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EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset

Kentaro Inamura[1,4], Kengo Takeuchi[1,4], Yuki Togashi[1], Satoko Hatano[1], Hironori Ninomiya[1], Noriko Motoi[1], Ming-yon Mun[2], Yukinori Sakao[2], Sakae Okumura[2], Ken Nakagawa[2], Manabu Soda[3], Young Lim Choi[3], Hiroyuki Mano[3] and Yuichi Ishikawa[1]

[1]*Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research (JFCR), Koto-ku, Tokyo, Japan;* [2]*Department of Thoracic Surgery, The Cancer Institute Hospital, Japanese Foundation for Cancer Research (JFCR), Koto-ku, Tokyo, Japan and* [3]*Division of Functional Genomics, Jichi Medical University, Tochigi, Japan*

A subset of lung cancers harbors a small inversion within chromosome 2p, giving rise to a transforming fusion gene, *EML4-ALK* (*echinoderm microtubule-associated protein-like 4 gene* and the *anaplastic lymphoma kinase gene*), which encodes an activated tyrosine kinase. We have earlier examined the presence of *EML4-ALK* by multiplex reverse transcription-polymerase chain reaction in 363 specimens of lung cancer, identifying 11 adenocarcinoma cases featuring the fusion gene. In this study, we clinicopathologically examined the characteristics of the *EML4-ALK*-positive cases, including the mutation status of *EGFR*, *KRAS*, and *TP53*, and whether they were of thyroid transcription factor-1 (TTF-1) cell lineage or not. Of 11 patients, 4 (36%) with *EML4-ALK*-positive lung adenocarcinomas who were below 50 years of age were affected by these diseases, as compared with 12 of 242 patients (5.0%) with *EML4-ALK*-negative lung adenocarcinomas ($P=0.00038$). *EML4-ALK*-positive lung adenocarcinomas were characterized by less-differentiated grade ($P=0.0082$) and acinar-predominant structure ($P<0.0001$) in histology. Furthermore, the presence of *EML4-ALK* appears to be mutually exclusive for *EGFR* and *KRAS* mutations ($P=0.00018$), whereas coexisting with *TP53* mutations at a low frequency (1/11 = 9.1%), and correlating with non- or light smoking ($P=0.040$), in line with the TTF-1 immunoreactivity. Thus, *EML4-ALK*-positive tumors may form a distinct entity among lung adenocarcinomas, characterized by young onset, acinar histology, no or rare mutations in *EGFR*, *KRAS*, and *TP53*, and a TTF-1 cell lineage, all in agreement with the prevalence in non- or light smokers.
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Lung cancer is one of the leading causes of cancer death in both men and women worldwide. Activating mutations within *epidermal growth factor receptor* (*EGFR*) have been identified in lung cancers,[1,2] and chemical inhibitors for the kinase activity of EGFR have been found effective in treatment of a subset of lung cancer patients harboring such mutations. Interestingly, the tumors for which EGFR inhibitors are effective develop preferentially in populations of Asian ethnicity and non-smokers, and generally lack *KRAS* mutations.[2–4] Furthermore, such tumors have low rates of smoking-specific *TP53* mutations, such as G/C to T/A transversions.[5,6]

Recently, we have found a novel transforming fusion gene joining the *echinoderm microtubule-associated protein-like 4 gene* (*EML4*) and the *anaplastic lymphoma kinase gene* (*ALK*) in four lung adenocarcinomas and one squamous cell carcinoma.[7] The *EML4-ALK* fusion gene is formed by a small inversion within chromosome 2p. The encoded protein contains the N-terminal part of *EML4* and the intracellular catalytic domain of *ALK*. Replacement of the extracellular and transmembrane domain of ALK with a region of EML4 results in constitutive dimerization of the kinase domain and thereby a consequent increase in its catalytic activity.[7]

