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ORIGINAL ARTICLE

Clinicopathological features of younger (aged ≤ 50 years) lung adenocarcinoma patients harboring the *EML4-ALK* fusion gene

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Abstract

Background: The *EML4-ALK* fusion gene has recently been identified as a driver mutation in a subset of non-small cell lung cancers. In subsequent studies, *EML4-ALK* has been detected in a low percentage of patients, and was associated with a lack of *EGFR* or *KRAS* mutations, younger age, and adenocarcinoma with acinar histology. Cases with the *EML4-ALK* fusion gene were examined to clarify the clinicopathological characteristics of young adenocarcinoma patients.

Methods: Between December 1998 and May 2009, 85 patients aged ≤ 50 with lung adenocarcinoma were treated at our hospital. We examined 49 samples from adenocarcinoma patients who underwent surgical resection, chemotherapy, and/or radiotherapy for the *EML4-ALK* gene. None of the patients received ALK inhibitors because these drugs had not been approved in Japan before 2012. *EML4-ALK* fusion genes were screened using multiplex reverse-transcription PCR assay, and were confirmed by direct sequencing.

Results: The *EML4-ALK* fusion gene was detected in five tumors (10.2%). One patient had stage IB disease, one had stage IIIA, and three had stage IV. Histologically, there was one solid adenocarcinoma, two acinar adenocarcinomas, and two papillary adenocarcinomas. *EML4-ALK* fusion genes were mutually exclusive to *EGFR* and *KRAS* mutations. The five-year survival rate was 59.4% in patients without *EML4-ALK* fusion and was not reached in patients with *EML4-ALK* fusion.

Conclusion: The *EML4-ALK* fusion gene may be a strong oncogene in younger patients with lung adenocarcinoma.

Introduction

Lung cancer is one of the most prevalent cancers worldwide, and the mortality rate is expected to remain very high for several decades. Although a combination of surgery, chemotherapy, and/or radiotherapy can be used to treat non-small cell lung cancer (NSCLC), the prognosis for patients remains dismal. While the histologic subtype is an important factor for choosing between standard cytotoxic chemotherapies, tyrosine kinase-based therapeutics also play a key role, particularly in genetically defined subsets of patients. Following the discovery of activating mutations in *EGFR* associated with sensitivity to EGFR-tyrosine kinase inhibitors (TKIs),[1] therapy with gefitinib, erlotinib, or afatinib has become a first-line treatment for patients with *EGFR* mutations.[2,3]

In 2007, Soda *et al.* identified another type of tyrosine kinase with accelerated activity in a fusion gene formed between *EML4* and *ALK* located within chromosome 2p.[4] Previous studies have reported that 1.6–13.5% of lung tumors harbor *EML4-ALK* fusions.[4–15] Large-scale screening using reverse-transcription (RT)-PCR in 7344 NSCLC specimens showed *EML4-ALK* fusion genes in 200 cases (2.7%), with 94% of such cases involving adenocarcinoma.[8] *ALK* fusion genes, including fusion to *EML4*, *KIF5B*, *TFG*,

and *KLC1*, have been reported to be associated with a history of light/never smoking, young age, lack of *EGFR* or *KRAS* mutations, and adenocarcinoma with an acinar histology.[5–7,11,12]

ALK kinase inhibitors have been developed and are reported to suppress the growth of *EML4-ALK* fusion-positive cells.[4,7] Thus, treatment with ALK inhibitors can be effective for NSCLC patients whose tumors contain an *EML4-ALK* fusion.[16] Clinical trials for *EML4-ALK* positive lung cancer with ALK-TKI crizotinib have demonstrated that TKI treatment is superior to standard chemotherapy in patients with previously untreated advanced NSCLC associated with *ALK* fusion genes.[17]

In this study, we determined the frequency of *EML4-ALK* fusion genes to clarify the clinicopathological characteristics of patients aged ≤ 50 years with lung adenocarcinoma and *EML4-ALK* fusion to identify useful information regarding patient selection for ALK-TKI therapy.

