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Tumorigenesis and Neoplastic Progression

EML4-ALK Rearrangement in Non-Small Cell Lung Cancer and Non-Tumor Lung Tissues

Maria Paola Martelli,* Gabriella Sozzi,† Luis Hernandez,‡ Valentina Pettirossi,* Alba Navarro,‡ Davide Conte,† Patrizia Gasparini,† Federica Perrone,† Piergiorgio Modena,† Ugo Pastorino,† Antonino Carbone,† Alessandra Fabbri,† Angelo Sidoni,§ Shigeo Nakamura,¶ Marcello Gambacorta,‖ Pedro Luis Fernández,‡ Jose Ramirez,‡ John K.C. Chan,** Walter Franco Grigioni,†† Elias Campo,‡ Stefano A. Pileri,†† and Brunangelo Falini*

From the Institutes of Hematology, and Pathology,§ Policlinico Monteluce, University of Perugia, Italy; the Istituto Nazionale Tumori,† Milan, Italy; Hospital Clinic,‡ the Institut d'Investigacions Biomediques August Pi i Sunyer, University of Barcelona, Barcelona, Spain; the Department of Pathology and Clinical Laboratories,¶ Nagoya University Hospital, Nagoya, Japan; the Institute of Pathology,‖ Policlinico Niguarda, Milan, Italy; the Department of Pathology,** Queen Elizabeth Hospital, Kowloon, Hong Kong; and the Units of Surgical Pathology and Hematopathology,†† S. Orsola Hospital, University of Bologna, Bologna, Italy*

A fusion gene, echinoderm microtubule associated protein like 4 – anaplastic lymphoma kinase (*EML4-ALK*), with transforming activity has recently been identified in a subset of non-small cell lung cancer (NSCLC), but its pathogenetic, diagnostic, and therapeutic roles remain unclear. Both frequency and type of *EML4-ALK* transcripts were investigated by reverse transcription PCR in 120 frozen NSCLC specimens from Italy and Spain; non-neoplastic lung tissues taken far from the tumor were used as controls. In cases carrying the fusion transcript, we determined *EML4-ALK* gene and protein levels using fluorescence *in situ* hybridization, Western blotting, and immunoprecipitation. We also analyzed ALK protein levels in paraffin samples from 662 NSCLC specimens, including the 120 cases investigated in the molecular studies. *EML4-ALK* transcripts (variants 1 and 3) were detected in 9 of 120 NSCLC samples but were not specific for NSCLC since they were also found in noncancerous lung tissues taken far from the tumor. Notably, no transcripts were detected in matching tumor samples from these patients. Fluorescence *in situ* hybridization analysis of cases expressing *EML4-ALK* transcripts showed that only a minority of cells harbored the *EML4-ALK* gene. None of these cases was found to express the EML4-ALK protein as examined by immunohistochemistry, Western blotting, and immunoprecipitation. The *EML4-ALK* transcript cannot be regarded as a specific diagnostic tool for NSCLC. Our results show therefore that the causal role and value of EML4-ALK as a therapeutic target remain to be defined. ***(Am J Pathol 2009, 174:661–670; DOI: 10.2353/ajpath.2009.080755)***

The anaplastic lymphoma kinase (*ALK*) gene fuses with nucleophosmin (*NPM1*)[1] or other gene partners[2,3] in ana-

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M.P.M., G.S. and L.H. equally contributed to this work.

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Address reprint requests to Brunangelo Falini, M.D., Institute of Hematology, University of Perugia, 06122 Perugia, Italy. E-mail: faliniem@unipg.it.

plastic large cell lymphoma (ALCL). These tumors, named ALK+ALCL, exhibit distinctive biological and clinical features.[4,5] *ALK* gene recombinations were also detected in a rare subset of diffuse large B-cell lymphoma[6] and in inflammatory myofibroblastic tumors.[7] Consequent to these rearrangements, ALK is constitutively expressed as a phosphorylated fusion product displaying tumorigenic activity.[8,9] ALK fusion proteins serve as specific immunohistochemical markers[2,3] and are potential therapeutic targets for ALK-kinase inhibitors.[10]

Soda et al[11] recently identified a transforming *EML4-ALK* fusion gene in 6.7% of smoker non-small-lung cancer (NSCLC) patients from Japan. This chimeric gene, which was generated by a small inversion within the chromosome 2 short arm, encoded a 1059-amino acid fusion protein. The N-terminal portion was identical to the human echinoderm microtubule associated protein like 4 (EML4)[12,13] and the C-terminal portion was the same as the intracellular domain of human ALK.[1,11] The EML4-ALK protein localized in the cytoplasm of transfected cells and induced transformation of mouse 3T3 cells, which, when injected into nude mice, gave rise to tumors.[11] *In vitro*, a specific ALK inhibitor significantly reduced growth of *EML4-ALK* transformed BA/F3 cells.[11]

Expression of the *EML4-ALK* transcript in NSCLC, although at lower frequencies (0.9% to 2.6%) than originally reported,[11] was subsequently confirmed by several investigators in a total of 381 cases from Japan[14–16]; other solid tumors were consistently negative for the *EML4-ALK* transcript.[15] More recently, another molecular variant of *EML4-ALK* rearrangement (the so-called variant 3) was identified in some patients with NSCLC and in the human NSCLC cell line H2228.[17–19] Therefore, *EML4-ALK* was proposed as a new diagnostic marker and therapeutic target in NSCLC.[11,19,20]

Although the frequency of *EML4-ALK* transcript expression in NSCLC seems low, it could potentially impact many patients, since NSCLC constitutes about 80% of all lung cancers, the leading cause of cancer deaths in developed countries.[21] Information on the expression of *EML4-ALK* fusion transcripts is, however, limited to mainly Japanese patients,[11,14–16,18] and no data are available on EML4-ALK fusion protein expression in primary NSCLC samples. Furthermore, to date, the *EML4-ALK* rearrangement has not been sought in non-tumor lung tissues. Since these issues could have a major impact on understanding the role of the *EML4-ALK* rearrangement in the pathogenesis, diagnosis, and molecularly targeted therapy of NSCLC, we investigated expression of the *EML4-ALK* fusion gene, transcript, and protein in 120 NSCLC frozen specimens from Italy and Spain, using non-neoplastic lung tissues taken at a distance from the tumor as controls. Moreover, ALK protein expression was analyzed by immunostaining of paraffin sections from 662 NSCLC specimens, which included the 120 cases we investigated in molecular studies.