Correspondence: Dr Y Ishikawa, Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan.
E-mail: ishikawa@jfcr.or.jp
[4]These authors contributed equally to this work.
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More recently, we have identified novel variants for *EML4-ALK* fusion gene with cDNA screening and multiplex reverse transcription-polymerase chain reaction (RT-PCR), capturing all possible in-frame fusions of *EML4* to exon 20 of *ALK*. By carrying out cDNA screening, we identified variant 3,[8] and using multiplex RT-PCR assays, we identified variants 4 and 5[9] after the first identification of variants 1 and 2. In variant 3, exon 6 of *EML4* is joined to exon 20 of *ALK*. cDNA from variant 4 ligates exon 14 of *EML4* to a position within exon 20 of *ALK*, whereas another cDNA from a variant 5 tumor connects exon 2 of *EML4* to exon 20 of *ALK*. All the new three isoforms of *EML4-ALK* have a marked oncogenic activity *in vitro* as well as *in vivo*.[8,9] The variant 3 was also identified by Rikova *et al*[10] and another new variant, in which exon 15 of *EML4* is jointed to a position within exon 20 of *ALK*, was identified by Koivunen *et al*.[11]

Earlier we conducted the first large scale-study to detect *EML4-ALK* (3.4%) in lung adenocarcinomas and found five fusion-positive cases (two variant 1 and three variant 2) in 149 adenocarcinoma samples.[12] At that point in time, only two variants were recognized, and we investigated their clinicopathological characteristics. However, with development of multiplex RT-PCR for detecting all possible in-frame variants, we captured theoretically all *EML4-ALK* variants and found 11 *EML4-ALK*-positive cases among 363 lung cancers.[9] In this study, we examined the clinicopathological and genetic features of the 11 tumors, and found *EML4-ALK* lung cancers to be characterized by a lack of *EGFR* and *KRAS* mutations, a low rate for *TP53* mutations, a thyroid transcription factor-1 (TTF-1)-positive cell lineage, an acinar histology, and young onset.

Materials and methods

Clinical Samples and Pathological Review

The clinical specimens for this study were 11 lung tumors detected in our earlier study, using multiplex RT-PCR and fluorescent *in situ* hybridization.[9] Briefly, samples were obtained from 363 individuals who underwent surgery at the Cancer Institute Hospital (Tokyo, Japan) between May 1997 and February 2004. The 363 lung cancers comprised 253 adenocarcinomas, 7 adenosquamous carcinomas, 72 squamous cell carcinomas, 7 large-cell carcinomas (including 4 large-cell neuroendocrine carcinomas), 2 pleomorphic carcinomas, and 22 small-cell carcinomas. This project was approved by the Institutional Review Board of the Japanese Foundation for Cancer Research, and informed consent was obtained from each study subject. Histological diagnoses were made based on the World Health Organization (WHO) classification.[13] However, with its subdivision of lung adenocarcinomas, more than 80% tumors fell into the mixed subtype category. We therefore additionally used a predominance classification of invasive components, which is mostly based on the WHO classification except for the mixed subtype, such as papillary predominant, acinar predominant, etc. In the predominance classification of invasive components, we diagnose by a component that makes up the predominant portion of invasive lesion even if it is $<50\%$. In addition, we used a differentiation grading that was basically according to the former version of the Japanese Lung Cancer Society criteria,[14] as performed earlier.[15]

Immunohistochemical Analysis

Unstained paraffin-embedded sections were depleted of paraffin with xylene, rehydrated through a graded series of ethanol solutions, and subjected to immunohistochemical staining with a mouse monoclonal antibody (ALK1, 1:20, Dako, Carpinteria, CA, USA). Heat-induced antigen retrieval pretreatment was performed with Target Retrieval Solution pH 9.0 (Dako). Immune complexes were detected with the EnVision + DAB system (Dako) with minor modifications.[16] TTF-1 was also immunostained using a mouse monoclonal antibody (clone 8G7G3/1, 1:100, Dako), as described earlier.[15] Tumors were considered negative if staining was found in $<5\%$ of neoplastic cells, partly positive if present in 5–50%, and positive if in more than 50%. The results of immunostaining with TTF-1 were based on nuclear staining of neoplastic cells.

