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Genetic alterations in lung adenocarcinoma with a micropapillary component

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Abstract. Pulmonary adenocarcinoma (PA) with a micropapillary component (PA-MPC) is known as an aggressive subtype of PA. The molecular profiles of PA-MPC have not been well characterized. The pathological reports of patients who underwent surgical resection for lung cancer between April, 2004 and May, 2012 were reviewed. Of the 674 patients diagnosed with PA, 28 were found to have MPC. A total of 138 resected PAs without MPC were selected in the same period to serve as age-, gender- and smoking status-matched controls to the PA-MPC group. Mutational status was determined by the following two methods: SNaPshot assay based on multiplex polymerase chain reaction (PCR), primer extension and capillary electrophoresis that was designed to assess 38 somatic mutations in 8 genes [*AKT1, BRAF,* endothelial growth factor receptor (*EGFR*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), mitogen-activated protein kinase kinase 1, neuroblastoma RAS viral oncogene homolog, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α (*PIK3CA*) and phosphatase and tensin homolog]; and a PCR-based sizing assay that assesses *EGFR* exon 19 (deletions), *EGFR* exon 20 (insertions) and human epidermal growth factor receptor 2 exon 20 (insertions). Echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase fusion gene (*EML4-ALK*) was screened by ALK immunohistochemistry and confirmed using the reverse transcription PCR assay and the break-apart fluorescence *in situ* hybridization assay. Regarding genetic alterations, 13 (46.4%) of the 28 PA-MPCs harbored mutually exclusive mutations: 9 (32.1%) *EGFR* mutations, 1 (3.6%) *KRAS* mutation and 3 (10.7%) *EML4-ALK* fusion genes. PAs without MPC harbored 42 (30.4%) *EGFR* mutations, 17 (12.3%) *KRAS* mutations, 3 (2.2%) *EML4-ALK* fusion genes and 1 (0.7%) *PIK3CA* mutation. *EML4-ALK* fusion genes appeared to occur significantly more frequently in PA-MPCs compared with PAs without MPC (P=0.027). Although the sample size was small, our study suggests that the molecular pathogenesis of PA-MPC may be different from that of other adenocarcinomas.

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Key words: micropapillary adenocarcinoma, lung cancer, endothelial growth factor receptor gene, Kirsten rat sarcoma viral oncogene homolog, echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase fusion gene

Introduction

Lung cancer is one of the most refractory malignancies and the leading cause of cancer-related mortality worldwide (1-3). Lung cancer is mainly classified into two categories, small-cell lung cancer (SCLC) and non-SCLC (NSCLC). Recent advances in lung cancer research have identified several novel therapeutic agents, such as pemetrexed and bevacizumab (4), which target non-squamous cell carcinomas, i.e., mainly adenocarcinomas; thus, an accurate subclassification of NSCLC is required. Furthermore, the use of molecular-targeted agents, such as gefitinib and erlotinib, necessitated the subclassification of adenocarcinomas from the aspect of molecular characteristics (5,6). Thus, the existing classifications of the World Health Organization (WHO) in 2004 required a revision. The new international, multidisciplinary classification of lung adenocarcinoma, was proposed by the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATC) and the European Respiratory Society (ERS) (7). In this classification, pulmonary adenocarcinoma (PA) with a micropapillary component (PA-MPC) was recommended as a new subtype of PA in addition to the lepidic, acinar, papillary and solid subtypes defined in the 2004 WHO classification (8). MPC was defined as tumor cells growing in papillary tufts lacking fibrovascular cores that may float within alveolar spaces (Fig. 1) (9,10). PA-MPC has been associated with an aggressive clinical course compared with traditional papillary adenocarcinoma and bronchioloalveolar carcinoma (9,11-13). PA-MPC is frequently encountered in non-smokers, with intralobar satellites, and frequently metastasizes to the contralateral lung, mediastinal lymph nodes, bone and adrenal

glands, with a high mortality rate (11-14). Although PA-MPC represents a unique form of PA, its molecular profile is yet to be elucidated. In the present study, PA-MPC was analyzed for the common genetic mutations in PA, including endothelial growth factor receptor gene (*EGFR*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase fusion gene (*EML4-ALK*) to determine whether a distinct genetic profile was associated with this histopathological growth pattern.

