

## Mutation-Specific Antibodies for the Detection of *EGFR* Mutations in Non – Small-Cell Lung Cancer

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**Abstract Purpose:** Activating mutations within the tyrosine kinase domain of epidermal growth factor receptor (EGFR) are found in approximately 10% to 20% of non – small-cell lung cancer (NSCLC) patients and are associated with response to EGFR inhibitors. The most common NSCLC-associated EGFR mutations are deletions in exon 19 and L858R mutation in exon 21, together accounting for 90% of EGFR mutations. To develop a simple, sensitive, and reliable clinical assay for the identification of EGFR mutations in NSCLC patients, we generated mutation-specific rabbit monoclonal antibodies against each of these two most common EGFR mutations and aimed to evaluate the detection of EGFR mutations in NSCLC patients by immunohistochemistry.

**Experimental Design:** We tested mutation-specific antibodies by Western blot, immunofluorescence, and immunohistochemistry. In addition, we stained 40 EGFR genotyped NSCLC tumor samples by immunohistochemistry with these antibodies. Finally, with a panel of four antibodies, we screened a large set of NSCLC patient samples with unknown genotype and confirmed the immunohistochemistry results by DNA sequencing.

**Results:** These two antibodies specifically detect the corresponding mutant form of EGFR by Western blotting, immunofluorescence, and immunohistochemistry. Screening a panel of 340 paraffin-embedded NSCLC tumor samples with these antibodies showed that the sensitivity of the immunohistochemistry assay is 92%, with a specificity of 99% as compared with direct and mass spectrometry – based DNA sequencing.

**Conclusions:** This simple assay for detection of EGFR mutations in diagnostic human tissues provides a rapid, sensitive, specific, and cost-effective method to identify lung cancer patients responsive to EGFR-based therapies.

Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future. Lung cancer is broadly divided into small-cell lung cancer (20% of lung cancers) and non – small-

cell lung cancer (NSCLC; 80% of lung cancers). Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are found in a subset of NSCLC adenocarcinomas and are associated with sensitivity to the small-molecule EGFR tyrosine kinase inhibitors gefitinib (1, 2) and erlotinib (3). Different EGFR mutations have been reported, but the most common NSCLC-associated EGFR mutations are in-frame deletions in exon 19 (E746\_A750del) and the point mutation replacing leucine with arginine at codon 858 in exon 21 (L858R; refs. 3 – 5). These two mutations represent 85% to 90% of EGFR mutations in NSCLC patients. Data from clinical research have confirmed that patients with these mutations are highly responsive to EGFR inhibitors including gefitinib and erlotinib (5 – 8).

Based on these clinical findings, EGFR mutational analysis in lung adenocarcinoma may now be used to guide treatment decisions and to enroll patients in specific arms of clinical trials. Direct DNA sequencing of PCR-amplified genomic DNA has been developed to detect EGFR mutations in patient tumor tissue. However, the adoption of this technique as a clinical test suffers from high costs of equipment and reagents, technical difficulties of performing the assay, and length of the procedure. In addition, direct DNA sequencing has a limited sensitivity for the detection of tumor cells containing an EGFR

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## Translational Relevance

Somatic mutations in the epidermal growth factor receptor (EGFR) correlate with increased response in patients with non-small-cell lung cancer treated with EGFR tyrosine kinase inhibitors. EGFR mutation testing in lung adenocarcinoma has already been used to help guide treatment decisions and/or to enroll patients in specific arms of clinical trials. To date, significant efforts have been made to develop DNA-based clinical mutation detection testing. Here we present a novel assay involving the development of mutation-specific antibodies to identify mutant protein, offering a rapid, sensitive, accurate, and low-cost immunohistochemistry assay that we believe can be rapidly implemented and thus have an impact on the treatment of patients worldwide.

mutation against a background of nonmutant cells. Therefore, the sensitivity of DNA sequencing is dependent on the percentage of tumor cells in the sample and, importantly, on the percentage of tumor cells carrying the mutation. Finally, the DNA obtained from paraffin specimens of standard biopsies is commonly not sufficient or is of poor quality for DNA sequencing. Recently, other DNA-based methods have been developed to improve the detection of EGFR mutation in lung cancer specimens, including allelic-specific PCR, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS)-based genotyping, denatured high-performance liquid chromatography, and single molecule sequencing (9, 10). However, these methods are not routine procedures in clinical labs and remain expensive and time-consuming. Furthermore, these methods do not take into account tumor heterogeneity.