Table 1. Frequency of the *EML4-ALK* Fusion Transcript in NSCLC Tumors and its Association with Clinical and Pathologic Features

	EML4-ALK, *n* (%)		*p**
Clinico-pathological characteristics	+	−	
All tumors	9 (7.5)	111 (92.5)	
Sex			
Male	8 (6.6)	88 (73.3)	0.69
Female	1 (0.83)	23 (19.2)	
Smoking			
Never	1 (0.83)	15 (12.5)	1
Smoker	8 (6.6)	93 (77.5)	
Age, median (y)	64	67	
Stage			
I	4 (3.33)	61 (51.26)	0.73
II	2 (1.68)	19 (15.96)	
III	2 (1.68)	20 (16.8)	
IV	1 (0.84)	10 (8.40)	
Histology			
Adenocarcinoma	3 (2.5)	60 (50)	0.46
Squamous carcinoma	4 (3.33)	44 (36.66)	
Adenosquamous carcinoma	2 (1.66)	0 (0)	
Large cell	0 (0)	3 (2.59)	
Others	0 (0)	4 (3.33)	

*Fisher's exact test, two tailed.

Stage: I versus II-IV; Histology: adenocarcinoma versus squamous carcimoma.

Materials and Methods

Tissue Specimens and Cell Lines

Frozen material for molecular studies included 120 NSCLC specimens (60 from the Istituto Nazionale Tumori, INT; 60 from the Institute of Pathology, University of Barcelona) and 67 non-tumor lung tissues (58 paired and 9 unpaired) from INT. All tumors were resected from series of consecutive patients treated in the two Institutions. All samples were collected following Institutional Review Board guidelines. Tissues were freshly collected during surgery, snap-frozen in liquid nitrogen, and stored at −80°C. The clinical and pathological features of the 120 NSCLC patients are shown in Table 1.

Paraffin-embedded specimens for immunohistochemical studies were from 662 NSCLC patients, including the 120 cases for which frozen material was studied (see above). NSCLC paraffin samples were from Caucasians (n = 481 from Italy; n = 60 from Spain), and Asian patients (n = 101 from Japan; n = 20 from Hong Kong). The 662 patients included 511 males and 151 females. The histological subtypes were: 294 adenocarcinoma, 258 squamous-cell carcinoma, 71 undifferentiated large-cell carcinoma, 29 bronchiolo-alveolar carcinoma, 6 adeno-squamous carcinoma, and 4 small-cell/large-cell carcinoma.

Cell Lines

The NSCLC human cell line H2228 (American Type Culture Collection, ATCC, Rockville, MD) was used as positive control for expression of the shorter variant 3 of *EML4-ALK*.[17] The ALCL (Karpas 299) and rhabdomyosarcoma (Rh30) human cell lines were used as positive

controls for expression of NPM-ALK and full-length ALK proteins, respectively.[22]

EML4-ALK Gene Construct Production and Cell Transfection

The coding sequence of human *EML4-ALK* variant 1 fusion gene (3192 bp) was synthesized by Genscript (Genscript Corporation, Piscataway, NJ) based on the GenBank accession number sequence AB274722; EcoRI cloning sites were added at 5′ and 3′ of the cDNA. cDNA was cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA). pcDNA3_EML4-ALK (10 μg) was transfected into Phoenix cells, a human embryonic kidney derived cell line (ATCC), by the calcium phosphate/DNA co-precipitation method. Phoenix cells expressing EML4-ALK were harvested, washed and cell pellets were either lysed for Western blot and immunoprecipitation assays or fixed (in B5 and formalin) and embedded in paraffin for immunohistochemical studies. These samples were used as positive controls for expression of EML4-ALK, variant 1.

Antibodies

The following anti-ALK monoclonal antibodies (mAbs) were used: ALK1,[23] ALKc,[22] Clone 5A4 (Thermo Fisher Scientific, Fremont CA); and rabbit mAb ALK/p80 (clone SP8; Thermo Fisher Scientific). The monoclonal antibody against CD34 was purchased from Dako (Glostrup, Denmark).

Reverse Transcription-PCR Analysis

Total RNA was extracted from cells or frozen tissues using RNA isolation TRIZOL Gibco (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA concentration was determined on a photospectrometer and quality was assessed by 1% agarose gel electrophoresis. To search for *EML4-ALK* transcripts in NSCLC and non-tumor lung specimens, 1 μg of total RNA was retrotranscribed using Random Primer and 200 U of Superscript III Reverse Transcriptase (Invitrogen) followed by a PCR with the following primers, which, as previously described,[11,14] detect variants 1 and 2: Fusion-RT-S 5′-GTGCAGTGTTTAGCATTCTTGGGG-3′ and Fusion-RT-AS 5′-TCTTGCCAGCAAAGCAGTAGTTGG-3′, using 1/20th of the cDNA.

To analyze the *EML4-ALK* variant 3 transcript displayed by H2228 cell line,[17] the *ALK* Fusion-RT-AS primer was combined with a forward primer located in exon 6 of *EML4*: *EML4-ex6F* 5′-GCATAAAGATGTCATCATCAACCAAG-3′.

We also used PCR primers glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-S 5′-ACCACAGTCCATGCCATCAC-3′ and GAPDH-AS 5′-TCCACCACCCTGTTGCTGTA-3′ for glyceraldehyde-3-phosphate dehydrogenase cDNA (452bp) and primers epidermal growth factor receptor (EGFR)-S 5′-CCTGACTCCGTCCAGTATTGATC–3′ and EGFR-AS 5′-CTGTGGATCCAGAGGAGGAGTATG–3′ for EGFR cDNA, as controls for cDNA integrity. Samples were processed in a Gene-Amp PCR system 9700 thermal cycler through 25 cycles for *GAPDH* (Ta 58°C, 30-minute elongation) and 40 cycles *EGFR* (Ta 60°C, 1-minute elongation), *EML4-ALK* (Ta 60°C, 1-minute elongation) and *ALK* wild-type (Ta 58°C, 30-minute elongation). Nucleotide sequencing of PCR products was performed to confirm identity of amplified fragments.

EGFR *and* KRAS *Mutational Analysis*

Analysis of *EGFR* and *KRAS* mutations was performed on DNA extracted from NSCLC specimens, as previously described.[24]

Fluorescence in Situ Hybridization

Fluorescence *in situ* hybridization (FISH) studies were performed on 2 to 3 μm thick paraffin sections from 20 NSCLCs and 1 ALCL specimen with t(2;5), on touch imprints from 8 non-tumor lung samples and in Carnoy's fixed metaphases and interphase nuclei of the H2228 cell line. The commercially labeled LSI ALK Dual Color (split-apart) Probe (Downers Grove, IL) was used to detect any rearrangement involving the *ALK* gene. The probe hybridizes to band 2p23, on either side of the *ALK* gene breakpoint.

