Assessment of *EGFR* Mutation Status in Lung Adenocarcinoma by Immunohistochemistry Using Antibodies Specific to the Two Major Forms of Mutant *EGFR*

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EGFR mutations are the best predictors of response to EGFR kinase inhibitors in lung adenocarcinoma. We evaluated two mutation-specific monoclonal antibodies for the detection of EGFR mutations by immunohistochemistry (IHC), generated respectively against the L858R mutant and the exon 19 mutant with the common 15bp/5AA deletion. These two mutations account for approximately 90% of all EGFR mutations. IHC staining performed on 218 paraffin-embedded lung adenocarcinomas was assessed on a 0 to 3+ scale, and positivity cutoffs of 1+ and 2+ were compared. All cases were studied by standard molecular methods for these two mutations, and selected cases were also studied using higher sensitivity molecular assays. The EGFR L858R mutant antibody showed a sensitivity of 95% and a positive predictive value (PPV) of 99% with a positivity cutoff of 1+ and a sensitivity of 76% and a PPV of 100% with a positivity cutoff of 2+. The EGFR exon 19 mutant-specific antibody showed reduced sensitivity for exon 19 deletions other than 15bp. A positivity cutoff of 1+ resulted in a sensitivity of 85% and a PPV of 99%, whereas a 2+ cutoff gave a sensitivity of 67% and a PPV of 100%. IHC with EGFR mutant-specific antibodies could be used as a screen to identify most candidates for EGFR inhibitors. (*J Mol Diagn* 2010, 12:169–176; DOI: 10.2353/jmoldx.2010.090140)

Somatic mutations within the tyrosine kinase domain of EGFR are found in approximately 20% of lung adenocarcinomas and are the most reliable predictors of response to EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib (Sharma et al, 2007).¹ Multiple studies support that, in addition to their predictive value in treatment selection, EGFR mutations are also prognostic for survival benefit.^{2,3} Specifically, patients with these tumors survive significantly longer on EGFR TKIs than with conventional cytotoxic chemotherapy.⁴ EGFR-mutant lung adenocar-

cinomas also form a distinct clinically favorable biological subset, regardless of EGFR TKI therapy.² Mutated EGFR is more often found in better differentiated adenocarcinomas with or without a bronchioloalveolar component.^{5,6} It is virtually absent in other lung cancer subtypes except for adenosquamous carcinoma.^{7,8}

In-frame deletions in exon 19 and the exon 21 L858R substitution are the most common EGFR mutations and, combined, represent approximately 90% of all mutants.⁹ Analysis for common EGFR mutations is now performed in many institutions to help direct treatment decisions. Direct DNA sequencing is a common detection method but has well-known sensitivity limitations depending on the proportion of tumor cells present in the material available for DNA extraction. Other DNA-based methods have been developed to address issues of sensitivity and turnaround time associated with direct sequencing.¹⁰ However, the cost and complexity of molecular methods has slowed their widespread implementation outside of major academic centers and commercial laboratories and drives the continued interest in less robust predictors of response such as EGFR copy number and conventional immunohistochemistry (IHC) for total EGFR. IHC for total EGFR is an especially poor substitute as it correlates poorly or not at all with the presence of mutations.^{11,12}

Another more challenging IHC strategy is to develop antibodies that react only with the mutant form of a given oncoprotein. Interest in this approach is driven by the fact that IHC is a technology available to essentially all pathology departments, can be automated, and can be performed on samples where the number or proportion of tumor cells poses challenges for molecular tests based on bulk DNA extraction from tissue. Cell Signaling Technology has recently developed two mutant-specific antibodies for IHC directed against the most common mutant forms of EGFR: the 15-bp/5-aa deletion (E746_A750del) in

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Table 1. Primers Listed by Assay

