Higher Dosage of the Epidermal Growth Factor Receptor Mutant Allele in Lung Adenocarcinoma Correlates with Younger Age, Stage IV at Presentation, and Poorer Survival

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**Introduction:** The clinical significance of epidermal growth factor receptor (EGFR) mutant allele specific imbalance (MASI) in lung adenocarcinomas is unknown.

**Methods:** EGFR MASI was characterized by sequencing electropherograms (SEs) and EGFR fluorescence in situ hybridization (FISH) in 96 prospectively tested lung adenocarcinoma patients with a median follow-up of 20 months (all cases were EGFR mutation-positive).

**Results:** In 25 cases, the mutant allele (MA) peak was higher than the wild-type allele (WA) peak, indicating the presence of EGFR MASI (25/96, 26%). The adenocarcinomas with EGFR MASI had a 4.4-fold higher average EGFR/Chromosome Enumeration Probe 7 ratio than carcinomas without MASI (7.9 ± 3.8 versus 1.8 ± 0.6, p = 0.01). A high degree of correlation between the MA/WA ratio (SE) and the EGFR/CEP7 ratio (FISH) (p = 0.757, p = 0.003) validated the quantitative nature of SE. Amplification was the most common mechanism of EGFR MASI (13/21, 62%). EGFR MASI was more commonly associated with exon 19 mutations than with exon 21 mutations (19/53, 36%, versus 6/43, 14%, p = 0.015, odds ratio [OR] = 3.4) and in patients younger than 65 years (17/46, 37%, versus 8/50, 16%, p = 0.019, OR = 3.1). Patients with EGFR MASI presented with stage IV disease more frequently (p = 0.01, OR = 3.5) and had a poorer disease-specific survival rate (p = 0.021, 54% versus 83% at 31 months).

**Conclusions:** EGFR MASI in lung adenocarcinomas can be assessed based on SE and can be used to identify younger patients with more aggressive disease.

**Key Words:** Adenocarcinoma, EGFR, Sequencing, Allelic imbalance.

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The development of lung adenocarcinoma is a multistep process characterized by the accumulation of genomic alterations. Approximately 10% of lung adenocarcinomas in Western populations harbor epidermal growth factor receptor (EGFR) mutations, and 20% are characterized by EGFR gene copy number gain (CNG). The combination of EGFR mutations and EGFR CNG has rarely been studied.

Similar to other oncogenes, EGFR mutations are heterozygous in up to 80% of lung adenocarcinomas. Even when the combined effect of EGFR mutations and EGFR CNG has been studied, the individual contributions from mutant alleles (MAs) and wild-type alleles (WAs) have largely been ignored. The MA/WA ratio is maintained after transcription, and the dosage of EGFR MA may be a critical biological predictor of tumor behavior. The dosage of MA can be equal to or greater than that of the WA. The situation when MA is in excess of WA was defined as mutant allele specific imbalance (MASI).

Mechanistically, the partial dominance of MA may be due to CNG of the MA. CNG may result from the preferential amplification of MA. When amplification involves a relatively large DNA segment, it is likely to be detected by fluorescence in situ hybridization (FISH), a method most commonly used to study CNG in clinical samples. Polysomy of the chromosome carrying MA may also lead to partial dominance of the MA over WA (i.e., partial MASI) and is also routinely identified by FISH.

In some cases, the excess of MA may be seen in neoplastic cells with diploid karyotype and in the absence of amplification. These cases are characterized by the virtual absence of the WA, were defined as complete MASI, and develop through acquired uniparental disomy (UPD). UPD was first described during meiosis and results from the inheritance of both copies of a chromosome from the same parent (isodisomy). Since its description, acquired UPD (arising in mitosis through trisomy rescue or monosomy duplication) was recently shown in colorectal and ovarian carcinomas. Importantly, UPD affects short DNA segments and cannot be detected by FISH.

The advantage of term “MASI” is in its all-inclusive nature—it reflects all major currently known genetic mechanisms leading to the excess of MA (i.e., amplification, polysomy, and UPD).