Methods

Patients and sample collection

Between December 1998 and May 2009, 85 patients (male/female: 38/47) aged ≤ 50 were diagnosed with lung adenocarcinoma at the National Kyushu Cancer Center Hospital. We examined 17 frozen and 32 formalin-fixed samples available for RNA analysis (male/female: 23/26) from patients who underwent resection, chemotherapy, or radiotherapy for the presence of the *EML4-ALK* gene. Biopsy specimens were obtained before chemotherapy or radiotherapy. Histological diagnosis of the tumors was based on World Health Organization (WHO) criteria, and tumor node metastasis (TNM) stage was determined according to Union for International Cancer Control TNM criteria version 7. Our institutional review board approved the genetic analyses conducted in the present study. All specimens were subjected to hematoxylin-eosin staining in the Department of Diagnostic Pathology of our hospital. Two board-certified pathologists independently reviewed the slides and made the diagnoses according to the WHO classification of lung tumors.

Nucleic acid extraction

Total RNA was extracted from frozen and formalin-fixed paraffin-embedded (FFPE) tissues using an RNeasy Kit (Qiagen, Valencia, CA, USA). Genomic DNA from frozen tissues was extracted using the phenol-chloroform method. Genomic DNA from FFPE tissues was extracted using TakaRa DEXPAT (TaKaRa Bio.Inc., Kusatsu, Shiga, Japan). Quantification of extracted nucleic acids and measurement of the A260/A280 ratio were performed using an ultraviolet spectrophotometer (DU800, Beckman-Coulter, Tokyo, Japan).

ALK fusion analysis by multiplex reverse transcription-PCR and sequencing

Complementary (c)DNA synthesis from total RNA was performed using random primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). To detect *EML4-ALK* fusion cDNA, multiplex PCR was performed using the Amplitaq Gold DNA 360 Master Mix (Applied Biosystems, Foster City, CA, USA). Primer sets for variants of *EML4-ALK* fusion were used as reported previously.[18,19] Amplification of *EML4-ALK* fusion cDNA was performed for 35 cycles (1 minute at 94°C, 1 minute at 64°C, and 1 minute at 72°C) using the TGRADIENT system (Biometra, East Lyme, CT, USA). GAPDH cDNA was amplified by PCR with the primers 5′-TGTCAGTGGTGGACCTGACC-3′ and 5′-TGAGCTTGACAAAGTGGTCG-3′ using TaKaRa Ex-Taq (TaKaRa Bio. Inc.) Amplification of GAPDH cDNA was performed for 35 cycles (30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 72°C) using the TGRADIENT system (Biometra). Agarose gel electrophoresis was performed to detect PCR products, and the results were observed using Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). PCR products were purified and labeled for sequencing using the BigDye v1.1 kit (Applied Biosystems) according to the manufacturer's protocol. Sequencing was performed using a 310 Genetic Analyzer (Applied Biosystems).

Mutation analysis for *EGFR* and *KRAS* by sequencing

Genomic DNA from each sample was used for sequencing analysis of *EGFR* exons 19 and 21 and *KRAS* exon 1. The sequencing primers used for PCR were: *EGFR* exon 19: 5′-TGGCACCATCTCACAATTGC-3′(forward), 5′-GAAAAG GTGGGCCTGAGGTTC-3′(reverse); *EGFR* exon 21: 5′-CA TGAACTACTTGGAGGACC-3′ (forward), 5′-CAGGAAA ATGCTGGCTGACC-3′ (reverse); and *KRAS* exon 1: 5′-GACTGAATATAAACTTGTGG-3′ (forward) 5′-CTATTG TTGGATCATATTCG-3′ (reverse). Each PCR was run for 35 cycles, and the annealing temperatures were 64°C (*EGFR* exon 19), 60°C (*EGFR* exon 21), and 56°C (*KRAS* exon 1) using TaKaRa Ex-Taq (TaKaRa Bio. Inc.).

Statistical analysis

The overall survival (OS) duration was calculated from the date of initial therapy of the patients. Survival curves were prepared using the Kaplan–Meier method, and comparisons among the survival curves were made using the log-

rank test. The Cox proportional hazards model was used to assess the following factors: age, gender, smoking history, pathology, stage, and *EGFR* and *KRAS* mutation status. Data were considered significant at $P \leq 0.05$.

