Epidermal Growth Factor Receptor Gene Amplification Is Acquired in Association with Tumor Progression of EGFR-Mutated Lung Cancer

Yasushi Yatabe, Takashi Takahashi, and Tetsuya Mitsudomi

Departments of Pathology and Molecular Diagnostics and Thoracic Surgery, Aichi Cancer Center; and Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan

Abstract

Both mutation and amplification of epidermal growth factor receptor (EGFR) in lung cancers have been reported in association with clinical responses to tyrosine kinase inhibitors. We have reported evidence implicating mutation specifically in the “terminal respiratory unit” type of adenocarcinoma, which is characterized by expression of thyroid transcription factor 1, a lineage marker of peripheral airway cells. However, little is known about the role of gene amplification in the molecular progression of lung adenocarcinoma. In this study, we examined the topographical distribution of amplification in three microdissected portions each of 48 individual lung cancers with confirmed mutations. Relative copy number of the gene was analyzed using Taq Man–based gene dosage analysis and fluorescent in situ hybridization technique. Gene amplification was found in 11 lung cancers. Strikingly, nine of the cancers showed heterogeneous distribution, and amplification was associated with higher histologic grade or invasive growth. Because it was likely that the high-grade lesions were the origin for metastases, metastatic lymph nodes corresponding to five tumors with heterogeneous distribution were analyzed. Unexpectedly, amplification status of the metastatic sites was not always associated with gene amplification of the primary tumors, suggesting that selection of the metastatic clone may be defined by other factors. We also examined 17 precursor lesions and 21 in situ lung adenocarcinomas, and found that only one in situ carcinoma harbored gene amplification. Taken together, our results show that mutation occurs early in the development of lung adenocarcinoma, and that amplification may be acquired in association with tumor progression. [Cancer Res 2008;68(7):2106–11]

Introduction

Epidermal growth factor receptor (EGFR) mutations are correlated with a subset of non–small cell lung cancers, along with specific characteristics such as nonsmoking status, adenocarcinoma histology, being female, and of East Asian ethnicity (1, 2). EGFR mutations are clinically relevant for making treatment decisions (3); they are closely associated with a high response rate for the treatment with EGFR tyrosine kinase inhibitors. However, the association of the mutation with the response and survival has not been confirmed in clinical trials. On the other hand, findings from several clinical trials have indicated that EGFR gene copy number is a better response predictor than mutation status (4–7). In such studies, high polysomy, defined as the presence of >40% of tetraploid cells, along with gene amplification, is regarded as significant gene alteration, although the biological role of high polysomy has not been elucidated. Interestingly, EGFR amplification is closely associated with EGFR mutation. Takano et al. (8) described 13 tumors with gene copy number more than six harbored gene mutations, and Cappuzzo et al. (9) reported that all of the examined tumors with gene amplification were EGFR mutated.

MET is a receptor tyrosine kinase similar to EGFR, and germline mutations at 7q31 have been detected in patients with hereditary papillary renal carcinoma (10). Hereditary papillary renal carcinoma is cytogenetically characterized by trisomy of chromosome 7, for which duplication of the mutant allele is nonrandomly selected (11). In terms of EGFR mutation, a single mutant signal of sequencing electropherograms in some tumors suggested that a nonrandom increase in the number of the EGFR mutant allele caused the amplification (12–14), similar to increases in mutated MET gene copies. This was confirmed in a single case with fluorescent in situ hybridization (FISH) and sequencing, as reported by Ma et al. (15).

Thus, a convergence of evidence suggests that EGFR mutation and amplification are closely associated. However, little is known about EGFR mutation and its relationship with the molecular pathogenesis of lung adenocarcinoma. Therefore, in this study, we examined EGFR amplification in lung cancers with EGFR mutations, using both FISH and Taq Man–based gene dosage analyses. We further addressed whether EGFR amplification was associated with tumor progression and metastasis.

