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Detection of *EGFR* and *K-ras* mutations for diagnosis of multiple lung adenocarcinomas

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1. ABSTRACT

The incidence of multiple primary lung adenocarcinoma (MPLA) is increasing, and it is important to distinguish MPLA from intrapulmonary metastasis (IPM) in order to determine the therapeutic strategy. However, there is no reliable method to differentiate between the two. The purpose of this study was to distinguish MPLA from IPM based on the gene status of *EGFR* and *K-ras* and the morphological Noguchi classification system. Sixty-eight tumors from 34 cases of clinical MPLA were evaluated. Of them, 11 cases (32.4%) were diagnosed as biological MPLA (bMPLA) by *EGFR*/*K-ras* mutation analyses, and 12 cases (35.3%) by morphological analysis. In all, 23 of the 34 cases (67.6%) were diagnosed as bMPLA. The remaining 11 cases were diagnosed as biological IPM (bIPM). The 5-year survival rates of bMPLA and bIPM were 90.9% and 63.6%, respectively ($p = 0.04$). These findings suggest that the combination method including gene mutation and morphological analysis can guide treatment decisions and that there is a need for systemic chemotherapy, and surveillance monitoring.

2.INTRODUCTION

Lung cancer is the leading cause of cancer mortality in developed countries including Canada, France, Italy, UK, USA, and Japan (1). Lung tumors are classified into 2 broad classes, namely, small cell lung cancer (SCLC), accounting for about 15% of cases, and non-small cell lung cancer (NSCLC), which is the most common form of lung cancer, accounting for up to 85% of all cases (2, 3). The frequency of occurrence of the adenocarcinoma subtype of NSCLC has increased in recent years (4). New developments, such as high-resolution CT, have enabled the detection of small lung tumors and synchronous multiple primary lung cancers (MPLC) (5, 6). The incidence of a second lung cancer in patients who survive after resection of NSCLCs is also increasing (7).

Multiple genetic and epigenetic alterations, such as oncogene and tumor suppressor gene mutations, DNA repair gene variants, microsatellite instability, hypermethylation, and potential inherited predispositions are involved not only in tumor carcinogenesis and tumor progression (8-10) but also in preneoplastic lesion (11).

Mutations in K-ras or in the epidermal growth factor receptor (EGFR) gene arise early in the pathogenesis of lung adenocarcinoma and are related with the appearance of the malignant phenotype (12, 13).

Recently, *EGFR* has emerged as an attractive therapeutic molecular target in patients with adenocarcinoma harboring *EGFR* mutations (14, 15). In addition, mutations in *K-ras*, a downstream effector of EGFR signaling, are mutually exclusive with *EGFR* mutations, and are associated with resistance to EGFR tyrosine kinase inhibitors (16, 17).

Noguchi *et. al*. histologically classified small peripheral adenocarcinomas measuring 2 cm or less at their greatest dimension into 6 types (types A–F) on the basis of tumor growth patterns (i.e., Noguchi type) (18, 19). Noguchi types A, B, and C exhibit replacement growth of alveolar-lining epithelial cells and are classified as bronchioloalveolar carcinomas (BAC). Noguchi types A and B are considered noninvansive carcinomas *in situ* (CIS) and should be diagnosed as a primary cancers, and not metastatic cancers.

In patients with multiple lung adenocarcinomas, discrimination between multicentric lung adenocarcinoma and intrapulmonary metastasis (IPM) is important for determining treatment strategies although it is sometimes difficult to distinguish between these 2 malignancies (20). The aim of this study was to differentiate multiple primary lung adenocarcinomas (MPLA) from IPM in 34 cases by determining the mutational status of the *EGFR* and *K-ras* genes and by histological classification into Noguchi types A and B.

