Epidermal Growth Factor Receptor Mutations in Multicentric Lung Adenocarcinomas and Atypical Adenomatous Hyperplasias

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Background: The mechanisms of generation and progression of multicentric lung adenocarcinoma (AD), bronchioloalveolar carcinoma (BAC), and atypical adenomatous hyperplasia (AAH) in the peripheral lung is not well known. In this study, we analyzed epidermal growth factor receptor (EGFR) mutations in the cases of multicentric AD, BAC, and AAH to reveal the role of EGFR mutation in their generations and progressions.

Method: Ninety-seven AAH, BAC, or AD lesions less than 3 cm in size in 26 patients were surgically resected. Of these, EGFR mutations of the nodules with the highest and the second highest grade of histologic malignancy were examined in each patient by using the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp method.

Results: EGFR mutations could be examined in 48 nodules in the 26 patients. The EGFR mutations were found more frequently in lesions with higher histologic malignancy, ie, 9 of 10 ADs (90%), 16 of 28 BACs (57%), and one of 10 AAHs (10%). In 22 patients who could be examined of EGFR mutations for the two lesions in each patient, only two patients (9%) had the same mutation patterns between the two lesions, whereas 15 patients (68%) had the different statuses and the remaining five (23%) had no mutations.

Conclusion: Our data demonstrated that EGFR mutations seem to contribute to the acquisition of malignant potential in the AAH-AD sequence and occur independently in each lesion and in the cases of multicentric AD, BAC, and AAH.

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Recent advances in high-resolution computed tomography and the prevalence of CT screening for lung cancer have increased the detection of multiple ground-glass opacity (GGO) nodules,[1–4] which are often atypical adenomatous hyperplasia (AAH), bronchioloalveolar carcinoma (BAC), and adenocarcinoma (AD). However, the mechanisms of generation and progression of these multiple lesions are still unknown.

The concepts of multistep carcinogenesis during which genetic mutations are sequentially accumulated resulting in the development of invasive tumors have been well established in a number of cancers, including colon and breast cancers.[5,6] Although a hypothesis of multistep carcinogenesis has been also proposed for lung AD, ie, AD develops from AAH to invasive AD through BAC,[7] the signals that cause to either transform into invasive AD or to remain AAH or BAC are still unknown.

It has been reported that ADs with BAC features frequently have epidermal growth factor receptor (EGFR) mutations.[8,9] Recently, the possibility of significant role of EGFR mutation in the carcinogenesis of lung AD was also described. Tang et al. showed that EGFR mutation were frequently observed in normal epithelium of patients with EGFR mutant lung AD and described that EGFR mutation could occur in the early step of AD development.[10] Ji et al. showed a close relationship between EGFR mutation and carcinogenesis of lung AD in the experiments on the transgenic mice.[11] From these reports, we speculate that AAH or BAC lesions in patients with multiple AAH, BAC, and AD lesions could already have EGFR mutations. If the EGFR mutations occurred in an early step of AD development, EGFR tyrosine kinase inhibitors (TKIs) could be one of the treatments for patient with multiple AAH, BAC, and AD lesions, because they are frequently effective for AD with EGFR mutations.[12,13]

In this study, to investigate the role of EGFR mutations in generation and progression of multicentric AD, BAC, and AAH lesions, we examined the EGFR mutations in patients with these lesions.

PATIENTS AND METHODS

Patients

Between January 1999 and December 2006, 276 patients with AD, BAC, or AAH lesions underwent surgical

resection in Kumamoto University Hospital. Of these, 30 patients had multiple AAH, BAC, and AD lesions, whereas the 246 patients had single lesion. In the 30 patients with multiple lesions, to rule out intrapulmonary metastasis, two patients with moderately or poorly differentiated AD and two patients with lesions more than 3 cm in size were excluded from this study. As a result, 26 patients with multiple AAH, BAC, and AD lesions that showed GGO images on CT were selected for this study. The pathologic diagnoses were made by two different pathologists (Y.H. and K.I.)according to the 1999 World Health Organization histologic classification.[14,15] The multiple lesions in the present case were denied as metastatic ones, according to the criteria of Martini and Melamed.[16] None of the patients received adjuvant chemo- or radiotherapy before surgery.

The study protocol for examining EGFR mutation from the resected lesions was approved by the Ethics Committee of Kumamoto University Hospital in May 2005. Written informed consent was obtained from all patients for investigation of their EGFR mutations of tumors. The tumors with the highest and the second highest grade of histologic malignancy were defined as the first and second tumors, respectively, in each patient. Then, the DNA extracted from the first and the second tumors was investigated for EGFR mutations.