Materials and Methods

Patients. A total of 48 patients with EGFR gene mutations were selected for this study, including 47 with adenocarcinoma and one with adenosquamous carcinoma. A KRAS mutation was not detected in any of these tumors, which is in accordance with previous reports (13, 16, 17). To address the role of EGFR mutation, EGFR amplification and KRAS mutation in association with schema of lung cancer progression, 107 lesions were examined. These comprised 17 precancerous lesions (atypical adenomatous hyperplasia; AAH), 21 in situ adenocarcinomas (bronchioloalveolar carcinoma), 23 minimally invasive adenocarcinomas, and 46 invasive adenocarcinomas with subsequent recurrence. Some of these data have been published previously (18). Clinicopathologic data were obtained from patient records at the Department of Pathology and Molecular Diagnostics at the Aichi Cancer Center in Nagoya, Japan. For this analysis, written informed consent was obtained from each patient according to the protocol approved by the Institutional Review Board.

Mutational analysis of the EGFR tyrosine kinase domain. EGFR mutation analysis was performed as previously described (16). Briefly, frozen tissue from tumor specimens was grossly dissected into extraction solution, followed by total RNA extraction using an RNeasy kit (Qiagen).
The EGFR tyrosine kinase domain (exons 18–24) was amplified by reverse transcription-PCR (SuperScript One-Step RT-PCR System with Platinum Taq DNA Polymerase; Invitrogen), and the products were directly sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The primer sequences were as follows: forward, 5'-AGTCATGGGAGAAAACAACACCC-3' and reverse, 5'-ATCCCCCTGAATGACAAGGTAG-3'. For the analysis of small portions of paraffin sections, the Cycleave PCR technique and fragment analysis were applied. This method has also been previously described in detail (19). The Cycleave PCR technique was also used for mutational analysis of KRAS.

**Analysis of EGFR gene amplification.** To examine the topographical distribution of EGFR amplification, we obtained paraffin sections containing both tumor and normal lung tissue. Three independent portions from each tumor were selected to reflect the morphologic characteristics. DNA was extracted from microdissected tissue and used as a template for Taq Man–based gene dosage using Taq Man Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The primer and probe sequences used for gene copy analysis of EGFR are as follows: 5'-ACTGGAAAAAACTGTTTGGGACCT-3' (forward primer), 5'-AGCTGTTCCTCTCTGTTGCTTAT-3' (reverse primer), and 5'-CCGGTCAGAAAACCA-3' (MGB probe). Fold increase in copy number was calculated as the ratio of the EGFR signal to control gene signals (COG5 and POP7), normalized to the corresponding normal lung tissue in the same section. The primer and probe sequences of these control genes were as follows: for COG5, 5'-CTTGTTCTCTGATCATGGAAGGAGCTA-3' (forward primer), 5'-AAAGCAAAAAATCCAACTTAAAGGTGT-3' (reverse primer), and 5'-CTCTGGCAAATAAACCTT-3' (MGB probe); for POP7, 5'-GCCACACATCCTTCTTTCT-3' (forward primer), and 5'-CCGCCAGGGAAGTCA-3' (MGB probe). Gene dosage analysis results were confirmed by FISH, using the LSI EGFR Spectrum-Orange/CEP 7 SpectrumGreen probe (Vysis; Abbott Laboratories) according to the manufacturer's protocol. FISH was performed on serial paraffin sections and in the same tissue areas as the gene dosage analysis. More than two or more increase of EGFR gene signals relative to CEP7 signals was considered as gene amplification.

**Results**

**EGFR gene amplification is heterogeneously distributed in most individual tumors.** Previous studies have shown that EGFR

