chromatography fractionation and sequencing, all seven patients were found to be heterozygous for the common Q787Q polymorphism. Two patients had an exon 21 SNP (R836R).

Combined analyses using SURVEYOR and denaturing high-performance liquid chromatography for EGFR mutation detection. To determine the sensitivity and specificity of using SURVEYOR and the WAVE HS denaturing high-performance liquid chromatography system as a method for EGFR mutation detection, we combined our tumor specimens for these analyses. We only considered tumors that had undergone both direct sequencing and SURVEYOR analyses for a total of 160 tumor specimens. In this combined sample set, the sensitivity of our analysis was 100% and the specificity was 87%. The negative predictive value was 100% whereas the positive value was 74%. Among the 160 specimens, 102 tumors contained no detectable mutations in EGFR exons 18 to 21. Seven of 160 (4%) mutations were detected using SURVEYOR which were not previously detected by direct sequencing. In addition, in three of four patients who achieved a partial response to gefitinib, we were able to detect mutations in EGFR using this technique.

Discussion

The close correlation between EGFR mutations and radiographic regression in NSCLC patients treated with gefitinib was first reported in the spring of 2004 (1, 2). Since then, EGFR mutations have been examined in thousands of patients and

Table 1. Fractionation and sequencing of seven SURVEYOR positive patient samples showed 10 genetic variations undetected by direct sequencing

Specimen	Direct sequencing	SURVEYOR positive exon(s)	Fractionation and sequencing	Additional mutations
TT10	Exon 19 deletion	19	Exon 19 deletion	
	Exon 20 WT	20	V769M, I789_L792del	Two mutations exon 20
TT24	WT	20	K823K	SNP exon 20
TT53	WT	20	T790M	Mutation exon 20
TT55	G719A	18	G719A	Mutation exon 20
	R776H	20	R776H,T783I	
TT56	WT	20	L799L, K806K	Two SNPs exon 20
TT60	failed	20	L815L	SNP exon 20
		21	E866K	Mutation exon 21
TT90	WT	20	G779F	Mutation exon 20

NOTE: Out of these 10 variants, 5 were mutations and 5 were SNPs. WT, wild-type.

Table 2. Summary of EGFR mutations in patients treated with gefitinib

No.	Sex	Histology	Response	Specimen	% Tumor	Sequence	Mutation
1	F	BAC	PR	Lung wedge	50	Yes	L747_S752del, P753S, A755G
2	F	Adeno	PR	Lymph node	25	QNS	G719C
3	F	Adeno	PR	Lymph node	20	Yes	del747_752
4	F	NSCLC	PR	Pleura	5	QNS	WT
5	F	NSCLC	PR	Transbronch	N/A	QNS	G719S, L861Q
6	F	NSCLC	PR	Effusion block	55	Yes	Del746_750
7	F	NSCLC	PR	Lung mass	20	Yes	L858R
8	F	BAC	PR	Lung mass	5	No	L858R
9	F	Adeno	PR	FNA	70	QNS	Del747_750
10	F	NSCLC	PR	Lung mass	70	No	L746Q,Del747_749,A750P
11	F	Adeno	PR	Brain	80	Yes	Del746_750
12	F	Adeno	PR	Brain	50	Yes	Del747_752, E746V
13	M	Adeno	PD	Spleen	90	Yes	WT
14	F	Adeno	PD	Lymph node	30	Yes	WT
15	F	Adeno	PD	Lung	65	Yes	WT
16	F	NSCLC	PD	Lung	60	Yes	WT
17	F	Adeno	PD	Lung	90	Yes	WT
18	F	Adeno	PD	Lung	80	Yes	WT
19	M	NSCLC	PD	Lymph node	15	Yes	WT
20	M	NSCLC	PD	Lung	15	No	WT
21	F	Squamous	SD	Lung	20	No	WT
22	F	Adeno	SD	Lung	75	No	WT
23	F	Adeno	SD	Lung	30	No	WT
24	M	BAC	SD	Lung	30	No	WT
25	F	Adeno	SD	Brain	40	Yes	WT
26	M	BAC	SD	Pericardium	5	No	WT

NOTE: Data for patients 1, 12, and 13 to 17 have been previously published (1).
Abbreviations: Adeno, adenocarcinoma; BAC, bronchioloalveolar carcinoma; PR, partial response; PD, progressive disease; SD, stable disease; N/A, not available; QNS, quantity not sufficient.