In contrast, immunohistochemistry is a well-established method routinely applied in solid tumor diagnosis in clinical laboratories. Immunohistochemistry also allows for the simultaneous analysis of expression level of other proteins or protein modifications. Finally, immunohistochemistry also allows for the analysis of small tissue samples or individual cells obtained from body fluids, bronchial washings, fine needle aspirates, as well as circulating tumor cells. Thus, the development of antibodies that specifically detect mutant EGFR protein by immunohistochemistry would be a valuable addition to the current protocols used in the diagnosis and treatment of lung cancer. In this work, we generated mutation-specific rabbit monoclonal antibodies (mAb) against EGFR with the E746\_A750 deletion in exon 19 or the L858R point mutation for immunohistochemistry application. These antibodies were extensively evaluated by Western blotting, immunofluorescence, and immunohistochemistry to determine their sensitivity and specificity in NSCLC patient tumor samples.

## Materials and Methods

**Generation of rabbit mAbs.** New Zealand rabbits were immunized with synthetic peptides matching the EGFR sequence with E746\_A750del or L858R mutations. Positive immunoreactive rabbits were identified by ELISA and Western blots and chosen for rabbit monoclonal preparation. Supernatants from newly generated clones were screened by ELISA for reactivity with the immunogen peptide.

Supernatants with specificity for EGFR with E746\_A750del and L858R point mutation were tested by Western blots, flow cytometry, and immunohistochemistry.

**Cell lines.** H1975 and H1650 cell lines were obtained from American Type Culture Collection. HCC827 and two human esophageal squamous cell carcinoma cell lines (Kyse450 and Kyse70) were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). The H3255 cell line was provided by Dr. Lew Cantley (Beth Israel Deaconess Medical Center, Boston, MA).

**Western blot analysis.** Cultured cells were washed twice with cold  $1 \times$  PBS and then lysed in  $1 \times$  cell lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L  $\text{Na}_2\text{EDTA}$ , 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L  $\beta$ -glycerophosphate, 1 mmol/L  $\text{Na}_3\text{VO}_4$ , 1  $\mu\text{g/mL}$  leupeptin] supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail (Roche). Lysates were sonicated and centrifuged at 14,000 rpm for 5 min. The protein concentration was measured using Coomassie protein assay reagent (Pierce Chemical Co.). Equal amounts of total protein were resolved by 8% precast Tris-glycine gels (Invitrogen). Proteins were blotted onto nitrocellulose membranes and incubated overnight at  $4^\circ\text{C}$  with the rabbit mAb following standard CST protocols. Specific binding was detected with HRP-conjugated species-specific secondary antibody and visualized by using LumiGLO development and exposed to X-ray film.

**Fluorescence immunocytochemistry.** Xenografts (H1975, H3255, H1650, and HCC827) or cell pellets (Kyse70 and Kyse450) were fixed overnight in 10% formalin (Richard Allen Scientific), dehydrated in a graded series of ethanol concentrations, and embedded in paraffin. Paraffin sections (4  $\mu\text{m}$ ) were deparaffinized in xylene and rehydrated in an ethanol series. Antigen retrieval was done by incubating sections in 1 mmol/L EDTA for 10 min at  $95^\circ\text{C}$ . All subsequent incubations were done on slides lying flat in a humid chamber. Sections were rinsed in PBS and blocked in 5% normal goat serum (Sigma-Aldrich) in PBS containing 0.3% Triton X-100 (Mallinckrodt Baker) for 1 h at room temperature. The blocking solution was aspirated and sections were incubated overnight at  $4^\circ\text{C}$  in primary antibodies diluted in PBS with 0.3% Triton and 1% bovine serum albumin (American Bioanalytical). Sections were rinsed in PBS (3  $\times$  5 min) and then incubated for 1 h at room temperature in AlexaFluor 488-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen) diluted 1:1,000 in PBS with 0.3% Triton and 1% bovine serum albumin. Sections were rinsed in PBS (3  $\times$  5 min), incubated in DRAQ5 DNA stain (Biostatus Ltd.), diluted 1:1,000 in PBS for 5 min, rinsed briefly in PBS, and coverslipped with Prolong Gold antifade mounting medium (Invitrogen). Sections were imaged on a Nikon C1 confocal microscope.

