

# Identification of Novel Isoforms of the *EML4-ALK* Transforming Gene in Non–Small Cell Lung Cancer

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## Abstract

The genome of a subset of non–small-cell lung cancers (NSCLC) harbors a small inversion within chromosome 2 that gives rise to a transforming fusion gene, *EML4-ALK*, which encodes an activated protein tyrosine kinase. Although breakpoints within *EML4* have been identified in introns 13 and 20, giving rise to variants 1 and 2, respectively, of *EML4-ALK*, it has remained unclear whether other isoforms of the fusion gene are present in NSCLC cells. We have now screened NSCLC specimens for other in-frame fusion cDNAs that contain both *EML4* and *ALK* sequences. Two slightly different fusion cDNAs in which exon 6 of *EML4* was joined to exon 20 of *ALK* were each identified in two individuals of the cohort. Whereas one cDNA contained only exons 1 to 6 of *EML4* (variant 3a), the other also contained an additional 33-bp sequence derived from intron 6 of *EML4* (variant 3b). The protein encoded by the latter cDNA thus contained an insertion of 11 amino acids between the *EML4* and *ALK* sequences of that encoded by the former. Both variants 3a and 3b of *EML4-ALK* exhibited marked transforming activity *in vitro* as well as oncogenic activity *in vivo*. A lung cancer cell line expressing endogenous variant 3 of *EML4-ALK* underwent cell death on exposure to a specific inhibitor of *ALK* catalytic activity. These data increase the frequency of *EML4-ALK*-positive NSCLC tumors and bolster the clinical relevance of this oncogenic kinase. [Cancer Res 2008;68(13):4971–6]

## Introduction

Lung cancer is the leading cause of cancer deaths in the United States, with >160,000 individuals dying of this condition in 2006 (1). The efficacy of conventional chemotherapeutic regimens with regard to improving clinical outcome in lung cancer patients is limited. Activating mutations within the epidermal growth factor receptor gene (*EGFR*) have been identified in non–small-cell lung cancer (NSCLC), the major subtype of lung cancer (2, 3), and chemical inhibitors of the kinase activity of *EGFR* have been found to be effective in the treatment of a subset of NSCLC patients harboring such mutations. However, these somatic mutations of

*EGFR* are prevalent only among young women, nonsmokers, and Asian populations (3, 4).

We recently identified a novel transforming fusion gene, *EML4* (echinoderm microtubule-associated protein-like 4)-*ALK* (anaplastic lymphoma kinase), in a clinical specimen of lung adenocarcinoma from a 62-year-old male smoker (5). This fusion gene was formed as the result of a small inversion within the short arm of chromosome 2 that joined intron 13 of *EML4* to intron 19 of *ALK* (transcript ID ENST00000389048 in the Ensembl database<sup>5</sup>). The *EML4-ALK* protein thus contained the amino-terminal half of *EML4* and the intracellular catalytic domain of *ALK*. Replacement of the extracellular and transmembrane domains of *ALK* with this region of *EML4* results in constitutive dimerization of the kinase domain of *ALK* and a consequent increase in its catalytic activity (5).

Whereas this *EML4-ALK* fusion gene was detected in 3 of 75 individuals with NSCLC, we further identified another isoform of *EML4-ALK* in two patients of the same cohort (5). In these two individuals, intron 20 of *EML4* was disrupted and joined to intron 19 of *ALK*, with the fusion protein thus consisting of the amino-terminal two thirds of *EML4* and the intracellular domain of *ALK*. This larger version of *EML4-ALK* was referred to as variant 2, with the original smaller version being termed variant 1. A total of 5 of the 75 (6.7%) patients in the cohort were thus positive for *EML4-ALK*.

Given that detection of *EML4-ALK* cDNA by the PCR would be expected to provide a highly sensitive means for diagnosis of lung cancer, and given that inhibition of the catalytic activity of *EML4-ALK* may be an effective approach to treatment of this disorder, we have examined whether other isoforms of *EML4-ALK* are associated with NSCLC. We now describe a third isoform of *EML4-ALK* (variant 3) that is smaller than variants 1 and 2.

## Materials and Methods

**PCR.** This study was approved by the ethics committees of Jichi Medical University and The Cancer Institute of the Japanese Foundation for Cancer Research. Total cDNA of NSCLC specimens was synthesized with PowerScript reverse transcriptase (Clontech) and an oligo(dT) primer from total RNA purified with the use of an RNeasy Mini RNA purification kit (Qiagen). Reverse transcription-PCR (RT-PCR) to amplify the fusion point of *EML4-ALK* variant 3 mRNA was done with a QuantiTect SYBR Green kit (Qiagen) and the primers 5'-TACCAGTGCTGTCTCAATTGCAGG-3' and 5'-TCTTGCCAGCAAAGCAGTAGTTGG-3'. A full-length cDNA for *EML4-ALK*