Patients and methods

Patients. The pathological reports of patients who underwent surgical resection for lung cancer between April, 2004 and May, 2012 at the Okayama University Hospital (Okayama, Japan) were reviewed. Of the 674 patients diagnosed with PA, 28 were found to have MPC. The ratio of MPC varied widely (3-80%) among these 28 patients. A total of 138 resected PAs without MPC were randomly selected in the same period to serve as age-, gender- and smoking status-matched controls to the PA-MPC cases (Table I). Our Institutional Review Board approved this study's protocol and informed consent was obtained from all the patients.

DNA and RNA extraction. Genomic DNA was obtained from primary tumors by standard phenol-chloroform (1:1) extraction followed by ethanol precipitation, or by using the DNeasy Tissue kit (Qiagen, Valencia, CA, USA). Total RNA was extracted from primary tumors using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Oligo(dT)-primed cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) with DNase treatment.

Genotype screening. Using DNA derived from frozen tumor specimens, genotyping was performed by SNaPshot, a targeted mutational analysis assay designed by Su *et al* (15). The platform involves two methods: a screen (SNaPshot) based on multiplex polymerase chain reaction (PCR), primer extension and capillary electrophoresis that was designed to assess 38 somatic mutations in 8 genes [(*AKT1*, *BRAF*, *EGFR*, *KRAS*, mitogen-activated protein kinase kinase 1 (*MEK1*), neuroblastoma RAS viral oncogene homolog (*NRAS*), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α (*PIK3CA*) and phosphatase and tensin homolog (*PTEN*)]; and a PCR-based sizing assay that assesses *EGFR* exon 19 (deletions), *EGFR* exon 20 (insertions) and human epidermal growth factor receptor 2 (*HER2*) exon 20 (insertions).

Detection of EGFR and KRAS mutations. PCR-based assays or direct sequencing were performed to confirm the results if mutations of *EGFR* and *KRAS* were detected by the SNaPshot assay.

The *EGFR* mutational status was determined using a PCR-based length polymorphism and restriction fragment length polymorphism assay, as previously reported (16). Briefly, the common deletions of exon 19 were distinguished from the wild-type based on PCR product length polymorphisms using 12% polyacrylamide gel electrophoresis (PAGE) via ethidium bromide staining. For the exon 21 *L858R* mutation, *Sau*96I digestion, which specifically digests the mutant type, was performed prior to 12% PAGE.

The *KRAS* mutations in codons 12 and 13 were examined using PCR-based direct sequencing on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems), as previously reported (17-19).

Detection of EML4-ALK fusion events. *EML4-ALK* fusion was screened by ALK immunohistochemistry (IHC) and confirmed by reverse transcription (RT)-PCR assay and fluorescence *in situ* hybridization (FISH) analysis.

ALK IHC. Unstained paraffin-embedded sections were deparaffinised in xylene, hydrated through, and rinsed in distilled water. Heat-induced epitope retrieval was performed with EnVision FLEX Target Retrieval Solution, High pH (Dako, Carpinteria, California, USA). The slides were then incubated at room temperature with mouse anti-ALK monoclonal antibody (clone 5A4; dilution, 1:100; cat. no. ab17127; Abcam) for 30 min. The slides were incubated at room temperature with EnVision FLEX+Mouse Linker (Dako) for 15 min. The immune complexes were then detected with the dextran polymer reagent (Fig. 2A) (20,21).

RT-PCR. The primers used to identify the *EML4-ALK* fusion transcript were selected to enable the detection of all possible in-frame fusions of *EML4* to exon 20 of *ALK*, in which the kinase domain of *ALK* would be preserved. The forward primers used were *EML4* 72F (5'-GTCAGCTCTTGAGTC ACGAGTT-3') and fusion-RT-S (5'-GTGCAGTGTTTAGCA TTCTTGGGG-3'); the reverse primer was *ALK* 3078RR (5'-ATCCAGTTCGTCCTGTTCAGAGC-3') (22). PCR was performed for EML4-ALK under the following conditions: 94˚C for 10 min, followed by 35 cycles of denaturation at 94˚C for 1 min, annealing at 64˚C for 1 min and polymerization at 72˚C for 1 min, with a final extension step at 72˚C for 7 min. RT-PCR for GAPDH expression as an internal control was performed under the same conditions in each tumor sample.