Results

Identification of the *EML4-ALK* fusion gene

Eighty-five patients aged ≤ 50 with lung adenocarcinomas were treated at our hospital during the study period. We examined 49 samples (17 frozen and 32 formalin-fixed samples) available for RNA analysis for the presence of the *EML4-ALK* fusion gene. Using multiplex RT-PCR and direct sequencing, *EML4-ALK* transcripts were detected in 5 of the 49 tumors (10.2%). Table 1 shows the clinical and pathological profiles of all patients with the *EML4-ALK* fusion gene. There were four cases of variants with fusion points between *EML4* exon 20 and *ALK* exon 20 (variant 2). One tumor involved *EML4* exon 6, which included a splice form that differed by 32 nucleotides from intron 6 of *EML4* (variant 3b).

Clinicopathological characteristics of patients with *EML4-ALK* fusion genes

Table 2 summarizes the clinicopathological characteristics of these patients in relation to *EML4-ALK* status. The five patients with *EML4-ALK* fusion genes included three women and two men, ranging in age from 37 to 50 years. One patient had stage IB disease, one had stage IIIA, and three had stage IV disease with N3 lymph node metastases. The *EML4-ALK* fusion gene in these younger (≤ 50 years) patients with lung adenocarcinoma was associated with higher stage tumors. The *EML4-ALK* fusion gene was mutually exclusive to *EGFR* and *KRAS* mutations. Histologically, there was one solid adenocarcinoma, two acinar adenocarcinomas, and two papillary adenocarcinomas (Fig 1). None of the tumors with *EML4-ALK* fusion genes had a lepidic predominant adenocarcinoma component.

Table 2 Relationship between *EML4-ALK* gene fusion and clinicopathological profiles in younger (≤ 50 years) patients with lung adenocarcinoma

	Total		*EML4-ALK*				
			Positive		Negative		
	(*n* = 49)		(*n* = 5)		(*n* = 44)		
Variable	No.	(%)	No.	(%)	No.	(%)	*P*
Age, years							
Median	48		47		48		0.560
Range	31–50		37–50		31–50		
Gender							
Male	23	(47)	2	(40)	21	(48)	0.743
Female	26	(53)	3	(60)	23	(52)	
Smoking history							
Non-smoker	27	(55)	3	(60)	24	(55)	0.816
Ever smoker	22	(45)	2	(40)	20	(45)	
Pathology							
With lepidic growth	16	(33)	0	(0)	16	(36)	0.100
Without lepidic growth	33	(67)	5	(100)	27	(64)	
Stage							
I	23	(47)	1	(20)	22	(50)	0.002
II	4	(8)	0	(0)	4	(9)	
III	15	(31)	1	(20)	14	(32)	
IV	7	(14)	3	(60)	4	(9)	
EGFR							
Wild type	36	(73)	5	(100)	31	(70)	0.156
Mutation	13	[27]	0	[0]	13	[30]	
KRAS							
Wild type	48	[98]	5	[100]	43	[98]	0.733
Mutation	1	[2]	0	[0]	1	[2]	

Stages I–III versus IV.

Clinical outcome of patients with and without *EML4-ALK*

Of the five patients with *EML4-ALK* fusion genes, three patients with stage IV disease received platinum-based chemotherapy, such as carboplatin + paclitaxel, cisplatin + gemcitabine + vinorelbine, or cisplatin + S1. None of the patients received ALK inhibitors because these drugs were not approved in Japan before 2012. The overall response to

Table 1 Clinicopathological profile of patients with the *EML4-ALK* fusion gene

Case No.	*EML4-ALK*	Gender	Age	Smoking history	T factor	N factor	M factor	Stage	Treatment	Pathology	*EGFR*	*KRAS*
1	Variant 2	F	47	S	4	1	0	III A	C + R, Surg	Acinar	WT	WT
2	Variant 2	F	49	NS	1	3	1	IV	C	Acinar	WT	WT
3	Variant 2	M	37	S	4	3	1	IV	C	Solid	WT	WT
4	Variant 3	M	50	NS	2	0	0	I B	Surg	Papillary	WT	WT
5	Variant 2	F	39	NS	2	3	1	IV	C	Papillary	WT	WT

C, chemotherapy; NS, nonsmoker; R, radiotherapy; S, smoker; Surg, surgery; WT, wild type.