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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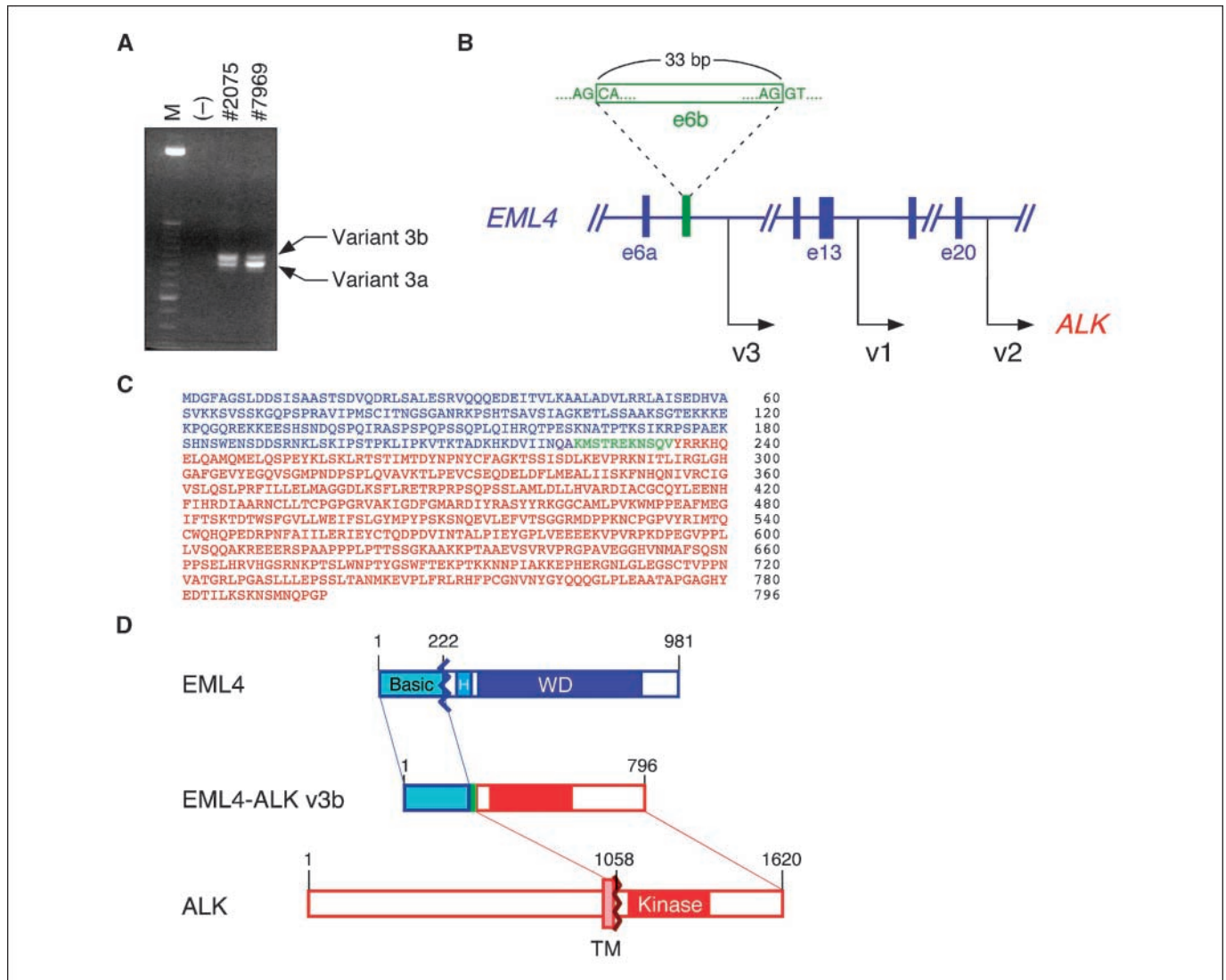
<sup>5</sup> <http://www.ensembl.org/index.html>

variant 3 was amplified from total cDNA of a NSCLC specimen (ID no. 2075) with PrimeSTAR HS DNA polymerase (Takara Bio) and the primers 5'-ACTCTGTCGGTCCGCTGAATGAAG-3' and 5'-CCACGGTCTTAGG-GATCCCAAGG-3'; PCR was done for 35 cycles of 98°C for 10 s and 68°C for 6 min. The fusion point of *EML4-ALK* in the genome was amplified by PCR with genomic DNA of NSCLC specimens, PrimeSTAR HS DNA polymerase, and the primers 5'-GGCATAAAGATGTCATCATCAAC-CAAGG-3' and 5'-AGCTTGCTCAGCTTGACTCAGGG-3'. The nucleotide sequences of the *EML4-ALK* variant 3a and 3b cDNAs have been deposited in DDBJ/EMBL/GenBank under accession nos. AB374361 and AB374362, respectively.