In many laboratories, EGFR mutation analysis is performed using direct sequencing, which allows the visualization of both MA and WA when EGFR mutations are present. To the best of our knowledge, the EGFR MA/WA ratio has never been characterized in clinical samples of lung adenocarcinoma and correlated with the outcome of lung adenocarcinoma patients. We hypothesized that the semiquantitative assessment of sequencing electropherograms (SEs) performed on clinical tumor samples is representative of the actual EGFR MA/WA ratio in the tumor and identifies a
PATIENTS AND METHODS

Clinicopathological Characteristics of Studied Patients

Ninety-six consecutively diagnosed lung adenocarcinomas positive for EGFR exon 19 (n = 53) or exon 21 (n = 43) mutations were included in this study. These cases were identified at the University of Pittsburgh Medical Center (UPMC) from 2005 to 2010 through routine EGFR testing of all newly diagnosed lung adenocarcinomas. Sixty-nine resected adenocarcinomas (51 lobectomies, 7 wedge resections, 7 segmentectomies, 3 pneumonectomies, and 1 bilobectomy), 66 of which were stage I through III, and 27 biopsied adenocarcinomas, 21 of which were stage IV, were studied. All cases were restaged according to the seventh edition of the American Joint Committee on Cancer manual. Eighty-nine patients were White, 4 were African American, 2 were Asian, and 1 was Middle Eastern descent. Only 10 patients received neoadjuvant chemotherapy before EGFR testing, and none of these patients had prior treatment with tyrosine kinase inhibitors. The median follow-up was 20 months. Smoking history was available for 95 of 96 patients. There were 45 never smokers and 50 ever smokers (former or current). The high prevalence of smokers in this cohort is consistent with previously reported smoking rates among patients treated at UPMC (41 never smokers and 296 smokers). The clinicopathological features of the studied patients are summarized in Table 1.

This article is the result of a quality improvement project and was approved by the UPMC Total Quality Council.

EGFR Exon 19 and Exon 21 Mutation Analysis

EGFR exon 19 and 21 mutation analysis was performed as previously described. Briefly, for resected tumors, targets containing more than 75% tumor cells were manually microdissected from 4-µm unstained histological sections obtained from formalin-fixed paraffin-embedded tissue. DNA was isolated from each formalin-fixed paraffin-embedded target using the DNaseasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. To detect mutations, the DNA was amplified with primers flanking exon 19 (forward primer 5'-CCAGCATAATCAGCCTTAGGTG-3' and reverse primer 5'-CCACTAGAGCTAGAAGGGAAAGAC-3') or exon 21 (forward primer 5'-CCTCAGACGAGGTCTTCTTC-3' and reverse primer 5'-CTCTGGTGTCAGGAAATGCTG-3') of the EGFR gene in separate reaction tubes for each primer set. PCR products (40 cycles) were sequenced in both the sense and antisense directions using the BigDye Terminator v3.1 cycle sequencing kit on an ABI 3130 automated sequencer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer’s instructions. The sequences were analyzed using Mutation Surveyor software (SoftGenetics, LLC, State College, PA) and compared with reference sequence NM_005228.3 along the full length of each exon.

EGFR FISH Analysis

EGFR FISH analysis was successfully performed on 81 cases as previously described using a standard method with a dual-color EGFR SpectrumOrange/CEP7 SpectrumGreen probe (Vysis, Inc., Downers Grove, IL). EGFR FISH was not performed on 14 biopsies due to insufficient material, and in one resection specimen EGFR FISH failed despite three hybridization attempts. Sections were deparaffinized, dehydrated in ethanol, air-dried, and then digested with protease K (0.5 mg/ml) at 37°C for 28 minutes. The slides were denatured at 75°C for 5 minutes and dehydrated in ethanol; the probes were also denatured for 5 minutes at 75°C before hybridization. Slides were hybridized overnight at 37°C and washed in 2x SSC/0.3% NP40 at 72°C for 2 minutes, and the nuclei were counterstained with DAPI/Antifade 1 (Vysis, Inc., Downers Grove, IL). Each FISH assay included normal lung tissue sections as a negative control. Analyses were performed using a fluorescence microscope (Nikon Optiphot-2 and Quips Genetic Workstation, Melville, NY). The histological areas previously selected on the hematoxylin and eosin-stained sections were identified on the FISH slides. Only individual and well-delineated cells were scored, and overlapping cells were excluded from the analysis. At least 60 cells were scored for each case, and the EGFR/CEP 7 ratio was recorded for each case. Amplification was defined as an EGFR/CEP7 ratio of >2. High polysomy was defined as ≥40% of cells with ≥4 EGFR copies (highly correlated with CEP7 hyperdiploidy, i.e., >2 CEP7 signals per nucleus, data not shown).
Determination of the Ratio between the MA and WA by Semiquantitative Assessment of the MA and WA Peak Heights on SEs