**Xenografts, cell pellets, and human NSCLC tumor tissues.** H3255, H1975, H1650, and HCC827 cells were inoculated s.c. in the right thigh of nude mice ( $1 \times 10^7$  cells per mouse) and grown until a tumor diameter of  $\sim 10$  mm was reached. Kyse70 and Kyse450 cells were formalin fixed and paraffin embedded for immunohistochemistry analysis.

Institutional Review Board approval was granted by the Second Xiangya Hospital, Central South University (Changsha, Hunan, P.R.China). Human samples of NSCLC paraffin blocks were provided by the Second Xiangya Hospital. The tissues were examined with H&E to confirm histopathologic diagnosis and for further analysis. Immunohistochemistry with an antibody to total EGFR was used to screen for EGFR-positive samples for molecular studies.

**DNA extraction and EGFR sequencing.** H&E-stained sections of formalin-fixed paraffin-embedded tissue were reviewed to identify regions of tissue composed of at least 50% tumor cells. Cases where tumor cells composed less than 50% of the total tissue, or where the amount of tumor tissue was too small, were excluded from immunohistochemistry study of EGFR genotyped NSCLC patient samples. Genomic DNA was isolated using the FormaPure kit (Agencourt) according to the manufacturer's instructions.

Exon sequences for EGFR (kinase domain) were amplified with specific primers by a nested PCR. Molecularly pretyped samples were

selected based on results from direct DNA sequencing of *EGFR* (exon 19 and exon 21). Direct DNA sequencing results from some samples were further validated by MS-based DNA analysis (Sequenom).

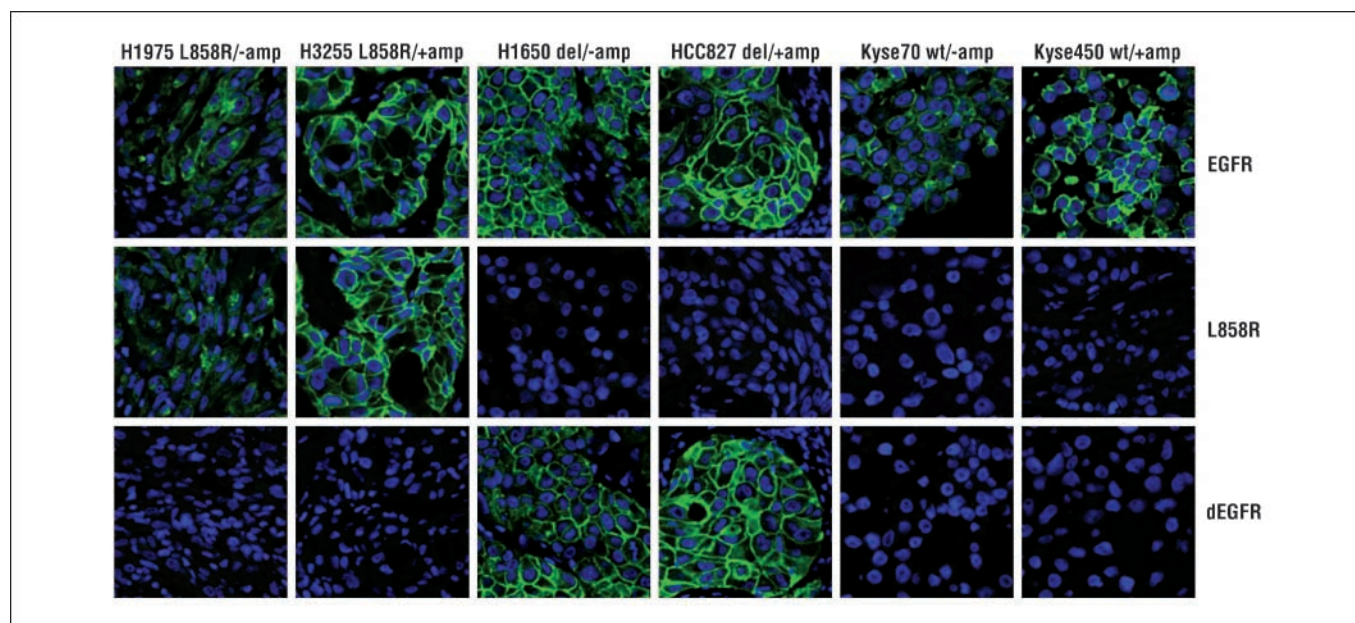
**Immunohistochemistry.** All analyses were done on formalin-fixed, paraffin-embedded blocks. Serial 4- $\mu$ m-thick tissue sections were cut from tissue microarrays for immunohistochemistry study. The slides were baked at 55°C overnight, then deparaffinized in xylene and rehydrated through a graded series of ethanol concentrations. Antigen retrieval (microwave boiling for 10 min in 1 mmol/L EDTA) was done. Intrinsic peroxidase activity was blocked by 3% hydrogen peroxide for 10 min. Goat serum (5%; Sigma) solution was used for blocking nonspecific antibody binding, and the optimally diluted primary antibodies were applied to cover the specimen. Slides were incubated at 4°C overnight. After three washes in TBS-T for 5 min each, slides were incubated for 30 min with labeled polymer-HRP antirabbit secondary antibody at room temperature. Following three additional washes in TBS-T, slides were visualized using substrate-chromagen (Envision + kit, Dako). Sections were scanned at low magnification. The intensity of the staining as well as the percentage of positive cells was recorded. Staining intensity was scored from 0 to 3+; the intensity score was established as follows: 0 if tumor cells had complete absence of staining or faint staining intensity in <10%; 1+ if >10% of tumor cells had faint staining; 2+ if tumor cells had moderate staining; and 3+ if tumor cells had strong staining. Tumors with 1+, 2+, and 3+ expression were interpreted as positive for dEGFR or L858R EGFR antibodies expression, and tumors with no expression (0 score) were interpreted as negative. The distribution of staining, membrane or cytoplasm, was also recorded and assessed at high magnification. For the scores of the images, see Supplementary Table S1.