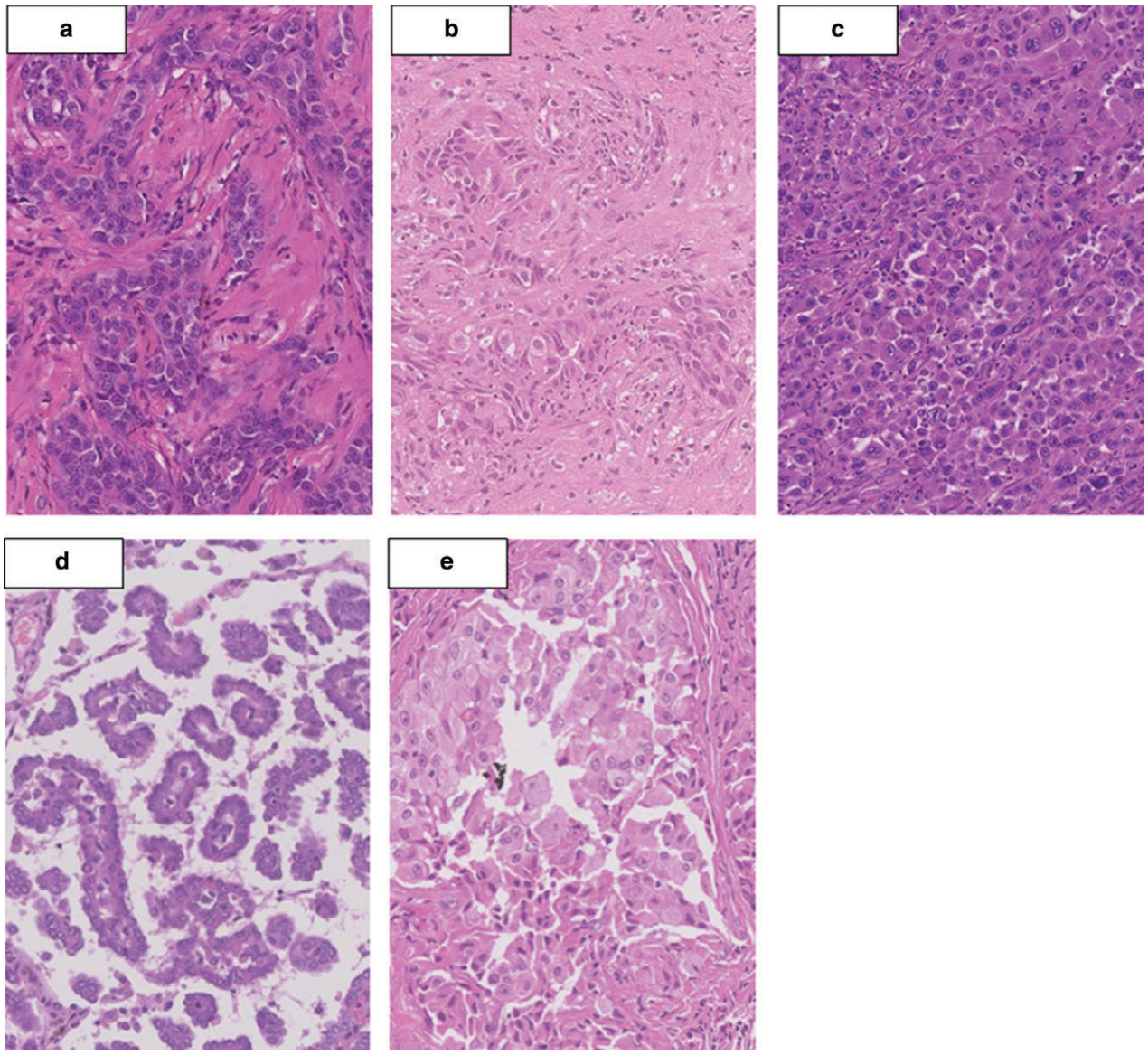

Figure 1 Histopathological results of *EML4-ALK* fusion-harboring tumors: (**a**,**b**) two acinar adenocarcinomas (cases 1 and 2), (**c**) one solid adenocarcinoma (case 3), and (**d**,**e**) two papillary adenocarcinomas (cases 4 and 5).