Fragment analysis	
A1	EGFR-Ex19-FW1: 5'-GCACCATCTCACAATTGCCAGTTA-3'
A2	EGFR-Ex19-REV1: 5'-Fam-AAAAGGTGGGCTGAGGTTCA-3'
B1	EGFR-Ex 19-FW2: 5'-ATCCAGAAGGTGAGAAAGATAAAATTC-3'
B2	EGFR-Ex 19-REV2: 5'-CCTGAGGTTTCAGAGCCATGGA-3'
B3	EGFR-Ex 19-REV3: 5'-Fam-CCTGAGGTTTCAGAGCCATGGA-3'
C1	EGFR-Ex 19-FW4: 5'-CCCAGCAATATCAGCCTTAGGTG-3'
C2	EGFR-Ex 19-REV4: 5'-CCACTAGAGCTGGAAAGGGAAGA-3'
D1	EGFR-Ex21-FW1: 5'-CCTCACAGCAGGGTCTTCTCTGT-3'
D2	EGFR-Ex21-REV1: 5'-Fam-TCAGGAAAATGCTGGCTGACCTA-3'
Sequenom Mass Spectrometry assay	
E1	2573-FW 5'-ACGTTGGATGACGTACTGGTGAAAACACCG-3'
E2	2573- REV: 5'-ACGTTGGATGTCTTCTCTTCCGCACCCAG-3'
E3	2573 extension primer-REV: 5'-CACCCAGCAGTTTGGCC-3'
Mass of extension primer: 5131.3 Da	
Mass of extension product Call T: 5402.6 Da	
Call G: 5378.5 Da	

exon 19 and the L858R point mutation in exon 21.¹³ In the present study, we performed an independent evaluation of these two antibodies on a large series of lung adenocarcinomas with molecular data available for *EGFR* mutation status. We provide a careful assessment of putative false-positive and false-negative results, including a detailed analysis of how they relate to the molecular heterogeneity in *EGFR* exon 19 deletions and we propose an algorithm for their possible clinical implementation.

Materials and Methods

Tumor Samples

Two hundred eighteen lung adenocarcinoma samples, procured at Memorial Sloan-Kettering Cancer Center under IRB-approved protocols, between the years 1999 and 2008 were used for this study. The vast majority of cases were classified as adenocarcinoma, mixed subtype. A total of 194 formalin-fixed paraffin-embedded

(FFPE) lung adenocarcinoma samples with available *EGFR* molecular data were selected for tissue microarray (TMA) construction. These included 18 *EGFR* L858R mutants, 31 cases with *EGFR* exon 19 deletions (deletion sizes: 9 bp [$n = 4$], 12 bp [$n = 1$], 15 bp [$n = 20$], 18 bp [$n = 3$], 24 bp [$n = 3$]), and 145 cases without either *EGFR* mutation. The TMAs were constructed using triplicate 0.6-mm tissue cores. Three cores from different areas were selected from each tumor. Serial 4- μ m-thick tissue sections were freshly cut from the TMAs for IHC. To more thoroughly evaluate the mutation-specific IHC on tumors bearing a variety of exon 19 deletions, 24 tumors harboring less common exon 19 deletions (9, 12, 18, 24 bp) were identified, and unstained slides were prepared using 4- μ m-thick tissue sections cut directly from the FFPE tumor blocks.

DNA Extraction

Hematoxylin and eosin-stained sections of FFPE tissue were reviewed for each sample to identify areas of tumor.

Table 2. Main Types of *EGFR* Exon 19 Deletions and Corresponding Amino Acid Sequences

Deletion size	AA mutation	AA sequence	COSMIC ($n = 1531$)
Del-9	No mutation	IKELREATSPKANKEIL	
	L747_E749del	IK. . .EATSPKANKEIL	6.4%
	K745_L747del	I. . .REATSPKANKEIL	0.2%
Del-12	E746_E749del	IK. . .ATSPKANKEIL	0.2%
Del-15	E746_A750del*	IK. . . .TSPKANKEIL	68.1%
	L747_T751del	IKE. . . .SPKANKEIL	6.4%
	K745_E749del	I. . . .ATSPKANKEIL	0.9%
Del-18	A750_K754del	IKELRE. . . .ANKEIL	0.12%
	E746_T751del	IK.SPKANKEIL	0.7%
	L747_S752del	IKE.PKANKEIL	2.4%
	E746_S752>X [†]	IK.XPKANKEIL	3.8%
	L747_P753del	IKE.KANKEIL	0.4%
	L747_P753>Q	IKE.QKANKEIL	1.1%
Del-24	L747_P753>S	IKE.SKANKEIL	6.7%
	E746_P753del	IK.KANKEIL	0.4%
	L747_K754del	IKE.ANKEIL	0.12%
	A750_K757del	IKELRE.EIL	0.12%
	T751_E758del	IKELREA.IL	0.06%
	S752_I759del	IKELREAT.L	0.7%