DNA Extraction and Mutation Analysis of *EGFR*, *KRAS*, and *TP53*

Of 253 patients with adenocarcinomas, both *EGFR* and *KRAS* data were available for 68 patients and *EGFR* data alone for further 12 patients, including all the patients with *EML4-ALK*-positve cases.[12,17] DNA extraction and mutation analysis of *EGFR* and *KRAS* were performed as described earlier.[17] Mutation analysis of *TP53* was also performed as described earlier.[18] Genomic DNAs from fresh tumor samples were prepared and exons 4–8 and 10 of the *TP53* gene were analyzed by the PCR – single-strand conformation polymorphism and DNA sequencing. For case #4808, *TP53* mutation analysis was performed using DNA extracted from formalin-fixed paraffin-embedded tissue and a method based on direct sequencing, because no fresh sample was available for this study at the time of the current study.

Results

Histologically, the 253 adenocarcinomas comprised 213 mixed subtypes, 19 acinar, 9 papillary, 4 solid, 1 other, and 7 bronchioloalveolar carcinomas based on the WHO classification. According to the subdivision

of lung adenocarcinomas with the WHO criteria, more than 80% of tumors fell into the mixed subtype category. However, this contains very varied lesions; for example, a tumor comprising solid and acinar adenocarcinoma elements would be expected to have a very different prognosis from one composed of bronchioloalveolar carcinoma, with only a small amount of papillary adenocarcinoma. We therefore additionally used a predominance classification and also paid attention to the minor components. According to the predominance subtypes in adenocarcinomas, 6 of 11 *EML4-ALK*-positive lung cancers (54.5%) were subclassified as acinar adenocarcinomas ($P=0.000044$, Table 1), as compared with 4 based on the WHO classification (36.4%, $P=0.0018$, Table 2). In other words, 6 of 34 (18%) acinar-predominant adenocarcinomas, as well as 4 of 19 (21%) acinar adenocarcinomas based on the WHO classification, have *EML4-ALK* fusion. In adenocarcinomas not subclassified as acinar adenocarcinomas based on the WHO criteria, acinar structures were also frequently observed (Figure 1). In differentiation grading, *EML4-ALK* lung cancers were less differentiated (Table 3, $P=0.0082$, 10/11). In addition, they often featured mucin production, as proven by Alcian Blue staining (Figure 1b) with acinar structures. As for the cell types originally proposed by Hashimoto *et al*,[18] the columnar cell type was characteristic of *EML4-ALK* lung cancers (Figure 1).

EML4-ALK-positive lung adenocarcinomas were also found to be significantly smaller than other lung adenocarcinomas (Table 3, $P=0.031$), in line with the lack of bronchioloalveolar components.

Patients with *EML4-ALK* lung cancers tended to be young (56 *vs* 64 years for other tumor types, $P=0.0062$). We defined early-onset lung cancers by classifying patients as below or above 50 years of age. In 253 patients with lung adenocarcinomas, 16 patients were affected by the disease at below 50 years of age. Four of 11 patients (36%) with *EML4-ALK*-positive lung cancers were affected by these diseases at below 50 years of age, as compared with 12 of 242 patients (5.0%) with *EML4-ALK*-negative lung cancers ($P=0.00038$).

It is true that the *EML4-ALK* translocation was first found in a smoker's lung cancer, but overall there was no significant difference between smokers' and non-smokers' tumors with regard to *EML4-ALK* fusion ($P=0.37$). Smoking habits can be classified into the following two grades of cumulative smoking based on the smoking index (SI), a product of the number of cigarettes per day, and the duration in years: (a) non-smokers and light smokers (SI <400); and (b) heavy smokers (SI $=400$ or above). Ten of the 11 (91%) *EML4-ALK*-positive lung cancer patients had SI <400, as compared with 109 of 241 (45%) *EML4-ALK*-negative lung cancer patients ($P=0.040$). In this study, *EML4-ALK* fusion was detected in only one heavy smoker's lung cancer (1/11 = 9.1%).