## Results

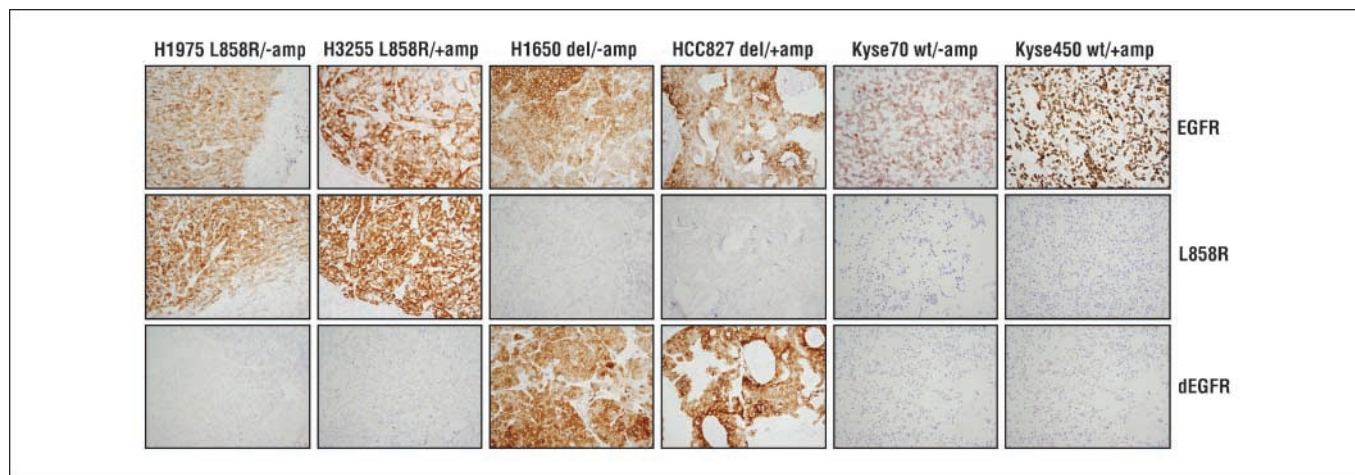
**Development of mutation-specific EGFR antibodies.** Clonal cell lines expressing rabbit IgG were derived from rabbits injected with mutation-specific antigens and screened by Western blot analysis using a panel of six human cancer cell lines expressing either wild-type (wt) *EGFR*, with or without

amplification, or *EGFR* mutation E746\_A750del or L858R. As shown in Supplementary Fig. S1, one rabbit mAb (clone 43B2) was found to be highly specific for the point mutation, detecting the L858R mutation in H3255 cells (*EGFR* amplification with L858R point mutation) and H1975 cells (*EGFR* with L858R point mutation) but not reacting significantly with the deletion mutation, whereas another rabbit mAb (clone 6B6) detects *EGFR* (E746\_A750del) in H1650 and HCC827 cells (*EGFR* amplification with E746\_A750del). On the other hand, these two mutation-specific antibodies do not react with *EGFR* in two human esophageal squamous cell carcinoma cell lines (Kyse450 and Kyse70) that contain wt sequences for exons 19 and 21 of *EGFR*. As expected, a control *EGFR* rabbit mAb (clone 86) reacted with *EGFR* in all cases (Supplementary Fig. S1).

