Research Article

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

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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).

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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′-AGTCATGGGAGAAAACAACA-CC-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′-AGCTGTT-TTCACCTCTGTTGCTTAT-3′ (reverse primer), and 5′-CCGGTCAGAAAAC-CA-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′-CTTGTTCTCTGTACATGAAGGAGCTA-3′ (forward primer), 5′-AAGACAAAATCCAAGCATTCAAAGTGTT-3′ (reverse primer), and 5′-CT-CTGGCAATGAAACCT-3′ (MGB probe); for POP7, 5′-GCACCCACATCCT-TCTTTCTCT-3′ (forward primer), 5′-CCGCCAGGCCACTCAA-3′ (reverse primer), and 5′-CCGGCCAGCCCAAGTA-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 manufacture'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

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

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

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 homogenously 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.

Table 2. Histological features and *EGFR* amplification

	n	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.

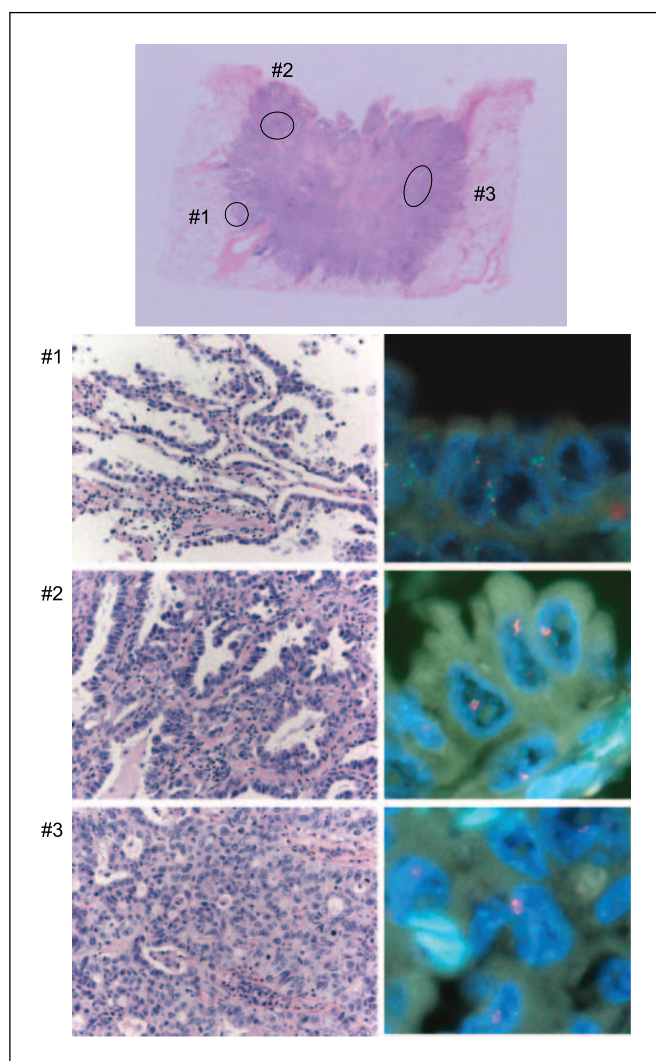

Figure 1. Representative morphologic features at three portions and corresponding FISH results (case ID 5 in Table 1). *Portion 1,* a noninvasive lesion, which frequently has bronchioloalveolar features. Papillary growth and solid glandular aggregates are characteristic in portions 2 and 3, respectively. *EGFR* gene amplification was seen in portions 2 and 3. All the portions have a confirmed 9-bp deletion in exon 19.

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 (bronchioalveolar 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

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Table 3. *EGFR* amplification in metastatic lymph nodes

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

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

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.