Before hybridization, paraffin sections were deparaffinized in xylene (3 times, 10 minutes. each), followed by two 5 minutes washes in 100% ethanol and two 5-minute washes in 96% ethanol. Sections were pretreated in Tris EDTA (5mmol/L Tris and 1 mmol/L EDTA) at 96°C for 15 minutes, followed by treatment in 0.01N HCL + 0.4% pepsin. Touch imprints from the 8 non-tumor lung samples were placed in methanol for 30 minutes, transferred into 100% ethanol, stored at 4°C overnight, and treated for 10 to 15 minutes, with 0.005% pepsin in 0.01N HCl. For paraffin-embedded and touch preparations, co-denaturation with Hybrite (Vysis, Dwoners Grove, IL) at 72°C for 2 minutes, was followed by overnight storage at 37°C. Posthybridization washes were performed following the Vysis protocol.

Criteria for probe signal interpretation in at least 100 interphase nuclei were as follow: i) separated green and orange signals or single red signals identified cells with rearranged *ALK*; ii) overlapping of red and green signals (yellowish) indicated cells in which *ALK* was not rearranged.

Western Blot and Immunoprecipitation Studies

Frozen material (about 150 mg of tissue) for Western blotting and immunoprecipitation studies was available from the following samples: seven NSCLCs harboring *EML4-ALK* transcript (6 with variant 1 and one case with variant 3); and three non-tumor lung specimens (one carrying *EML4-ALK* transcript variant 1 and two carrying variant 3). All tissues had been mechanically disrupted

Table 2. Characteristics of NSCLC Specimens Carrying the *EML4-ALK* Transcripts

Patient	Sex	Age	Histotype	Stage	KRAS mutation	EGFR mutation	FU*	Smoking habit†	EML4-ALK variants‡
424T§	M	54	Adenocarcinoma	IIIA	Gly12Cys	wt	DOD 19 mo	S	V1
440T§	M	74	Squamous cell carcinoma	IA	wt	wt	NED 14 mo	S	V1
447T§	M	61	Adenosquamous carcinoma	IIB	wt	wt	NED 12 mo	S	V1
B/252T¶	M	78	Adenosquamous carcinoma	II	wt	wt	Alive 18 mo	S	V1
B/5796T¶	M	74	Squamous cell carcinoma	II	wt	wt	DOD 8 mo	S	V1
B/8237T¶	M	71	Squamous cell carcinoma	IV	wt	wt	DOD 60 mo	S	V1
B/9020T¶	M	68	Squamous cell carcinoma	I	wt	wt	Alive 68 mo	S	V1
470T§	F	64	Adenocarcinoma	IA	wt	wt	NED 7 mo	NS	V3 short
435T§	M	85	Adenocarcinoma	IB	wt	wt	NED 15 mo	S	V3 long

*FU = follow up; DOD = dead of disease; NED = no evidence of disease; mo = months.
†S = smoker; NS = never smoker.
‡V1 = *EML4-ALK* variant 1 [*EML4* exon 13-*ALK* exon 20]; V3 = *EML4-ALK* variant 3 [*EML4* exon 6-*ALK* exon 20 with (long) or without (short) the insertion of 33 nucleotides from intron 6 as alternative splicing variant].
§From INT (Istituto Nazionale Tumori, Milan, Italy) series.
¶From University of Barcelona (Barcelona, Spain) series.

using a rotor-stator homogenizer in cell lysis buffer. Controls included lysates from Phoenix cells transfected with *EML4-ALK* variant 1 or empty vector, the cell line H2228 (expressing EML4-ALK variant 3), the ALCL cell line Karpas 299 (expressing NPM-ALK), and the rhabdomyosarcoma cell line Rh30 (expressing full-length ALK). Cell lysis buffer was 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 0.5% deoxycholic acid (DOC), 0.1% SDS, 1 mmol/L sodium orthovanadate; plus a protease inhibitor cocktail (leupeptin, aprotinin, pepstatin A, and pefabloc; RIPA lysis buffer). Proteins were separated by SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (*Trans*-Blot 0.2 μm, Bio-Rad Laboratories, Hercules, CA), and probed with either ALKc or ALK/p80 (clone SP8; Thermo Fisher Scientific) mAb, followed by horseradish peroxidase–conjugated secondary antibodies. Polypeptides were detected using the enhanced chemiluminescence method (GE Healthcare, Uppsala, Sweden).

To enrich for ALK fusion proteins, samples were also studied by immunoprecipitation. Lysates from cell lines, homogenized NSCLC and non-tumor lung tissue samples, were clarified by centrifugation and incubated with ALKc mAb pre-coupled to protein A/G Plus–Agarose beads (SantaCruz Biotecnology, Santa Cruz, CA) rocking overnight at 4°C. After washing, immunoprecipitates were separated by SDS–polyacrylamide gel electrophoresis. Immunoprecipitates were then analyzed by Western blot as specified above. Immunoprecipitation of Hsp90 protein, using the anti-Hsp90 rabbit Ab (Stressgen, Victoria, BC, Canada), served as controls for protein extraction and immunoprecipitation.

Immunohistochemical Studies

Paraffin sections from 662 NSCLC were microwave-heated (750-W, three 5-minute cycles) in 0.01 mol/L citrate, pH 6.0, or 1-mmol/L EDTA, pH 8.0, and immunostained with anti-ALK antibodies (ALK1, ALKc, Clone 5A4) using the sensitive Dako-REAL, Alkaline-Phosphatase/RED detection system (Dako, Glostrup, Denmark). NSCLC and non-tumor lung specimens expressing the *EML4-ALK* transcript were also immunostained in parallel with the Envision + DAB system (Dako).

Results

Frequency and Type of EML4-ALK Transcripts in European Patients with NSCLC

The *EML4-ALK* fusion mRNA was detected as a 247-bp product (variant 1) in 7/120 (5.8%) of NSCLC (Table 2) and representative examples are shown in Figure 1A. Reverse transcriptase (RT)-PCR experiments on *EML4-ALK* positive cases were independently replicated thrice. In half of the cases, one replicate experiment did not confirm the fusion transcript was present, suggesting it was expressed at very low level in tumor samples.

We did not detect any case showing *EML4-ALK* fusion variant 2 (~1 kb),[11,14] even though in all cases we were able to amplify an *EGFR* transcript of similar size (1.3 kb), which confirmed mRNA integrity (Figure 1).