**Antibody characterization with xenografts and cell pellets.** Next, we performed immunofluorescence (Fig. 1) and immunohistochemical analyses (Fig. 2) using the L858R, dEGFR, and control *EGFR* antibodies on xenografts of H3255, H1975, H1650, and HCC827 cell lines. Cell pellets of Kyse70 and Kyse450 cells were used as controls for both immunofluorescence and immunohistochemistry analyses. Immunofluorescence staining of cells and xenograft tissues with the control *EGFR* antibody resulted in labeling of all tumor-derived cells (Fig. 1, top row). The fluorescent stain was localized to the plasma membrane and cytoplasm. The fluorescence intensity was proportional to *EGFR* expression; that is, cells with amplified expression (+amp) had a brighter signal than those with lower expression levels (-amp). Staining with mutation-specific antibodies was only seen in cancer cells and not in normal tissue, and its localization correlated with control *EGFR* antibody staining. The L858R antibody labeled only the cells expressing L858R mutation (H3255 and H1975; middle row). No signal was detected in cells harboring the *EGFR* deletion mutation (HCC827 and H1650). Similarly, the deletion-specific antibody



**Fig. 1.** Immunofluorescence staining of xenografts. Control *EGFR* antibody stains all six cell lines regardless of *EGFR* mutational status (top). L858R-specific antibody stains only the cancer cells with L858R point mutation (H1975 and H3255; middle). dEGFR-specific antibody stains only the cancer cells with E746A750 mutant *EGFR* (H1650 and HCC827; bottom). Abbreviations: del, *EGFR* exon 19 deletion (E746\_A750); +/- amp, *EGFR* gene amplification status; *EGFR*, *EGFR* antibody; L858R, L858R mutation specific antibody; dEGFR, *EGFR* exon 19 deletion (E746\_A750) specific antibody.



**Fig. 2.** Immunohistochemical staining of xenografts. Control EGFR antibody stains all six cell lines (*top*). L858R-specific antibody stains only the cancer cells with the L858R point mutation (H1975 and H3255; *middle*). dEGFR-specific antibody stains only the cancer cells with the exon 19 deletion (H1650 and HCC827; *bottom*).

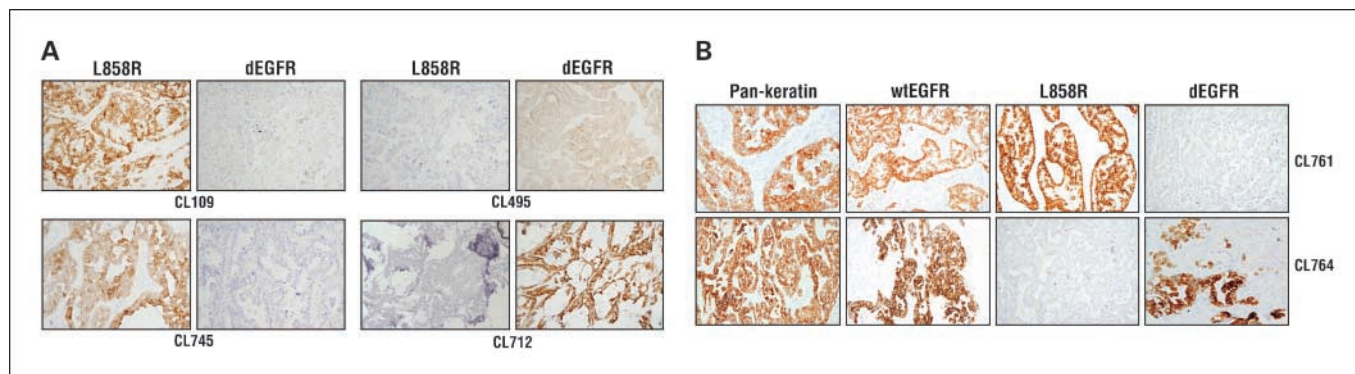
only labeled the cell lines expressing EGFR deletion (HCC827 and H1650; *bottom row*) but not the L858R mutant cell lines (H3255 and H1975). As expected, Kyse450 and Kyse70 cell lines were negative with both mutation-specific antibodies.