3. MATERIALS AND METHODS

3.1. Patients

A total of 944 patients with primary lung cancer who had undergone surgical resection between April 1994 and March 2007 at the University of Occupational and Environmental Health, Kitakyushu, Japan were included in the study. Of the 944 patients with primary lung cancer, 42 (4.4%) were diagnosed as synchronous or metachronous clinical multiple primary lung adenocarcinomas (cMPLA) according to the criteria proposed by Martini and Melamed (21). Eight patients were excluded from this study because the corresponding tumor DNA was unavailable. Finally, 68 adenocarcinoma samples from 34 patients with cMPLA (24 patients: synchronous cMPLA, 10 patients: metachronous cMPLA) were examined for *EGFR* and *K-ras* mutations and evaluated according to the histological classification of Noguchi types A and B.

The characteristics of the patients with cMPLA are shown in Table 1. The patients consisted of 22 men and 12 women, with an average age of 68.3 years (range, 46 to 81 years old). There were 13 never-smokers, and 21 ever-smokers, including 7 former and 14 current smokers and smoking status was evaluated as pack-years (PY; pack/day × years). Among the 24 synchronous tumors, a tumor larger than the other tumors in the greatest dimension was defined as the first tumor. Ten patients were metachronous with a median interval of 59.6 months (range, 26.3 to 144.6 months). The tumor stage was classified according to the Revisions in the International System for Staging Lung Cancer (22). All of the second tumors were evaluated as stage IA. The study was approved by the institutional review board and informed consent for the use of the tumor specimens was obtained from all the patients.

3.2. Histological classification of tissues into Noguchi types A and B

All tissues were formalin-fixed and processed in the same manner according to standard histological practices. A 3 μm thick formalin-fixed paraffin-embedded tissue section was prepared from each of the 68 tumors from 34 patients. All of the sections were stained with hematoxylin and eosin and then examined by light microscopy. Each specimen was diagnosed histologically, using World Health Organization criteria (2) and the Noguchi classification (19). Pulmonary adenocarcinomas 20 mm in diameter or less are defined in the Noguchi classification as follows. Type A: Localized bronchioloalveolar carcinoma (Figure 1), Type B: Localized bronchioloalveolar carcinoma with foci of alveolar structure collapse, Type C: Localized bronchioloalveolar carcinoma with foci of active fibroblastic proliferation, Type D: Poorly differentiated adenocarcinoma, Type E: Tubular adenocarcinoma, Type F: Papillary adenocarcinoma with a compressive growth pattern (Figure 2).

3.3. Detection of *EGFR* and *K-ras* mutations

All tissues were formalin-fixed and processed in a similar manner according to standard histological practices. Mutations in the *EGFR* and *K−ras* genes were examined in 2 cancer tissue specimens from each of the 34 patients. Formalin-fixed paraffin-embedded tissue sections of 3 μm were prepared from 68 samples obtained from 34 patients. The relevant areas of adenocarcinoma were precisely microdissected from hematoxylin and eosin-stained slides of paraffin-embedded sections. Genomic DNA was extracted from microdissected adenocarcinoma cells using a QIAamp DNA Micro Kit (QIAGEN, Valencia, CA). Mutational analysis of the exon 19 deletion and exon 21 L858R point mutation of the *EGFR* gene was performed as previously described (23). As shown in Figure 3, both mutations were easily detectable. A modification of the designed RFLP method using nested PCR reactions was used to detect point mutations in codon 12 of the *K-ras* gene (13, 24).

3.4. Statistical analysis

Surgical curves were plotted according to the Kaplan-Meier method, and differences between the curves were analyzed by the log-rank test. A statistical difference was considered to be significant if the P value was less than 0.05. All data were analyzed with the use of Abacus Concepts, Survival Tools for StatView (Abacus Concepts, Inc., Berkeley, CA).