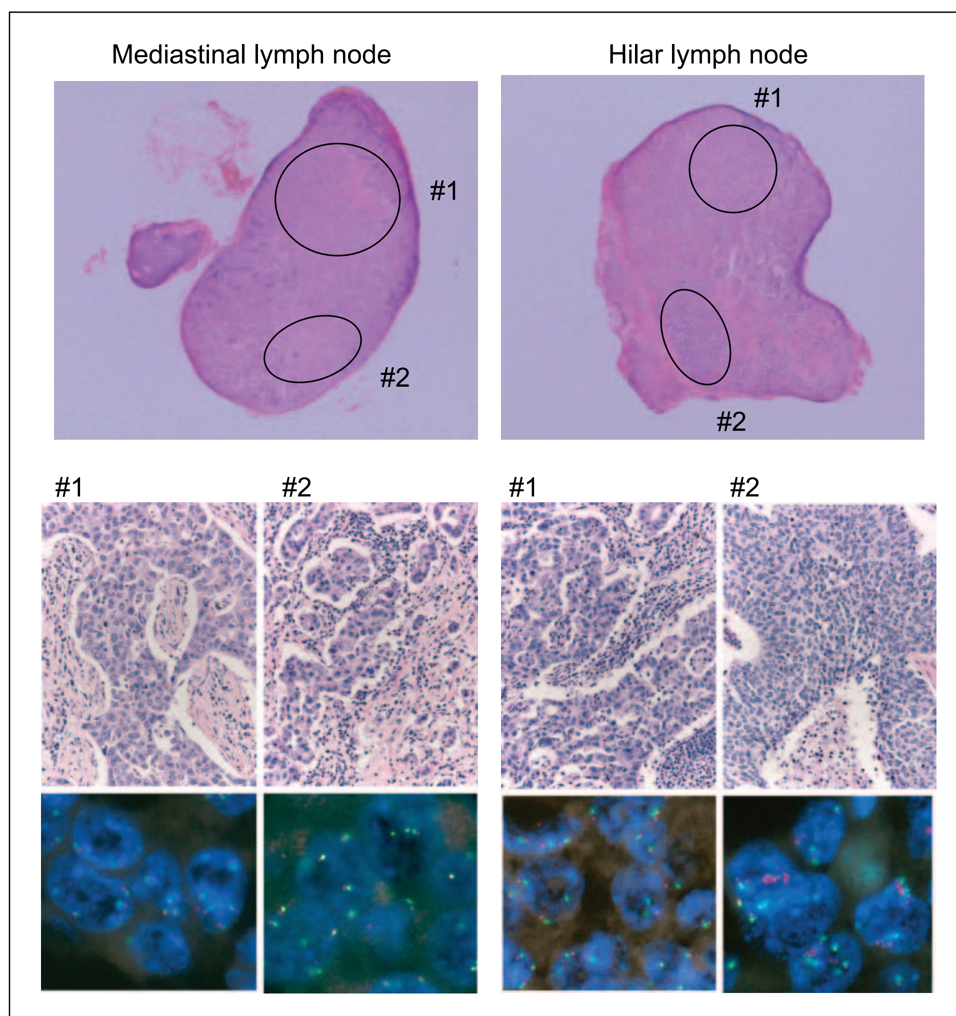

Figure 2. Representative results of *EGFR* amplification in mediastinal and hilar lymph nodes with metastasis (case ID 5). Two portions in individual lymph nodes were selected for analysis to represent morphologic differences. Only one of the four portions (hilar 2) harbors *EGFR* gene amplification.

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

	EGFR			*KRAS* (%)
	Total	Mutation (%)	Amplification (%)	
Precancerous lesion (AAH)	17	3 (18)	0 (0)	5 (29)
In situ lesion (BAC)	21	7 (40)	1 (5)	2 (10)
Minimally invasive adenocarcinoma	23	14 (61)	2 (9)	1 (4)
Overtly invasive adenocarcinoma*	46	28 (61)	4 (9)	4 (9)

*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 cell lung cancer 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 β (*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 α 1 and PDGFRB [t(17;22;q21;q13)]. 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 "bronchioid" (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.

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 *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.

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

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diversity of the tumors, suggesting that lung adenocarcinoma with *EGFR* mutations may acquire the additional gene amplification during the progression to invasive cancer.

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