**Fluorescence in situ hybridization.** Fluorescence *in situ* hybridization (FISH) analysis of the fusion gene was done with archival pathology specimens and with bacterial artificial chromosomes containing genomic DNA corresponding to *EML4* or *ALK* and their flanking regions as probes. In brief, surgically removed lung cancer tissue was fixed in 20% neutral

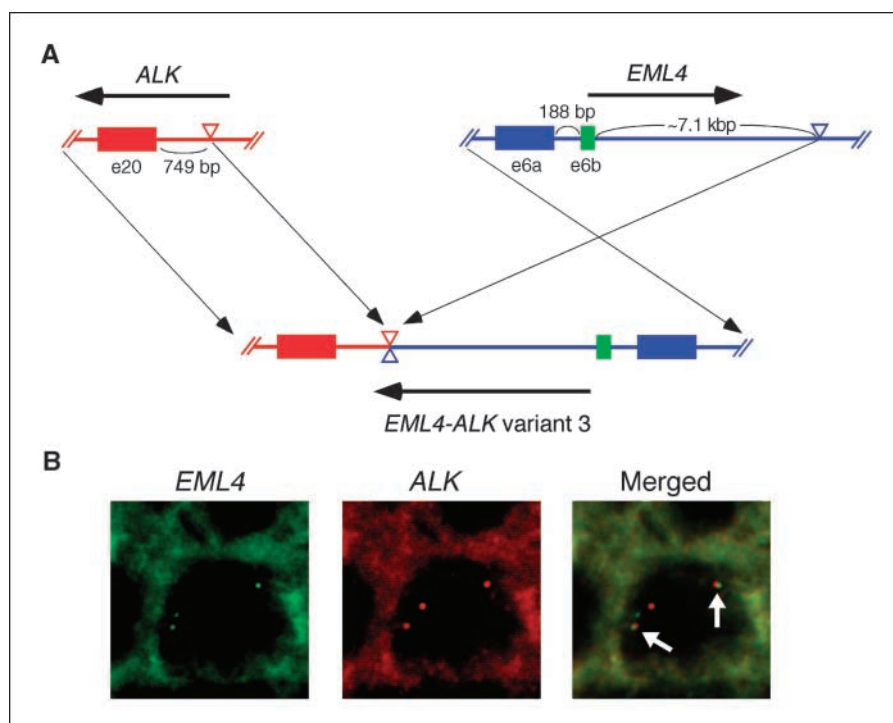
buffered formalin, embedded in paraffin, and sectioned at a thickness of 3 μm. The sections were placed on glass slides and processed with a Histology FISH Accessory Kit (DakoCytomation) before hybridization with the *EML4* and *ALK* probes and examination with a fluorescence microscope (BX61, Olympus).

**Transforming activity of *EML4-ALK* variant 3.** Analyses of the function of *EML4-ALK* variant 3 were done as described previously (5). In brief, the cDNA for *EML4-ALK* variant 3a or 3b was fused with an oligonucleotide encoding the FLAG epitope tag and then inserted into the retroviral expression plasmid pMXS (6). The resulting plasmids as well as similar pMXS-based expression plasmids for *EML4-ALK* variant 1, variant 1 (K589M), or variant 2 were individually introduced into mouse 3T3 fibroblasts by the calcium phosphate method for a focus formation assay and assay of tumorigenicity in nu/nu mice. The same set of *EML4-ALK* proteins was expressed in HEK293 cells and assayed for kinase activity *in vitro* with the YFF peptide (7).



**Figure 1.** Identification of *EML4-ALK* variant 3. **A**, detection of fusion cDNAs linking exon 6 of *EML4* to exon 20 of *ALK* by RT-PCR analysis. Two RT-PCR products of 548 bp (corresponding to variant 3b) and 515 bp (corresponding to variant 3a) were detected by agarose gel electrophoresis with total RNA from two NSCLC specimens (tumor ID nos. 2075 and 7969). Lane (-), no-template control; lane M, size markers (50-bp ladder). **B**, genomic organization of *EML4*. Intronic sequences downstream of exons (e) 6, 13, and 20 of *EML4* are fused to intron 19 of *ALK* to generate variants (v) 3, 1, and 2 of *EML4-ALK*, respectively. Exon-intron boundary sequences as well as the size of exon 6b are indicated. **C**, predicted amino acid sequence of *EML4-ALK* variant 3b. Blue, green, and red, amino acids corresponding to exons 1 to 6a of *EML4*, exon 6b of *EML4*, and *ALK*, respectively. Amino acid number is indicated on the right. **D**, fusion of an amino-terminal portion of *EML4* [which consists of a basic region (Basic), HELP domain (H), and WD repeats] to the intracellular region of *ALK* (containing the tyrosine kinase domain) generates *EML4-ALK* variant 3b. Green, the region of the fusion protein encoded by exon 6b of *EML4*. TM, transmembrane domain.