Table 1. Characteristics of 34 patients with clinical multiple primary lung adenocarcinomas

Factors	Number of cases	Age	Total
Gender			
Male	22		
Female	12		
Age (range)		68.3 (46-81)	
Smoking status		Smoking index	
Never smoker	13	0.0 PY[1]	
Smoker	21	40.2 PY	
Former	7	30.7 PY	
Current	14	45.0 PY	
Multiple status		Age (range)	
Synchronous	24	69.6 (52-77)	
Metachronous	10	62.4 (46-77): first tumor	
		67.5 (50-81): second tumor	
Pathological stage			
Synchronous	first tumor	second tumor	
IA	13	24	
IB	3	0	
IIA/B	2	0	
IIIA	2	0	
IIIB	3	0	
IV	1	0	
Metachronous	first tumor	second tumor	
IA	6	10	
IB	1	0	
IIIA	2	0	
IIIB	1	0	
Mutations	1st tumor (n=34)	2nd tumor (n=34)	Total (n=68)
EGFR	13 (38.2%)	12 (35.3%)	25 (36.7%)
K-ras codon12	2 (5.9%)	2 (5.9%)	4 (5.9%)

PY[1]: pack-years

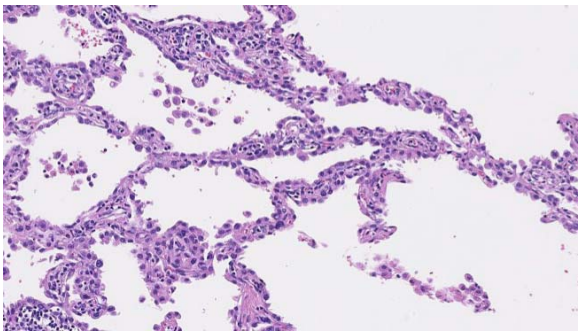

Figure 1. Histology of Noguchi type A reveals bronchioloalveolar carcinoma. A replacement growth pattern is shown (H and E, ×100).

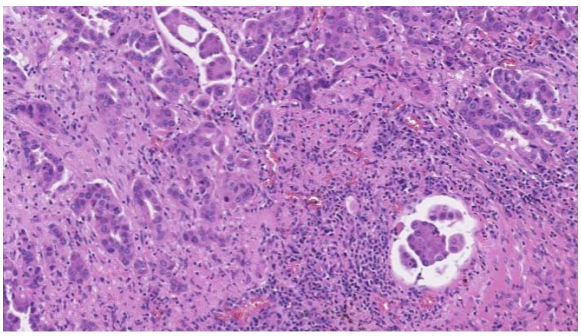

Figure 2. Histology of Noguchi type F reveals papillary adenocarcinoma. The tumor shows papillary structures and expansive and compressive growth (H and E, ×100). Note that there is no area of replacement-type growth.

4. RESULTS

4.1. Morphological analysis based on histological classification into Noguchi types A and B

The tumor sizes ranged from 3 to 68 mm in diameter. Tumors measuring 2 cm or less in their greatest dimension were observed in 50 cases. Seventeen tumors were classified as Noguchi's type A or B (Table 2). Three tumors were identified as Noguchi type A and 7 tumors were designated as Noguchi type B among the first tumor. Three tumors were determined to be Noguchi type A and 4 tumors as Noguchi type B among the second tumors. One patient was diagnosed as Noguchi type A in both the first and the second tumors. Fifteen patients were diagnosed as Noguchi type A or B either in the first tumor or in the second tumor. These 16 (47.1%) patients were diagnosed as having biological multiple primary lung adenocarcinomas (bMPLA), because Noguchi types A and B tumors were considered as non-invasive CIS.

4.2. *EGFR* and *K-ras* mutations in the first and the second tumors

EGFR mutations were detected in 25 of 68 tumors (36.7%) from 34 patients. The *EGFR* mutations were detected in 13 of 34 first tumors (38.2%) and in 12 of 34 second tumors (35.3%) (Table 1). An L858R mutation in *EGFR* was detected in both the first and the second tumors in 4 patients. A deletion (2236-2250) in exon19 of *EGFR* in both the first and the second tumors was detected in 2 patients (Table 3). The *EGFR* mutations detected in the second tumor were the same as the *EGFR* mutations of the first tumor in these 6 patients. The *EGFR* genotypes of the

Table 2. Morphological analyses in the first and the second tumors, and *EGFR* and *K-ras* mutations