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Sex</th>
<th>Smoking status</th>
<th>Histology</th>
<th>pTNM</th>
<th>EGFR mutation</th>
<th>Histological grade and EGFR amplification in the portions examined</th>
<th>Histological remarks</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>SMK</td>
<td>AD</td>
<td>pT2N0M0</td>
<td>E746-A750del</td>
<td>Grade 3 Amplified</td>
<td>1–3, throughout high-grade acinar carcinoma</td>
</tr>
<tr>
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<td>F</td>
<td>NSMK</td>
<td>AD</td>
<td>pT4N2M0</td>
<td>L858R</td>
<td>Grade 2 Grade 2</td>
<td>1, papillary with molura; 2, papillary; 3, papillary</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>NSMK</td>
<td>AD</td>
<td>pT2N2M0</td>
<td>L858R</td>
<td>Grade 1 Grade 2</td>
<td>1, BAC; 2, papillary; 3, solid</td>
</tr>
<tr>
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<td>F</td>
<td>NSMK</td>
<td>AD</td>
<td>pT4N2M0</td>
<td>L858R</td>
<td>Grade 1 Grade 1+2</td>
<td>1, BAC; 2, papillary; 3, acinar</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>NSMK</td>
<td>AD</td>
<td>pT1N2M0</td>
<td>E747-E749del, insP</td>
<td>Grade 2 Grade 2</td>
<td>1, BAC; 2, papillary; 3, solid</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
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<td>AD</td>
<td>pT2N1M0</td>
<td>L858R</td>
<td>Grade 1 Grade 2+3</td>
<td>1, BAC; 2, acinar; 3, acinar</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>SMK</td>
<td>AD</td>
<td>pT1N2M0</td>
<td>E746-A750del</td>
<td>Grade 2 Grade 2</td>
<td>1, BAC; 2, acinar; 3, solid</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>SMK</td>
<td>AD</td>
<td>pT2N1M0</td>
<td>L858R</td>
<td>Grade 2 Grade 2</td>
<td>1, acinar; 2 acinar; 3 acinar</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>SMK</td>
<td>AD</td>
<td>pT2N0M0</td>
<td>E746-A750del</td>
<td>Grade 1 Grade 2</td>
<td>1, BAC; 2 papillary; 3 acinar</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>NSMK</td>
<td>AD</td>
<td>pT4N1M1</td>
<td>E746-A750del</td>
<td>Grade 1 Grade 2</td>
<td>1, papillary; 2 papillary; 3 papillary</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
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<td>AS</td>
<td>pT4N1M0</td>
<td>E746-S752del, insA</td>
<td>Grade 2 Grade 2</td>
<td>1, squamous ca; 2, squamous; 3, adenocarcinoma</td>
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</tbody>
</table>

**Table 1.** Clinicopathological features of lung cancer with both EGFR mutation and amplification

NOTE: SMK, smoker; NSMK, nonsmoker. Underlining denotes in situ lesion in the periphery of cancer nodule. Blanks in the amplification column indicate no EGFR amplification.

*EGFR mutation was detected in all the portions.*
amplification is closely associated with EGFR mutations (8, 9). Thus, we selected 48 samples from patients with lung carcinomas and EGFR mutations. A total of three independent portions of each tumor were selected to represent morphologic differences. Gene amplification status was first screened using Taq Man–based gene dosage analysis. When a tumor portion showed a 2-fold or greater increase in copy number relative to normal tissue, gene amplification was confirmed by FISH. Using this procedure, we identified 11 tumors with EGFR amplification, summarized in Table 1. The amplification signals were loosely clustered (Fig. 1), suggesting that the amplification occurred as homogeneously staining regions. The incidence of gene amplification was consistent with those reported in the literature (8, 9, 20). Strikingly, EGFR amplification in 9 of the 11 tumors was distributed heterogeneously, whereas all three portions of the other two tumors showed equal levels of amplification throughout the tumor, suggesting homogeneous distribution. Regardless of the presence or absence of the amplification, EGFR mutation was detected in all of the individual portions from the 11 tumors.