An *EML4-ALK* fusion variant 3 (155/188 bp) representing *EML4* exon 6 – *ALK* exon 20 fusion transcript was detected in 2/120 (1.7%) NSCLC (Table 2 and Figure 1B). This variant presents two fusion transcript isoforms of 155 and 188 bp, with the long one including 33bp from intron 6 of the *EML4* gene. Tumor samples presented either the short (case 435T) or the long (case 470T) isoforms whereas the H2228 cell line consistently showed an abundantly expressed variant 3 transcript with both isoforms (Figure 1B). Similar type and frequencies of positive cases were obtained independently in two different laboratories (INT and Barcelona).

Sequencing of PCR products amplified from each of the 9 NSCLC samples confirmed *EML4-ALK* variant 1 was present in seven cases and variant 3 in two (Figure 1C). None of these 9 tumors showed *EGFR* mutations; a *KRAS* mutation (Gly12Cys substitution) was detected in one lung adenocarcinoma carrying *EML4-ALK* variant 1 (Table 2). No significant associations were found be-

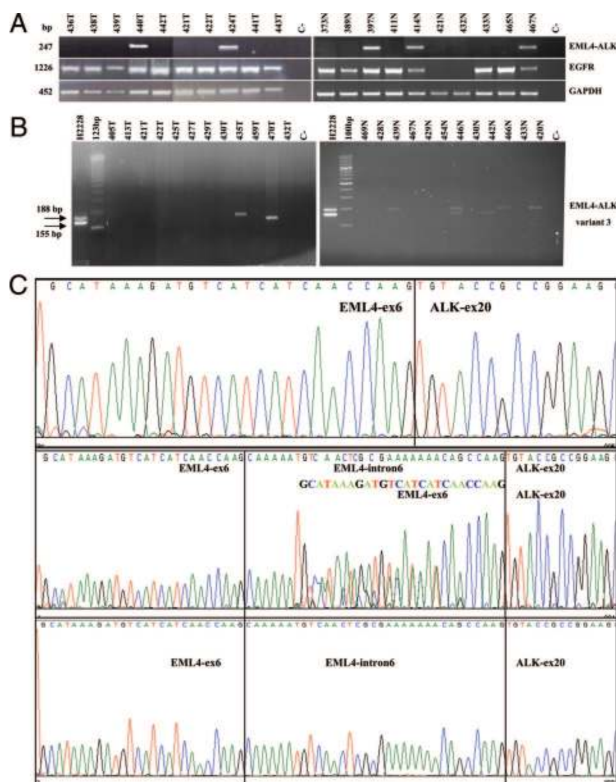


Figure 1. *EML4-ALK* transcripts in NSCLC and non-tumor lung samples. **A:** Analysis of variant 1 (*EML4* exon13-*ALK* exon 20) and (**B**) variant 3 (*EML4* exon 6 – *ALK* exon 20) fusion transcripts in tumor and non-tumor lung samples. Genes analyzed are indicated on the **right**. Amplicon size in bp is indicated on the **left**. Case IDs are reported on **top**; suffix "T" indicates lung cancer samples, while suffix "N" indicates non-tumor lung samples from cancer patients. "C" is the no-template negative control of PCR. H2228 lung cancer cell line served as positive control. **C:** Sequence electropherogram of *EML4-ALK* variant 3 isoforms representing fusion of *EML4* exon 6 – *ALK* exon 20. The fusion transcripts generated may contain a short isoform of variant 3 fusion gene (155 bp, **upper panel**), a long isoform (188 bp, including 33bp from intron 6 of *EML4* gene, **lower panel**) or both (155/188 bp, **middle panel**).

tween the presence of *EML4-ALK* fusion transcript and clinical-pathological features including sex, age, smoking habits, tumor stage, and histology (Table 1).

Our results demonstrate that a subset (7.5%) of NSCLC from non-Japanese patients expresses *EML4-ALK* transcripts (variants 1 and 3).

Expression of EML4-ALK Transcript in Non-Tumor Lung Tissues

As ideal targets for cancer diagnosis and treatment need to be specific to tumor cells and absent in normal tissues, we investigated whether the *EML4-ALK* transcript was expressed in non-tumor lung tissues. To address this issue that had not been investigated in previous studies, we analyzed by RT-PCR non-tumor lung tissues from 67 patients with NSCLC (60 with paired tumor and 7 unpaired). As a routine practice for TNM staging in the Pathology Department of Isituto Nazionale Tumori, non-tumor lung specimens are sampled at a distance from the tumor to guarantee that the tissues are free from cancerous cells, atelectasis, and obstructive pneumonia.[25]