Noguchi classification in tumor 2cm or less in diameter	Total	1st tumor	2nd tumor	Number of tumor with EGFR mutation (1st/ 2nd tumor)	%	Number of tumor with K-ras mutation (1st/ 2nd tumor)	%
Type A and B							
Type A	6	3	3	2 (2/0)	33.3	0 (0/0)	0
Type B	11	7	4	4 (2/2)	36.3	1 (1/0)	9.1
Other types							
Type C	16	5	11	7 (3/4)	43.7	1 (0/1)	6.2
Type D	5	5	0	2 (2/0)	40	1 (1/0)	20
Type E	2	2	0	0 (0/0)	0	0 (0/0)	0
Type F	10	7	3	1 (0/1)	10	0 (0/0)	0

Table 3. *EGFR* and *K-ras* mutations in clinical multiple primary lung adenocarcinomas

Mutations		Number of patients			
1st tumor	2nd tumor	Total		Synchronous	Metachronous
The mutations of the second tumor were detected as the same mutations of the first tumor.		7		6	1
EGFR 21 L858R	*EGFR* 21 L858R		4	3	1
EGFR 19 del 2236-2250 (E746-A750)	*EGFR* 19 del 2236-2250 (E746-A750)		2	2	0
K-ras codon 12 mutation	*K-ras* codon 12 mutation		1	1	0
The genotypes of the second tumor were detected as the different genotypes of the first tumor.		11		6	5
EGFR 19 del 2236-2250 (E746-A750)	*EGFR* 19 del 2235-2249 (E746-A750)		1	0	1
EGFR 19 del 2235-2249 (E746-A750)	*EGFR* 19 del 2239-2262 (L747-K754)		1	1	0
EGFR 21 L858R	*EGFR* 19 del 2240-2254 (L747-T751)		1	0	1
EGFR 21 L858R	*EGFR* 19 del 2239-2256 (L747-S752)		1	0	1
EGFR 19 del 2238-2255 (E747-S752)	Wild-type		1	0	1
EGFR 19 del 2235-2249 (E746-A750)	Wild-type		1	1	0
EGFR 21 L858R	Wild-type		1	1	0
Wild-type	*EGFR* 19 del 2238-2258 (L747-P753)		1	0	1
Wild-type	*EGFR* 21 L858R		1	1	0
(Total of different *EGFR* gene status)			(9)	(4)	(5)
K-ras codon 12 mutation	Wild-type		1	1	0
Wild-type	*K-ras* codon 12 mutation		1	1	0
(Total of different *K-ras* gene status)			(2)	(2)	0
Patients without mutation		16		12	4

second tumor were different from the *EGFR* genotypes of the first tumor in 9 patients (Table 3).

K-ras mutations were detected in 4 out of 68 tumors (5.9%) in 3 patients (Table 1). The *K-ras* mutation detected in the second tumor was the same as the *K-ras* mutation of the first tumor in 1 patient. The codon 12 mutation of *K-ras* was detected in the first tumor but not in the second tumor in 1 patient. The codon 12 mutation of *K-ras* was not detected in the first tumor, but was detected in the second tumor in 1 patient (Table 3).

4.3. Combined analyses by mutational status of *EGFR* and *K-ras* genes and according to Noguchi types A and B

EGFR or *K-ras* mutations were detected in 18 patients. The genotypes of the second tumor were different from the genotypes of the first tumor in 11 of 18 patients and these 11 patients were confirmed to have bMPLA (Table 3 and Figure 4). The mutations of the second tumor were the same as the mutations of the first tumor in 7 patients (Table 3 and Figure 4). Patients were confirmed to have bMPLA by histological classification according to Noguchi types A and B in 4 of the 7 patients (Figure 4). On the other hand, there were no mutations in *EGFR* or *K-ras* in 16 patients. A total of 8 out of 16 patients were confirmed to have bMPLA based on histological classification into Noguchi types A and B (Figure 4). In total, 23 of 34 (67.6%) patients were confirmed to have bMPLA through combined analysis by mutational status of *EGFR* and *K-ras* genes and by Noguchi types A and B classification. Another 11 cases were confirmed as biological intrapulmonary metastasis (bIPM). The 5-year survival rate for cMPLA was 81.7% (Figure 5A). The 5-year survival rates in the case of bMPLA and bIPM were 90.9% and 63.6%, respectively (P = 0.04, Figure 5A). The 5-year survival rates for bMPLA with a different genotype (n = 11) and bMPLA with Noguchi's A or B at least in 1 tumor (n = 12) were 90.0% and 91.7%, respectively (P = 0.58, Figure 5B).