The proportions among the 1531 Exon 19 deletion cases collected in COSMIC are indicated.

*15-bp deletion mutant used to generate the antibody.

[†]Complex deletion encoding novel amino acid (X), which varies between samples.

Table 3. Prevalence of the Main *EGFR* Exon 19 Deletion Sizes in the COSMIC Database (among 1531 Cases with Exon 19 Deletion or Complex-Deletion), in our TMA (among the 31 Cases with Exon 19 Deletion), and in our Clinical Data at MSKCC during the Year 2008 (among the 107 Cases with Exon 19 Deletion)

Size of <i>EGFR</i> exon 19 deletion	COSMIC*	MSKCC TMA	MSKCC clinical data
9 bp	6.6%	12.9%	2.8%
12 bp	0.2%	3.2%	2%
15 bp	75.5%	64.5%	65%
18 bp	15.2%	9.7%	29%
24 bp	1.4%	9.7%	1%

*Total = 98.9%. Some cases with very rare deletion sizes are not included.

Macrodissection was performed on corresponding unstained sections to ensure greater than 50% tumor volume for each case. Genomic DNA was extracted using the QIAamp MiniKit kit (Qiagen) according to the manufacturer's protocol.

Fragment Analysis for *EGFR* Exon 19 Deletion

Detection of the small in-frame deletions in exon 19 of *EGFR* was performed by fragment analysis of fluorescently labeled PCR products as previously described.¹⁴ Briefly, a 207-bp genomic DNA fragment encompassing the entire exon 19 was amplified using the primers A1 and A2 (Table 1). PCR products were subjected to capillary electrophoresis on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). This assay can detect an *EGFR* exon 19 deletion in as little as approximately 5% to 10% tumor cells in a given sample.¹⁴

Mutant-Enriched PCR Assay for *EGFR* Exon 19 Deletion

Cases showing discordant results between mutation-specific IHC and the standard *EGFR* exon 19 mutational assay described above were further studied using a more sensitive mutant-enriched PCR assay.¹⁵ A 138-bp genomic fragment of exon 19 was amplified with the primers B1 and B2 (Table 1) for 17 cycles at 60°C. After purification (QIAquick Purification kit, Qiagen), PCR products were subjected to restriction enzyme digestion by the *MseI* enzyme (New England BioLabs) for 4 hours at 37°C. On an *EGFR* allele with an exon 19 deletion, the restriction site would be absent yielding an undigested PCR product. During a second round of amplification

(primers B1 and B3, 40 cycles at 60°C) only the mutant and the undigested wild-type fragments are amplified. The PCR products were analyzed by fragment analysis on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). This assay has been reported to detect an *EGFR* exon 19 deletion at a level of 0.1%.¹⁵

Sequencing for *EGFR* Exon 19 Deletion

Twenty cases with 15-bp and 17 cases with 18-bp Exon 19 deletion were also sequenced to characterize the exact structure of the deletion. The PCR used primers C1 and C2 (35 cycles at 55°C). Direct sequencing was performed on these purified PCR products on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA).

