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EGFR Mutations in Lung Adenocarcinomas

Clinical Testing Experience and Relationship to EGFR *Gene Copy Number and Immunohistochemical Expression*

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Lung adenocarcinomas responsive to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors possess *EGFR* mutations and often increased *EGFR* copy number. We prospectively studied 334 clinical cases using polymerase chain reaction-based assays to detect deletions within exon 19 and the L858R mutation in exon 21, which together account for approximately 90% of *EGFR* mutations. Seventy-eight (23%) of these tumors had an *EGFR* mutation, with 55 (71%) exon 19 deletions and 23 (29%) exon 21 L858R mutations. We were able to compare mutant and normal *EGFR* alleles and found a preferential amplification of the mutant allele. The association of mutations with *EGFR* amplification (determined by chromogenic *in situ* hybridization) and EGFR expression (determined by immunohistochemistry) was further examined in a subset of 60 tumors. *EGFR* amplification (≥5 *EGFR* signals per nucleus) was seen in 15 of 29 (52%) *EGFR*-mutated tumors but in only five of 31 (6%) non-mutated tumors ($P = 0.006$). EGFR overexpression was strongly associated with amplification but was statistically independent of *EGFR* mutation. Most patients with *EGFR* mutations (17 of 29, 59%) never smoked compared with 13% (four of 31) of patients lacking such mutations ($P = 0.0003$). The association of amplification with smoking status was marginal and was nonexistent with EGFR expression. Thus, these results indicate that *EGFR* amplification, preferentially of the mutant allele, often accompanies *EGFR* mutation, whereas EGFR immunohistochemical staining associates with amplification but cannot predict *EGFR* mutation status. *(J Mol Diagn 2008, 10:242–248; DOI: 10.2353/jmoldx.2008.070178)*

Recent studies have shown that somatic mutations in the *EGFR* TK domain in patients with lung adenocarcinoma are associated with sensitivity to the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib.[1–3] The epidermal growth factor receptor (EGFR, HER-1/ErbB1) is a receptor tyrosine kinase (TK) of the ErbB family, which consists of four closely related receptors: HER-1/ErbB1, HER-2/neu/ErbB2, HER-3/ErbB3, and HER-4/ErbB4. The two most common *EGFR* mutations are short in-frame deletions of exon 19 and a point mutation (CTG to CGG) in exon 21 at nucleotide 2573, which results in substitution of leucine by arginine at codon 858 (L858R). Together, these two mutations account for ~90% of all *EGFR* mutations in non-small cell lung cancer (NSCLC). Screening for these mutations in patients with NSCLC can be used to predict which patients will respond to TKIs. So far, most large series have used direct sequencing to detect these mutations. We developed polymerase chain reaction (PCR)-based clinical diagnostic tests for the two *EGFR* hotspot mutations[4] and report our initial experience with 334 prospective clinical cases. We also report on the associations of *EGFR* mutation, amplification, and protein expression in NSCLC.

Materials and Methods

Tumor Samples

Three hundred thirty-four lung cancer samples, mainly adenocarcinoma, were received in the Memorial Sloan-Kettering Cancer Center Laboratory of Diagnostic Molecular Pathology over a consecutive 12-month period. Tumor DNA was extracted from formalin-fixed paraffin-embedded tissue or frozen fine needle biopsies or frozen surgical resections using standard methods.

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EGFR Mutational Analysis

EGFR Exon 19 Deletion Assay

The assay is based on length analysis of fluorescently labeled PCR products.[4] By its design, it should also detect the much rarer *EGFR* exon 19 insertions.[5] Briefly, a 207-bp genomic fragment including all of exon 19 is amplified, using the following primers: EGFR-Ex19-FWD1, 5′-GCACCATCTCACAATTGCCAGTTA-3′, and EGFR-Ex19-REV1, 5′-Fam-AAAAGGTGGGCCTGAGG-TTCA-3′.[4] PCR products were subjected to capillary electrophoresis on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA). For all cases with exon 19 deletions, the proportion of mutant and normal *EGFR* alleles was determined by comparison of peak heights on the electrophorogram. The ratio between mutant and normal *EGFR* alleles was calculated. As clinical samples always contain admixed non-neoplastic elements, sometimes abundant, any ratio >1.5 was interpreted as evidence of *EGFR* mutant allele amplification in this assay. We have found this ratio to be highly reproducible for a given tumor DNA sample (results not shown).

