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Year: 2008

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Originally published at:
Neoplasia 2008, 10(3):298-302.

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Abstract

A recurrent gene fusion between EML4 and ALK in 6.7% of non-small cell lung cancers (NSCLCs) and NKX2-1 (TTF1, TITF1) high-level amplifications in 12% of adenocarcinomas of the lung were independently reported recently. Because the EML4-ALK fusion was only shown by a reverse transcription-polymerase chain reaction approach, we developed fluorescent in situ hybridization assays to interrogate more than 600 NSCLCs using break-apart probes for EML4 and ALK. We found that EML4-ALK fusions occur in less than 3% of NSCLC samples and that EML4 and/or ALK amplifications also occur. We also observed that, in most cases in which an EML4/ALK alteration is detected, not all of the tumor cells harbor the lesion. By using a detailed multi-fluorescent in situ hybridization probe assay and reverse transcription-polymerase chain reaction, we have evidence that other, more common mechanisms besides gene inversion exist including the possibility of other fusion partners for ALK and EML4. Furthermore, we confirmed the NKX2-1 high-level amplification in a significant subset of NSCLC and found this amplification to be mutually exclusive to ALK and EML4 rearrangements.

EML4-ALK Fusion Lung Cancer: A Rare Acquired Event

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Abstract

A recurrent gene fusion between *EML4* and *ALK* in 6.7% of non-small cell lung cancers (NSCLCs) and *NKX2-1* (*TTF1*, *TTF1*) high-level amplifications in 12% of adenocarcinomas of the lung were independently reported recently. Because the *EML4-ALK* fusion was only shown by a reverse transcription–polymerase chain reaction approach, we developed fluorescent *in situ* hybridization assays to interrogate more than 600 NSCLCs using break-apart probes for *EML4* and *ALK*. We found that *EML4-ALK* fusions occur in less than 3% of NSCLC samples and that *EML4* and/or *ALK* amplifications also occur. We also observed that, in most cases in which an *EML4/ALK* alteration is detected, not all of the tumor cells harbor the lesion. By using a detailed multi-fluorescent *in situ* hybridization probe assay and reverse transcription–polymerase chain reaction, we have evidence that other, more common mechanisms besides gene inversion exist including the possibility of other fusion partners for *ALK* and *EML4*. Furthermore, we confirmed the *NKX2-1* high-level amplification in a significant subset of NSCLC and found this amplification to be mutually exclusive to *ALK* and *EML4* rearrangements.

Neoplasia (2008) 10, 298–302

Introduction

The paucity of recurrent gene fusions in common epithelial malignancies may be due to an inability to discover these events rather than any true lack of such events [1]. Supporting this is the recent discovery that the majority of prostate cancers harbor a *TMPRSS2-ETS* fusion [2]. Finding yet unidentified recurrent rearrangements in other common epithelial cancers could provide important insights into carcinogenesis and accelerate rational drug design.

Soda et al. [3] described a novel recurrent gene fusion between *ALK* (2p23) and *EML4* (2p21) in 5 (6.7%) of 75 non-small cell lung cancer (NSCLC) cases. Using an *in vitro* model, they demonstrated fusion of the kinase domain of *ALK* to a poorly characterized gene, *EML4*, to generate an oncogenic fusion (*EML4-ALK*). *ALK*, the anaplastic lymphoma kinase, has been previously observed to fuse with nucleophosmin (*NPM*) and multiple alternative partner genes in anaplastic large cell lymphoma [4]. The resulting highly expressed fusion proteins possess constitutive kinase activity and transforming capacity.

An intriguing but unexplained result was their finding that wild-type *ALK* mRNA was detected in one third of all NSCLCs. They suggested that simple inversion generated the *EML4-ALK* fusion gene (*ALK* and *EML4* are 12 Mb apart with opposite orientations). However, more complex cytogenetic events could be at play, but may have been overlooked, because of the lack of an *in situ* analysis. To resolve this, we developed break-apart fluorescent *in situ* hybridization (FISH) assays for *EML4* and *ALK* (Figure 1A) and analyzed two population-based NSCLC cohorts from Switzerland and the USA. Another recently reported recurrent genetic alteration is the high-level amplification of *NKX2-1* (*TTF1*) in 12% of adenocarcinomas of the lung [5]. Using the same cohorts, we validated the recently reported *NKX2-1* high-level amplification in lung cancers and assessed for correlation with *EML4* and *ALK* alterations.