EGFR Exon 21 L858R Assay

This mutation was detected by a PCR-restriction fragment length polymorphism assay (PCR-RFLP), based on a *Sau96I* restriction site created by the mutation 2573T>G as previously described.¹⁴ Briefly, a 222-bp genomic fragment encompassing the entire exon 21 was amplified using primers D1 and D2 (Table 1). Digestion of the mutant PCR product with *Sau96I* enzyme (New England BioLabs) generates a shorter 87-bp fragment. The digested fluorescently labeled PCR products were analyzed by capillary electrophoresis on an ABI 3730 Genetic analyzer (Applied Biosystems, Foster City, CA). The detection sensitivity of this assay for the *EGFR* exon 21 L858R mutation is approximately 5% to 10% tumor cells.¹⁴

EGFR Exon 21 L858R Mutation Analysis by Mass Spectrometry

Cases with discordant mutation status between mutation-specific IHC and the *EGFR* L858R PCR-RFLP assay were further studied by mass spectrometry-based DNA analysis (Sequenom Inc, San Diego, CA), as described elsewhere.¹⁶ Briefly, tumor DNA was subject to a first PCR amplification following the recommendations of Sequenom (total volume: 5 μ l, 1.25 $\times$ buffer, 1.625 mmol/L *MgCl*₂, 500 μ mol/L dNTP, 100 nmol/L from each primer, 0.5 U HotStar TaqDNA Polymerase [Qiagen]) using primers E1 and E2 (Table 1). Alkaline Phosphatase treatment was performed in a total volume of 2 μ l (final volume: 7 μ l), incubated at 37°C for 40 minutes and 85°C for 5 minutes. A single allele base extension reaction was performed using the E3 primer (Table 1). Two microliters of a solution containing 0.74 μ l ddH₂O, 0.2 μ l Buffer (Sequenom Inc.), 0.1 μ l Ter-

Table 4. Intensity of the Staining with Antibody to Total *EGFR* versus Mutation Status

Total <i>EGFR</i>	0	1+	2+	3+
Wild-type (<i>n</i> = 145)	86 (59.3%)	13 (8.9%)	25 (17.2%)	21 (14.5%)
L858R + (<i>n</i> = 18)	7 (38.8%)	2 (11.1%)	3 (16.6%)	6 (33.3%)
Exon 19 del + (<i>n</i> = 55)	15 (27.3%)	4 (7.3%)	11 (20%)	25 (45.6%)

P value for 2 $\times$ 2 table defined by staining 0-1+ vs 2-3+ and *EGFR* wild-type vs either mutant = 0.0002 (Fisher exact test).

Figure 1. Immunohistochemistry staining of lung adenocarcinoma samples. **A:** Example of EGFR L858R mutant tumor. Cytokeratin AE1/AE3, total EGFR, and the EGFR L858R mutant-specific antibody show a positive staining, whereas the EGFR Exon 19 deletion mutant-specific antibody is negative. **B:** Conversely, in a tumor harboring the *EGFR* Exon 19 15-bp deletion, Cytokeratin AE1/AE3, Total EGFR, and the Exon 19 mutant-specific antibody show a positive staining whereas the EGFR L858R mutant-specific antibody is negative.

mination Mix (Sequenom Inc.), 0.94 μ l of 7 μ mol/L extension primer, and 0.02 μ l of Thermo Sequenase (Sequenom Inc.) were added to the 7 μ l SAP (Shrimp Alkaline Phosphatase) treated PCR products. Thermal cycling was performed following the recommendations of Sequenom. The detection sensitivity of this Sequenom assay for *EGFR* L858R is estimated to be 5% tumor cells (not shown).

Mutation-Specific Antibodies and Immunohistochemistry

Two rabbit monoclonal antibodies were kindly provided by Cell Signaling, one with specificity for the exon 21 L858R *EGFR* mutation and the other for the 15bp, E746-750 deletion in exon 19.¹³ IHC was done manually using the following protocol: after overnight incubation at 55°C and deparaffinization, antigen retrieval was performed using EDTH pH 9 (Dako) for 30 minutes. Primary antibodies were applied (dilution 1:100 for both antibodies) and the slides incubated for 30 minutes. For detection, the EnVision + kit (Dako) was used following the manufacturer's recommendations. Counterstain was realized using hematoxylin, 4 minutes, and Bluing reagent, 4 minutes.