EGFR Exon 21 L858R Mutation Assay

This mutation is detected by a PCR-restriction fragment length polymorphism assay, based on a Sau96I restriction site created by the mutation (2573T→G).[4] Briefly, a 222-bp genomic fragment including all of exon 21 was amplified using primers EGFR-Ex21-FWD1, 5′-CCTCACAGCAGGG-TCTTCTCTGT-3′, and EGFR-Ex21-REV1, 5′-Fam-TCAGGAAAAT-GCTGGCTGACCTA-3′. If the 2573T→G mutation is present, after digestion, a 173-bp wild-type product and 87-bp mutant PCR product were produced. The digested fluorescently labeled PCR products are analyzed by capillary electrophoresis. These two assays are more sensitive than direct sequencing and can detect mutations in the presence of up to 90% non-neoplastic cells.[4]

Construction of Tissue Microarray

A subset of 60 tumors (29 *EGFR* mutated, 31 *EGFR* non-mutated) was selected for tissue microarray construction based on paraffin block availability for tumors with *EGFR* mutations. The 31 cases without *EGFR* mutation were matched for age, sex, and histology to the 29 *EGFR* mutated cases. The tissue microarray was constructed using triplicate 0.6-mm tissue cores. Three cores from different areas were selected for each tumor.

EGFR Chromogenic in Situ *Hybridization (CISH)*

CISH for *EGFR* was performed according to the manufacturer's instructions (Zymed Laboratories Inc., South San Francisco, CA). Briefly, 4- to 5-μm sections were incubated at 55°C overnight. After deparaffinization in xylene and graded ethanols, heat pretreatment was carried out in the pretreatment buffer (Zymed Laboratories Inc.) at 98–100°C for 15 minutes. The tissue was digested with pepsin for 10 minutes at room temperature. After application of Zymed SpotLight digoxigenin-labeled *EGFR* probe (Zymed Laboratories Inc.), the slide was coverslipped and edges sealed with rubber cement. The slide was heated at 95°C for 5 minutes followed by overnight incubation at 37°C using a moisturized chamber. A posthybridization wash was performed the next day and followed by immunodetection using the CISH polymer detection kit (Zymed laboratories Inc.) and the signal enumerated on a standard light microscope using a 40X objective. Gene copy numbers in 30 tumor cell nuclei were counted for each tissue core, and the average gene copies per nucleus were used as CISH result for that tissue core. The highest CISH score among all cores was used as the final result for that tumor. The results of CISH were interpreted as follows: <5 gene copies per nucleus, no amplification; 5–10 gene copies per nucleus, low-level amplification; and >10 gene copies per nucleus, high-level amplification.[6]

EGFR Immunohistochemistry

Immunohistochemistry (IHC) staining for EGFR was performed using monoclonal EGFR antibody 31G7 (Zymed Laboratories Inc.) 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. Tumors with a score 2+ or 3+ were interpreted as positive for overexpression.[6,7] The highest score obtained among different cores of the same tumor was used as the final EGFR IHC result of that tumor.

Statistical Analysis

Statistical analyses were performed using Fisher's exact test or χ^2 test. Statistical significance was determined by a two-tailed $P < 0.01$.