**Figure 2.** Chromosomal rearrangement responsible for generation of *EML4-ALK* variant 3. **A**, schematic representation of the chromosomal rearrangement underlying the generation of *EML4-ALK* variant 3. Exon 6b of *EML4* is located 188 bp downstream of exon 6a. In NSCLC specimen ID no. 7969, *EML4* is disrupted at a position ~7.1 kbp downstream of exon 6b and is ligated to a position 749 bp upstream of exon 20 of *ALK*, giving rise to the *EML4-ALK* (variant 3) fusion gene. Horizontal arrows, direction of transcription. **B**, FISH analysis of a representative cancer cell in a histologic section of lung adenocarcinoma (ID no. 7969) with differentially labeled probes for *EML4* (left) and *ALK* (center). Two fusion signals (arrows) and a pair of green (corresponding to *EML4*) and red (corresponding to *ALK*) signals are present in the merged image (right).



The cDNA for FLAG-tagged *EML4-ALK* variant 3b was also inserted into pMX-iresCD8 for the expression of both *EML4-ALK* and mouse CD8 (8), and the resulting recombinant retroviruses were used to infect mouse BA/F3 cells (9). CD8-positive cells were then purified with the use of a miniMACS magnetic bead-based separation system (Miltenyi Biotec) and cultured in the absence or presence of mouse interleukin-3 (IL-3; Sigma) or 2,4-pyrimidinediamine (Example 3-39, a specific inhibitor of ALK enzymatic activity that was developed by Novartis<sup>6</sup> and synthesized by Astellas Pharma).

Mouse 3T3 fibroblasts and NCI-H2228 lung cancer cells (both from American Type Culture Collection) as well as 3T3 cells expressing v-Ras were plated in 96-well spheroid culture plates (Celltight Spheroid, Sumilon) at a density of  $1 \times 10^3$  per well. Cell growth was examined with the WST-1 Cell Proliferation Reagent (Clontech) after culture for 5 d with 2,4-pyrimidinediamine.

**Luciferase reporter assays.** The promoter fragments of *Fos*, *Myc*, and *Bcl-x<sub>l</sub>* genes were ligated to a luciferase cDNA to generate pFL700 (10), pHxLuc (11), and pBclxl-Luc (12) reporter plasmids, respectively. Luciferase cDNA ligated to the DNA binding sequence for nuclear factor  $\kappa$ B (NF- $\kappa$ B) or to the GAS sequence was obtained from Stratagene. HEK293 cells were transfected with these various reporter plasmids together with the expression plasmid for *EML4-ALK* variant 3b or the empty vector, as described previously (13). The pGL4 plasmid (Promega) for expression of *Renilla* luciferase was also included in each transfection mixture. After culture of the cells for 2 d, luciferase activity in cell lysates was measured with a Luciferase Assay system (Promega).

## Results and Discussion

**Detection of *EML-ALK* variant 3.** The *EML4-ALK* variant 1 and 2 proteins are produced as a result of genomic rearrangements that

lead to the juxtaposition of exons 13 and 20 of *EML4*, respectively, to exon 20 of *ALK*. It is theoretically possible that exon 2, 6, 18, or 21 of *EML4* also could undergo in-frame fusion to exon 20 of *ALK*. We therefore examined whether transcripts of any such novel *EML4-ALK* fusion genes are present in NSCLC cells by RT-PCR analysis with primers that flank each putative fusion point (data not shown). With the primer set for amplification of the *EML4* (exon 6)-*ALK* (exon 20) fusion cDNA, we detected a pair of PCR products in two individuals with lung adenocarcinoma (Fig. 1A). Although one of the patients (tumor ID no. 7969) had a smoking index of 540, the other patient (tumor ID no. 2075) had never smoked. Nucleotide sequencing of each PCR product from both patients revealed that the smaller product of 515 bp corresponded to a fusion cDNA linking exon 6 of *EML4* to exon 20 of *ALK*, whereas the larger product of 548 bp contained an additional sequence of 33 bp that was located between these exons of *EML4* and *ALK* and which mapped to intron 6 of *EML4* (Fig. 1B). The larger cDNA would thus be expected to encode a fusion protein with an insertion of 11 amino acids between the *EML4* and *ALK* sequences of the protein encoded by the smaller cDNA.

Although we did not detect human mRNAs or expressed sequence tags containing this cryptic exon of *EML4* in the nucleotide sequence databases, it is likely that this exon is physiologic and functional because (a) the fusion cDNA containing this exon was identified in two independent patients and in amounts no less than those of the corresponding cDNA without it (Fig. 1A); (b) the intron-exon boundary sequence for this exon conforms well to the AG-GU rule for mRNA splicing (Fig. 1B); and (c) *EML4* cDNAs or expressed sequence tags containing this exon were detected in the sequence databases for other species (for instance, GenBank accession no. AK144604 corresponding to a mouse *EML4* cDNA). We thus refer to this cryptic exon as exon 6b and to the original exon 6 as exon 6a (Fig. 1B). The novel isoforms of *EML4-ALK* transcripts containing exons 1 to 6a or 1 to 6b of *EML4* were also designated variants 3a and 3b, respectively.