Peak heights of the MA and WA were compared and grouped into two categories: MA higher than WA (MA > WA) and MA lower than WA (MA < WA) (Figure 1). Any increase of the MA over WA was defined as MASI. For cases with EGFR exon 19 deletions, the MA/WA ratio was determined by comparing the first five different peaks starting from the deletion point. To test whether the MA > WA ratio as determined using SEs is an accurate semiquantitative representation of allelic imbalance in the tumor, peak heights of MA and WA waves were measured using a ruler from the midpoint of the peak to the SE baseline. For deletion mutations, the average of the first five different peaks was calculated using the modification of Soh et al. (MA height/WA allele height). Cases with MA > WA were selected to test the correlation between the MA/WA ratio and EGFR/CEP7 ratio as determined using FISH. The validation set included cases with MA > WA and EGFR/CEP7 FISH ratio > 2. Cases with MA > WA and an EGFR/CEP7 FISH ratio < 2 were excluded from the correlation analysis because the likely mechanism of EGFR MASI in these cases is chromosome 7 polysomy, UPD, or homozygous mutation; UPD and homozygous mutations involve short segments of DNA and are not detected by FISH.

Statistical Analysis

DSS was measured from the date of surgery to the date of death. Living patients were censored at the date of the last available clinical information. Survival probabilities were calculated using the Kaplan-Meier method and compared among different groups and subgroups (log-rank test). Statistical analysis was performed using SPSS 19 (Sommers, New York, NY).

RESULTS

Identifying EGFR MASI: Comparing SEs and FISH

In 25 cases, the MA peak was higher than the WA peak, indicating the presence of EGFR MASI (25/96, 26%). Twenty-one case (21/25) had sufficient material for EGFR FISH. On average, the EGFR/CEP7 ratio was 4.4-fold higher in EGFR MASI cases (7.9 ± 3.8 versus 1.8 ± 0.6, p = 0.01, Table 2). Thirteen of 21 (62%) cases with EGFR MASI showed an EGFR/CEP7 FISH ratio of > 2, indicating that amplification of the EGFR MA is the most common mechanism of EGFR MASI. We noted that 10 of 13 cases showed an EGFR/CEP7 ratio > 5. Both EGFR/CEP7 ratio cutoffs (≥ 2 and > 5) were associated with EGFR MASI (≥ 2, p < 0.001, χ² test; > 5, p < 0.001, Fisher’s exact test).

The MA/WA ratio, as assessed using SE, showed a high degree of correlation with the EGFR/CEP7 ratio (Figure 2; Spearman’s correlation coefficient, p = 0.757, p = 0.003). In 1 of 25 cases with EGFR MASI, the dominant sequence after the deletion point was that of the MA (Figure 3A), and the EGFR/CEP7 ratio in this case was 39 (Figure 3B).

Six of 21 (29%) cases with EGFR MASI showed an EGFR/CEP7 FISH ratio of < 2 and EGFR high polysomy (Figures 3C, D).

Two of 21 EGFR MASI cases did not show EGFR amplification or high polysomy. The types of observed EGFR MASI are summarized in Table 3.

TABLE 2. EGFR Mutant Allele Specific Imbalance and Clinicopathological Features of Patients with Lung Adenocarcinoma (n = 96)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>MA &gt; WA (n = 25)</th>
<th>MA &lt; WA (n = 71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (yr)</td>
<td>59</td>
<td>67</td>
</tr>
<tr>
<td>Age groups (yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65 (n = 46)</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>&gt;65 (n = 50)</td>
<td>8</td>
<td>42</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-III (n = 72)</td>
<td>14</td>
<td>58</td>
</tr>
<tr>
<td>IV (n = 24)</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>EGFR/CEP7 FISH ratio (average)</td>
<td>7.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

aStatistically significant, two-tailed independent groups Student’s t test for means, p < 0.001.
bThe association between age (< 65 yr) and MA > WA is statistically significant (χ² method, p = 0.019, odds ratio [OR] = 3.1, 95% CI, 1.2–8; with hypothesis-driven directionality).
cThe association between stage IV and MA > WA is statistically significant (χ² method, p = 0.01, OR = 3.5, 95% CI, 1.3–9.5; with hypothesis-based directionality).
dStatistically significant, Student’s t test with unequal variance (two-tailed, p = 0.01).

EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; MA, mutant allele; WA, wild-type allele.
Clinicopathological Correlates of EGFR MASI

Patients with EGFR MASI carcinomas were 8 years younger and more likely to present with stage IV disease \((p = 0.01, \text{odds ratio} = 3.5, 95\% \text{ confidence interval [CI]} \ 1.3–9.5\) (Table 2). EGFR MASI was seen more frequently in carcinomas that harbored EGFR exon 19 mutations (19/25, 76\%).

The patient’s gender, smoking history, exon harboring mutation (19 versus 21), and EGFR/CEP7 ratio were not significantly associated with DSS. As expected, stage IV patients had worse DSS than stage I–III patients (Figure 4A). Importantly, patients with carcinomas characterized by EGFR MASI showed worse DSS (Figure 4B). As EGFR MASI was associated with younger age and stage IV disease at the time of diagnosis, a subgroup analysis was performed to determine whether EGFR MASI held an independent prognostic value. In a subset of patients with stage I–III adenocarcinoma, patients with EGFR MASI showed a trend of more aggressive disease (Figure 4C). Finally, even among patients younger than 65 years, carcinomas characterized by EGFR MASI showed poorer DSS (Figure 4D).

DISCUSSION

Lung adenocarcinomas are characterized by complex genetic alterations. For instance, EGFR may be activated by mutations or CNG, either alone or in combination. A variety of methods are used to characterize EGFR status, including chro-

FIGURE 2. Correlation between the EGFR mutant and wild-type allelic ratio as determined by sequencing electrophrogram (SE) and the EGFR/CEP7 ratio as determined by fluorescence in situ hybridization (Spearman’s bivariate correlation test). The correlation coefficient \((p)\) is 0.757 and indicates a high degree of correlation \((p = 0.003)\).

FIGURE 3. Correlation of EGFR fluorescence in situ hybridization (FISH) with the semiquantitative evaluation of sequencing electropherograms (SEs). Portions of SE surrounding the deletion points (red arrows) were scanned. The top row was typed in and represents the wild-type sequence, and the second row represents the actual sequence of the dominant polymerase chain reaction product. A, SE of a case with an EGFR exon 19 deletion and without an easily identifiable wild-type allele after the deletion point. These laboratory values belonged to a deceased 44-year-old white nonsmoking woman who presented with stage IIIb (pT2b N2) lung adenocarcinoma and developed recurrence associated with distant metastases 8 months after the initial surgery. B, EGFR FISH corresponding to the SE in 3A (EGFR/CEP7 ratio = 39). C, Mutant allele peaks are higher than the wild-type allele peaks. However, mutant allele specific imbalance is not accompanied by amplification (see panel D). D, The EGFR/CEP7 ratio is 1.1. The presence of four EGFR and CEP7 signals per nucleus indicates tetraploidy. Polysomy and amplification represent two mechanisms of mutant allele specific imbalance that are detectable by FISH. Red signal, Texas red—EGFR probe; green signal, fluorescein isothiocyanate—CEP7 probe; blue signal, 4’,6-diamidino-2-phenylindole—outlining the nuclei.
mogenic in situ hybridization, Southern blot analysis, qPCR, and, most commonly, EGFR FISH. In routine clinical application, however, none of these techniques can characterize the individual contributions of MA and WA to EGFR protein levels.

Soh et al. demonstrated that direct sequencing is a valid method of quantifying the MA/WA ratio. The quantitative nature of SE was demonstrated using several techniques, including subcloning, plasmid mixture experiments, and restriction fragment length polymorphism combined with band intensity measurement on gel electrophoresis. The correlation between the MA/WA ratio and EGFR FISH (i.e., amplification) presented here further demonstrates that SE can detect EGFR MASI in clinical samples.