In the immunohistochemical analysis, for all the antibodies, we used a scale of intensity from 1 to 3 in describing the increase in protein expression (Fig. 2). Total EGFR staining was positive in all the samples. L858R staining was detected in either H3255 or H1975 xenografts, with higher intensity in H3255 xenograft where the high L858R EGFR expression is due to *EGFR* gene amplification, whereas the staining was not detected in xenografts lacking L858R mutation. In a similar way, dEGFR staining was only observed in HCC827 and H1650 xenografts, and the intensity was higher in HCC827 cells bearing *EGFR* amplification.

**Immunohistochemistry on EGFR genotyped NSCLC patient samples.** We next tested the two mutation-specific antibodies on a panel of 40 paraffin-embedded human NSCLC tissues with known EGFR mutational status by sequencing (Fig. 3A): 20 cases with wt EGFR, 10 cases with L858R, and 10 cases with E746\_A750 deletion. Tumor samples expressing EGFR E746\_A750del or L858R point mutation were positive with control EGFR antibody

as well as with the respective mutant EGFR antibody. None of the wt-EGFR cases were stained by either mutation-specific EGFR antibody. Thus, we observed a 100% correlation between immunohistochemistry data and EGFR mutational status data among these 40 tumor samples (results not shown).

**Immunohistochemistry on NSCLC tumors of unknown genotype.** Paraffin-embedded tumor specimens from 340 patients with primary NSCLC were screened for the presence of EGFR deletion and EGFR L858R point mutation by immunohistochemistry with a panel of four antibodies. The panel included the two EGFR mutation-specific antibodies, a control EGFR antibody, and a pan-cytokeratin antibody to verify the tissue quality of the paraffin blocks (Fig. 3B). Of 340 NSCLC samples, 24 (7.1%) cases were scored positive with E746\_A750 deletion antibody and 28 (8.2%) cases were scored positive with L858R antibody using the scoring criteria previously described (Supplementary Table S1). Therefore, 52 (15.3%) patients were positive with both EGFR mutation-specific antibodies (Table 1). We observed moderate to strong staining with the control EGFR antibody in 84.6% of the mutant-EGFR-positive cases. To confirm the immunohistochemistry results, we performed direct DNA sequence analysis of the *EGFR* gene



**Fig. 3.** Immunohistochemical staining of NSCLC tumor samples. *A*, pre-genotyped NSCLC patient samples were stained with L858R- and dEGFR-specific rabbit mAbs by immunohistochemistry. CL109 and CL745 with the L858R point mutation stained positive with the L858R antibody but negative with the dEGFR antibody. CL495 and CL712 with the E746A750 deletion stained positive with the dEGFR antibody but negative with the L858R antibody. *B*, NSCLC patient samples with unknown genotype were stained with a panel of four antibodies: pan-cytokeratin, control EGFR, L858R, and dEGFR antibodies. Tumor CL761 showed positive staining for the pan-cytokeratin, control EGFR, and L858R antibodies, but negative staining with the dEGFR antibody. In contrast, tumor CL764 stained positive for the pan-cytokeratin, control EGFR, and dEGFR antibodies but negative with the L858R antibody. DNA sequence analysis confirmed the presence of the L858R mutation in CL761 and the E746A750 deletion in CL764.

**Table 1.** Immunohistochemistry and direct DNA sequencing analysis of NSCLC tumor samples with unknown genotype**(A) IHC results of tumor samples with unknown genotype**

Pathology	n	L858R (+)	dEGFR (+)
AC	217	28	23
SCC	112	0	1
LCC	11	0	0
Total	340	28	24

**(B) Direct DNA sequencing results of tumor samples with unknown genotype**

Pathology	n	L858R (+)	dEGFR (+)	Wt	Failed	
					L858R	dEGFR
AC	217	29	23	143	25	22
SCC	19	0	1	17	1	1
LCC	8	0	0	7	1	1
Total	244	29	24	167	27	24

Abbreviations: IHC, immunohistochemistry; AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma.