EGFR and *KRAS* mutations are mutually exclusive in usual cases while being two major oncogenic drivers of lung adenocarcinoma development. *EML4-ALK*-positive lung cancers lacked both *EGFR* and *KRAS* mutations ($P=0.00018$), and only 1 of 11 (9.1%) harbored a *TP53* mutation (Table 2). It is noteworthy that the single mutation was a G/A transition (GTG→ATG) (V→M) in codon 273, exon 8. This is known to be a spontaneous rather than a tobacco-carcinogen-induced mutation, usually seen in non-smokers' lung cancers.

In the *EML4-ALK*-positive 11 cases, immunohistochemical assays with the anti-ALK antibody ALK1 consistently showed definite staining. As illustrated

Table 1 *EML4-ALK* fusion and histology of adenocarcinomas classified by predominant subtypes

Histology	*Total (363)*	*EML4-ALK*(+)	*EML4-ALK*(−)
Adenocarcinoma	253	11 (4.3%)	242 (96%)
Subtype by predominance classification			
Invasive carcinoma			
Papillary adenocarcinoma	206	5 (5/11 = 45%)	201 (201/242 = 83%)
Acinar adenocarcinoma	34	6 (6/11 = 55%)[a]	28 (28/242 = 12%)
Solid adenocarcinoma with mucin	5	0 (0%)	5 (5/242 = 2.1%)
Others	1	0 (0%)	1 (1/242 = 0.41%)
Noninvasive carcinoma			
Bronchioloalveolar carcinoma	7	0 (0%)	7 (7/242 = 2.9%)
Adenosquamous carcinoma	7	0 (0%)	7 (100%)
Squamous cell carcinoma	72	0 (0%)	72 (100%)
Large-cell carcinoma	7	0 (0%)	7 (100%)
Large-cell neuroendocrine carcinoma	4	0 (0%)	4 (100%)
Pleomorphic carcinoma	2	0 (0%)	2 (100%)
Small-cell carcinoma	22	0 (0%)	22 (100%)

Acinar-predominant adenocarcinomas *vs* the other adenocarcinomas.
[a]Fisher's exact test, $P<0.0001$ ($P=0.000044$).

Table 2 *EML4-ALK* variants detected by multiplex RT-PCR analysis and clinicopathologic and genetic data

V	*Tumor ID*	*Sex*	*Age (years)*	*p-Stage*	*LKD*	*Survival (days)*	*Size (mm)*	*SI*	*WHO subtype*	*Pred-subtype*	*diff.*	*Histological components*	*ALK IHC*	*TTF-1 IHC*	*KRAS mut*	*EGFR mut*	*TP53 mut*
1	#9034	F	43	IA	Alive	1714	12	10	Acinar	Acinar	Por		+	P+	–	–	–
1	#4808	F	58	IA	Alive	2246	27	0	Mixed	Pap	Well	Papillary adenocarcinoma with BAC	+	+	–	–	–
1	#9968	F	66	IIIA	Alive	1036	33	0	Mixed	Pap	Mod	Papillary adenocarcinoma with BAC	+	+	–	–	–
2	#4180	M	43	IV	Dead	527	23	160	Acinar	Acinar	Por		+	P+	–	–	–
2	#3121	M	64	IIIB	Alive	2673	18	220	Acinar	Acinar	Por		+	+	–	–	–
2	#2374	F	66	IA	Alive	1632	28	0	Acinar	Acinar	Mod		+	P+	–	–	–
3	#7969	M	47	IIIA	Alive	1328	17	540	Mixed	Pap	Por	Papillary adenocarcinoma with BAC	+	+	–	–	–
3	#2075	F	62	IIA	Dead	522	19	0	Mixed	Acinar	Mod	Acinar+papillary+solid adenocarcinoma	+	P+	–	–	+[a]
3	#9616	M	73	IA	Alive	1465	13	300	Pap	Pap	Mod		+	+	–	–	–
4	#8398	F	52	IA	Alive	1834	24	0	Mixed	Acinar	Mod	Acinar+papillary adenocarcinoma with BAC	+	P+	–	–	–
5	#8993	M	44	IA	Alive	1730	15	0	Pap	Pap	Mod		+	+	–	–	–

acinar, acinar adenocarcinoma; BAC, bronchioloalveolar carcinoma; diff., differentiation; *EGFR* mut, *EGFR* mutation; IHC, immunohistochemistry; *KRAS* mut, *KRAS* mutation; LKD, lung cancer death; mixed, adenocarcinoma with mixed subtype; P+, Partly +; pap, papillary adenocarcinoma; Pred-subtype, predominance subtype; p-Stage, pathological-Stage; SI, smoking index; *TP53* mut, *TP53* mutation; V, *EML4-ALK* variant.