IHC Scoring

Slides were scanned at low magnification and scored based on cytoplasmic and/or membrane staining intensity as follows: 0 = no staining or faint staining intensity in <10% of tumor cells; 1+ = faint staining in >10% of tumor cells; 2+ = moderate staining; 3+ = strong staining. Triplicate tumor tissue cores from the TMA were scored independently, and a final composite grade was given for each

case. For TMA cases, a case was considered positive if at least 1 core showed 2+ or 3+ staining or at least 2 cores showed 1+ staining. If only 1 of the 3 cores showed 1+ staining, the case was scored as negative.

Control IHC staining for total EGFR protein and Cytokeratin AE1/AE3 were performed using the monoclonal EGFR mouse antibody (Dilution 1:100, Clone 31G7, Zymed Laboratories Inc.) and the anti-cytokeratin cocktail AE1/AE3 (Biogenex) according to the manufacturer's instructions. EGFR results were scored as follows: 0, no membrane staining; 1+, faint, partial membrane staining; 2+, weak, complete membrane staining in >10% of tumor cells; 3+, intense complete membrane staining in >10% of tumor cells. A case was considered positive if at least one core was scored 1, 2, or 3+ (1+ cutoff) or 2 or 3+ (2+ cutoff).

Table 5. Summary of IHC Results on TMA Cases and Mutation Status Based on PCR-Based Fragment Analysis for *EGFR* Exon 19 Deletion

	Exon 19 mutation analysis (PCR-based fragment analysis)		
	Mutation+	Mutation–	Total
A. 15-bp deletion cases only			
IHC+	20	2	22
IHC–	0	161	161
Total	20	163	183
B. Exon 19 deletion cases			
IHC+	23	2	25
IHC–	8	161	169
Total	31	163	194

Sensitivity in A is 100% (20/20). Sensitivity in B is 74.2% (23/31). Specificity is 98.8% (161/163), considering true negatives as cases lacking any type of exon 19 deletion.

Table 6. Staining Intensity with the Exon 19 Deletion Mutant-Specific Antibody versus Exon 19 Deletion Size

	0	1+	2+	3+	Sensitivity
Exon 19 9-bp del (<i>n</i> = 10)	8 (80%)	1 (10%)	0	1 (10%)	20%
Exon 19 12-bp del (<i>n</i> = 3)	1 (33.3%)	0	2 (66.7%)	0	67%
Exon 19 15-bp del (<i>n</i> = 20)	0	2 (10%)	11 (55%)	7 (35%)	100%
Exon 19 18-bp del (<i>n</i> = 17)	7 (41.2%)	6 (35.3%)	2 (11.8%)	2 (11.8%)	58.8%
Exon 19 24-bp del (<i>n</i> = 5)	2 (40%)	1 (20%)	2 (40%)	0	60%
False positive for A746-750 del Ab (<i>n</i> = 2)	—	2 (100%)	—	—	—

Note: study group enriched for non-15-bp exon 19 deletions.

Results

Baseline EGFR Mutation Profile of Test Set

To provide a thorough evaluation of the sensitivity of these two mutant-specific antibodies, the evaluation set was enriched for *EGFR* mutant cases, in particular cases with *EGFR* exon 19 deletions. Thus, *EGFR* mutations were identified in 25.3% (49/194) of the cases present in the TMA and in 33.5% (73/218) of all cases, based on fragment analysis (compared with a prevalence of about 20% among adenocarcinomas tested at our institution). The *EGFR* L858R mutation was detected in 18 samples (18/73, 23.7% of mutant cases), whereas 55 cases had exon 19 deletions (55/73, 75.3% of mutant cases), reflecting enrichment for non-15-bp *EGFR* exon 19 deletions. Deletion sizes were as follows: 9 bp (*n* = 10), 12 bp (*n* = 3), 15 bp (*n* = 20), 18 bp (*n* = 17), 24 bp (*n* = 5). The heterogeneity in the exact structure of these in-frame deletions is shown in Table 2, based in data from the COSMIC database. To obtain unbiased data on the distribution of different types of *EGFR* exon 19 deletions, we also reviewed the cases collected in the COSMIC database and we reviewed our in-house prevalence based on 898 cases consecutively studied during the year 2008 (Table 3). This confirmed that only approximately 65% to 75% of *EGFR* exon 19 deletions are 15 bp.