Results

Clinical Testing Experience in 334 Consecutive Cases

EGFR exon 19 deletions or exon 21 L858R were identified in 23% (78 of 334) of our consecutive lung cancer samples, over 90% of which were pure adenocarcinomas (Figure 1). Among the cases with mutations, 55 (71%) had an exon 19 in-frame deletion [deletion sizes: 9 bp ($n = 3$), 15 bp ($n = 41$), 18 bp ($n = 9$), 24 bp ($n = 2$)] and 23 (29%) had the exon 21 L858R point mutation. One patient had two synchronous primaries with different exon 19 alterations, a 15-bp deletion and an 18-bp insertion. The majority (64%) of the patients with mutations were women. Histologically, 42 (54%) of 78 cases were invasive adenocarcinoma, and 31 (40%) cases were either bronchioloalveolar carcinoma (BAC) or adenocarci-

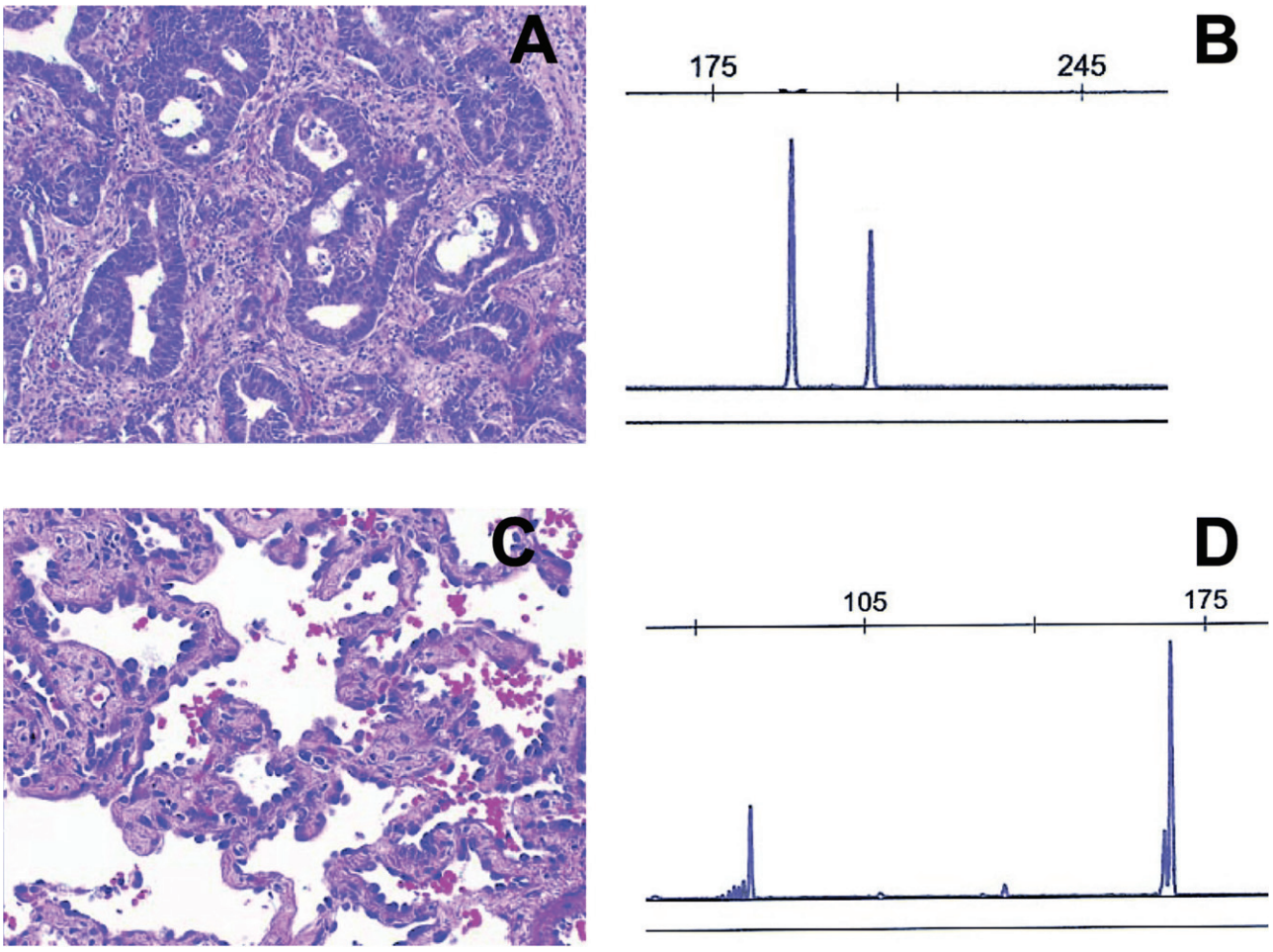

Figure 1. **A** and **B:** Adenocarcinoma with 15-bp *EGFR* exon 19 deletion. The mutant peak is to the left of the 207-bp normal peak. **C** and **D:** BAC with *EGFR* exon 21 L858R mutation. The 87-bp product of this PCR-RFLP assay represents the mutant allele (see Materials and Methods for details).

noma, mixed subtype with BAC (Figure 1). Age, sex, and histology did not differ significantly between patients whose tumors had exon 19 deletions compared to exon 21 L858R mutations.

Operationally, all assays were performed in duplicate (ie, total four PCRs per sample) and all results were concordant in the duplicate reactions. The assays can be completed in 2 days on a rush basis but the turnaround time was generally 7 days because of weekly batching. Most of the specimens were submitted as paraffin-embedded material from surgical resections or, less commonly, from Tru-cut biopsies. Paraffin blocks containing more than 50% tumor were selected by a pathologist for DNA extraction. Ten 5-μ-thick sections were collected as curls in 1.5-ml tubes in each case. If the proportion of the tumor was less than 50%, then the tumor was manually microdissected using a sterile scalpel from 15 