[a]G/A transition (GTG→ATG) (V→M) in codon 273, exon 8.

in Figure 2a, the cytoplasm of tumor cells harboring the variant 2 (tumor ID #2374) was strongly stained with fine granular accentuation. Although we performed the immunostaining of 88 *EML4-ALK*-negative lung adenocarcinoma specimens, we could discriminate all the fusion-negative specimens from the fusion-positive ones by our refined immunohistochemical condition.[16] All the 11 cases were also positive (six cases) or partly positive (five cases) for TTF-1 immunohistochemistry (Figure 2b), a characteristic of alveolar type II cells, which is featured in non-smokers' cancers.

Discussion

With the present large-scale screen for *EML4-ALK* fusion in lung cancers, we detected 11 adenocarcinomas with an *EML4-ALK* translocation. In the current study, we revealed a relatively young occurrence and a typically less-differentiated acinar histology, which might be used as clinical pointers. It is of great interest that *EML4-ALK* translocation is associated with young onset, whereas *EGFR* mutation status is not associated with the patient's age at diagnosis.[4]

Currently, anaplastic large-cell lymphomas (ALCLs) are divided into three entities, namely primary systemic *ALK* (+) ALCL, primary systemic *ALK* (−) ALCL, and primary cutaneous ALCL. The ALK expression is caused most commonly t(2;5) by chromosomal translocations, and *ALK* (+) ALCL predominantly affects young male patients and, if treated with chemotherapy, has a favorable prognosis.[19] This might similarly be applicable to *EML4-ALK* lung cancers. Presently, the primary treatment for lung cancers is surgery where possible. However, for *EML4-ALK* lung cancers, chemotherapy or a targeted therapy with an ALK inhibitor might be effective, given that *EML4-ALK*-dependent cells are known to undergo apoptosis in response.[7–9,11]

Here, *EML4-ALK* fusion was found to be mutually exclusive for *EGFR* or *KRAS* mutations, thus pointing to a distinct genetic subtype of lung adenocarcinoma. The possibility of a genetic classification of lung adenocarcinomas based on oncogene mutations has already been considered. In fact, one-third to nearly half of Japanese adenocarcinomas harbor *EGFR* mutations,[4,20] about 10% have *KRAS* mutations[21–23] and about 4% have *EML4-ALK* translocations, implying that two-thirds of adenocarcinomas feature mutually exclusive oncogenic mutations. The mutation rate of *TP53* (1/11 = 9.1%) was also low compared with that of lung adenocarcinomas in general (41%),[18] and the single mutation found was G to A transition, which was not related to smoking. Strong *in vitro* as well as *in vivo* oncogenic activity of *EML4-ALK* fusion products[8,9] might account for the lack of other genetic alterations.

All 11 *EML4-ALK* lung cancers were positive or partly positive for TTF-1 immunostaining. TTF-1