5. DISCUSSION

The incidence of clinical multiple primary lung cancers (cMPLC) has been reported to be 4% (25). Retrospective data have shown an increased risk of developing a second lung cancer following the diagnosis of the first lung neoplasm: patients with NSCLC have a 1–2% per year risk of developing a second primary lesion (7, 26).

With the progress of imaging diagnostic technology, the

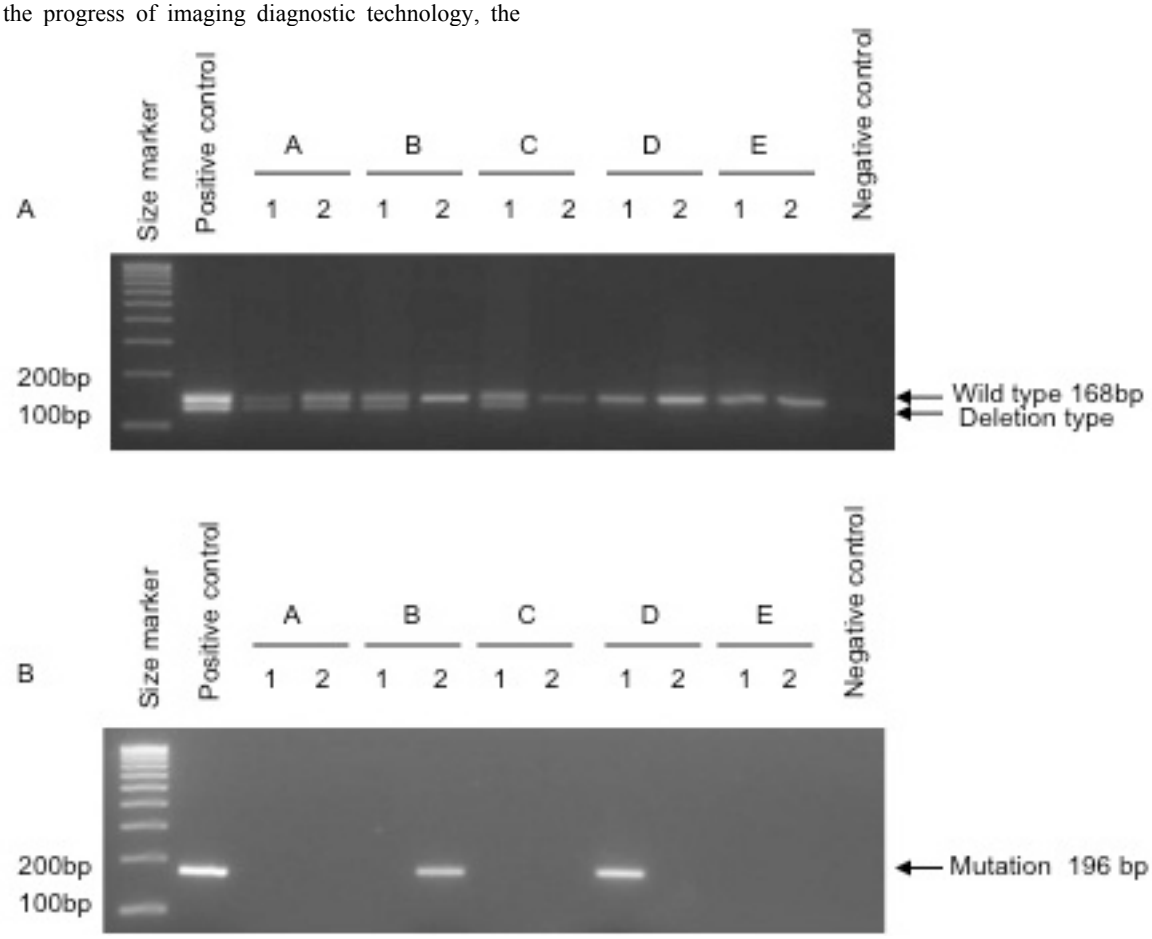

Figure 3. PCR analysis of representative cases with *EGFR* exon 19 deletion (upper panel) and L858R of exon 21 (lower panel). Patient A had a deletion in exon 19 in both the first and the second tumors. Patient B had a deletion in exon 19 in the first tumor and a L858R in the second tumor. Patient C had a deletion in exon 19 in the first tumor and a wild type in the second tumor. Patient D had a L858R in the first tumor and a wild type in the second tumor. Patient E had a wild type in exons 19 and 21 in both tumors. Positive control: sample with 15bp deletion in exon 19, and sample with L858R. Negative control: no DNA in the PCR analysis.

detection of a synchronous combination of well differentiated adenocarcinomas in cMPLA is increasing (6). Lung cancers of the same pathological type are found in 50–68% of patients with cMPLC (27-30), while multiple adenocarcinomas of the same subtype are present in 61–68% of patients with cMPLC (30, 31), suggesting that the majority of cMPLAs have the same etiology.