FISH. FISH was performed on formalin-fixed, paraffin-embedded tumor tissues using a break-apart probe to the *ALK* gene (Vysis LSI ALK Dual Color, Break Apart Rearrangement Probe; Abbott Molecular, Abbott Park, IL, USA) as per the manufacturer's instructions. Cases were defined as FISH-positive when there was a >15% split signal in the tumor cells (Fig. 2B) (23,24).

Statistical analysis. Differences in statistical significance among the categorized groups were compared using the Chi-square test or the Student's t-test. An analysis of overall survival and disease-free survival was performed using the Kaplan-Meier method with the log-rank test. The data were analyzed using SPSS v22.0 software (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference for each analysis.

Results

Genetic alterations in clinical samples. Regarding genetic alterations, 13 (46.4%) of the 28 PA-MPCs harbored mutually

Table I. Patient characteristics.

Characteristics	MPC Positive (n=28)	MPC Negative (n=138)	P-value
Age (years)	65.3±11.0	66.0±9.8	NS
Gender			NS
Male	21	101	
Female	7	37	
Smoking history			NS
Smoker	20	100	
Non-smoker	8	38	
Pathological stage			<0.001[a]
I	13	104	
II	7	12	
III	41	8	
IV	4	4	

[a]Comparing pathological stage I with stages II-IV. MPC, micropapillary component; NS, non-significant.

Table II. Association between MPC and genetic alterations.

Genes	MPC, patient no. (%) Positive (n=28)	MPC, patient no. (%) Negative (n=138)	P-value
EGFR	9 (32.1)	42 (30.4)	0.9
KRAS	1 (3.6)	17 (12.3)	0.2
EML4-ALK	3 (10.7)	3 (2.2)	0.027

MPC, micropapillary component; *EGFR*, endothelial growth factor receptor gene; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *EML4-ALK*, echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase fusion gene.

exclusive mutations: 9 (32.1%) *EGFR* mutations, 1 (3.6%) *KRAS* mutation and 3 (10.7%) *EML4-ALK* fusion genes. PAs without MPC harbored 42 (30.4%) *EGFR* mutations, 17 (12.3%) *KRAS* mutations, 1 (0.7%) *PIK3CA* mutation and 3 (2.2%) *EML4-ALK* fusion genes in a mutually exclusive manner, except that 1 case had *EGFR* G719A and L861Q mutations. There were no mutations in the *AKT1*, *BRAF*, *MEK1*, *NRAS*, *PTEN* or *HER2* genes in either group. *EML4-ALK* fusion genes appeared to occur significantly more frequently in PA-MPCs compared with PA without MPC (P=0.027) (Table II). Regarding the *EGFR* and *KRAS* mutational status, the results of the SNaPshot assay were consistent with those of PCR-based assays or direct sequencing.

Effect of PA-MPCs on clinical outcome. To confirm the clinical outcome of PA-MPC, 11 cases with pathological stage IA PA-MPC were compared with 65 cases with pathological stage IA PA without MPC (Table III). As of December, 2013, 13 (17.1%) patients had succumbed to the disease, with a median follow-up period of 62.0 months; 2 (18.2%) of the patients with PA-MPC had succumbed to PA during follow-up; 11 (16.9%) patients with PA without MPC had succumbed (7 to PA and 4 to other causes). The 5-year overall survival rates of all pathological stage IA patients (n=76), patients with PA-MPC (n=11), and patients with PA without MPC (n=65) were 82.0, 87.5 and 81.0%, respectively. A total of 13 (17.1%) patients developed disease relapse (4 patients with PA-MPC and 7 patients with PA without MPC). The 5-year disease-free survival rates of all pathological stage IA patients, patients with PA-MPC, and patients with PA without MPC were 80.7, 58.4 and 84.2%, respectively. Patients with PA-MPC exhibited a significantly poorer disease-free survival rate compared with those with PA without MPC (log-rank test, P=0.04) (Fig. 3).