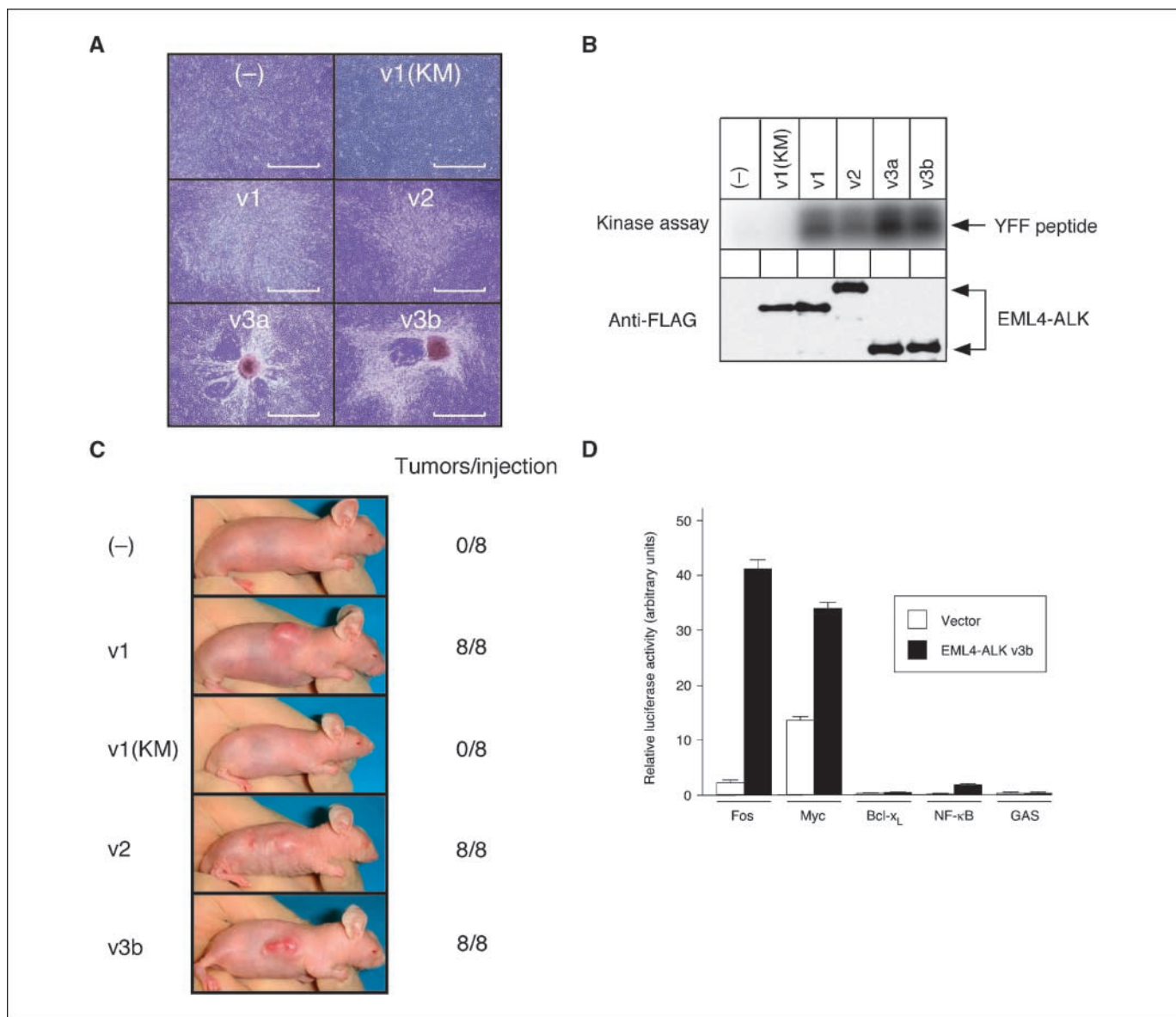
<sup>6</sup> Patent information: Garcia-Echeverria C, Kanazawa T, Kawahara E, Masuya K, Matsuura N, Miyake T, et al., inventors; Novartis AG, Novartis Pharma GmbH, IRM LLC, applicants. 2,4-Pyrimidinediamines useful in the treatment of neoplastic disease, inflammatory and immune system disorders. PCT WO 2005016894. 2005 Feb 24.