Detection of EGFR Mutations

We analyzed exons 18, 19, and 21, because most mutations were known to be clustered in these exons.[17] Because most of the examined nodules were less than 2 cm and the amount of extracted DNA was relatively small, the EGFR mutation statuses were analyzed with the use of peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp methods, which was reported to be more sensitive than conventional direct sequencing.[18,19] Surgically resected specimens were fixed with formalin and embedded in paraffin. All sections were cut from paraffin blocks at a thickness of 5 μm. DNA extraction and analysis of EGFR mutations were performed by Mitsubishi Kagaku Bio-chemical Laboratories Inc (Tokyo, Japan). Tumor cells were visually dissected, and samples were incubated in 200 μL of lysis buffer (10 mMTris–HCl pH 8.0, 1 mM EDTA, 0.5% SDS) with 40 μg of glycogen and 2 μL of proteinase K (20 mg/mL) and incubated at 56°C overnight. DNA was purified by phenol and chloroform extraction and dissolved in 20 μL TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA). The details of PNA-LNA clamp methods and the design of PCR primers was followed by Nagai et al.[19] Briefly, all PCR reaction solutions (25 μL) were based on the Basic Mixture containing 25 mmol/L TAPS (pH 9.3), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mmol/L 2-mercaptoethanol, 200 μmol/L each of deoxynucleotide triphosphates, and 1.25 units of Takara Ex *Taq*HS (Takara Bio, Shiga, Japan). For conventional PCR, PCR primers (200 nmol/L each) were added to the Basic Mixture. For PNA-LNA PCR clamp, PCR primers (200 nmol/L each), fluorogenic probes (100 nmol/L each), and a PNA clamp primer (5 μmol/L) were added to the Basic Mixture. The real-time amplification monitoring for both conventional and the PNA-LNA PCR clamp were done using Smart Cycler II (Cepheid, Sunnyvale, CA). PCR cycling was a 30-second hold at 95°C followed by 45 cycles of 95°C for 3 seconds and 62°C (exon 18 and 19) or 56°C (exon 21) for 30 seconds. Nested PCR for the PNA-LNA PCR clamp was done using the same reaction conditions except that the inner primers and 1 μL of a $1:10^6$ dilution of the first PCR reaction were used.

Detection of K-ras mutations

We also analyzed the mutation of K-*ras* exon 2 in the same extracted DNA samples. We used the enriched PCR methods to analyze the K-*ras* mutations, which was reported to be able to detect K-*ras* mutation from small amount of DNA samples.[20]

Statistical Analysis

All values in the text and tables are given as mean ± SD. Category data were compared using the Fisher exact test. The numeric data were analyzed for significance using the two-tailed Student *t* test. Bonferroni test was used to determine significance for comparisons among three parts. Values of $p < 0.05$ were accepted as significant.

RESULTS

Clinicopathological Characteristics

Clinicopathological characteristics of the 26 patients with multiple AAH, BAC, and AD lesions are shown in Table 1. There were 6 men and 20 women. Twenty patients (77%) were never smokers. Two patients (8%) had a familial history of lung cancer within the second degree of kinship. The number of resected lesions in each patient was 1 in 3 patients, 2 in 12, 3 in 4, 4 in 2 and more than 5 (up to 27) in 5.

TABLE 1. Patient's Characteristics

Mean age (yr; range)	65 (49–79)
Sex	
Male	6
Female	20
Smoking status	
Smoker	6
Never smoker	20
Number of lesions in CT	
2	8
3	4
4	3
≥5	11
Number of resected lesions	
1	3
2	12
3	4
4	2
≥5	5
Location	
Bilateral	10
Unilateral	16
Total	26

CT, computed tomography.

Pathologic diagnoses of the 97 resected nodules were AAH in 34, BAC in 52, and AD in 10. All of the ADs were well differentiated one with BAC or AAH features except for one, which showed a pure papillary pattern. The mean size was 5 ± 3 mm in AAH, 9 ± 8 mm in BAC, and 16 ± 8 mm in AD. There was a significant difference in the mean sizes among the three groups ($p < 0.05$). Of the 26 patients, two patients had multiple AAH lesions. Six patients had multiple BAC lesions. Eight patients had multiple BAC and AAH lesions. Ten patients had AD with BAC or AAH or both. Of the 97 lesions, 54 were resected by lobectomy, 13 by segmentectomy, and 30 by wedge resection. Bilateral lesions were resected in 10 patients, seven of whom underwent simultaneous bilateral resections. Although lymph node dissections or samplings were performed in all patients, none of the patients had lymph node metastasis. All patients are alive without recurrence (mean observation period: 661 days) except for one patient, who died of another disease. In 10 patients, all of lesions could not be resected because of too many lesions, although all of the nonresected nodules have not grown after surgery.