(exon 19 and 21) on tumor specimens from 244 patients, including all adenocarcinoma samples and a small number of the squamous and large cell carcinoma samples (Table 1B). In addition, all samples positive with the control EGFR antibody but showing a discrepancy between immunohistochemistry and direct DNA sequencing results were genotyped on the Sequenom MS-based system. This technology has been reported to have higher accuracy than direct DNA sequencing in the genotyping of low-quality DNA obtained from formalin-fixed paraffin-embedded tissues (11). We found a higher correlation between the Sequenom and immunohistochemistry results than between direct DNA sequencing and immunohistochemistry (Table 2), suggesting that EGFR mutation-specific immunohistochemistry might be more accurate than EGFR direct DNA sequencing. Overall, the detection of these two EGFR mutations by immunohistochemistry was confirmed in 47 of 52 cases by either direct DNA sequencing or Sequenom analysis. Overall, the sensitivity of the immunohistochemistry assay using mutation-specific antibodies is 92%, with a specificity of 99%. DNA sequence analysis identified an additional five cases containing EGFR mutations that were negative for immunohistochemistry by EGFR mutant-specific antibodies (Table 3). However, these samples were negative for immunohistochemistry by either control EGFR or pan-cytokeratin staining, suggesting that the quality of these samples was too poor for immunohistochemistry. This suggests that PCR amplification and DNA sequencing may improve mutation detection for cases involving poorly preserved tissue.

## Discussion

Selective administration of EGFR inhibitor gefitinib as first-line therapy to advanced NSCLC patients carrying EGFR-activating mutations has shown very favorable clinical outcomes (12), emphasizing the importance of identifying patients carrying EGFR mutations. To address this need, we generated two mutation-specific rabbit mAbs able to specifically detect the

**Table 2.** MS sequencing results from the nine tumor samples that showed a mismatch between immunohistochemical staining and direct DNA sequencing**(A) EGFR mutant status from IHC, direct DNA sequencing, and MS sequencing**

No.	IHC	Direct seq	MS
Exon 19 deletions			
CL182	Wt	Del	Wt
CL193	Wt	Wt	Wt
CL472	Wt	Failed	Wt
CL508	Del	Wt	Del
CL720	Del	Wt	Del
CL736	Wt	Del (E746_A750)	Del
CL742	Wt	Del	Wt
CL761	Wt	Wt	Wt
CL781	Wt	Wt	Del
L858R mutations			
CL182	L858R	T847A	Failed
CL193	Wt	L858R	Wt
CL472	L858R	Wt	Failed
CL508	Wt	Failed	Wt
CL720	Wt	L858R	Wt
CL736	Wt	Wt	Wt
CL742	Wt	Wt	Wt
CL761	L858R	Wt	Failed
CL781	Wt	L858R	Wt

**(B) Correlation of MS sequencing to detect EGFR mutation with IHC and direct DNA sequencing**

Correlative no.	Exon 19 del	L858R	%Correlation
IHC/MS seq	7/9	6/6 (3 MS seq failed)	87
Direct seq/MS seq	4/9	2/6 (3 MS seq failed)	40

Abbreviations: seq, sequencing; MS, mass spectrometry; L858R, EGFR L858 mutation; dEGFR, EGFR exon 19 deletion (E746\_A750).

**Table 3.** Summary of immunohistochemistry and DNA sequencing results

<b>(A) L858R mutation</b>			
<b>IHC</b>	<b>DNA sequencing</b>		
	<b>L858R</b>	<b>Wt</b>	<b>Failed</b>
L858R (+)	24	2	2
L858R (-)	2	193	25

<b>(B) Exon 19 deletion</b>			
<b>IHC</b>	<b>DNA sequencing</b>		
	<b>dEGFR</b>	<b>Wt</b>	<b>Failed</b>
dEGFR (+)	23	0	1
dEGFR (-)	3	196	23

two most common EGFR mutations in NSCLC. We showed that these antibodies are highly sensitive and specific and can be used in a conventional immunohistochemistry assay to identify the presence of cells carrying EGFR mutations.

These antibodies should prove valuable clinical tools to clarify questions that have been difficult to address by other means. In fact, because immunohistochemistry technique preserves tissue morphology, the EGFR mutation-specific antibodies should be useful in determining the stage at which mutations arise during tumor progression, as well as in verifying the presence of "field effects" or the presence of tumor-surrounding normal tissue bearing mutant EGFR (13).