this correlation continues to be observed. Patients with EGFR mutations treated with gefitinib have significantly greater response rate, time to disease progression, and survival compared with those without EGFR mutations treated with gefitinib (5, 28, 29). The outcomes observed in these studies are substantially different than those achieved with second-line systemic chemotherapy (30, 31). The median time to progression and survivals are in the order of 8 to 10 months and 2 years for patients with EGFR mutations treated with gefitinib (5, 28, 29). In contrast, for patients treated with docetaxel or pemetrexed, the median time to progression and survivals are 2 to 3 months and 8 months retrospectively (30, 31). At the present time, both gefitinib and erlotinib are approved in the United States only for patients who have failed systemic chemotherapy. The outcomes observed in the retrospective studies suggest that for patients with EGFR mutations, these agents should be examined as initial therapies and such studies are under way.

At the present time, EGFR mutation detection is most commonly done by direct DNA sequencing of the EGFR kinase domain. For patients with NSCLC, biopsies are often small, the tumor cellularity may be low, and the specimen may be very heterogeneous. These types of samples may not be amenable to direct sequencing. For example, in a recent large study, mutation analysis was possible only in 177 of 731 (24%) patients and in only 107 (15%) tumors were exons 18 to 21 analyzed whereas in 70 specimens only exons 19 and 21 were analyzed (26). Of the 177 analyzed samples, 104 required microdissection. These findings highlight the need for more sensitive and widely applicable methods of EGFR mutation detection.

To exclude patients who do not have EGFR mutations and identify those likely to harbor EGFR mutations, we have explored a more sensitive and efficient method. In our study of 188 specimens, this method provided clear results for 95% (178) of the samples. The remaining 10 specimens were unable to be amplified due to inconsistent PCR amplifications. This method uses exon specific PCR followed by treatment with an endonuclease that digests variant-containing heteroduplexes and separates the digestion products using the WAVE HS system. No a priori knowledge of the specific type of variation (missense, deletion, or insertion) or its exonic location is required. This method has several advantages over direct sequencing and other recently published methods for EGFR mutation detection which either use allele-specific PCR (11, 12) or have been only used on DNA isolated from frozen tumor specimens (15). First, it can be done using formalin-fixed, paraffin-embedded tumor specimens from patients diagnosed with advanced NSCLC. Second, it is amenable to use with small diagnostic biopsies that cannot be sequenced directly. Third, due to the sensitive nature of the method, it can be done

without the need for dissection of tumor material and from heterogeneous specimens which contain significant amounts of normal cells. This speeds up the process of mutation detection and requires less manipulation of the specimen. Finally, it is capable of detecting mutations that are missed by direct sequencing.

Using DNA derived from frozen tumors, dissected formalin-fixed, paraffin-embedded tumor specimens or undissected formalin-fixed, paraffin-embedded tumor specimens we were able to show 100% sensitivity and negative predictive value for this method compared with direct sequencing. More importantly, specimens not amenable to conventional sequencing due to small specimen size or low tumor content (Table 2) could be analyzed using this technique. This provides the potential to significantly increase the number of patients whose EGFR mutation status can be assessed. Furthermore, we identified seven mutations in patients previously characterized by direct sequencing as wild-type for EGFR. For example, in one patient we detected a rare T790M mutation (Table 1) that is associated with resistance to gefitinib (13). We also found one novel mutations (I789_L792del) although its clinical significance remains to be determined. Without appropriate clinical correlation, these findings should be interpreted with caution. Mutation analysis from paraffin-embedded tumor specimens has previously been shown to produce false positives when analyzing for BRCA1 mutations (32, 33). However, such false positives would not be unique to our mutation detection method as all of the mutation detection methods to date, including direct sequencing, involve PCR amplification of the source DNA material.

A potential limitation in this technique is that it would miss a homozygous mutation. However, few homozygous EGFR mutations have been described to date. Moreover, gross dissected specimens rarely contain exclusively tumor cells and our method of preparing DNA from the entire tumor section guarantees that the DNA is derived from a heterogeneous cell population. Furthermore, we have been able to detect the L858R mutation in the H3255 cell line in which the mutant allele is amplified >10-fold over the wild-type allele (34).