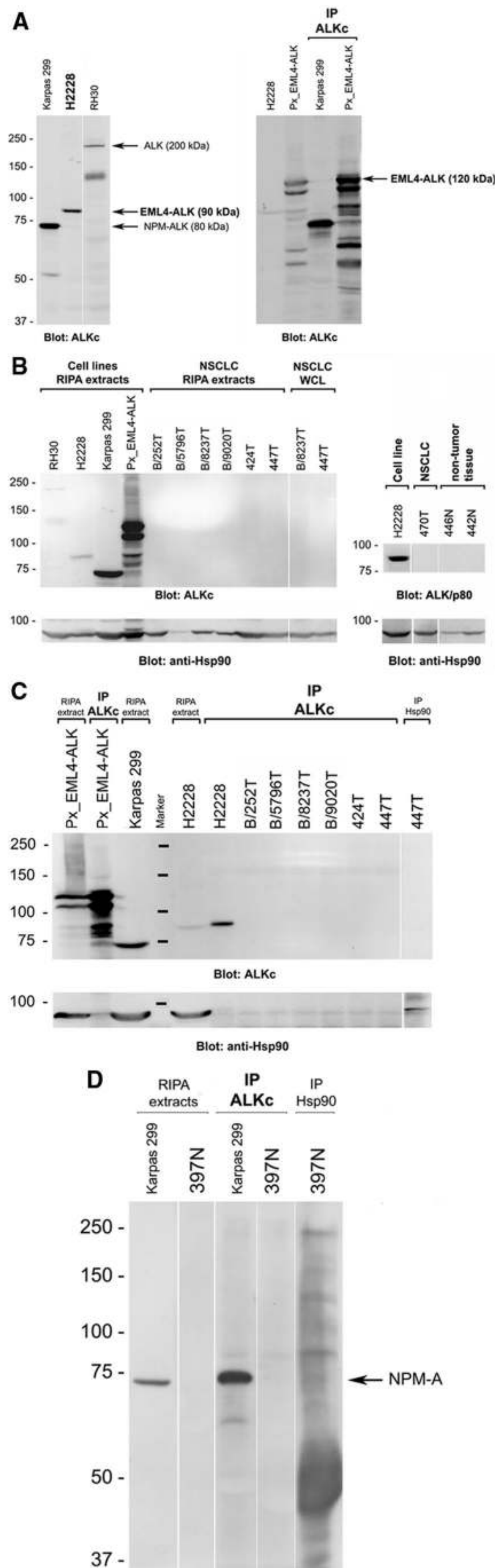
Unexpectedly, 4/67 (about 6%) non-tumor lung samples displayed the presence of *EML4-ALK* transcript (variant 1) and 6/67 (about 9%) showed *EML4-ALK* variant 3 transcripts confirmed by sequence analysis (Figure 1, A–C). The frequency of *EML4-ALK* transcripts did not differ significantly in non-tumor lung tissues and tumor samples ($P = 0.27$, Fisher's exact test). Careful histological analysis of frozen sections showed only normal lung tissue without any preneoplastic or neoplastic foci in all of these cases, except one (n. 397N) that contained some alveolar hyperplasia foci. Interestingly, the *EML4-ALK* transcript was not detected in matching tumor samples from the same patients. As for the tumor samples, in one of three replicate experiments neither the Milan nor the Barcelona laboratories were able to confirm the presence of the fusion transcript in half of the cases. To assess sensitivity of the RT-PCR assay, we serially diluted H2228 cell line cDNA and in 3/3 replicate experiments observed that as little as 0.1 ng of cDNA consistently amplified the variant 3 fusion transcript (Supplementary Figure S1 available at *http://ajp.amjpathol.org*). In RT-PCR assays performed on tissue samples this amount corresponds to 1/500 cells carrying the fusion gene if expression was equivalent to the H2228 cell line. Low fusion transcript expression in some normal and tumor samples, even though FISH detected the fusion gene in 1% to 3% of cells, suggests that fusion-positive cells in tissues express lower levels of fusion transcript than the H2228 cell line.

Our results indicate that *EML4-ALK* transcripts (variants 1 and 3) are not tumor specific for NSCLC, since they are detected in about 15% of distant non-tumor lung tissues and are not retained in the paired NSCLCs.

EML4-ALK Protein Expression in NSCLC Harboring the EML4-ALK Transcript

Studies on EML4-ALK protein expression in NSCLC harboring *EML4-ALK* mRNA are scarce. To address this issue, we first assessed the capability of anti-ALK mAbs to recognize the EML4-ALK protein by Western blot and immunoprecipitation in lysates from the H2228 cell line and *EML4-ALK* transfected Phoenix cells. All anti-ALK mAbs recognized the EML4-ALK products at the expected molecular weights (about 90 kDa and 120 kDa, respectively). A representative example using the ALKc mAb is shown in Figure 2A (left panel). The same antibody also immunoprecipitated the fusion protein from *EML4-ALK* transfected Phoenix cells (Figure 2A, right panel). In control lysates from Karpas 299 and Rh30 cell lines, anti-ALK antibodies recognized proteins with the expected molecular weights of NPM-ALK (about 80 kDa) and full-length ALK (about 200 kDa), respectively (Figure 2A).

We then sought the EML4-ALK protein in 6 NSCLCs carrying the *EML4-ALK* transcript variant 1, for which enough material was available for analysis. Neither Western blotting nor immunoprecipitation of NSCLC lysates (cases 424T, 447T, B/252T, B/5796T, B/8237T, and B/9020T) with ALKc mAb and subsequent Western blot-



ting with ALKc or ALK/p80 (Clone SP8) mAb revealed the EML4-ALK protein in cancer specimens (Figures 2B, left panel, and 2C). Identical results were obtained in one non-tumor lung sample with *EML4-ALK* transcript variant 1 (case 397N; Figure 2D). Similarly, no specific EML4-ALK band (about 90 kDa) was detected in the single NSCLC specimen (case 470T) or in two non-tumor tissues (cases 446N and 442N) harboring the *EML4-ALK* variant 3 transcript by either Western blot (Figure 2B, right panel) or immunoprecipitation (data not shown). In contrast, hybrid EML4-ALK proteins of the expected molecular weight were strongly expressed in, and immunoprecipitated from, the H2228 cell line and *EML4-ALK* transfected Phoenix cells (Figure 2, B and C).

These results demonstrate that Western blot and immunoprecipitation did not detect the EML4-ALK protein in NSCLC and non-tumor lung samples expressing *EML4-ALK* transcripts.

FISH Analysis of EML4-ALK Gene in NSCLC Harboring EML4-ALK Transcript

Inability to detect the EML4-ALK protein could be due to: i) tumor cells producing a low amount of, or no, fusion protein; ii) a minority of tumor cells carrying the *EML4-ALK* gene; or iii) a combination of the two events.

To clarify this issue, we used the ALK Dual Color Probe split-apart probe to search for *ALK* rearrangements in paraffin sections of 20 NSCLC (8 positive and 12 negative for the *EML4-ALK* transcripts), in touch imprints from frozen tissue of 8 non-cancerous lung samples (histologically verified to be normal) carrying the *EML4-ALK* transcript variants, in Carnoy's fixed metaphases and interphase nuclei of the H2228 cell line, and in 1 paraffin-embedded ALCL specimen with the *NPM-ALK* rearrangement.

In 12 NSCLC where RT-PCR did not detect the *EML4-ALK* transcripts, FISH analysis revealed only a background level of abnormal signals (about 0.8%). Six