chemotherapy was progressive disease in two cases (cases 3 and 5), and stable disease in one case (case 2). One patient (case 1) received preoperative chemoradiotherapy with cisplatin + S1, and achieved stable disease. The patient (case 4) with the variant 3-*ALK* fusion received left lower lobectomy. Four months after surgery, multiple pulmonary metastases appeared, however, he was not treated for the recurrence because of his poor performance status.

Overall, the five-year survival rate of the 49 patients was 54.9%. The five-year survival rate was 59.4% in the patients without *EML4-ALK* fusion. In the patients with *EML4-ALK* fusion, the five-year survival rate was not reached, while the one-year survival rate was 60% and the two-year survival rate was 40% (Fig 2). After univariate analysis of eight factors, subgroups consisting of pathological features without lepidic growth, higher stage, and positive status of *EML4-ALK* fusion showed significantly shorter survival, with *P* values of 0.0315, 0.0003, and 0.0037, respectively. Although gender and *EGFR* status were likely to affect survival, no significance was observed in this analysis. Multivariate analysis identified that stage was the only significant prognostic factor, with a hazard ratio of 4.975, and *EML4-ALK* fusion was not identified as significant (Table 3).

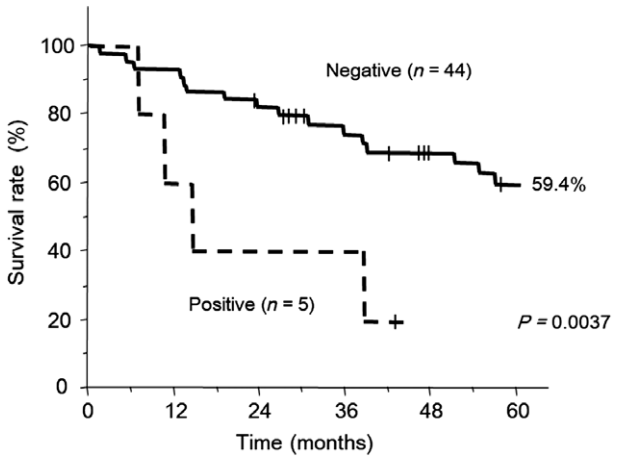

Figure 2 Kaplan–Meier plots of the overall survival of *EML4-ALK*-positive compared to *EML4-ALK*-negative patients. Overall survival was calculated from the date of initial therapy of the patients.

Discussion

It remains controversial whether younger patients with NSCLC have a better or worse prognosis than older patients.[20–23] In the present study conducted to detect *EML4-ALK* fusion genes in 49 samples from patients aged ≤ 50 with lung adenocarcinomas, five adenocarcinomas (10.2%) proved positive for fusion messenger RNA. Previous studies have reported that between 1.6% and 13.5% of lung tumors harbor *EML4-ALK* fusions (Table 4).[4,6,7,9–15] The frequency of *EML4-ALK*-positive patients in our study was very high compared to the results of previous studies. *EML4-ALK* fusions may be more common in younger patients with lung adenocarcinomas. Inamura *et al.* reported that 4 out of 16 patients (25%) aged < 50 had *EML4-ALK* fusions, while seven of 237 patients (3%) aged ≥ 50 had *EML4-ALK* fusions.[5] Shaw *et al.* demonstrated that the median age of NSCLC patients with *EML4-ALK* fusion, *EGFR* mutation, and wild type genes was 52, 66, and 64 years, respectively.[11]