Mutation Analysis

In 4 of the 26 patients, only one lesion could be examined for EGFR mutations because only one nodule was resected in two patients and the second tumor were too small to extract sufficient amount of DNA in two patients. As a result, EGFR mutations could be examined for 48 nodules in the 26 patients. EGFR mutations were detected in one of the 10 AAHs (10%), 16 of the 28 BACs (57%), and 9 of the 10 ADs (90%) (Table 2). Thirty-eight lesions of AD or BAC harbored EGFR mutations more frequently than 10 AAH lesions ($p = 0.01$). AD harbored EGFR mutations more frequently than BAC with marginal significance ($p = 0.06$). One AD without EGFR mutation showed a pure papillary pattern without BAC features (Case 8 in Table 3). One AAH with EGFR mutation was histologically high grade one (Case 17 in Table 3).

Of the 48 lesions, 26 (54%) harbored EGFR mutations. The type of mutations in these 26 lesions was exon 21 L858R in 15 lesions (58%), exon 19 deletions in 10 (38%), and exon 21 L861Q in one (4%). Tables 3 and 4 show the mutation statuses of the lesions of the 22 patients who could be examined for the presence of EGFR mutations in both first and second tumors, two patients (9%) showed the same pattern of mutations in each lesion. Fifteen patients (68%) showed different mutational statuses of mutations among the lesions, of whom five patients showed different patterns of mutations between the two lesions and 10 patients had EGFR mutations in only one of the two lesions. Five patients (23%) showed no mutation in both lesions.

K-*ras* mutations were detected in one AAH (10%) and one BAC (4%). Both of them were transversion that changes G to T at codon 12. These K-*ras* mutations were not overlapped with the EGFR mutations.

TABLE 2. EGFR Mutations in Each Histological Type

Histological Type	Number of Nodules (%)		Total
	With Mutations	Without Mutations	
AAH	1 (10%)	9 (90%)	10
BAC	16 (57%)	12 (43%)	28
AD	9 (90%)	1 (10%)	10
Total	26 (54%)	22 (46%)	48

EGFR, epidermal growth factor receptor; AAH, atypical adenomatous hyperplasia; BAC, bronchioloalveolar carcinoma; AD, adenocarcinoma.

DISCUSSION

It has been reported that ADs with BAC features harbor EGFR mutations frequently, ie, 9 of 18 (50%) by Blons et al.,[8] and 14 of 21 (66%) by Hsieh et al.[9] The significant high rate of EGFR mutation was also reported in ADs of East Asian patients (270 of 563, 48%), females (203 of 411, 49%), and nonsmokers (232 of 433, 54%).[21] Although the mutation rate of ADs in our study was 90% (9 of 10), which was higher than those in previous reports, it could be because of the following reasons: (1) All of the patients were East Asian; (2) Twenty of the 26 patients (77%) were female; (3) Twenty of the 26 patients (77%) were nonsmokers; and (4) Nine of the 10 ADs (90%) were well differentiated AD with BAC features.

Our result showed that the EGFR mutation patterns of the first and second tumors were same in only 2 of the 22 patients (9%) and the mutation statuses were different in 15 of the 22 patients (68%). This result demonstrated that EGFR mutations would be independently acquired after multicentric generation of the AAH, BAC, and AD lesions.

A hypothesis of multistep carcinogenesis has been proposed for lung AD, which develops from AAH to invasive AD through BAC.[7] We expected that EGFR mutations might occur in an early step of the AAH-AD sequence, and therefore that the mutation might frequently be detected already in AAH or BAC in patients with multiple AAH, BAC, and AD lesions. However, our results showed that most AAHs did not have EGFR mutations and that BACs had the mutations less frequently than ADs. This result corresponded to that of Yoshida et al., who examined EGFR mutations mostly in solitary AAH, BAC, and AD lesions.[22] We therefore consider that the EGFR mutations would not cause the occurrence of multiple AAH, BAC, and AD lesions and would be acquired independently after multicentric generation of the lesions, and contribute to acquirement of malignant potentials during AAH-AD sequence.