unstained sections using a stained section as a guide. In some cases, cell blocks from fine needle aspiration cytology specimens were used (after confirmation of adequacy of tumor content by a cytopathologist). In one case, only air-dried Giemsa-stained fine needle aspiration cytology smears were available. In this case, DNA was extracted from the smear using the PUREGENE kit (Gentra, Minneapolis, MN). Overall, only two samples received during this 12-month period gave no result due to insufficient DNA for PCR amplification. Both were fine needle aspiration cell blocks with inadequate cellularity. All cases received as frozen samples were successfully studied.

Association between EGFR Mutation and EGFR Amplification

In the 55 cases with exon 19 deletions, the relative levels of mutant and wild-type allele were determined by comparing peak heights in the electrophoretogram tracings of the exon 19 deletion assay. These ratios were highly reproducible (not shown) and were greater than, for instance, those typically associated with preferential PCR amplification of the shorter allele at a given polymorphic short tandem repeat locus (ratios generally <1.5). Therefore ratios >1.5 were interpreted as evidence of increased copies of the mutant allele in the tumor cells, given that the mutations are almost always heterozygous and that admixed non-neoplastic cells contain two normal alleles. In 15 of 55 cases (27%) the peak height of the mutant allele was greater than that of the normal *EGFR* allele (Figure 2). The ratios of peak heights ranged from 1.6 to 7.0 (mean, 2.5; median, 2.1). However, this may be

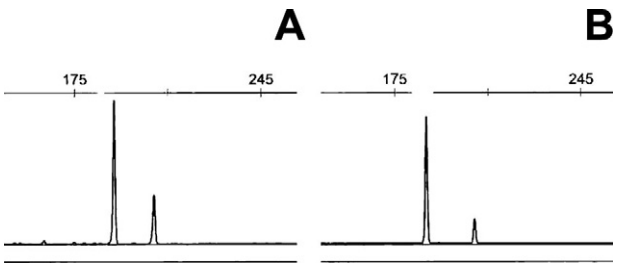

Figure 2. **A** and **B:** Cases with *EGFR* exon 19 deletions (of different sizes) showing three- and fivefold copy number gains of the mutant allele relative to the 207-bp normal allele.

an underestimation of the prevalence of *EGFR* mutant allele copy number gains because of varying degrees of dilution with DNA from admixed non-neoplastic cells.