To isolate a full-length cDNA for EML4-ALK variant 3, we performed RT-PCR with total cDNA of a positive specimen (ID no. 2075) and with a sense strand primer targeted to the 5' untranslated region (UTR) of *EML4* mRNA and an antisense strand primer targeted to the 3' UTR of *ALK* mRNA. One-step PCR analysis yielded cDNA products for both *EML4-ALK* variants 3a and 3b (Fig. 1C; Supplementary Fig. S1).

The EML4 protein contains an amino-terminal basic domain followed by a hydrophobic echinoderm microtubule-associated protein-like protein (HELP) domain and WD repeats (14). Given

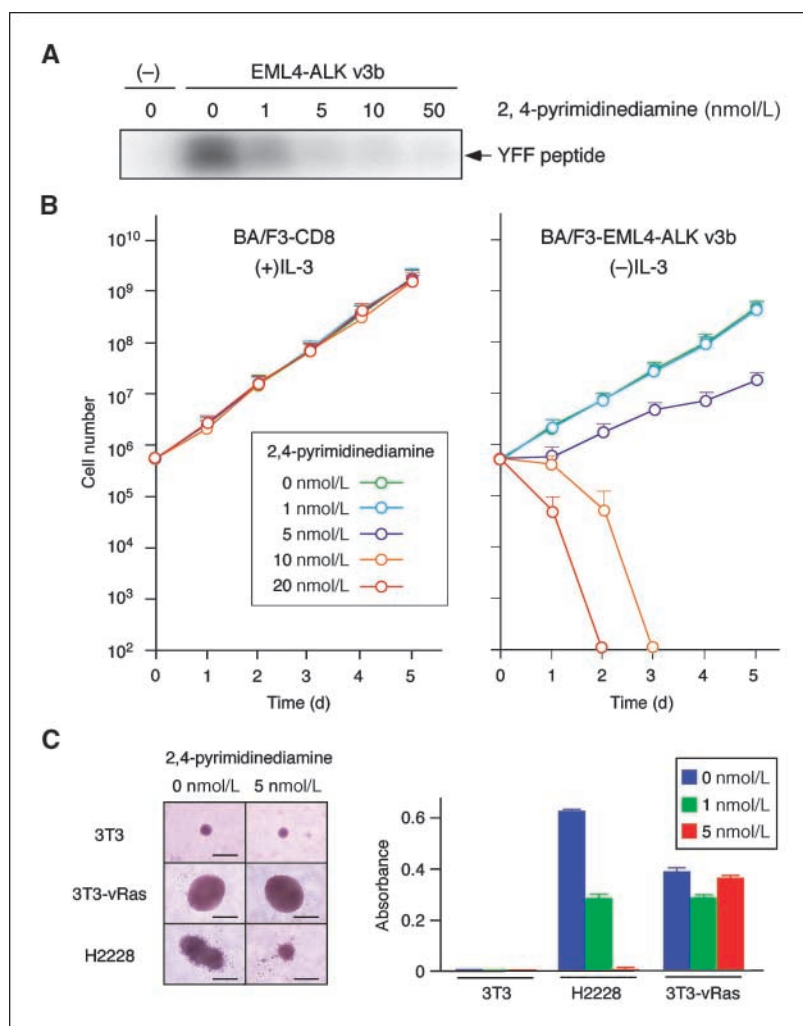
that exons 1 to 6 of *EML4* encode the basic domain, the proteins encoded by the variant 3 cDNAs contain the entire basic domain of EML4 directly linked to the catalytic domain of ALK (Fig. 1D). The fact that the basic domain was found to be essential for both the self-dimerization and oncogenic activity of EML4-ALK (5) suggested that the variant 3 isoforms likely also possess transforming activity.

**Chromosome rearrangement responsible for generation of *EML4-ALK* variant 3.** To show the presence of a chromosome rearrangement responsible for the generation of *EML4-ALK* variant



**Figure 3.** Transforming potential of EML4-ALK variants. *A*, focus formation assay. Mouse 3T3 fibroblasts were transfected with the empty expression plasmid [(-)] or with plasmids for wild-type (*v1*) or K589M mutant [*v1(KM)*] forms of variant 1, variant 2 (*v2*), variant 3a (*v3a*), or variant 3b (*v3b*) of FLAG-tagged EML4-ALK. The cells were photographed after culture for 18 d. *Bar*, 1 mm. *B*, *in vitro* kinase assay. HEK293 cells expressing the various FLAG-tagged variants of EML4-ALK were lysed and subjected to immunoprecipitation with antibodies to FLAG, and the resulting precipitates were assayed for kinase activity with the synthetic YFF peptide (*top*) or subjected to immunoblot analysis with antibodies to FLAG (*bottom*). *C*, *in vivo* assay of tumorigenicity. 3T3 cells expressing the indicated EML4-ALK variants were injected s.c. into nu/nu mice, and tumor formation was examined after 20 d. The number of tumors formed per eight injections is indicated on the right. *D*, analysis of EML4-ALK signaling with luciferase-based reporter plasmids. HEK293 cells were transfected with an expression plasmid for EML4-ALK variant 3b (or with the empty vector) together with reporter plasmids containing the promoter fragment of *Fos*, *Myc*, or *Bcl-x<sub>L</sub>* gene; the DNA binding sequence for NF-κB; or the GAS sequence. Cells were cultured for 2 d, lysed, and assayed for luciferase activity. The activity of firefly luciferase was normalized by that of *Renilla* luciferase. *Columns*, mean of three experiments; *bars*, SD.