Previous studies on EGFR MASI in lung adenocarcinomas made an extensive effort to account for the DNA derived from non-neoplastic cells (Table 4). “Contamination” by non-neoplastic cells is of concern when determining the MA/WA ratio in clinical samples. For instance, the median proportion of tumor DNA extracted from nonmicrodissected clinical samples of non-small cell lung carcinoma was 57% and from control cell lines 89 to 95%. The review of hematoxylin and eosin slides and manual microdissection limits the proportion of the nontumor DNA. The smaller remaining amount of non-neoplastic tissue would randomly affect all clinical samples. Nontumor DNA increases the WA peak, decreases the MA/WA ratio, and decreases the ability of SE to detect MASI.

Considering this limitation, we show for the first time that EGFR MASI as identified by SE correlates with distinct clinical features. The presence of EGFR MASI identified a subset of patients who were younger, more likely to present with stage IV disease, and had shorter DSS, overall and in a subset of patients younger than 65 years (Figure 4).

It is difficult to compare the incidence of EGFR MASI in this study with that in other studies because (1) previous studies did not routinely specify which EGFR exon (19 or 21) showed MASI and (2) the numbers of patients of Asian descent in those studies easily exceeded that in our study (Table 4). The combined interpretation of EGFR FISH and SE indicates that the most common mechanism of MASI is CNG by amplification (as previously described by Soh et al.) (Table 3). Yokoyama et al. anecdotally commented that “sequence analysis demonstrated that the mutation signals of

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**TABLE 3. Types of EGFR Mutant Allele Specific Imbalance: Combined Interpretation of EGFR FISH and Sequencing Electropherograms (n = 21)**

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>No. of Cases, n (%)</th>
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<tbody>
<tr>
<td>Amplification</td>
<td>13/21 (62)</td>
</tr>
<tr>
<td>Polysomy</td>
<td>6/21 (29)</td>
</tr>
<tr>
<td>Unknown, presumed UPD, or homozygous mutation</td>
<td>2/21 (10)</td>
</tr>
</tbody>
</table>

*Percentages do not add up to 100% due to rounding. EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization.

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**FIGURE 4.** Disease-specific survival (DSS) analysis based on the Kaplan-Meier method. A, DSS by stage: stages I–III (events [i.e., death from disease] = 6/55, 24%; estimated mean survival = 53 months, 95% CI 47–60) versus stage IV (events = 4/17, 24%; estimated mean survival = 36 months, 95% CI 24–47). B, DSS and the dosage of the EGFR mutant allele. MA < WA (mutant allele peak height lower than that of the wild-type allele; events = 4/52, 8%; estimated mean survival = 55 months, 95% CI 48–62) versus MA > WA (mutant allele peak height higher than that of the wild-type allele, EGFR mutant allele specific imbalance; events = 6/20, 33%, estimated mean survival = 37 months, 95% CI 29–45). C, To control for stage, the DSS analysis was performed in a subgroup of patients with stage I to III. MA < WA: events = 3/43, 7%; estimated mean survival = 56 months, 95% CI 50–63 versus MA > WA: events = 3/12, 25%, estimated mean survival = 34 months, 95% CI 28–39. D, To control for patient age, the DSS was analyzed in a subgroup of patients younger than 65 years. MA < WA: events = 2/21, 10%; estimated mean survival = 57 months, 95% CI 50–65 versus MA > WA: events = 5/15, 33%, estimated mean survival = 32 months, 95% CI 25–38.
[amplified lung carcinomas] were dominant, with 5 being completely lost for wild-type signal,\textsuperscript{a} concluding that the MA was selectively amplified.

Two \textit{EGFR} MASI cases showed an \textit{EGFR}/CEP7 ratio of \textless2 and no chromosome 7 polysomy. Assuming that the WA signal is derived entirely from the admixed normal tissue, it is possible that MASI is the result UPD in these cases. If so, then the incidence of UPD in our report (2/21, or about 10%) is comparable to that reported previously by Soh et al. (22%).\textsuperscript{a}

The number of cases analyzed in this study was limited by the low prevalence of \textit{EGFR} mutations in lung adenocarcinomas affecting Western population. Our conclusions require further validation in larger cohorts including patients of Asian descent and treated with tyrosine kinase inhibitors.

In summary, \textit{EGFR} MASI as determined using SE is a valid way to further subclassify \textit{EGFR} exon 19- and 21-mutated lung adenocarcinomas. \textit{EGFR} MASI in lung adenocarcinomas also identified younger patients with more aggressive disease.

ACKNOWLEDGMENTS

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REFERENCES


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