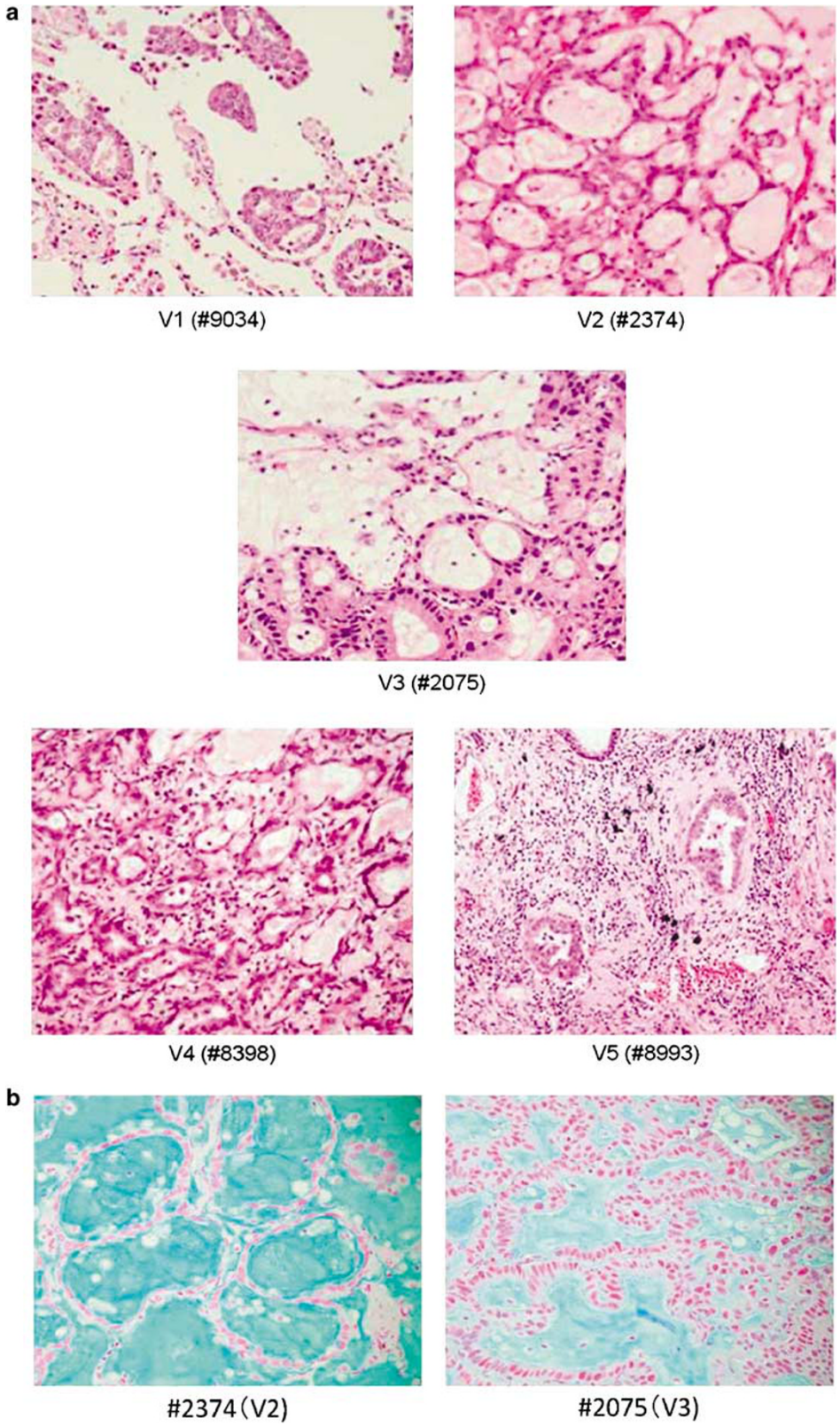

Figure 1 (**a**) Representative appearance of all the five variants of the *EML4-ALK* lung cancers (hematoxylin and eosin staining). Histologically, acinar structures with some mucin production are characteristic. (**b**) Alcian Blue staining shows the abundant mucin production.

Table 3 Clinicopathologic and genetic comparisons between *EML4-ALK* fusion-positive and -negative lung adenocarcinomas