**Figure 4.** Essential role of EML4-ALK kinase activity in malignant transformation. *A*, lysates of HEK293 cells expressing FLAG-tagged EML4-ALK variant 3b (v3b) were divided into five equal portions, and each portion was subjected to immunoprecipitation with antibodies to FLAG. The immunoprecipitates were washed with kinase buffer [10 mmol/L HEPES-NaOH (pH 7.4), 50 mmol/L NaCl, 5 mmol/L MgCl<sub>2</sub>, 5 mmol/L MnCl<sub>2</sub>, 0.1 mmol/L Na<sub>3</sub>VO<sub>4</sub>] containing 0, 1, 5, 10, or 50 nmol/L of 2,4-pyrimidinediamine and then incubated for 30 min at room temperature for assay of kinase activity with the YFF peptide in the continued absence or presence of 2,4-pyrimidinediamine. The same amount of lysate of cells transfected with the empty vector was also subjected to immunoprecipitation and assayed as a negative control (-). *B*, mouse BA/F3 cells expressing CD8 alone were cultured in the presence of IL-3 (1 ng/mL) and the indicated concentrations of 2,4-pyrimidinediamine (*left*). BA/F3 cells expressing both CD8 and EML4-ALK variant 3b were cultured with the indicated concentrations of 2,4-pyrimidinediamine but without IL-3 (*right*). Cell number was counted at the indicated times. Points, mean of three separate experiments; bars, SD. *C*, mouse 3T3 fibroblasts expressing (or not) v-Ras or NCI-H2228 cells were cultured in a spheroid culture plate for 2 d, after which 2,4-pyrimidinediamine was added to the culture medium at a concentration of 0, 1, or 5 nmol/L. The cells were photographed after culture for an additional 5 d (*left*). Bar, 4 mm. Cell number in each well was also assessed at the same time with the use of the WST-1 assay (*right*). Columns, mean of three wells from a representative experiment; bars, SD.



3, we attempted to amplify the fusion point between the two genes from the genome of positive NSCLC cells. PCR with primers targeted to regions flanking the putative fusion point yielded a product of ~8 kbp with the genomic DNA of tumor ID no. 7969 (data not shown). Our failure to detect an unambiguous PCR product with genomic DNA of tumor ID no. 2075 may indicate that the breakpoint in intron 6 of *EML4* in this specimen is too distant from exon 6 to be readily amplified by PCR (intron 6 of *EML4* is >16 kbp). Nucleotide sequencing of the PCR product for tumor ID no. 7969 revealed that intron 6 of *EML4* was disrupted at a position ~7.1 kbp downstream of exon 6b and was joined to a point 749 bp upstream of exon 20 of *ALK* (Fig. 2A).

We also confirmed the chromosome rearrangement involving *EML4* and *ALK* by FISH analysis of cells from tumor ID no. 7969 (Fig. 2B) and tumor ID no. 2075 (data not shown) with differentially labeled probes for the two genes. Both genes map to the short arm of chromosome 2 within a distance of ~12 Mbp. The tumor cells exhibited fusion signals (corresponding to *EML4-ALK*) in addition to a pair of isolated green and red signals (corresponding to the two genes on the normal chromosome 2). The chromosome rearrangement involving the *ALK* locus was further verified with a different set of fluorescent probes (Supplementary Fig. S2).

**Transforming activity of EML4-ALK variant 3.** To compare the transforming potential of variants 1, 2, 3a, and 3b of EML4-ALK,

we introduced expression plasmids for each variant into mouse 3T3 fibroblasts for assay of focus formation. No transformed foci were detected for cells transfected with the empty plasmid or with a plasmid for a kinase-inactive mutant (K589M) of EML4-ALK variant 1 (5) in which Lys<sup>589</sup> in the ATP binding site of the catalytic domain is replaced with Met (Fig. 3A). In contrast, variants 3a and 3b of EML4-ALK each exhibited marked transforming activity that was not less than that of variant 1 or 2. To examine directly the tyrosine kinase activity of EML4-ALK variants, we subjected HEK293 cells expressing each of these variants to an *in vitro* kinase assay with a synthetic YFF peptide (7). Again, both variants 3a and 3b exhibited marked kinase activity that was not less than that of variant 1 or 2 (Fig. 3B). Similarly, in a tumorigenicity assay with nude mice, 3T3 cells expressing EML4-ALK variant 3b formed large subcutaneous tumors at all injection sites (Fig. 3C). Consistent with our previous observations (5), cells expressing variant 1 or 2 of EML4-ALK also formed tumors.