To further assess the prevalence of *EGFR* amplification, we performed *EGFR* CISH analysis on a tissue microarray containing 29 *EGFR* mutated and 31 *EGFR* non-mutated cases. The clinical characteristics of the NSCLC cases represented on the tissue microarray are listed in Table 1. These cases were matched for age, sex, and histology. Twenty cases were scored as amplified (≥5 signals per nucleus) but only four of 20 showed 7 or more signals (7, 7.2, 8, and 11 signals). Fifty-two percent of tumors with *EGFR* mutations had *EGFR* amplification (15 of 29, 52%) as compared to 16% (five of 31) of non-mutated cases ($P = 0.006$) (Figure 3). There was no significant difference in the frequency of *EGFR* amplification between *EGFR* exon 19 deletion cases and L858R cases: among the 10 cases *EGFR* L858R, seven (70%) were amplified, compared to eight of 19 (42%) cases with *EGFR* exon 19 deletion ($P = 0.25$). The proportion of exon 19 deletion cases amplified by CISH was not significantly higher (eight of 19 versus 15 of 55; $P = 0.26$) than that determined above by the comparison of peak heights in the PCR-based assay. This suggests that most or all of the extra *EGFR* gene copies seen by CISH in cases with exon 19 deletions are gains of the mutant allele.

Association between EGFR Amplification and EGFR Protein Expression

Eighty percent (16 of 20) of *EGFR*-amplified tumors were IHC-positive as compared with 13 of 40 (33%) *EGFR* nonamplified tumors ($P = 0.0008$) (Table 2 and Figure 3). The proportion of *EGFR*-amplified cases increased from 11% to 67% as EGFR IHC staining increased from 0 to 3+ (Table 2). All four *EGFR*-amplified cases showing 7 or more signals per nucleus were also IHC-positive (one 2+, three 3+). Notably, the EGFR IHC results showed no significant association with *EGFR* mutation status (Table 3). These interrelationships are depicted in Figure 3.

Association between EGFR Mutation and Smoking History

Smoking history data were available for all 60 patients represented on the tissue microarray. Most patients (17 of 29, 59%) whose tumors bore *EGFR* mutations were never smokers, but this was the case for only four of 31 (13%) without *EGFR* mutations ($P = 0.0003$). In contrast,

Table 1. Characteristics of 60 Patients Included on Tissue Microarray

	Non-mutated EGFR ($n = 31$, 52%)	Mutated EGFR ($n = 29$, 48%)	*P* value
Age (years)			
Median	70	69	
Mean	68	66	
Range	42–85	38–88	
Sex			
Male	7 (23%)	7 (24%)	
Female	24 (77%)	22 (76%)	1.00
Smoking history			
Former and current	27 (87%)	12 (41%)	
Never	4 (13%)	17 (59%)	0.0003
Stage at presentation			
I	13 (42%)	9 (31%)	
II	5 (16%)	4 (14%)	
III	9 (29%)	13 (45%)	
IV	4 (13%)	3 (10%)	0.43*
Site of tumor			
Primary	31 (100%)		27 (93%)
Metastasis	0 (0%)	2 (7%)	0.23
Histology			
Adenocarcinoma	12 (39%)	12 (41%)	
BAC or adenocarcinoma with BAC feature	18 (58%)	16 (55%)	
Other	1 (3%)	1 (4%)	1.00†
EGFR gene amplification			
Amplified	5 (16%)	15 (52%)	
Not amplified	26 (84%)	14 (48%)	0.006

*Fisher's exact test result for comparison of stage I versus II, III, and IV.
†Fisher's exact test result for comparison of adenocarcinoma, BAC, or adenocarcinoma with BAC feature versus other.