Figure 2. Western blotting and immunoprecipitation assays on NSCLC harboring *EML4-ALK* fusion transcript. **A:** Testing of anti-ALK mAb (ALKc) in Western blot and immunoprecipitation. **Left:** Western blot analysis on cell lysates of Rh30, Karpas 299 and H2228 cell lines, expressing full-length ALK (about 200 kDa), NPM-ALK (80 kDa), and the short form of EML4-ALK (about 90 kDa), respectively. **Right:** ALKc is able to recognize and immunoprecipitate the *EML4-ALK* fusion protein variant 1 (about 120 kDa) from the Phoenix cells transfected with *EML4-ALK* fusion gene construct, variant 1 (Px_EML4-ALK). The lower molecular weight bands appearing in the membrane likely represent degradation products of the over-expressed protein. **B:** Western blot analysis on *EML4-ALK*-positive samples: a band corresponding to EML4-ALK fusion protein (variant 1, about 120 kDa, **left;** variant 3, about 90 kDa, **right**) is not detectable in either NSCLC (cases B/252T, B/5796T, B/8237T, B/9020T, 424T, 447T for variant 1; 470T for variant 3) or non-neoplastic patient samples (cases 446N and 442N for variant 3). RIPA extracts or whole cell lysates, WCL. The membrane was stripped and reblotted with anti-Hsp90 rabbit polyclonal antibody (lower panels). **C:** Absence of detectable EML4-ALK fusion protein in the ALK-immunoprecipitates from *EML4-ALK*-positive NSCLC patient samples. As positive controls for ALK-immunoprecipitation, the H2228 and the Phoenix cell line transfected with *EML4-ALK* fusion gene construct, variant 1 (Px_EML4-ALK) were used. As positive control, immunoprecipitation of Hsp90 from one out of six NSCLC samples was performed. The same membrane was blotted with ALKc mAb (**upper**) and then stripped and reblotted with anti-Hsp90 rabbit polyclonal antibody (**lower**). **D:** Same experiment as in (**C**) was conducted on one *EML4-ALK*-positive non-tumor lung tissue sample (397N).

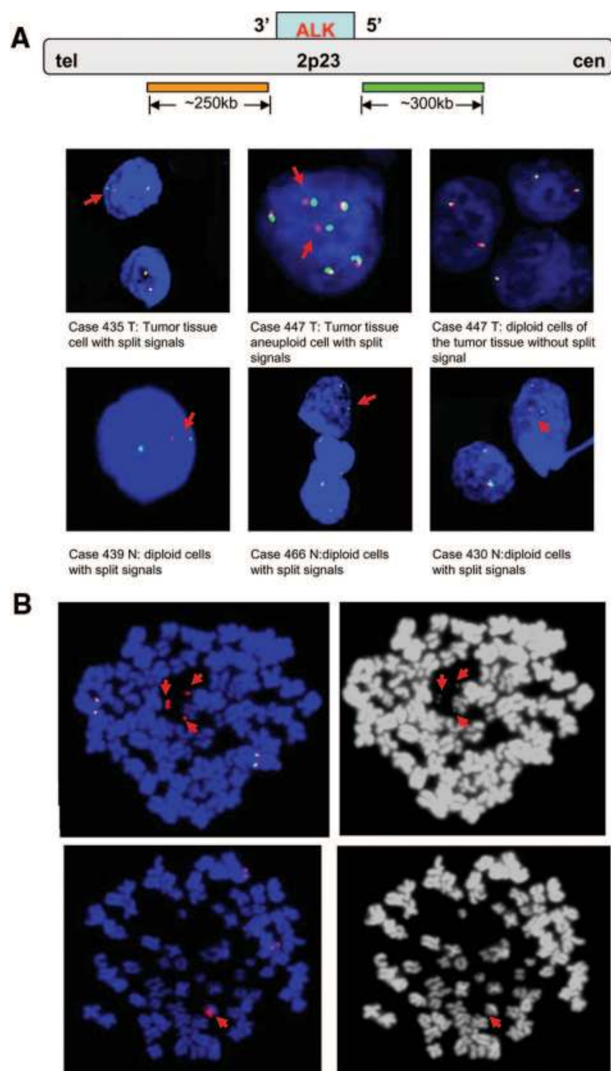


Figure 3. FISH analysis of *ALK* rearrangements in NSCLC and non-tumor lung samples positive for *EML4-ALK* transcripts by RT-PCR. The *ALK* break-apart FISH probe contains two differently labeled probes on opposite sides of the breakpoint of the *ALK* gene. The normal *ALK* appears as a yellow signal, while rearranged *ALK* at this locus will result in separate red and green signals or only one red signal. **A:** FISH analysis on paraffin-embedded section of NSCLC samples 435T and 447T and on touch imprints samples of non-tumor lung tissues of cases 439N, 466N, 430N. Red **arrows** indicate rearranged *ALK*. **B:** FISH analysis performed with the same probe on metaphases of cell-line H2228 (positive for *EML4-ALK* variant 3 by RT-PCR): two normal chromosome 2 (with a co-localization of the red and a green signal) and a deletion of the 5′ *ALK* (with only one red signal present) are visible indicating a rearranged *ALK* locus. The latter is localized on an extrachromosomal element, possibly a double minute, indicated by the red **arrow**, which is present in more than one copy per cell in 4% of the nuclei analyzed.

NSCLC positive for *EML4-ALK* transcript variant 1 (samples 424T, 440T, 447T, B/252T, B/5796T, and B/9020T) showed a low percentage of aneuploid cells containing an *ALK* split signal indicating rearranged ALK. These cells were scattered throughout the paraffin section and accounted for, respectively, 1.2%, 1.2%, 3.0%, 1.5%, 2.7%, and 1.2%, with an average of 1.8% (Figure 3A). Similar results were obtained in touch imprints of two non-tumor lung samples expressing *EML4-ALK* variant 1 (397N and 468N) that showed *ALK*-rearranged interphase nuclei in 9% and 4% of disomic cells, respectively (Figure 3A). The apparently slightly higher percentage of *ALK*-rearranged cells in non-tumor than in lung cancer tissues is probably due to analysis on touch imprints, which provides better morphology and staining of single nuclei. Since we used touch imprints from frozen non-tumor lung tissues so as to obtain optimal FISH signal resolution, we were unable to define what cell type carried the *EML4-ALK* fusion gene.

Similar FISH findings were observed in two paraffin-embedded tumors (average 2.0% positive cells) and in touch imprints from 6 non-tumor lung samples (average 4.25%, range 2.7% to 5.6% of positive cells) that harbored *EML4-ALK* transcript variant 3 (Figure 3A).

FISH findings differed remarkably in *EML4-ALK* positive NSCLC and in the H2228 cell line (Figure 3B). Two normal chromosome 2 (with co-localization of red and green signals) and deletion of the 5′ ALK (one red signal) indicated the *ALK* locus was rearranged in the H2228 cell line. Interestingly, metaphase FISH analysis showed the red signal (rearranged *ALK*) was found on an extra-chromosomal element possibly a double minute. About 4% of cells presented more than one red signal on double minutes, suggesting low-copy number amplification (Figure 3B, red arrows). FISH of a paraffin-embedded ALCL sample expressing NPM-ALK showed 74% of interphase nuclei were positive for an *ALK* rearrangement, where split signals were clearly visualized (Supplementary Figure 2 available at *http://ajp.amjpathol.org*).