Mutation-specific antibodies offer an alternative assay to detect NSCLC patients potentially responsive to EGFR kinase inhibitors. In contrast to the currently used DNA sequencing approach, the interpretation of immunohistochemistry results depends on the intensity of staining of individual cancer cells rather than on results obtained from a whole tissue lysate. As a result, mutations in tumor samples that have a low percentage of EGFR-mutant cancer cells are often missed by DNA sequencing but can be detected by immunohistochemistry with mutation-specific antibodies. This is important because small biopsy samples, which often do not provide enough high quality DNA

for sequencing, are routinely analyzed by immunohistochemistry. In addition, the spatial distribution of mutant cells (e.g., confined to the growing edge of the tumor, associated with *in situ* lesions, colocalization with cancer stem cell markers, etc.) can be missed when sequencing techniques are used. Thus, an immunohistochemistry assay with mutation-specific antibodies is a simple, rapid, sensitive, and reliable assay to identify the presence of the two most common EGFR mutations in NSCLC tissue samples.

The EGFR deletion specific antibody was generated against the common 15-bp deletion in exon 19 of EGFR and this is the most common deletion from exon 19. It represents more than 90% of all exon 19 deletions in our tumor specimens. However, rare exon 19 deletions of sizes 9, 12, 18, or 24 bp have been identified, each producing a slightly different epitope. Our DNA sequencing analysis identified only two of these rare deletions. One case (E746\_T751del) stained positive and another case (L747\_A750) was negative, indicating that the antibody detected the most common 15-bp deletion in exon 19 of EGFR and some but not all rare exon 19 deletions. A first-pass screening of NSCLC patients with these antibodies would rapidly identify 90% of patients responsive to EGFR inhibitors; to detect the remaining 10% will require the development of additional mutation-specific protein or DNA-based assays. In addition, tumors from other types of cancer have shown some responsiveness to EGFR inhibitors (14, 15), and low frequency of EGFR mutations (E746\_A750del and L858R) has been reported in esophageal, pancreatic, and ovarian cancers (15, 16). Immunohistochemistry with EGFR mutation-specific antibodies will be a more accessible assay than DNA sequencing to rapidly screen for EGFR mutations in these cancers.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### References

- Lynch TJ, Bell DW, Sordella R, 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;350:2129–39.
- Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–500.
- Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* 2004;101:13306–11.
- Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res* 2004;64:8919–23.
- Riely GJ, Politi KA, Miller VA, Pao W. Update on epidermal growth factor receptor mutations in non-small cell lung cancer. *Clin Cancer Res* 2006;12:7232–41.
- Inoue A, Suzuki T, Fukuhara T, et al. Prospective phase II study of gefitinib for chemotherapy-naïve patients with advanced non-small-cell lung cancer with epidermal growth factor receptor gene mutations. *J Clin Oncol* 2006;24:3340–6.
- Marchetti A, Martella C, Felicioni L, et al. EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol* 2005;23:857–65.
- Mitsudomi T, Kosaka T, Endoh H, et al. Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. *J Clin Oncol* 2005;23:2513–20.
- Ladanyi M, Pao W. Lung adenocarcinoma: guiding EGFR-targeted therapy and beyond. *Mod Pathol* 2008;21 Suppl 2:S16–22.
- Li AR, Chitale D, Riely GJ, et al. EGFR mutations in lung adenocarcinomas: clinical testing experience and relationship to EGFR gene copy number and immunohistochemical expression. *J Mol Diagn* 2008;10:242–8.
- Jaremko M, Justenhoven C, Abraham BK, et al. MALDI-TOF MS and TaqMan assisted SNP genotyping of DNA isolated from formalin-fixed and paraffin-embedded tissues (FFPET). *Hum Mutat* 2005;25:232–8.
- Sequist LV, Martins RG, Spigel D, et al. First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic EGFR mutations. *J Clin Oncol* 2008;26:2442–9.
- Tang X, Shigematsu H, Bekele BN, et al. EGFR tyrosine kinase domain mutations are detected in histologically normal respiratory epithelium in lung cancer patients. *Cancer Res* 2005;65:7568–72.
- Cohen EE, Kane MA, List MA, et al. Phase II trial of gefitinib 250 mg daily in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck. *Clin Cancer Res* 2005;11:8418–24.
- Schilder RJ, Sill MW, Chen X, et al. Phase II study of gefitinib in patients with relapsed or persistent ovarian or primary peritoneal carcinoma and evaluation of epidermal growth factor receptor mutations and immunohistochemical expression: a Gynecologic Oncology Group Study. *Clin Cancer Res* 2005;11:5539–48.
- Kwak EL, Jankowski J, Thayer SP, et al. Epidermal growth factor receptor kinase domain mutations in esophageal and pancreatic adenocarcinomas. *Clin Cancer Res* 2006;12:4283–7.

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