Mutations in the *EGFR* kinase domain are thought to be an early event in the development of adenocarcinoma that is generally unrelated to smoking (32, 33), while *K-ras* mutations may occur relatively early in the development of smoking-related adenocarcinoma (13, 34). Therefore, these 2 mutations are useful biomarkers to identify different etiologies in lung adenocarcinoma. *EGFR* and *K-ras* mutations were mutually exclusive in this study, as previously reported in several studies (23, 35-37). The KRAS-MAPK pathway is 1 of the EGFR downstream signaling pathways, indicating that at least 1 activating mutation in the EGFR-KRAS-MAPK signaling pathway may be sufficient for the carcinogenesis of lung adenocarcinoma.

Patients who have been clinically diagnosed with multiple primary lung cancers sometimes show poor 5-year survival rates (38). These results suggest that some of the cMPLCs might be metastatic lesions (bIPM). In addition, bMPLC and bIPM may show different biological behaviors. Because some of the patients with clinical intrapulmonary metastasis (cIPM) show poor prognosis, it is necessary to discriminate bIPM from cMPLC (39). The distinction between cMPLC and cIPM is often difficult because these 2 malignancies can show the same histological type. A multiple gene analysis has been proposed to identify the clonality in a combination of mutations in genes such as *p53*, *K-ras* and chromosomal LOH (40-42). Ono *et al.* showed that the protein expression profile of cancer-related genes (*p53*, *p16*, *p27*, and *c-erbB2*) is a useful tool to distinguish between bMPLA and bIPM and to determine the appropriate biological staging of lung cancer (39). The criteria for the molecular diagnosis of

bMPLC include a different type of mutation in the first and the second tumor, and a somatic mutation of a particular gene only in the first tumor. However, it is difficult to make