In conclusion, in our NSCLC primary samples expressing *EML4-ALK* transcripts, only a small percentage of tumor cells appeared to carry the *EML4-ALK* fusion gene, which might explain why Western blot and immunoprecipitation failed to detect the EML4-ALK protein.

Immunohistochemical Detection of ALK in NSCLC

To determine whether the few tumor cells harboring the *EML4-ALK* gene in NSCLC expressed the corresponding fusion protein, we immunostained paraffin sections from these samples with anti-ALK antibodies. Immunohistochemistry did not reveal even a low percentage ALK-positive tumor cells (Figure 4, A and B), in any NSCLC carrying the *EML4-ALK* fusion gene/transcript. Inability to detect EML4-ALK protein expression could not be due to denaturation of ALK epitopes since the same results were obtained using three different anti-ALK monoclonal antibodies (ALK1, ALKc, 5A4). Moreover, pellets of Phoenix ectopically expressing EML4-ALK fusion protein (variant 1; Figure 4, C and D) or H2228 cell line cells (not shown) that were fixed and embedded in paraffin like NSCLC primary samples, showed strong ALK positivity, with the expected cytoplasmic-restricted distribution of EML4-ALK.

Since immunostaining for ALK is a rapid, sensitive and specific method for detecting *ALK* rearrangements in several tumors,[3] we extended our immunohistochemical studies to 662 paraffin-embedded NSCLC samples from Italy, Japan, and Hong Kong. No specific expression for ALK protein was found in any of these cases. In contrast,

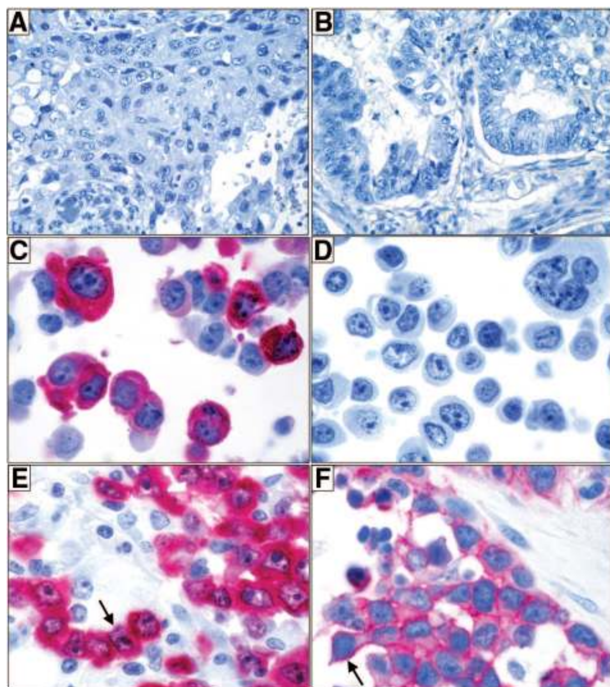


Figure 4. ALK protein expression in NSCLC. A lung squamous cell carcinoma (**A**, magnification = original ×400) and a lung adenocarcinoma (**B**, magnification = original ×400) positive for *EML4-ALK* fusion transcript do not express the ALK protein. **C:** Phoenix cells transfected with *EML4-ALK* show strong cytoplasmic-restricted ALK positivity (magnification = original ×800). **D:** No staining is observed in Phoenix cells transfected with the empty vector (magnification = original ×800). **E:** Anaplastic large cell lymphoma carrying t(2;5)/NPM-ALK shows cytoplasmic and nucleolar positivity for ALK (**arrow**; magnification = original ×800). **F:** ALK positivity in a rhabdomyosarcoma carrying full-length ALK is mainly restricted to the cell surface (**arrow**; magnification = original ×800). **A-F:** immunostaining with anti-ALK mAb clone 5A4; the same results (not shown) were obtained with the ALK1 and ALKc mAbs; Immuno-alkaline phosphatase anti-alkaline phosphatase technique.

all positive controls showed the expected subcellular ALK expression: cytoplasmic plus nuclear in ALCL with t(2;5)[4,22] (Figure 4E); cytoplasmic-restricted in Phoenix cells transfected with *EML4-ALK* (Figure 4, C and D) and in *EML4-ALK* positive H2228 cells (not shown); cell surface in a rhabdomyosarcoma carrying wild-type ALK[22] (Figure 4F). Paraffin samples from five NSCLC showed cytoplasmic ALK positivity that was clearly not specific since the same staining pattern was also observed with buffer or an unrelated mAb (anti-CD34).

Thus, immunohistochemistry did not reveal ALK-positive tumor cells, not even in a low percentage, in NSCLC specimens carrying *EML4-ALK* transcripts. Immunoscreening of a large series of cases from Europe and Eastern Asia suggested lack of ALK protein expression was a general feature in NSCLC.

Discussion

In this study, we found that about 7.5% of NSCLC from Italy and Spain carried variant 1 or 3 *EML4-ALK* transcripts. A similar frequency (6.7%) was previously reported for *EML4-ALK* variant 1 in Japanese patients.[11] These results suggest that, unlike mutations of *EGFR*[26], *EML4-ALK* rearrangements may not to be influenced by ethnic differences.

We also report for the first time that *EML4-ALK* transcripts are expressed in about 15% of non-tumor lung tissues, which implies that the *EML4-ALK* rearrangement is not tumor-specific. Moreover, finding that patients expressing the *EML4-ALK* mRNA in non-tumor lung tissues do not harbor the fusion transcript in the paired tumors raises the question of whether the *EML4-ALK* rearrangement is directly linked to NSCLC pathogenesis. In fact, the scenarios of *EML4-ALK* and *EGFR1* mutations in lung cancer appear to be quite different. *EGFR1* mutations were found in the normal respiratory epithelium of 43% patients with *EGFR*-mutated lung adenocarcinoma but not in patients with *EGFR*-mutation-free lung tumors, suggesting a localized field effect phenomenon.[27]

In our NSCLC patients carrying the *EML4-ALK* transcript, only about 2% of tumor cells harbored the corresponding fusion gene, as detected by FISH analysis of paraffin-embedded sections. Perner et al[28] also detected *ALK* gene rearrangements, with or without *EML4* involvement, in 9/603 (1.5%) NSCLC samples they studied by FISH in tissue microarrays. The percentage of tumor cells carrying the rearrangement was, however, higher (50% to 100%) than in the present study. Different numbers of cases and techniques in the two studies could, at least in part, account for the discrepancy. We concur, however, with Perner et al, who conclude that, because of its heterogenous expression, the *EML4-ALK* fusion gene may not confer a selective proliferative advantage on NSCLC cells.[28]