The acinar pattern is reported to be associated with *ALK*-rearranged lung adenocarcinoma in Asian populations,[5,15] whereas the signet-ring cell histology is reported most frequently in Western patients.[11] We previously reported a case of signet ring carcinoma (SRC) of the lung with an *EML4-ALK* fusion gene mimicking mucinous (colloid) adenocarcinoma.[18] Ou *et al.* demonstrated that patients with SRC of the lung were significantly younger than patients with adenocarcinoma, with the proportion of

Table 3 Results of univariate and multivariate analyses of the prognostic factors for overall survival in younger (≤ 50 years) patients with lung adenocarcinoma (*n* = 49)

			Univariate analysis		Multivariate analysis		
Variable	No.	(%)	Five-year survival (%)	*P*	Hazard ratio	95% confidence interval	*P*
Age, years							
≤ 40	7	(14)	42.9				
> 40	42	(86)	56.9	0.4053			
Gender							
Male	23	(47)	39.0				
Female	26	(53)	67.5	0.0777	0.410	0.146–1.157	0.0922
Smoking history							
Non-smoker	27	(55)	62.9				
Ever smoker	22	(45)	45.1	0.1379			
Pathology							
With lepidic growth	16	(33)	76.9				
Without lepidic growth	33	(67)	44.4	0.0315	1.965	0.495–7.813	0.3369
Stage							
I–III	42	(86)	62.1				
IV	7	(14)	14.3	0.0003	4.975	1.534–16.129	0.0075
EML4-ALK fusion							
Negative	44	(90)	59.4				
Positive	5	(10)	NR	0.0037	2.215	0.514–9.537	0.2856
EGFR							
Wild type	36	(73)	46.6				
Mutation	13	(27)	82.1	0.0625	2.058	0.405–0.417	0.3843
KRAS							
Wild type	48	(98)	54.6				
Mutation	1	(2)	NR	NE			

NE, not evaluable; NR, not reached.

Table 4 Studies evaluating the frequency of *EML4-ALK* gene rearrangements in lung cancer

First author	Histological characteristics	Detection method	Population	Total number of patients	Number of *EML4-ALK* positive patients	Percentage
Rodig *et al.*[9]	Adenocarcinoma	IHC, FISH	American (US)	358	20	5.6
Koivunen *et al.*[7]	NSCLC	RT-PCR	American (US) (138), Korean (167)	305	8	2.6
Sequist *et al.*[10]	NSCLC	Multiplex RT-PCR	White (503), Black (7), Asian (22)	546	27	4.9
Shaw *et al.*[11]	Enriched NSCLC	FISH	Non-Asian (132), Asian (9)	141	19	13.5
Soda *et al.*[4]	NSCLC	RT-PCR	Japanese	75	5	6.7
Inamura *et al.*[6]	Adenocarcinoma	RT-PCR	Japanese	149	5	3.4
Takeuchi *et al.*[14]	Adenocarcinoma	Multiplex RT-PCR	Japanese	253	11	4.3
Shinmura *et al.*[12]	NSCLC	RT-PCR	Japanese	77	2	2.6
Takahashi *et al.*[13]	NSCLC	RT-PCR	Japanese	313	5	1.6
Takeuchi *et al.*[15]	Adenocarcinoma	IHC, Multiplex RT-PCR	Japanese	130	7	5.4
Present study	Adenocarcinoma in patients aged ≤50 years	Multiplex RT-PCR	Japanese	49	5	10.2

FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NSCLC, non-small cell lung cancer; RT, reverse-transcription.

patients with SRC < 40 years at 5.0% compared to 1.3% of patients with adenocarcinoma.[24] However, there were no patients with primary SRC of the lung in the present study.