**High-grade tumor areas with invasive growth harbor gene amplification.** We next examined whether gene amplification was associated with particular morphologic features of tumors. As summarized in Table 1, EGFR amplification was correlated with high histologic grade and/or invasive growth. This association was statistically significant (Table 2; Fisher’s exact test; two-tailed, \(P < 0.01\)). As reported previously (21), adenocarcinoma with EGFR mutations frequently showed bronchioloalveolar features in the tumor periphery. In adenocarcinoma cases 3 to 7 and 9, the peripheral areas showing lepidic growth did not harbor the EGFR amplification (Fig. 1). Sample 11 was an adenosquamous cell carcinoma, and both adenocarcinoma and squamous cell carcinoma areas of the tumor harbored gene amplification. EGFR amplification was not always associated with metastatic clone. The metastatic lymph nodes corresponding to the five tumors with heterogeneous amplification were analyzed to determine if high-grade lesions within the tumor are potential sources of metastases (Table 3). A metastatic lymph node from an adenocarcinoma with homogeneous gene amplification (case ID 1) was also found to have a gene amplification equal to the primary tumors. For this metastatic carcinoma, two portions were microdissected and examined individually. The amplification was homogeneous even within the metastatic site of the tumor. In contrast, the EGFR amplification status of metastatic lymph nodes was diverse in the other four tumors showing heterogeneous distribution of the gene amplification. In case 4, gene amplification was detected only in a part of three independent lymph nodes examined, whereas the metastatic site showed a homogeneous distribution despite the heterogeneity of the primary tumor in case 5 (Fig. 2). Taken together, these results suggest that other factors contribute to the observed metastasis.

**EGFR gene amplification is very rare in precursor and in situ lesions for lung adenocarcinomas.** Because we observed EGFR gene amplification primarily in high-grade tumor regions, we hypothesized that precursor lesions and in situ lung adenocarcinoma would not show gene amplification. We examined the gene amplification status in 17 samples of precursor lesions (AAH) and 21 of in situ lesions (bronchioloalveolar carcinoma; BAC), 23 minimally invasive adenocarcinomas, and 46 invasive adenocarcinomas that recurred after surgery. In the schema of lung cancer progression, whereas EGFR was mutated from the precancerous stage, EGFR amplification was detected mostly in

| Table 2. Histological features and EGFR amplification |
|-----------------|-----------------|
| Feature          | EGFR amplification (%) |
| Noninvasive lesion | 6               | 0 (0) |
| Invasive lesion   | 27              | 19 (70) |
| Lowest grade*     | 11              | 1 (9)  |
| Higher grade*     | 13              | 10 (77) |

NOTE: EGFR mutation was detected in all the portions. *Excluding three tumors, showing no differences of the histological grade among the portions examined.

Figure 1. Representative morphologic features at three portions and corresponding FISH results (case ID 5 in Table 1). Portion 1, a noninvasive lesion, which frequently has bronchioloalveolar features. Papillary growth and solid glandular aggregates are characteristic in portions 2 and 3, respectively. EGFR gene amplification was seen in portions 2 and 3. All the portions have a confirmed 9-bp deletion in exon 19.
the invasive stage and after (Table 4). These findings are consistent with those obtained in the transsectional analysis and suggest that gene amplification occurs in the later stages of lung adenocarcinoma development. Similar to previous reports (8, 9), EGFR amplification was found exclusively in adenocarcinomas with EGFR mutation. The incidence of KRAS mutation was curiously on the decrease along with the progression schema from precancerous to minimally invasive carcinoma.

Table 3. EGFR amplification in metastatic lymph nodes

<table>
<thead>
<tr>
<th>ID of the primary tumor*</th>
<th>Gene amplification status in primary tumor</th>
<th>Site of metastasis</th>
<th>Portions examined in metastatic lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Homogeneous</td>
<td>Hilar node</td>
<td>Amplified</td>
</tr>
<tr>
<td>3</td>
<td>Heterogeneous (1/3)</td>
<td>Mediastinal node</td>
<td>Not amplified</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hilar node</td>
<td>Amplified</td>
</tr>
<tr>
<td>5</td>
<td>Heterogeneous (2/3)</td>
<td>Mediastinal node</td>
<td>Not amplified</td>
</tr>
<tr>
<td>7</td>
<td>Heterogeneous (1/3)</td>
<td>Hilar node</td>
<td>Amplified</td>
</tr>
<tr>
<td>8</td>
<td>Heterogeneous (2/3)</td>
<td>Hilar node</td>
<td>Amplified</td>
</tr>
</tbody>
</table>

NOTE: Each row corresponds an individual lymph node.
*Corresponding to Table 1. Blank boxes indicate no examination.