Variables category	*No. of samples (%)*	*EML4-ALK fusion*		P-*value*
		(+) (n = *11)*	*(−)* (n = *242)*	
Age (years; mean ± s.d.)	253	56 ± 11	64 ± 9	0.0062[a]
				0.00038[b]
<50	16 (6.3)	4 (36)	12 (5.0)	
≤50	237 (94)	7 (64)	230 (95)	
Sex				0.61[b]
Males	134 (53)	5 (45)	129 (53)	
Females	119 (47)	6 (55)	113 (47)	
Smoking habit				0.37[b]
Never smokers	105 (41)	6 (55)	99 (41)	
Ever smokers	147 (59)	5 (45)	142 (59)	
Heavy smokers or not				0.040[b]
Heavy smokers	110 (44)	1 (9.1)	109 (45)	
Not heavy smokers	142 (56)	10 (91)	132 (55)	
Tumor size (mm)		20.8 ± 6.7	31.8 ± 16.7	0.031[a]
				0.039[b]
<30	142 (56)	10 (80)	132 (55)	
≥30	111 (44)	1 (20)	110 (45)	
Differentiation grading				0.0082[b]
Well	98 (39)	1 (9.1)	97 (40)	
Less	155 (39)	10 (91)	145 (60)	
EGFR mutation				0.00085[b]
Mutation(+)	41 (52)	0 (0)	41 (60)	
Mutation(−)	39 (48)	11 (100)	28 (40)	
KRAS mutation				0.49[b]
Mutation(+)	7 (10)	0 (0)	7 (12)	
Mutation(−)	61 (90)	11 (100)	50 (88)	
EGFR or *KRAS mutation*				0.00018[b]
Mutation(+)	38 (59)	0 (0)	38 (67)	
Mutation(−)	30 (41)	11 (100)	19 (33)	
p-Stage				0.89[b]
I	143 (57)	6 (55)	137 (57)	
II–IV	110 (43)	5 (45)	105 (43)	

Percentages may not total 100, because of rounding.
We have no smoking history of one patient.
Smoking habits were classified into the following two grades based on the smoking index: (a) non-smokers and light smokers (smoking index <400); and (b) heavy smokers (smoking index = 400 or above).
[a]Student's *t*-test.
[b]Fisher's exact test.

has a decisive role as a master regulatory transcription factor in lung development and in maintenance of the functions of terminal respiratory unit (TRU) cells.[24] The TTF-1 positivity of *EML4-ALK* lung cancers suggests that this subtype might have a TRU histogenesis. TRU-type lung cancers with a TTF-1-positive cell lineage often occur in non- or light smokers, which frequently harbor *EGFR* mutations (61%) and have less-frequent *TP53* mutations (36%) as compared with non-TRU-types (57%).[22] *EML4-ALK* lung cancers also occur in non- or light smokers but do not harbor *EGFR* mutations. The low frequency of *TP53* mutations (9.1%) not only indicates strong oncogenic activity for *EML4-ALK* fusion products but also suggests an independence from smoking, because smoker's adenocarcinomas very frequently harbor *TP53* mutations.[18]

Histologically, less-differentiated acinar structures composed of columnar cells appear characteristic of *EML4-ALK* lung adenocarcinomas. Generally, the columnar cell type is also found in smoker's lung adenocarcinomas, whereas the hobnail cell type, characterized by cytoplasmic protrusions and with a tadpole shape, is often observed in non-smoker's lung adenocarcinomas.[18] Although *EML4-ALK* lung cancers are TTF-1-positive, their histology is