IHC Results

IHC was performed on the 194 samples on TMAs with a panel of four antibodies that included the two *EGFR*

mutant-specific antibodies, total *EGFR* antibody, and cytokeratin AE1/AE3 antibody as a control for tissue quality. The 24 additional samples with less common exon 19 deletions were analyzed with total-*EGFR* and the exon 19 mutant-specific antibody.

Of the 218 samples, 39 cases (17.9%) were scored positive for the exon 19 *EGFR* mutant-specific antibody and 22 cases (10.1%) were scored positive for the *EGFR* L858R mutant-specific antibody. Total *EGFR* was expressed (1+ to 3+) in 50.5% of all cases (110/218) and 69.7% of mutant cases (51/73). Although it was statistically correlated with the presence of mutation ($P < 0.0002$; Table 4), it is important to note that IHC for total *EGFR* is not usable as a test for mutation status as it would miss >30% of mutant cases and, conversely, most strongly positive cases are not mutated (Table 4). Cytokeratin was expressed in 100% of the samples, confirming the reactivity of the tissues for IHC. Figure 1A shows an example of positive staining for a case with L858R mutation, whereas Figure 1B shows an example of positive staining with the exon 19 mutant-specific antibody in a case with a 15-bp deletion in *EGFR* exon 19. For both mutant-specific antibodies, the staining was cytoplasmic and membranous.

Correlation between Mutation-Specific IHC and Mutation Status

All 20 cases with a 15-bp deletion in exon 19 by fragment analysis were positive by IHC with the mutant-specific antibody. Positive staining with the exon 19 mutant-specific antibody was also observed in two cases without mutations (specificity = 98.8%; Table 5). More sensitive

Table 7. Staining Intensity with the Exon 19 Deletion Mutant-Specific Antibody versus Sequence Results for the 15-bp and 18-bp *EGFR* Exon 19 Deletion Mutants

Deletion size	AA mutation	AA sequence	CDS mutation	n	IHC 0	IHC 1+	IHC 2+	IHC 3+
Del-15	E746_A750del*	IK. TSPKANKEIL	Del 2235-2249	14	0	1	7	6
	E746_A750del*	IK. TSPKANKEIL	Del 2236-2250	4	0	1	3	0
	L747_T751del	IKE. SPKANKEIL	Del 2240-2254	1	0	0	1	0
	NA			1	0	0	0	1
Del-18	L747_S752del	IKE. PKANKEIL	Del 2239-2256	1	0	0	0	1
	E746_S752>V	IK. VPKANKEIL	Del 2237-2255>T (complex)	4	1	2	1	0
	L747_P753>Q	IKE. QKANKEIL	Del 2239-2258>CA (complex)	1	0	0	0	1
	L747_P753>S	IKE. SKANKEIL	Del 2240-2257	10	5	4	1	0
	NA			1	1	0	0	0

*Deletion mutant used for monoclonal antibody production.

Table 8. Summary of IHC and Molecular Assays for the *EGFR* L858R Mutation

	Mutation +	Mutation –	Total
IHC results versus mutation analysis by PCR-RFLP assay only			
IHC+	17	5	22
IHC–	1	171	172
Total	18	176	194
IHC results versus combined results of PCR-RFLP and Sequenom assays			
IHC+	20	2	22
IHC–	1	171	172
Total	21	173	194

Based on the combined results, the sensitivity is 95.2% (20/21) and the specificity is 98.8% (171/173).

mutation assessment with mutant-enriched PCR in these two cases failed to detect mutations (data not shown).