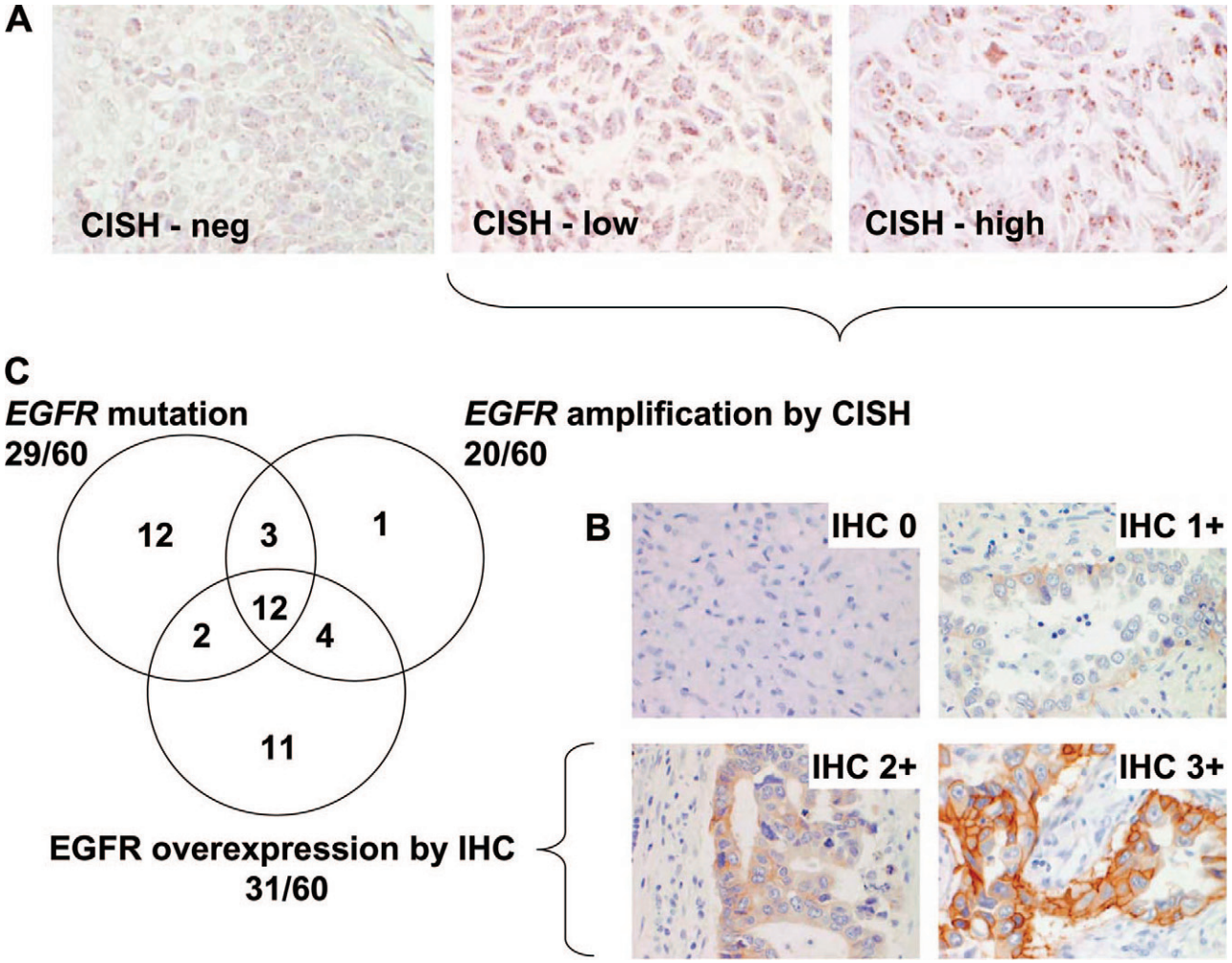

Figure 3. **A:** Chromogenic *in situ* hybridization showing no amplification, low-level amplification, and high-level amplification of the *EGFR* gene in three different lung adenocarcinomas. **B:** Lung adenocarcinomas with different levels of EGFR IHC staining: 0; 1+; 2+; 3+. Examples of both *EGFR* mutant and *EGFR* non-mutant cases are shown. **C:** Venn diagram showing relationships between *EGFR* mutations, *EGFR* amplification, and EGFR overexpression in a set of 60 lung tumors.

never-smoker status was not significantly associated with *EGFR* amplification status ($P = 0.10$) or EGFR IHC results ($P = 0.79$).

Discussion

Two *EGFR* mutations, exon 19 deletion and exon 21 L858R mutation, account for about 90% of all *EGFR* mutations reported in lung adenocarcinoma and are known to be predictive of response to the EGFR TKIs gefitinib and erlotinib.[8,9] Screening for these mutations in patients with lung adenocarcinoma can be used to predict which patients will respond to the EGFR TKIs, and numerous testing methods have been proposed.[10] With PCR-based tests for these mutations, our data showed a relatively high frequency of *EGFR* mutations (23%) for a North American patient population. This may reflect some re-

Table 2. Association of *EGFR* Gene Amplification and Protein Expression

IHC	Gene amplification	No gene amplification	Total	*P* value
0	1 (11%)	8	9	
1+	3 (14%)	19	22	
2+	6 (43%)	8	14	
3+	10 (67%)	5	15	0.0008*
Total	20 (33%)	40	60	

**EGFR* IHC: 0–1+, negative; 2–3+, positive. Fisher's exact test result for comparison of EGFR IHC score versus amplification status.

Table 3. Association of *EGFR* Mutations and Protein Expression

IHC	Mutation	No mutation	Total	*P* value
0	4 (44%)	5	9	
1+	11 (50%)	11	22	
2+	5 (36%)	9	14	
3+	9 (60%)	6	15	1.0*
Total	29 (48%)	31	60	