Figure 2. Representative results of EGFR amplification in mediastinal and hilar lymph nodes with metastasis (case ID 5). Two portions in individual lymph nodes were selected for analysis to represent morphologic differences. Only one of the four portions (hilar 2) harbors EGFR gene amplification.
As mentioned before, EGFR and PDGFB mutations are associated with fibrosarcomatous transformation, and gene amplifications of these genes in tumors are a consequence of a translocation of collagen type I isoform 1 in dermatofibrosarcoma protuberances (DFSP). DFSP is a low-grade sarcoma, characterized by continuous activation of the PDGFB gene. A recent article by Abbott et al. (22) reported a rare, simultaneous gene mutation and amplification event, which is quite rare. Sufficient to involve a single gene. Lack of p16 alteration in small lung adenocarcinomas was associated with a higher grade of individual tumors. EGFR can be mutated in precursor lesions of lung adenocarcinoma, but EGFR amplification is rather rare. Furthermore, EGFR mutation was uniformly detected in the nine tumors with heterogeneous EGFR amplification. Taking this together, we suggest that the mutation precedes the amplification and that EGFR gene amplification may occur during the progression to invasive cancer.

In addition to EGFR mutation, KRAS mutation also plays a crucial role in the molecular pathogenesis of lung adenocarcinoma, and is acquired in early step of the carcinogenesis. As shown in Table 4, a precancerous lesion, AAH, also harbored a KRAS mutation. However, the frequencies of the EGFR and KRAS mutation showed quite different trends in putative schema of adenocarcinoma development. As reported in a previous study (18), high frequencies of KRAS mutations in AAH but not in in situ carcinomas and early invasive carcinomas. In contrast, the incidence of EGFR mutation was higher in in situ and invasive cancers. It may be possible that the present cohort does not represent clinical disease; however, similar frequencies of these mutations in the spectrum of tumors have been reported (32). Therefore, this suggests that either KRAS or EGFR mutations were associated with the development of AAH, but AAH with KRAS mutations seemed to lack the capability to progress to invasive cancer. However, it could be possible that AAH is a lesion that failed to progress to invasive cancer, independent of the acquired gene alterations. In other words, when a lesion cannot progress to invasive cancer, it may turn into an AAH lesion, regardless of the acquired mutation. The preferential amplification of KRAS in high-grade areas within the individual EGFR-mutated tumors observed in this study might provide a new clue for this query. AAH with EGFR mutations may progress to invasive cancer through additional alterations, including EGFR gene amplification, whereas large barriers may hinder the progression of KRAS-mutated AAH.

Although progression-associated gene amplification was suggested, the observed amplification does not seem to contribute to metastasis. In this study, not all metastatic lesions were derived from the gene-amplified area within the primary tumors that showed heterogeneous distribution of gene amplification. Even in the metastatic site, heterogeneity of amplification status was observed in cases of adenocarcinoma development. As reported in a previous study (18), high frequencies of KRAS mutations in AAH but not in in situ carcinomas and early invasive carcinomas. In contrast, the incidence of EGFR mutation was higher in in situ and invasive cancers. It may be possible that the present cohort does not represent clinical disease; however, similar frequencies of these mutations in the spectrum of tumors have been reported (32). Therefore, this suggests that either KRAS or EGFR mutations were associated with the development of AAH, but AAH with KRAS mutations seemed to lack the capability to progress to invasive cancer. However, it could be possible that AAH is a lesion that failed to progress to invasive cancer, independent of the acquired gene alterations. In other words, when a lesion cannot progress to invasive cancer, it may turn into an AAH lesion, regardless of the acquired mutation. The preferential amplification of EGFR in high-grade areas within the individual EGFR-mutated tumors observed in this study might provide a new clue for this query. AAH with EGFR mutations may progress to invasive cancer through additional alterations, including EGFR gene amplification, whereas large barriers may hinder the progression of KRAS-mutated AAH.