Of 35 cases with exon 19 deletions other than the common 15-bp type, 48.6% stained positive with the mutant-specific antibody with the following distribution: 20% (2/10) of 9-bp deletions, 67% (2/3) of 12-bp deletions, 58.8% (10/17) of 18-bp deletions, and 60% (3/5) of 24-bp deletions (Table 5). Because of the low numbers of cases available for some of these deletion sizes, the percentages must be considered approximate; but it is

apparent that, overall, the sensitivity of this mutant-specific antibody is lower in non-15-bp deletion cases. In contrast to the generally robust staining in cases with 15-bp deletions, in most of these non-15-bp deletion cases, the staining quality was faint and heterogeneous and only three cases (one del-9 and two del-18) had strong and widespread staining (Table 6). To assess whether staining intensity could be related to the exact amino acid sequence of the deletion mutant, we sequenced all available 15-bp and 18-bp deletion cases;

Figure 2. Sequenom assay results for *EGFR* L858R in five cases with positive results by *EGFR* L858R mutant-specific IHC but negative results with the *EGFR* L858R PCR-RFLP assay. The H3255 cell line harboring the *EGFR* L858R mutation is a positive control, whereas the H358 cell line harboring the *KRAS* 34G/T mutation provides a negative control. A G call is visible, the mutant call (G) is visible at 5378.5 Da and is indicated by an **arrow**. The germline call (T) is indicated by an **asterisk**. In tumor samples **A** and **B**, only the T call is present and these cases were considered negative. In tumor samples **C**, **D**, and **E**, a mutant peak is present (**arrow**), confirming the presence of the *EGFR* L858R mutation.

Table 9. Intensity of Staining with the EGFR L858R Mutant-Specific Antibody

	0	1+	2+	3+
L858R (<i>n</i> = 21)	1 (4.8%)	4 (19%)	6 (28.6%)	10 (47.6%)
False positive for L858R Ab (<i>n</i> = 2)	—	2 (100%)	0	0

no simple relationship of the mutant amino acid sequence to the intensity of IHC staining was apparent (Table 7). Notably, 15-bp deletions can generate the common E746-750 deletion or, less commonly, the L747_T751 deletion but both showed robust staining. Finally, to estimate the sensitivity of the exon 19 deletion mutant-specific antibody in routine practice, we applied the resulting deletion type-specific sensitivities to our consecutive 2008 clinical testing data, which represent an unbiased distribution of exon 19 deletion sizes. In the year 2008, 898 adenocarcinomas were analyzed at MSKCC for *EGFR* mutations, and this identified 107 cases with exon 19 deletions, all with size information by our standard fragment analysis-based clinical assay.^{11,14} Based on this modeling, we would project a sensitivity of 84.6% (90.5/107) and a specificity of 98.9% (782.1/791) for this antibody based on the scoring used.

Regarding the EGFR L858R mutant cases, 17 of the 18 cases with an L858R mutation by PCR-RFLP were scored positive with the L858R mutant specific antibody (94%, Table 8). Five cases without the L858R mutation by PCR-RFLP were also scored as positive (Table 8). Mass spectrometry-based genotyping (Sequenom) was performed on these cases, which revealed an L858R mutation in three of the five cases (Figure 2). Based on the combined molecular data from the *EGFR* L858R PCR-RFLP assay and the *EGFR* L858R Sequenom assay, IHC with the EGFR L858R mutant-specific antibody showed a sensitivity of 95.2% and a specificity of 98.8% (Table 8). Both false positive cases presented only faint staining (1+) (Table 9). Therefore, changing the scoring to only categorize 2+ and 3+ cases as positive would raise the positive predictive value of the IHC assay to 100% with a minimal reduction in sensitivity.

Discussion

The diagnostic approach and management of patients with lung adenocarcinoma has changed significantly with the advent of TKIs targeting EGFR and the discovery of activating *EGFR* mutations, which predict sensitivity to these targeted drugs. As the determination of *EGFR* mutation status becomes standard of practice in this patient population, there is considerable interest in simple methods accessible to a wide range of laboratories. To address this need, two new antibodies suitable for IHC have been recently developed against the most common *EGFR* mutations, 15bp exon 19 deletions and the L858R mutation in exon 21.¹³