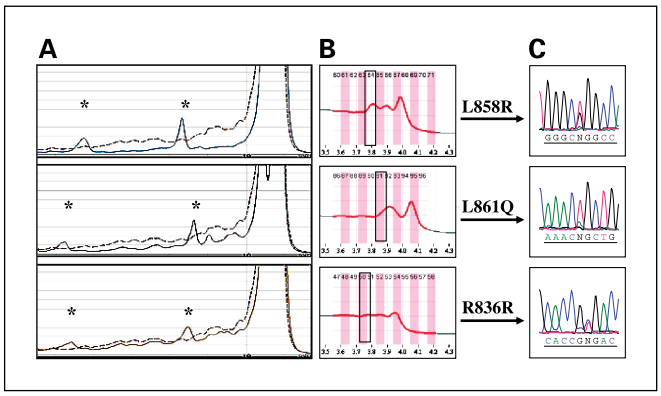

Fig. 3. SURVEYOR analysis of EGFR exon 21 followed by fractionation and sequencing of three patient specimens with three distinct variants. *A,* SURVEYOR analysis of three patient samples. DNA specimens isolated from formalin-fixed, paraffin-embedded tumor tissue were amplified for EGFR exon 21, subjected to SURVEYOR digestion, and separated on the WAVE HS system. All three specimens produce cleavage products (*) not observed in the wild-type control (*dashed lines*). *B,* fractionation of partially denaturing run. The specimens were reanalyzed under partially denaturing conditions and gave rise to three distinct elution profiles. Fractions were collected (*pink and white columns*) and sequenced (*black box*). *C,* sequencing of the fractions collected in (*B*). The three different profiles obtained in (*B*) revealed three distinct exon 21 variants: L858R, L861Q, and R836R.

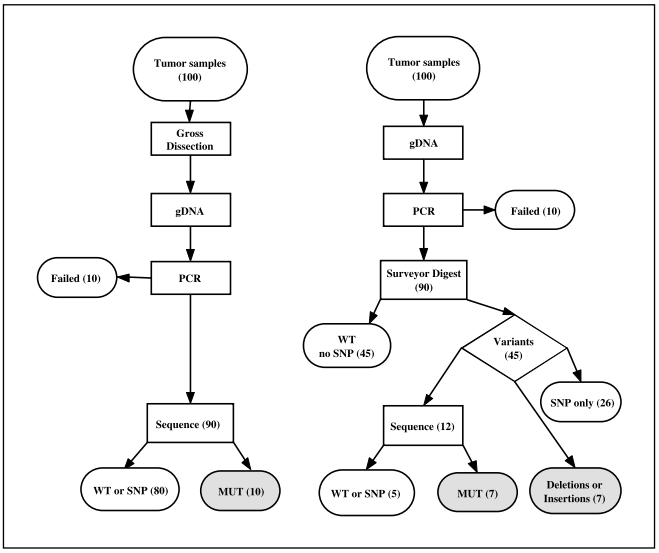

Fig. 4. Schema comparing direct sequencing with SURVEYOR analysis. Shown are 100 hypothetical tumor specimens which can be analyzed by either direct sequencing (*left*) or first by SURVEYOR (*right*). In both cases, 90% of the specimens are able to be PCR amplified for analyses. Following gross dissection and PCR amplification, all 90 specimens are sequenced. Using SURVEYOR, 50% (45 of 90) are initially excluded as being wild-type. Thirty-five of the remaining 45 (78%) specimens either contain SNPs or deletions in exon 19 or insertions in exon 20. The remaining 12 specimens are sequenced to confirm new digestion products produced by SURVEYOR. Additional mutations ($n = 14$ for SURVEYOR and $n = 10$ for sequencing) are detected using SURVEYOR due to the higher sensitivity. The numbers at each step are based on the data in the article. WT, wild-type; Mut, mutant; gDNA, genomic DNA.

EGFR mutation frequency is 10% to 15% in all patients from the United States and Europe and ranges from 20% to 30% in clinically enriched subsets of patients such as women, patients with adenocarcinomas, or nonsmokers (4, 15). Thus, the majority of all patients and even those from clinically enriched subsets will be wild-type for EGFR. The method of EGFR mutation detection described in our study can serve as an efficient screening test because the negative predictive value of the SURVEYOR analysis to date is 100%. A schema showing the utility of this approach is shown in Fig. 4. By conventional sequencing, 90% of tumor specimens, those in which there was successful PCR amplification following tumor dissection, undergo direct sequencing. In contrast, using SURVEYOR, the majority of specimens can be determined to be either wild-type or to contain SNPs (Fig. 4). Furthermore, using size fractionation on the WAVE HS system, exon 19 deletions and exon 20 insertions can be easily identified and do not need further confirmation by direct sequencing. Thus, only 12% of the original tumor specimens are subjected to sequencing. Rapid determination of EGFR mutation status will be particularly important in deciding whether or not to offer NSCLC patients EGFR tyrosine kinase inhibitors as their initial form of therapy.

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