Material and Methods

Fluorescent In Situ Hybridization Assays

To assess for rearrangements of both the *EML4* and *ALK* loci, two unique break-apart FISH assays were designed (because of the proximity of both genes, a fusion assay was not discriminative). For each gene, two bacterial artificial chromosome (BAC) FISH probes were created to hybridize with the neighboring centromeric and telomeric regions of each gene. BAC clones were selected from the March 2006 build of the human Genome using the University of California, Santa Cruz Genome Browser and were obtained from the BACPAC Resource Center (CHORI, Oakland, CA). For *EML4*, the centromeric BAC clone was biotin-14-deoxycytidine triphosphate (dCTP)-labeled RP11-368J11 (eventually conjugated to produce a red signal), and the telomeric BAC clone was digoxigenin-dUTP-labeled RP11-142M12 (eventually conjugated to produce a green signal). For *ALK*, the centromeric BAC clone was biotin-14-dCTP-labeled RP11-993C21 (red), and the telomeric BAC clone was digoxigenin-dUTP-labeled RP11-984I21 (green) (Figure 1A).

To assess for *NKX2-1* (*TTF1*) amplification, the same FISH assay as described previously was applied [5]. In brief, a probe spanning the *NKX2-1* gene locus (chr14q13.3) and a reference probe spanning a stable region in carcinomas of the lung on chromosome (chr14q24.1) were used. For the *NKX2-1* target probe, the biotin-14-dCTP-labeled BAC clone RP11-1083E2 (eventually conjugated to produce a red signal), and for the reference probe, the digoxin-dUTP-labeled BAC clone RP11-72J8 (eventually conjugated to produce a green signal) were applied as probes.

All samples were analyzed under a $\times 60$ oil immersion objective using a fluorescence microscope (Olympus, Center Valley, PA) equipped with appropriate filters, a charge-coupled device camera,

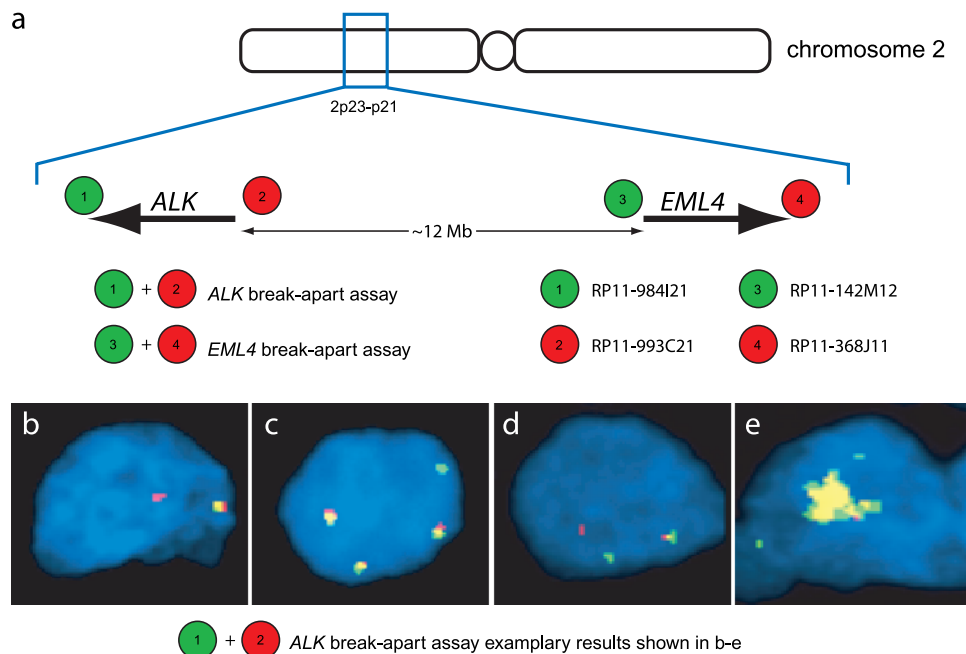


Figure 1. Schematic design of the break-apart FISH assays and exemplary findings. (A) Schematic of the break-apart FISH assays for *EML4* and *ALK*. (B) Loss of DNA on 2p outside the area encoding *EML4* and *ALK* (exemplary for *ALK*: loss of the green-labeled probe telomeric of *ALK*). (C) Loss of interstitial DNA between *EML4* and *ALK* (exemplary for *ALK*: loss of the red-labeled probe centromeric of *ALK*). (D) Break-apart of *ALK* (wild-type allele as one yellow, and one single red and green probe signal for the rearranged allele). (E) Cluster-like amplification of the *ALK* focus (multiple yellow signals).

and the Cytovision FISH imaging and capturing software (Applied Imaging, San Jose, CA). Evaluation of the tests was independently performed by two evaluators (S.P. and P.W.). For each case, we attempted to analyze at least 100 nuclei. We experienced no significant differences between the two independent evaluations.