**EGFR* IHC: 0–1+, negative; 2–3+, positive. Fisher's exact test result for comparison of EGFR IHC score versus mutation status.

ferral bias for patients more likely to have *EGFR* mutations (using histology, smoking history, or ethnicity). A recent report from another high-volume North American clinical laboratory also described a 23% *EGFR* mutation rate in routine testing.[11] Thus, judicious ordering of these tests can lead to more efficient use of clinical laboratory resources than originally estimated on the basis of a projected mutation rate of 10% in unselected populations of patients with non-small cell lung cancer. The prevalence of *EGFR* mutations in our clinical experience may also reflect the somewhat higher sensitivity of PCR-based tests compared to direct sequencing. These PCR-based tests can detect a lower proportion of mutant alleles than direct sequencing.[4] Others have noted that a small but significant proportion of mutated cases are missed by direct sequencing of clinical tumor samples but can be detected by more sensitive techniques.[12,13]

The interrelationships and clinical significance of *EGFR* mutation, amplification, and protein expression are complex and remain controversial.[7,14–17] In these studies, increased *EGFR* gene copy number/amplification has been reported in seven to 44% of cases. This range may be due to variations in techniques, criteria for determining amplification, and interobserver variability. In our study, CISH-detected *EGFR* amplification was found in 32% of lung adenocarcinomas, in keeping with previous studies based on fluorescence *in situ* hybridization. A recent systematic comparison of EGFR fluorescence *in situ* hybridization and CISH has validated the use of the latter in this setting.[18] Using CISH, we found that amplification of *EGFR* in tumors with *EGFR* mutation is common (52%) and is more frequent ($P = 0.006$) than in cases lacking *EGFR* mutations. We propose that the reported predictive value of *EGFR* amplification for EGFR TKI response is at least in part due to its strong association with *EGFR* mutation. Moreover, because *EGFR* mutation assays are susceptible to false-negative results due to admixed non-neoplastic cells but *EGFR* fluorescence *in situ* hybridization assays are not, we speculate that a subset of *EGFR*-amplified cases that lack mutations by direct sequencing may contain mutations that would be detectable by more sensitive methods.