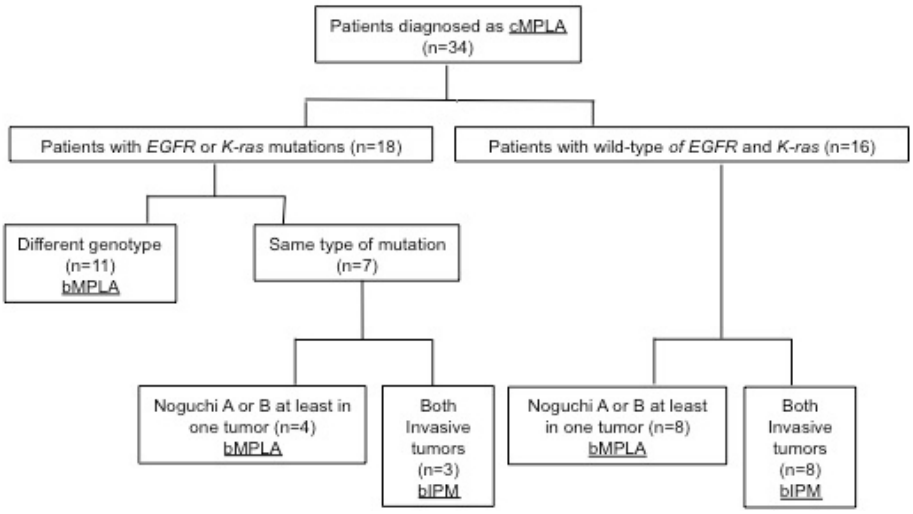

Figure 4. Flow chart for the determination of biological multiple primary lung adenocarcinomas (bMPLA) and biological intrapulmonary metastasis (bIPM). Clinical MPLA were divided into bMPLA and bIPM based on combined analyses by mutational status of *EGFR* and *K-ras* genes and by histologic classification of Noguchi type A and B. In total, 23 of 34 cMPLA patients (67.6%) were diagnosed as bMPLA.

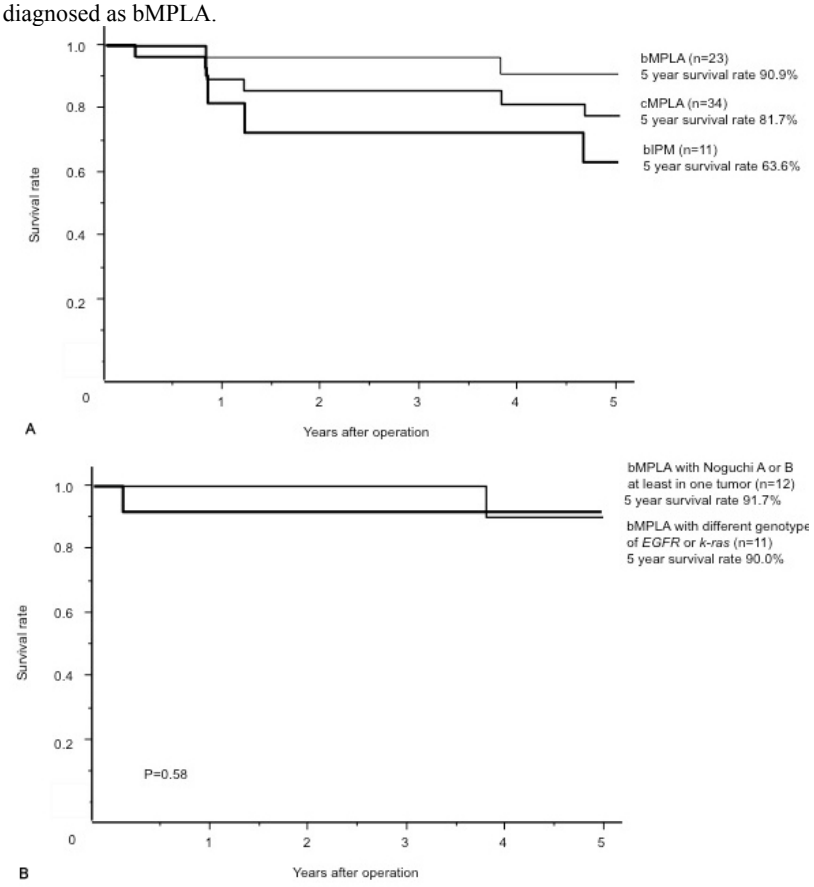

Figure 5. A. Five-year survival by subgroup. Five-year survivals of 34 clinical multiple primary lung adenocarcinoma (cMPLA), 23 biological multiple primary lung adenocarcinomas (bMPLA), and 11 biological intrapulmonary metastasis (bIPM) patients are shown by Kaplan-Meier curves. A significant difference was shown between these 2 groups (p=0.04). B. Five-year survival of

biological multiple primary lung adenocarcinomas (bMPLA). Comparison of survival rates between bMPLA with Noguchi A or B at least in one tumor (n=12) and bMPLA with different genotype of *EGFR* or *K-ras* (n=11) is shown by Kaplan-Meier curves. No significant difference was shown between these 2 groups (p=0.58).