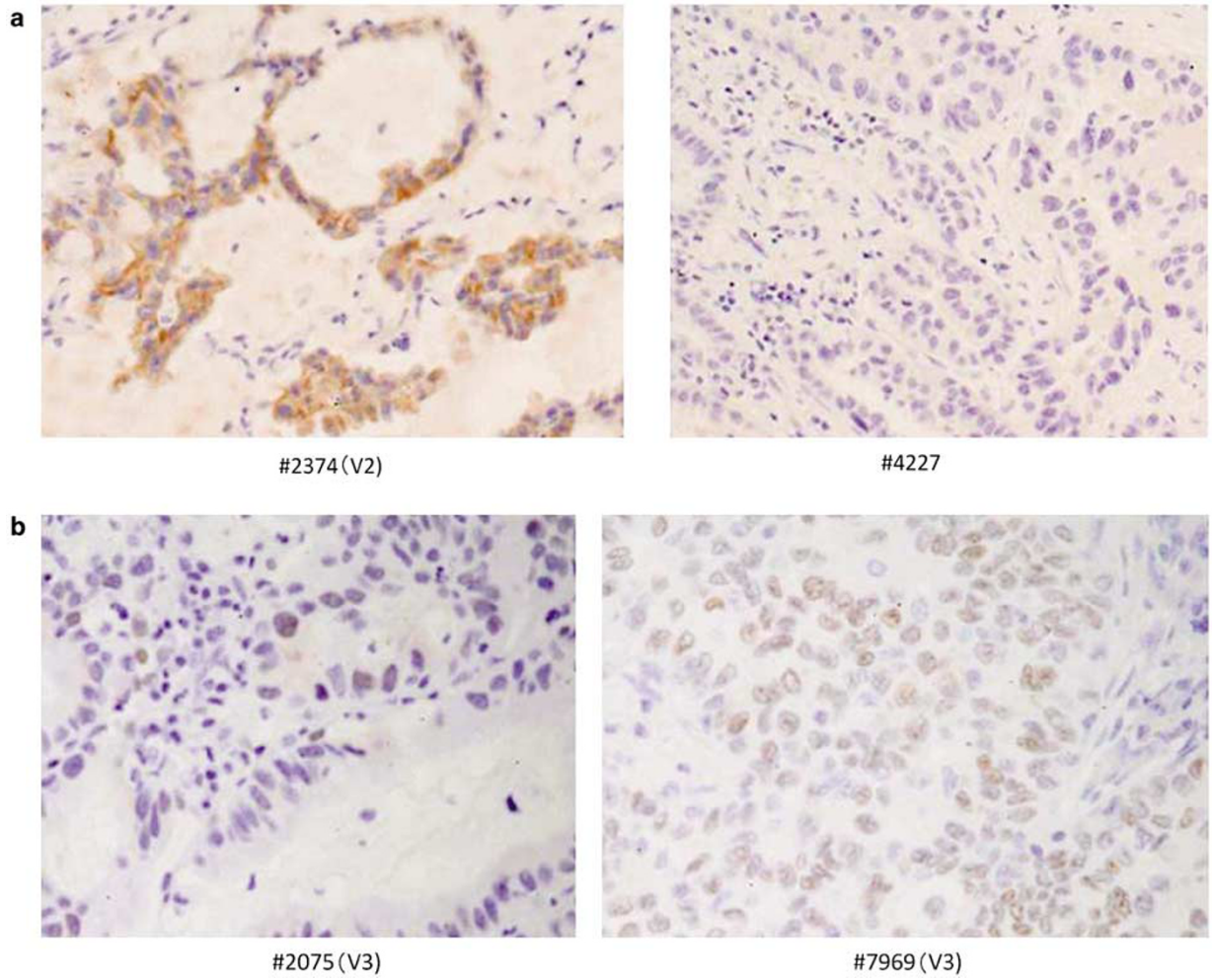

Figure 2 (**a**) Immunohistochemical analysis with a monoclonal anti-ALK antibody of lung adenocarcinoma specimens with (tumor ID #2374) and without (tumor ID #4227) *EML4-ALK* fusion. Note the diffuse staining in the cytoplasm with fine granular accentuation apparent for the *EML4-ALK*-positive tumor. (**b**) Immunohistochemical analysis of lung adenocarcinoma specimens with *EML4-ALK* fusion using a monoclonal anti-TTF-1 antibody. The *EML4-ALK*-positive tumors are partly (ID #2075) or diffusely (ID #7969) positive.

similar to lung cancers developing in smokers, which is interesting in the view of histology–etiology relationships.

Presently, lung adenocarcinomas may be genetically divided into *EGFR*-mutated, *KRAS*-mutated, and *EML4-ALK*-related subtypes. We here elucidated the clinicopathologic, histologic, and genetic characteristics of *EML4-ALK* lung cancers, bearing etiologic implications in mind. Just as some *EGFR*-mutated lung cancers can be successfully treated with EGFR inhibitors, *EML4-ALK* lung cancers may respond to a specific inhibitor treatment, allowing a good prognosis.

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Conflict of interest

K Takeuchi is a consultant providing advisory services to Dako for their antibodies.

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