Our evaluation of these two antibodies confirms that both perform remarkably well for the mutant proteins

against which they were raised, but that the clinical usefulness of one of them is reduced by the occurrence of non-15-bp exon 19 deletions. Specifically, the EGFR exon 19 mutant-specific antibody detects 100% of 15-bp deletions, with a specificity of 98.8%. However, for non-15-bp exon 19 deletion mutants, the sensitivity varied depending on the deletion size, ranging from 20% to 67%. In the original series, Yu et al reported IHC results on only two non-15-bp deletion cases, of which one was positive by IHC.¹³ Importantly, non-15-bp exon 19 deletion mutants account for 35% of exon 19 deletions in both the COSMIC database and in our clinical testing experience. Based on this intrinsic limitation of its sensitivity for exon 19 deletions, a negative result with this IHC assay could not be used to exclude patients from molecular testing. However, based on our specificity of 98.8%, a positive result could obviate the need for confirmatory molecular testing. Indeed, the positive predictive value of 2+ to 3+ staining is 100% in the present study. One caveat to this prospect is that we found strong (3+) staining in only a minority of true positives; the more frequent weak to moderate staining with this antibody may present problems with interpretation in routine practice, causing many cases to be considered equivocal.

In contrast, we found the IHC assay for the L858R mutation to be highly reliable and specific with sensitivity and specificity similar to the initial report.¹³ If a more stringent scoring is used (only 2+ or 3+ staining being considered positive), the positive predictive value is raised to 100% (with a sensitivity of 76%), and thus a positive result could obviate the need for confirmatory

Figure 3. Possible algorithm for the use of EGFR mutation-specific antibodies. Given its high PPV, IHC with EGFR mutant-specific antibodies could be used as a first pass screening method to identify most lung cancer patients eligible for EGFR-targeted therapy, thereby reducing the overall volume of *EGFR* molecular testing by an estimated 10 to 15%.

molecular testing and EGFR TKI therapy could be initiated on the basis on the IHC result alone, whereas a negative or equivocal result would trigger molecular testing for *EGFR* L858R. Given our clinical testing experience in which approximately 10% of lung adenocarcinomas tested contain *EGFR* L858R, this suggests that for 100 adenocarcinomas stained, 7 or 8 would be scored positive (all expected to be true positives), and therefore the molecular testing volume could be reduced by the same proportion (7% to 8%). A similar scenario can be proposed for the *EGFR* exon 19 deletion-specific antibody, reducing the need for testing for that mutation by a similar proportion. Based on these considerations, we present a schema for the possible use of these antibodies (Figure 3), which could reduce the molecular testing volume for *EGFR* mutations by about 15% while allowing faster treatment initiation for approximately 75% of patients with sensitizing *EGFR* mutations. The benefits of a testing algorithm such as this should be balanced with the potential delay caused by introducing another step before molecular testing and with the cost tradeoff of running IHC on all cases to reduce molecular testing by about 15%.

Aside from their potential to eliminate the need for molecular testing in some cases, these antibodies may also be implemented in other ways, for instance to guard against molecular false-negatives attributable to excessive admixed non-neoplastic elements. The IHC assays may also prove useful in settings where molecular testing may not be possible or reliable (ie, specimens with only a few clusters of tumor cells, such as certain cytologies, small biopsies, or metastatic lesions with tiny nests of tumor cells where microdissection would be technically challenging in routine practice). Both mutation-specific antibodies present intriguing possibilities for research applications, such as further exploration of issues of tumor heterogeneity, analysis of morphologically normal tissue adjacent to *EGFR*-mutant tumors, and others.

IHC has well-known advantages as a testing method, but the major drawbacks include, in comparison with molecular assays, generally higher interlaboratory variability in assay performance and higher interobserver variability in assay interpretation. These aspects of IHC lead to a higher risk of false positives than molecular assays, unless staining is scored in a consistent and rigorous fashion. A further consideration is that these IHC antibodies obviously cannot detect other clinically relevant mutations in *EGFR*, such as exon 18 G719 point mutations, or exon 20 mutations, including the T790M resistance mutation. In conclusion, the use of the *EGFR* mutant-specific antibodies for IHC could be incorporated into the routine IHC work-up of lung adenocarcinomas, leading to more rapid initiation of EGFR TKI therapy and a modest reduction in *EGFR* molecular testing volume.

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