a diagnosis of bMPLC when the first tumor shows a wild-type phenotype and the second tumor shows a mutated-type. In such a case, the second tumor may acquire the mutation during the metastatic process, or it may be ascribed to clonal expansion from a minor clone in the first tumor. Although *EGFR* and *K-ras* mutations occurred early in the process of carcinogenesis in the cases included in the present study, the genotypes of the second tumor were different from those of the first tumor in 11 of 18 patients and these 11 patients were confirmed to have bMPLA. The 5-year survival rates for bMPLA and bIPM were 90.9% and 63.6%, respectively, which was considered a statistically significant difference between these 2 groups (P = 0.04). One of the factors that may explain why bMPLA showed a better prognosis is the Noguchi type, i.e., types A or B are thought to show a good prognosis (19). In particular, the 5-year survival rate for 4 bMPLA patients who had different genotypes and Noguchi type A or B at least in 1 tumor was 100.0%.

Small peripheral adenocarcinomas measuring 2 cm or less in their greatest dimension were histologically classified into 6 types (Noguchi types A–F) on the basis of tumor growth patterns. Noguchi types A and B were considered noninvasive CIS, which are diagnosed as primary cancers. In total, 23 of 34 (67.6%) patients were confirmed as bMPLA by combined analysis of mutational status of *EGFR* and *K-ras* genes and by histological classification into Noguchi types A and B.

Among 18 patients with *EGFR* or *K-ras* mutations, 6 of 12 (50%) synchronous tumors exhibited the same mutation in *EGFR* or *K-ras* and 1 of 6 (17%) metachronous tumors exhibited the same mutation in *EGFR* or *K-ras*. These results suggest that the same mutation in *EGFR* or *K-ras* might be triggered by the same carcinogens as field cancerization (43).

The molecular mechanism underlying the development of lung adenocarcinoma with *EGFR* mutations is still poorly understood. *EGFR* mutations do not coexist with the G-to-T transversion in *p53* and *K-ras* mutations that are related with tobacco smoke. *EGFR* mutations may not be associated with tobacco smoke. Matsuo *et al.* showed that female gender was a risk factor for lung adenocarcinoma with an *EGFR* mutation (33). Recently, we demonstrated an association between estrogen receptor (ER) expression and *EGFR* mutations in lung adenocarcinoma, i.e. strong ER beta expression was more frequently detected in adenocarcinomas with *EGFR* mutations (44). These data may suggest that endogenous gonadal hormones could possibly induce a field cancerization effect in lung adenocarcinomas. In conclusion, the genetic analyses of *EGFR* and *K-ras* genes and Noguchi's histological classification are useful in to distinguishing between bMPLA and bIPM among cMPLAs.

6. ACKNOWLEDGMENT

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Abbreviations: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; MPLC, multiple primary lung cancers; EGFR, epidermal growth factor receptor; BAC, bronchioloalveolar carcinoma; CIS, carcinoma *in situ*; IPM, intrapulmonary metastasis; MPLA, multiple primary lung adenocarcinomas; cMPLA, clinical multiple primary lung adenocarcinomas; PY, pack-years; bMPLA, biological multiple primary lung adenocarcinomas; bIPM, biological intrapulmonary metastasis; cMPLC, clinical multiple primary lung cancers; cIPM, clinical intrapulmonary metastasis

Key Words: Multiple primary lung cancers, Multiple primary lung adenocarcinomas, Intrapulmonary metastasis, *EGFR*, *K-ras*, Bronchioloalveolar carcinoma, biological multiple primary lung adenocarcinoma, biological intrapulmonary metastasis, Noguchi classification

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