Detailed Fluorescent In Situ Hybridization Procedure

Escherichia coli containing the selected BACs were cultured overnight at 37°C on LB agar (Fisher Scientific, Hampton, NH). Single clones were selected and grown overnight at 37°C in LB broth (Fisher Scientific) in an incubator under constant shaking. BACs were selectively extracted from the cultures using Qiagen MiniPrep buffers (P1 with RNaseA, P2 and P3; Germantown, MD). Extracted BACs were precipitated with isopropyl alcohol and washed with 70% ethanol before resuspension in distilled, nuclease-free water. Resulting samples were amplified using the Repli-g Midi kit (Qiagen). Amplified BACs were labeled using the BioPrime DNA labeling system (Invitrogen, Carlsbad, CA) to incorporate biotin-14-dCTP into the target probes (eventually detected by streptavidin, Alexa Fluor 594 conjugate from Invitrogen) and digoxigenin-11-dUTP (Roche Applied Science, Nutley, NJ) into the reference probe (eventually detected by Anti-Digoxigenin, F'ab fragments from Roche Applied Science). Before cell line/tissue analysis, the integrity and purity of all probes were verified by hybridization to normal peripheral lymphocyte metaphase spreads (Vysis, Abbott Park, IL).

After deparaffinization, tissue samples were pretreated at 94°C in Tris/EDTA, pH 7.0, buffer for 0.5 hours before protein digestion with Zymed Digest-All (Invitrogen) and ethanol dehydration. After codenaturation of the probe and sample (3 minutes at 94°C for formalin-fixed paraffin-embedded (FFPE) tissue samples and 3.5 minutes at 85°C for cell lines), slides were immediately placed in a dark moist chamber to hybridize for at least 16 hours at 37°C. After incubation, samples were washed for 5 minutes at 75°C in 0.5× SSC buffer. Samples were washed three times for 10 minutes with PBS-T (PBS with 0.025% Tween-20) at room temperature. Blocking was performed in a dark, moist chamber with CAS-Block (Invitrogen) containing 10% goat serum (Invitrogen) for 10 minutes. Secondary detectors were diluted 1:500 in a blocking reagent, and samples were incubated for 1 hour at room temperature. Samples were washed three times with PBS-T buffer as above before mounting with Prolong Gold Antifade Reagent with DAPI (Invitrogen).

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Three FFPE biopsy cores (0.6 cm each) were used for RNA extraction using Trizol LS reagent (Invitrogen) as previously described [6]. Five hundred nanograms of total RNA were used as starting material for the reverse transcriptase (RT) reaction using the Omniscript RT kit (Qiagen) following the manufacturer's protocol. Fifty nanograms of the resulting cDNA template were used for polymerase chain reaction (PCR) amplification using the Platinum *Taq* DNA Polymerase kit (Invitrogen). All PCR reactions were performed using the master mix, the Platinum *Taq* DNA Polymerase supplied in the kit following the manufacturer's protocol, and a final MgCl₂ and each PCR primer (Integrated DNA Technologies, Inc., Coralville, IA) concentration of 1.5 and 0.2 mM, respectively. The reactions were incubated at 94°C for 4 minutes for an initial denaturation step followed by 45 cycles of 94°C for 30 seconds, with the annealing temperature specific to the primer pair used (see below) for 30 sec-

onds and elongation at 72°C for 1 minute. The reactions were then incubated at 72°C for an additional 10 minutes. All PCR reactions were performed using a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, CA). Polymerase chain reaction products were fractionated and visualized using an ethidium bromide (0.5 mg/ml; EMD Chemicals, Gibbstown, NJ) containing 2.5% agarose (EMD Chemicals) gel. Annealing temperatures for the *EML4-ALK* fusion transcript primers, downstream *ALK* primers and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were 55, 50, and 55°C, respectively.

In this RT-PCR assay, we used primers that target fusion variant 1 (as described in the study of Soda et al. [3]) which could also detect variant 2 which would have yielded a considerably longer amplicon. The latter variant may have been expressed in the fusion positive cases but missed in our assay because of the difficulties in amplifying long amplicons from RNA extracted from FFPE tissue.

The following are the forward and reverse primer sequences for: the *EML4-ALK* fusion transcript variant 1: 5'-GTGCAGTGTTTAGCA-TTCTTG GGG and 5'-TCTTGCCAGCAAAGCAGTAGTTGG, respectively; 3' *ALK*: 5'-TTGGAGAGAGGATTGAATACTGC and 5'-GACCAGGAGAGGAGGAACC, respectively; *GAPDH*: 5'-TGCACCACCAACTGCTTAGC and 5'-GGCATGGACTGTGGT-CATGAG, respectively. Independent PCR reactions were carried out for each primer pair.