Limited data exist to date on the efficacy of the currently available therapies in patients with *EML4-ALK* NSCLC. In a study by Shaw *et al.*, 12 patients with *ALK* genomic alterations were treated with platinum-based chemotherapy. The response rate, time to progression, and OS were similar to those of NSCLC patients harboring *EGFR* mutations or those that were wild type for both *EML4-ALK* and *EGFR*.[11] Camidge *et al.* demonstrated that *ALK*-positive patients have significantly longer progression-free survival (PFS) on pemetrexed compared to triple-negative (*EGFR*, *KRAS*, *ALK* wild-type) patients, whereas *EGFR* or *KRAS* mutant patients do not.[25] *ALK*-rearranged tumors demonstrate relatively high response rates to single-agent treatment with pemetrexed, with an objective response rate of 29% observed in a phase 3 study of *ALK*-positive patients, compared to ~10% in unselected NSCLC patients.[26] Li *et al.* found that the median thymidylate synthase RNA level, a biomarker of pemetrexed sensitivity, was significantly lower in *ALK*-positive than in *ALK*-negative lung adenocarcinomas.[8] In our study, although three *ALK*-positive patients received platinum-based chemotherapy, all were resistant to the treatment.

The prognosis and natural history of *ALK*-rearrangements in NSCLC have been explored retrospectively. For example, Rodig *et al.* demonstrated that patients with *ALK*-rearranged tumors often present at a higher stage, most commonly stage IV, compared to those with *ALK* germ-line tumors.[9] *ALK*-positive patients have also been reported to have a higher propensity for pericardial and pleural disease than triple-negative patients.[27] Notably, Shaw *et al.* demonstrated one and two-year OS rates of 74% and 54%, respectively, among 82 *ALK*-positive patients treated with crizotinib. In that study, survival of the *ALK*-positive controls did not differ significantly from that of the entire group of 252 wild-type controls, with a median OS duration of 20 versus 15 months.[16]

In our present study, patients with *EML4-ALK* fusion showed significantly shorter survival than those with negative status. The five-year survival rate was 59.4% in patients without the *EML4-ALK* fusion, although there were no five-year survivors with the *EML4-ALK* fusion. However, multivariate analysis identified that *EML4-ALK* fusion was not a prognostic factor in young (≤ 50 years) patients with lung adenocarcinoma in our study, because of the small number of patients with *EML4-ALK* fusions.

Several methods, including PCR, immunohistochemistry, and fluorescence in situ hybridization are currently being evaluated for the detection of *EML4-ALK* NSCLC. In this study, we used the multiplex RT-PCR method for screening because this method can rapidly identify *ALK* rearrangement. Of the five *EML4-ALK* fusion samples, there was one frozen sample from a resected tumor (case 4) and four FFPE samples (cases 1, 2, 3, and 5) from resected tumor and biopsy specimens. The RNA extracted from FFPE is highly degraded, and in general, more difficult to use for PCR relative to fresh-frozen tissue. In case 5, the commercially available chromosomal fluorescence in situ hybridization analysis showed split signals for *ALK*, which confirmed *EML4-ALK* fusion.[28] Immunohistochemical analysis of FFPE tissue specimens remains the mainstay of routine surgical pathology practice. Mino-Kenudson

et al. reported the use of an immunohistochemical test based on novel antibodies with increased sensitivity and specificity for detecting ALK protein expression in FFPE samples.[29] Takeuchi *et al.* developed an intercalated antibody-enhanced polymer method that incorporates an intercalating antibody between the primary antibody to ALK and dextran polymer-based detection reagents.[15] These methods should be used to detect *EML4-ALK* fusion in lung cancer specimens.

Crizotinib is a selective adenosine triphosphate-competitive small molecule oral inhibitor of ALK, c-MET/hepatocyte growth factor receptor, and ROS1 receptor tyrosine kinases. Solomon *et al.* conducted an open-label, phase 3 trial comparing crizotinib with chemotherapy in 343 patients with advanced *ALK*-positive NSCLC who had received no previous systemic treatment. Consequently, crizotinib significantly prolonged PFS compared to the standard chemotherapy regimen, with a median PFS of 10.9 months in the crizotinib versus 7.0 months in the chemotherapy group and a response rate of 74% for crizotinib versus 45% for chemotherapy.[17] To date, second-generation ALK inhibitors, such as ceritinib and alectinib, have been developed, demonstrating significant clinical activity in *ALK*-positive patients with NSCLC.[30–32]

In summary, the results of our study indicate that the *EML4-ALK* fusion gene may be an oncogene in younger patients with lung adenocarcinoma.

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Disclosure

No authors report any conflict of interest.

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