To examine the intracellular signaling pathways activated by EML4-ALK, we linked the luciferase cDNA to the promoter fragment of *Fos*, *Myc*, or *Bcl-x<sub>L</sub>* gene (10–12); the DNA binding sequence for NF-κB; or the GAS sequence [a target site of the transcription factors signal transducers and activators of transcription (STAT)-1 and STAT3; ref. 15]. The resulting constructs were then introduced into HEK293 cells together with an

expression plasmid for EML4-ALK variant 3b. EML4-ALK variant 3b markedly activated the promoters of the *Fos* and *Myc* genes (Fig. 3D), consistent with the transforming potential of EML4-ALK. In contrast, although STAT3 has been shown to be a downstream target of the NPM-ALK fusion protein (16), EML4-ALK did not activate the GAS sequence, suggesting that STAT3 is unlikely to be a major target of EML4-ALK, as was shown in an EML4-ALK-positive lung cancer cell line by a proteomics approach (17). The distinct subcellular localizations of the two ALK fusion proteins [EML4-ALK in the cytoplasm (5) and NPM-ALK in both the nucleus and cytoplasm (18)] may account for this difference. Whereas EML4-ALK did not activate the *Bcl-x<sub>L</sub>* gene promoter, it induced a small but significant increase in the activity of the NF- $\kappa$ B binding sequence ( $P = 1.86 \times 10^{-4}$ , Student's *t* test).

Several compounds have recently been identified as specific inhibitors of the kinase activity of ALK and as potential drugs for the treatment of lymphoma positive for *NPM-ALK* (19). We examined the effects of one such inhibitor, 2,4-pyrimidinediamine, on the transforming potential of EML4-ALK. We first determined the effect of this inhibitor on the kinase activity of EML4-ALK variant 3b immunoprecipitated from transfected cells. 2,4-Pyrimidinediamine inhibited the kinase activity of EML4-ALK in a concentration-dependent manner, with a concentration of 1 nmol/L reducing the kinase activity to <50% of the control value (Fig. 4A).

We also introduced EML4-ALK variant 3b and CD8 (or CD8 alone) into the IL-3-dependent hematopoietic cell line BA/F3 (9) and then purified the resulting CD8-positive cell populations. 2,4-Pyrimidinediamine, even at a concentration of 20 nmol/L, did not affect the IL-3-dependent growth of BA/F3 cells expressing only CD8 (Fig. 4B), indicating that this agent does not inhibit mitogenic signaling mediated by Janus kinase in BA/F3 cells. Expression of EML4-ALK rendered BA/F3 cells independent of IL-3 for growth, but the cells expressing the fusion protein also rapidly underwent cell death on exposure to 2,4-pyrimidinediamine (Fig. 4B).

Finally, we examined the effect of 2,4-pyrimidinediamine on lung cancer cells that express endogenous EML4-ALK variant 3. The human lung cancer cell line NCI-H2228 expresses EML4-ALK variants 3a and 3b (data not shown) and forms spheroids in a

three-dimensional spheroid culture system (Fig. 4C; ref. 20). Whereas 3T3 fibroblasts are unable to form such spheroids, expression of v-Ras in these cells results in the formation of large spheroids in culture. Whereas 2,4-pyrimidinediamine did not affect the proliferation of 3T3 cells expressing v-Ras in this system, it inhibited the growth of NCI-H2228 cells in a concentration-dependent manner (Fig. 4C). These data thus indicate that EML4-ALK is essential for the growth of cancer cells expressing this oncokinase.

In conclusion, we have identified novel isoforms of *EML4-ALK* in two patients with NSCLC. A chromosome inversion within 2p was shown to connect intron 6 of *EML4* to intron 19 of *ALK* and to be responsible for the generation of fusion cDNAs connecting exons 1 to 6a or exons 1 to 6b of *EML4* to exon 20 of *ALK*. Given that fusion cDNAs with or without exon 6b of *EML4* were each present in the two patients, EML4-ALK variant 3a and 3b proteins are likely to be coexpressed in NSCLC cells. Although RT-PCR analysis to detect *EML4-ALK* may provide a highly sensitive means to detect lung cancer, it is important that all variant forms of the fusion gene be assayed with appropriately designed primer sets. Given that all the identified variants possess prominent transforming activity, the newly revealed increased incidence of *EML4-ALK* fusion in NSCLC further increases the importance of the fusion gene as a therapeutic target for this intractable disorder.

## Disclosure of Potential Conflicts of Interest

K. Takeuchi: Consultant, DAKO. The other authors disclosed no potential conflicts of interest.

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