Study Populations

Tissue microarrays from the University of Zurich and the University of Michigan were used to access *EML4* and *ALK* rearrangement status. The University of Zurich cohort is a population based NSCLC cohort of 507 patients. The University of Michigan cohort represents a surgical series of 96 NSCLC patients. The samples were comprised in high-density tissue microarrays with one to three cores representing a case.

Results

In the two NSCLC cohorts examined, 16 of 603 total cases exhibited evidence for *EML4* and/or *ALK* rearrangement by FISH. Rearrangement patterns included loss of the interstitial probes between *EML4* and *ALK* (Figure 1B), loss of the probes on 2p outside the area encoding either *EML4* or *ALK* (Figure 1C), and break-apart of the genes (Figure 1D). Thus, the frequency of *EML4* and *ALK* rearrangements that potentially result in *EML4-ALK* fusion is 2.7%. We found that for the majority of positive cases only a subset of tumor cells (50-100%) harbored 2p rearrangements. We also found evidence of focal high-level amplification of *EML4* in five cases and of *ALK* in three cases. The amplification was similar to the recently reported *NKX2-1* (*TTF1*) amplification pattern seen in NSCLCs [5] (Figure 1E). In two cases, we found high-level amplification of both *EML4* and *ALK*, indicating a large-scale amplification of the region including both loci. Only two cases showed low-level amplification of one of the probes of the *ALK* break-apart assay, indicating a simultaneous occurrence of rearrangement and low-level amplification. No such event was observed with the *EML4* break-apart assay.

We used RT-PCR to assess for the presence of the *EML4-ALK* variant 1 fusion transcript and 3' *ALK* transcript in eleven cases that scored positive for *EML4* and/or *ALK* rearrangement by FISH (Table 1), as well as two cases (n1 and n2 in Figure 2) that showed no evidence for rearrangement. We detected *EML4-ALK* variant 1 fusion

Table 1. Summary of *EML4* and *ALK* Rearrangements by FISH as well as *EML4-ALK* Variant 1 Fusion Transcript and 3' *ALK* Transcript by RT-PCR.

Case No.	<i>EML4</i> FISH			<i>ALK</i> FISH			RT-PCR	
	Del Cent	Del Tel	Break-Apart	Del Cent	Del Tel	Break-Apart	<i>EML4-ALK</i> Variant 1 Fusion Transcript	3' <i>ALK</i>
1	-	-	-	+	-	-	-	-
2	+	-	-	+	-	-	+	+
3	+	-	-	-	-	+	+	+
4	+	-	-	-	-	-	-	-
5	-	+	-	-	-	-	-	+
6	+	-	-	-	-	-	-	-
7	-	+	-	-	-	-	-	-
8	-	-	-	-	-	+	-	-
9	-	+	-	-	-	-	-	-
10	-	+	-	+	-	-	+	+
11	-	-	-	-	+	-	N/A	N/A
12	-	+	-	+	-	-	N/A	N/A
13	-	-	-	-	+	-	N/A	N/A
14	-	-	-	+	-	-	N/A	N/A
15	+	-	-	-	-	-	N/A	N/A
16	+	-	-	-	-	-	N/A	N/A

RT-PCR data only available in cases where still tissue was available. *Del Cent* indicates loss of the centromeric probe; *Del Tel*, loss of the telomeric probe; *Break-Apart*, break-apart of the two signals of the FISH probes for the specific gene; *N/A*, samples not annotated by PCR because of the lack of available material.

transcript and 3' *ALK* transcript in all four cases that were positive for rearrangement in both genes (case nos. 2, 3, and 10 in Table 1 and Figure 2). Only one case with loss of the interstitial (telomeric) *EML4* FISH probe showed 3' *ALK* transcript but no variant 1 fusion transcript (case no. 5 in Table 1 and Figure 2). All other rearranged cases (case nos. 1, 4, 6, 7, 8, and 9 in Table 1 and Figure 2) and the two non-rearranged cases (case nos. n1 and n2 in Figure 2) did not yield detectable *EML4-ALK* variant 1 fusion transcript nor the 3' *ALK*-specific PCR product (see Table 1 for details of rearrangement status, *EML4-ALK* variant 1 fusion, and 3' *ALK* transcript detection).