Table III. Association among MPC, patient characteristics and genetic alterations in pathological stage IA lung adenocarcinoma.

Characteristics	MPC Positive (n=11)	MPC Negative (n=65)	P-value
Age (years)	67.3±9.2	67.2±9.5	NS
Gender			NS
Male	7	44	
Female	4	21	
Smoking history			NS
Smoker	6	42	
Non-smoker	5	23	
Tumor size (cm)	1.8±0.5	1.7±0.7	NS
EGFR mutations			NS
Positive	3	19	
Wild-type	8	46	
KRAS mutations			NS
Positive	0	6	
Wild-type	11	59	
EML4-ALK			NS
Positive	1	1	
Negative	10	64	

Data are presented as number of cases or mean ± standard deviation. MPC, micropapillary component; NS, non-significant; *EGFR*, endothelial growth factor receptor gene; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *EML4-ALK*, echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase fusion gene.

Discussion

The IASLC/ATS/ERS classification is the result of the advances in the research of PA. Although PA-MPC is newly classified in it, its detailed molecular characteristics, including *EML4-ALK* fusion, have not been determined. In this regard,

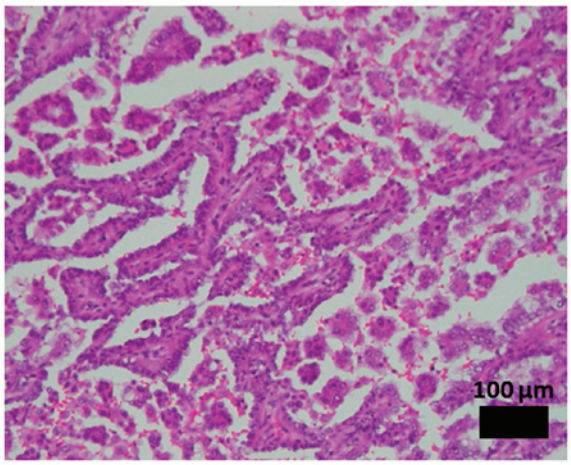

Figure 1. Pulmonary adenocarcinoma with a micropapillary component. Micropapillary growth, in which the papillary tufts lack a central fibrovascular core and extensively shed within the alveolar spaces (hematoxylin & eosin staining).

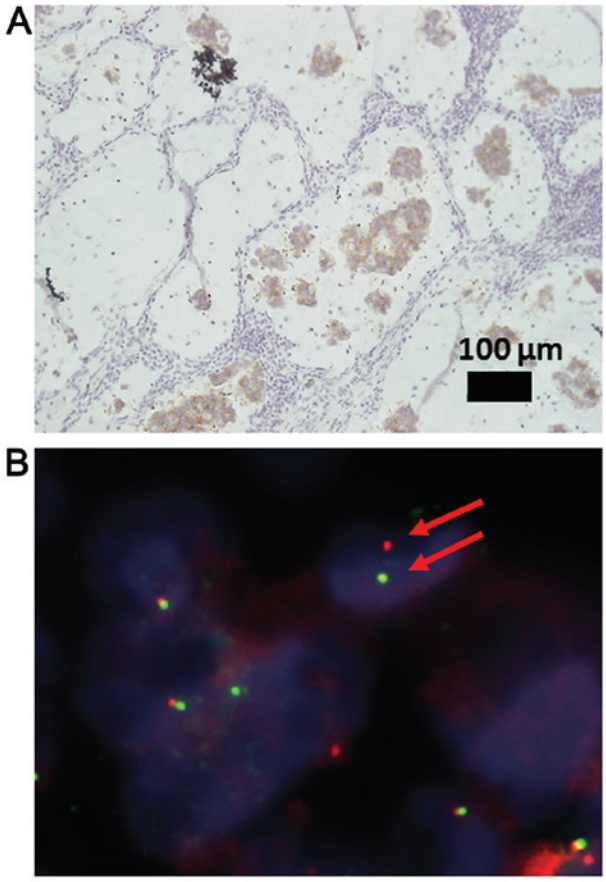

Figure 2. (A) Anaplastic lymphoma kinase (ALK) immunohistochemistry. displaying staining in *ALK*-rearranged lung adenocarcinoma with a micropapillary component. (B) ALK break-apart fluorescence *in situ* hybridization. Arrows mark split green 5' and orange 3' signals, indicating *ALK* rearrangement.