EGFR overexpression in NSCLC has been reported in 16 to 62% of cases.[7,15,19,20] This range in values likely reflects the use of a variety of antibodies, protocols, and interpretation criteria, as well as subjectivity in scoring. EGFR overexpression was seen in 48% of tumors in our analysis, which correlated well with *EGFR* gene amplification ($P = 0.0008$). This correlation is consistent with previous studies.[7,15,19] In contrast, we found no significant association between EGFR protein overexpression and *EGFR* mutation. Finally, it should also be noted that the 31 *EGFR* non-mutant cases were tested by direct sequencing for *KRAS* mutations, a known strong negative predictor of EGFR TKI response.[21,22] This revealed *KRAS* mutations in eight of 31 samples (results not shown), of which five were positive by EGFR IHC, further underscoring the drawbacks of EGFR IHC as a way of casting a "wider net" for patients potentially responsive to these agents.

EGFR mutation was significantly associated with a history of never smoking ($P = 0.0003$), which was similar to previous reports.[3,23] In contrast, never-smoker status was only marginally associated with *EGFR* amplification status ($P = 0.10$) and not at all with EGFR IHC results ($P = 0.79$).

Our clinical testing experience demonstrates that molecular testing of lung tumors for drug-sensitive *EGFR* mutations is a feasible, reliable, and relatively efficient process. Moreover, a routine turnaround time of about a week means that the results can be used to help guide treatment decisions regarding the use of gefitinib or erlotinib without clinically significant delays. To further ensure that this information is readily available in the chart at time of disease recurrence, we have recently implemented reflex *EGFR* mutation testing of all resected lung adenocarcinomas. While a negative test result does not currently eliminate the possibility of benefit from these drugs, a positive test can aid oncologists in several ways. First, as documented by several prospective clinical trials examining mutations and response rates, the presence of an *EGFR* mutation is associated with an aggregate 75% response rate.[8,9,24] Thus, clinicians can feel more confident in choosing EGFR TKIs even as first-line therapy. Second, although never-smoker patients are reported to have a higher incidence of *EGFR* mutations, only 50% of never-smokers have such mutations and correspondingly, only about half of never-smokers respond to EGFR TKIs. Thus, documenting the lack of an *EGFR* mutation provides better justification for a never-smoker to switch therapy earlier rather than later in the disease course. As patients with mutations who respond to therapy often experience rapid disease progression after discontinuation of drug, a positive test provides a rationale for clinicians to continue TKI treatment even if there is gradual disease progression; in this setting, rather than discontinuing TKI, additional agents are added but mutant EGFR suppression is maintained. Finally, combining *EGFR* mutation testing with testing for *KRAS* mutations, which are mutually exclusive with the former and with response to EGFR TKIs,[21,22] can help to further enhance response prediction and inform clinical decision-making.

In conclusion, lung adenocarcinoma with *EGFR* mutations is a distinct biological subset as evidenced by its strong association with never-smoker status and known high response rate to EGFR TKIs. Amplification of *EGFR* in NSCLC with *EGFR* mutation is common and, at least in exon 19 deletion cases, usually affects the mutant allele. Preferential amplification of the mutant allele has also been previously observed by other methods.[25] We propose that the predictive value of *EGFR* amplification for EGFR TKI response is more likely a result of its association with *EGFR* mutation. EGFR overexpression by IHC is associated with *EGFR* amplification but is of no utility in predicting the presence of *EGFR* mutations.

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