We found that none of the 662 NSCLC paraffin samples immunostained with specific anti-ALK antibodies expressed the ALK protein. Moreover, immunoprecipitation and immunohistochemistry were unable to demonstrate the presence of *EML4-ALK* protein in seven NSCLC specimens carrying EML4-ALK transcripts (six variant 1; one variant 3). Our findings diverge from those reported by Inamura et al[14] who detected ALK cytoplasmic positivity in five NSCLC carrying the *EML4-ALK* transcript using immunohistochemistry. However, their results cannot be taken as conclusive evidence that tumor cells expressed the EML4-ALK fusion protein, since transcripts encoding for native full length ALK have also been reported in NSCLC.[11] Moreover, Inamura et al did not rule out non-specific staining for proteins other than ALK, by immunostaining with more than one anti-ALK antibody and by Western blotting and/or immunoprecipitation and did not report the results of ALK expression in 144 *EML4-ALK* fusion-negative tumors.

Several reasons may underlie our failure to detect EML4-ALK protein in primary NSCLC specimens harboring the *EML4-ALK* fusion gene/transcript. One might argue that tumor cells could harbor the genetic recombination without producing any EML4-ALK fusion protein. This view is, however, contradicted by mass spectrometry studies on phosphopeptide enrichment with the PhosphoScan Kit (Cell Signaling Technology), which revealed ALK phosphopeptides (including EML4-ALK) in a small subset of NSCLC.[17] This is a rather sensitive method with the potential to detect minimal levels of specific peptides,

even when expressed by only a few cells in the tissue sample. Thus, the most likely explanation for our findings is that tumor cells from primary NSCLC specimens express such a low amount of the EML4-ALK protein that immunoprecipitation and immunohistochemical techniques cannot detect it.

Could such a low amount of EML4-ALK protein play a role in the pathogenesis of NSCLC? A minimal number of ALK phosphopeptides[17] might still possess sufficient tyrosine kinase activity to contribute to transformation. Should this be the case, it would contradict what is commonly observed in lymphoid and mesenchymal neoplasms carrying other *ALK*-rearrangements, including *NPM-ALK*, *TPM3-ALK*, *ATIC-ALK*, *TFG*extralong-*ALK*, *TFG*long-*ALK*, *TFG*short-*ALK*, *CLTC-ALK*, *MSN-ALK*, *TPM4-ALK*, *MYH9-ALK*, and *ALO17-ALK*. In all these tumors the transforming activity was always associated with constitutive expression of the corresponding phosphorylated ALK fusion proteins, which were easily detected by Western blotting[29] or immunohistochemistry.[2,3,30] Furthermore, since EML4-ALK proteins (variant 1 and 3) are clearly detectable by immunoblotting and immunohistochemistry in the H2228 and H3122 cell lines,[31] weaker activity of *EML4* than the *NPM1* promoter is unlikely to account for the differences in protein expression in NSCLC when compared with other tumors carrying *ALK* rearrangements. Finally, as in NSCLC, an *ALK* fusion transcript (ie, *NPM-ALK*) without the corresponding fusion protein, was observed in Hodgkin's disease and lymphomas other than ALK-positive ALCL,[32–34] whose pathogenesis is thought not to be related to *ALK* rearrangements. Thus, the role of EML4-ALK in the pathogenesis of NSCLC remains controversial and our findings call for further validation in experimental models fully mimicking EML4-ALK protein expression patterns found in primary NSCLCs. In this respect, the recently data published by Soda et al[35] may not reflect the situation in humans since the EML4-ALK protein is artificially expressed at higher levels than in primary human NSCLScs investigated in this study.

Our results also have implications for the diagnosis and targeted therapy of NSCLC. In reporting the *EML4-ALK* transcript was specific for NSCLC Soda et al[11] suggested that RT-PCR molecular screening of sputum specimens could be used as a highly sensitive means for early diagnosis of NSCLC with the *EML4-ALK* rearrangement.[11] Such a proposal would be also supported by the observation that *EML4-ALK* fusion transcripts, but no *NPM-*, *TPM3-*, *CLTC-*, *ATIC-* or *TFG-ALK* transcripts were detected in NSCLC,[16] and that solid tumors other than NSCLC do not carry *EML4-ALK* fusion transcripts.[15] However, the present findings that non-tumor lung tissues may carry *EML4-ALK* transcripts (both variants 1 and 3) that are otherwise undetectable in paired NSCLCs, cast doubts about the specificity of this diagnostic approach.

The kinase inhibitors erlotinib and gefinitib are effective in lung cancer patients carrying *EGFR* or *HER/neu* gene mutations.[24,26] Since the presence of *EML4-ALK* transcript is mutually exclusive of *EGFR* mutations,[11] *EML4-ALK* positive NSCLC may be another class of lung tumors that is susceptible to treatment with kinase inhibitors.[11,20] Indeed, an ALK inhibitor significantly reduced the growth of BA/F3 cells transfected with *EML4-ALK*.[11] More recently, the H2228 and H3122 cell lines were also shown to be highly sensitive to ALK inhibition,[31] using the specific NVP-TAE684 compound.[10] Indeed, treatment of these cell lines with the ALK inhibitor resulted in a potent suppression of Akt and Erk1/2 phosphorylation (suggesting that ALK activation is coupled with engagement of downstream survival effectors) and induction of cytotoxic or cytostatic responses.[31] Despite these encouraging results, our findings suggest that caution should be exerted in interpreting these *in vitro*[11] and *in vivo*[35] data as a sufficient evidence for predicting efficacy in the clinical setting. In fact, unlike EML4-ALK transfected cells and NSCLC cell lines (H2228 and H3122) and tumors induced in experimental models,[35] primary tumor cells do not express the *EML4-ALK* fusion protein at detectable levels and only a fraction appears to carry the *EML4-ALK* fusion gene. Moreover, we foresee difficulties in selecting NSCLC patients to test their sensitivity to ALK inhibitors because in routine biopsies the EML4-ALK protein is not detected by immunohistochemistry and RT-PCR amplification of the *EML4-ALK* transcript is usually weak, with most transcripts not consistently confirmed in independent PCR experiments. Finally, since this study shows *EML4-ALK* transcripts are expressed in non-tumor lung tissues, the design of molecularly targeted therapies will have to consider the potential toxicity of ALK inhibitors in cells other than the cancerous. Future clinical trials may ultimately answer questions on the effectiveness and toxicity of ALK inhibitors in NSCLC carrying an *EML4-ALK* rearrangement.

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