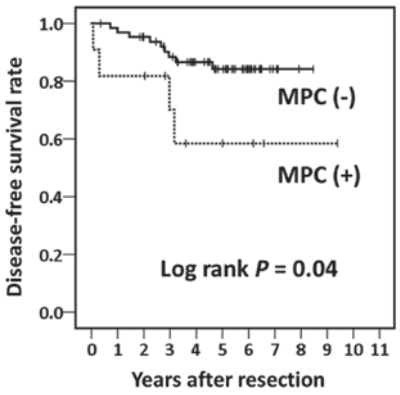

Figure 3. Disease-free survival of pathological stage IA patients with lung adenocarcinoma according to the presence or absence of the micropapillary component (MPC) (n=76). Of note, MPC-positive adenocarcinoma was associated with a significantly poorer prognosis (log-rank test, P=0.04).

this is a unique report describing a comprehensive gene mutational analysis, including *EML4-ALK* fusion gene, in patients with PA-MPC. In the present study, *EML4-ALK* fusion genes appeared to occur significantly more frequently in PA-MPC compared with PA without MPC. According to previous reports, the frequency of *EML4-ALK* fusion genes in PA is low (4.3-6%) (23,25,26). Furthermore, ALK-positive lung cancers tend to be more common among younger patients and marginally more common in women, which may be associated with the difference in smoking rates between the two genders (27). In our study, the 6 ALK-positive patients included 2 smoking men, 1 non-smoking woman with PA-MPC, and 3 non-smoking women with PA without MPC. The ALK-positive patients were younger than ALK-negative patients (56.0 vs. 66.3 years, respectively; P=0.013). Inamura *et al* reported that adenocarcinomas with *EML4-ALK* fusion were predominantly classified as the acinar or papillary subtypes (26). Although our sample size was small, our data suggest that the *EML4-ALK* fusion gene is one of the most common genetic alterations in PA-MPCs.

As regards the association between PA-MPC and gene status, De Oliveira Duarte Achcar *et al* reported the genetic alterations of 15 micropapillary-dominant cases, namely 5 (33%) *KRAS*, 3 (20%) *EGFR* and 3 (20%) *BRAF* mutations (28). A number of studies regarding patients with PA-MPC reported that they often harbored *EGFR* mutations (13,28,29). As previously reported, *EGFR* mutations are common in Asian, female, non-smoker PA patients (30,31) and the frequency of *EGFR* mutations in Japanese PA patients is ~44% (32). Conversely, in our series, the prevalence of *EGFR* mutations in PA was lower (51 of 166, 30.7%) compared with that previously reported, likely because the population of this study included several smokers (120 of 166, 72.3%) and men (122 of 166, 73.5%). In never-smoker patients, the prevalence of the *EGFR* mutation (24 of 46, 52.2%) was similar to our previous report (32).

Similar to previous studies (9,11-13), our data suggest a poorer prognosis for PA-MPC compared with that for PA without MPC. Miyoshi *et al* reported that a higher ratio (6-100%) of MPC was associated with a poorer prognosis compared with a lower ratio (1-5%) (12). The ratio of MPC in the 28 PA-MPC cases varied widely (3-80%) in this study. Our results indicated that PA-MPC had a tendency for relapse, even if the ratio of MPC was low. Thus, clinicians should bear in mind the possibility for metastasis when MPC is present in PA.

In conclusion, our study suggests that the molecular pathogenesis of PA-MPC may differ from that of other adenocarcinomas, which is associated with its aggressive clinical behavior. Further investigation is required to elucidate the characteristics of PA-MPC and lead to the development of new therapeutic strategies.

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