Table 4. Comparison of EGFR mutation, amplification, and KRAS mutation by histological subtypes along with the putative progression schema

<table>
<thead>
<tr>
<th>Subtype</th>
<th>EGFR</th>
<th>KRAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Mutation (%)</td>
</tr>
<tr>
<td>Precancerous lesion (AAH)</td>
<td>17</td>
<td>3 (18)</td>
</tr>
<tr>
<td>In situ lesion (BAC)</td>
<td>21</td>
<td>7 (40)</td>
</tr>
<tr>
<td>Minimally invasive adenocarcinoma</td>
<td>23</td>
<td>14 (61)</td>
</tr>
<tr>
<td>Overtly invasive adenocarcinoma*</td>
<td>46</td>
<td>28 (61)</td>
</tr>
</tbody>
</table>

*For this category, adenocarcinomas with subsequent recurrence were selected.

Discussion

Cancer develops as a result of many dysregulated signaling pathways, many of which are caused by genetic alterations as well as epigenetic changes. For impairment of one pathway, it is sufficient to involve a single gene. Lack of p16 alteration in small lung adenocarcinoma is such example, because RB, upstream regulator of p16, is already inactivated in nearly all the cancer. In this context, simultaneous gene mutation and amplification is quite rare. A recent article by Abbott et al. (22) reported a rare, simultaneous gene alteration of platelet-derived growth factor receptor beta (PDGFB) in dermatofibrosarcoma protuberances (DFSP). DFSP is a low-grade sarcoma, characterized by continuous activation of the PDGFB as a consequence of a translocation of collagen type I alpha 1 and PDGFB [t(17;22q21q13)]. On rare occasions, this sarcoma evokes fibrosarcomatous transformation, and gene amplifications of PDGFB in addition to the translocation were detected exclusively in the transformed lesion. These results suggest that gene amplification contributes to the high grade transformation of this tumor.

The EGFR locus can also have both mutation and amplification. As mentioned before, EGFR is amplified only in EGFR-mutated tumors, and the amplified allele is likely to be the mutated one. These findings suggest that mutation and amplification are closely associated with each other. We have reported that the EGFR mutation was involved specifically in the terminal respiratory unit type adenocarcinoma, characterized by expression of thyroid transcription factor 1 (TTF-1), a lineage marker of peripheral airway cells (23, 24). Recent identification of TTF-1 gene amplification in this subtype supports the hypothesis that terminal respiratory unit type adenocarcinoma is a distinct subset of lung adenocarcinoma (25). Furthermore, unsupervised hierarchical clustering based on expression profiles clearly show that this is a unique subset of adenocarcinoma (26), which was called "bronchioloid" (27), "terminal sac/alveolar stages-linked" (28), and "alveolar" (29) in the different studies, in addition to terminal respiratory unit type adenocarcinoma. Characteristically, this tumor frequently shows high morphologic diversity within individual tumors, with invasion in the center of the nodule and in situ lepidic growth in the periphery. These features are frequently called a representation of the adenoma-carcinoma sequence seen in colon cancer, and lung adenocarcinoma is considered to be developmentally analogous to colon carcinoma. Indeed, allelic loss was shown to be associated with progression (30, 31), although none of the other genetic alterations were found in lung cancer. The present study revealed that gene amplification was heterogeneously distributed in sections of adenocarcinoma tumor tissues, and EGFR amplification was associated with a higher grade of individual tumors. EGFR can be mutated in precursor lesions of lung adenocarcinoma, but EGFR amplification is rather rare. Furthermore, EGFR mutation was uniformly detected in the nine tumors with heterogeneous EGFR amplification. Taking this together, we suggest that the mutation precedes the amplification and that EGFR gene amplification may occur during the progression to invasive cancer.

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Although progression-associated gene amplification was suggested, the observed amplification does not seem to contribute to metastasis. In this study, not all metastatic lesions were derived from the gene-amplified area within the primary tumors that showed heterogeneous distribution of gene amplification. Even in the metastatic site, heterogeneity of amplification status was observed similar to the primary tumors. Thus, we suggested that selection of the metastatic clone may be defined by other factors (33, 34).

In summary, we examined the topographical distribution of EGFR amplification in individual tumors, using 48 lung adenocarcinomas with EGFR mutations. EGFR was amplified both heterogeneously and homogeneously in association with morphologic...
diversity of the tumors, suggesting that lung adenocarcinoma with EGFR mutations may acquire the additional gene amplification during the progression to invasive cancer.

Acknowledgments

References

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