The K-*ras* mutation in AAH was reported to be shown in 8 of 30 lesions (27%) by Yoshida et al.[22] and 13 of 40 (32%) by Sakamoto et al.,[23] of which frequency was higher than the one of 10 (10%) in our study. Although the reason of relatively low frequency of K-*ras* mutation in our study is not clear, it might be a character of our study with multiple neoplastic lesions.

In 10 of the 26 patients, all of the lesions could not be resected because of too many lesions. Because EGFR-TKIs would be effective for AD lesions with EGFR mutations,[12,13,24] we first expected that EGFR-TKIs could be effective for not only AD but also BAC or AAH in these patients. However, our results showed that the EGFR muta-

TABLE 3. Patterns of EGFR Mutations in Each Patient

Case	Age (yr)	Sex	First Tumor		Second Tumor	
			Histological Type	EGFR Mutation	Histological Type	EGFR Mutation
1	69	F	AD	exon19 delE746_A750	BAC	exon21 L858R
2	64	F	AD	exon21 L858R	BAC	exon21 L858R
3	72	F	AD	exon21 L858R	BAC	None
4	55	M	AD	exon21 L858R	BAC	None
5	53	F	AD	exon19 delL747_S752	BAC	None
6	71	F	AD	exon19 delL747_S752	BAC	None
7	49	F	AD	exon21 L858R	BAC	exon19 delE746_A750
8	79	F	AD	None	BAC	exon21 L858R
9	61	F	AD	exon19 delL747_S752	AAH	None
10	66	F	AD	exon21 L858R	AAH	None
11	63	F	BAC	exon19 delE746_A750	BAC	exon21 L858R
12	76	F	BAC	exon21 L858R	BAC	exon21 L858R
13	72	M	BAC	exon21 L861Q	BAC	exon21 L858R
14	65	F	BAC	exon21 L858R	BAC	None
15	79	F	BAC	exon21 L858R	BAC	None
16	54	F	BAC	None	BAC	None
17	73	M	BAC	exon19 delS752_I758	AAH	exon19 L747_T751
18	74	F	BAC	exon19 delE746_A750	AAH	None
19	57	M	BAC	None	AAH	None
20	47	F	BAC	None	AAH	None
21	58	F	BAC	None	AAH	None
22	57	M	AAH	None	AAH	None

EGFR, epidermal growth factor receptor; M, male; F, female; AAH, atypical adenomatous hyperplasia; BAC, bronchioloalveolar carcinoma; AD, adenocarcinoma; NE, not examined.

TABLE 4. Pattern of EGFR Mutations of the Two Lesions in Each Patient

Same mutation pattern	2 (9%)
Different mutation patterns	5 (23%)
Mutation in one of the two lesions	10 (45%)
No mutation in both lesions	5 (23%)
Total	22

EGFR, epidermal growth factor receptor.

tions were rare in AAH and were less frequent in BAC than in AD. Therefore, EGFR-TKIs might not be effective for AAH and BAC in the patients with multiple AAH, BAC, and AD lesions.

Although CT guided needle aspiration biopsy is a minimally invasive method for histologic diagnosis, it has some difficulties to differentiate boundary lesions. Our study showed that 90% of ADs in multiple GGO nodules had EGFR mutations, whereas 90% of AAHs did not. Because PNA-LNA PCR clamp method can detect EGFR mutations of small tumor tissues with higher sensitivity than the conventional direct sequencing,[18,19] we believe that the EGFR mutation analysis in the specimens obtained by needle biopsy would contribute to a pathologic diagnosis and prediction of malignant grade of GGO nodules. For example, when the EGFR mutations were positive in biopsy specimens of GGO nodules, which were suspected of AAH or BAC with histologic findings, surgical resection could be recommended because of high possibility of high grade of BAC. When EGFR mutations were negative for GGO nodules, which were suspected of AAH or BAC with histologic findings, follow-up could be one of the choices because of high possibility of AAH or low grade of BAC.

In conclusion, EGFR mutations independently occurred after mulicentric generation of lung cancers. In addition, there was close relationship between the histologic malignancy grade and EGFR mutations in patients with multiple AAH, BAC, and AD lesions. Although EGFR mutations would not to be involved in generation of multiple AAH, BAC, and AD lesions, they seem to contribute to the acquisition of malignant potential in the AAH-AD sequence. We believe that our results would contribute to a guideline of clinical management of patients with multiple GGO nodules.

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