We also assessed the two NSCLC cohorts for the recently described *NKX2-1* amplification status by FISH [5]. Of these, 35 cases (5.8%) demonstrated *NKX2-1* high-level amplification. Of note, all cases

showing evidence for *EML4* and *ALK* rearrangements were mutually exclusive to the cases showing *NKX2-1* high-level amplification.

Discussion

Whereas the true frequency of *EML4-ALK* fusion events remains to be determined, our FISH results from two independent population-based cohorts (Zurich and Michigan), comprising more than 600 patients, suggest that the *EML4-ALK* fusion may be less frequent (2.7%) than reported that by Soda et al. [3] (6.7%). Although ethnic or other patient differences could account for the discrepancy in frequency, Inamura et al. [7] also report a frequency of 3.4% of *EML4-ALK* fusion by RT-PCR in a Japanese cohort of 221 lung cancer patients. Based on the findings by our FISH assays, more complex cytogenetic *EML4/ALK* alterations result in *EML4-ALK* fusion rather than by a unique simple inversion as suggested by Soda et al. [3]. In most cases, we observed that only one of the assessed genes exhibited rearrangement, suggesting that other, potentially more common, fusion partners exist. This would not be surprising, because in anaplastic large cell lymphoma, *ALK* rearrangements involve at least 10 alternative fusion partners in addition to *NPM* [4]. Proof of this hypothesis was recently published by Rikova et al. [8], who have identified *TFG* as an *ALK* fusion partner in NSCLC. We also observed the 3' *ALK* product in one case (sample no. 5) that was negative for the *EML4-ALK* fusion variant 1 transcript, which may be an example of another *EML4-ALK* fusion transcript variant or another fusion event involving other partners [3,8].

In addition, amplification of either *EML4* or *ALK* could represent an important mechanism of overexpression in NSCLCs, in spite of the fact that such changes were not detected in a recently released large single nucleotide polymorphism dataset [5], likely due to intratumor lesion heterogeneity. It remains to be determined whether these amplified genes contain additional oncogenic abnormalities as in the case of epidermal growth factor receptor [9], or whether overexpression of wild-type *EML4* and/or *ALK* is itself tumorigenic.

From this and other studies, we conclude that a range of alterations of 2p21-p23 occurs in NSCLCs. However, within a given tumor, not all tumor cells (between 50% and 100%) harbor 2p rearrangements/amplifications, indicating that they may represent late-stage aberrations or different simultaneous coproliferation of clones within the tumor, rather than as early clonal events as recently observed in *TMPRSS2-ETS* prostate cancers [10]. While remaining to be proven, our observations support the hypothesis that 2p21-p23 alterations are a rare acquired but not a driving molecular event in lung cancer development and progression.

Furthermore, we found *NKX2-1* (*TTF1*) high-level amplification in almost 6% of NSCLCs. This is slightly less compared to the results of Weir et al. [5], who reported *NKX2-1* high-level amplification in ~12% of adenocarcinomas of the lung. However, unlike the study of Weir et al., our cohort is a mixture of adenocarcinomas (75%) and squamous cell carcinomas (25%). More importantly, all cases showing evidence for *EML4* and *ALK* rearrangements were mutually exclusive to the cases showing *NKX2-1* high-level amplification, indicating that these events are associated with two independent pathways resulting in lung cancer formation/progression.

Acknowledgments

We thank Nishi Singhal, Scott A. Tomlins, Nallasivam Palanisamy, and Michele LeBlanc (all from the Michigan Center for Translational Pathology, Department of Pathology, University of Michigan Medical

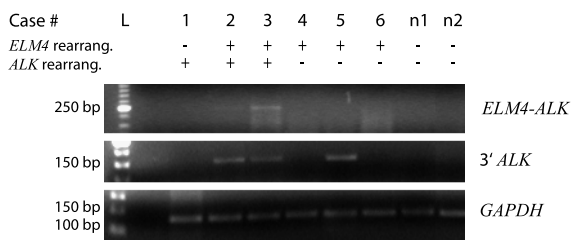


Figure 2. RT-PCR results of selected cases that were found to be positive or negative for *EML4* and/or *ALK* rearrangement by FISH. Primers specific to the *EML4-ALK* variant 1 fusion transcript (*EML4-ALK*, exon 6 of *EML4* and exon 20 of *ALK1*), downstream *ALK* (3' *ALK*, exons 28 and 29), and *GAPDH* were used in combination with PCR of cDNA derived from RNA extracted from FFPE tissue of the indicated cases or of two cases (n1 and n2) that were negative for rearrangements. Results from FISH of *EML4* or *ALK* rearrangement are given below the case number. Positive indicators of rearrangement were attributed to cases if break-apart and/or deletion was observed for gene-specific FISH probes. Refer to Table 1 for specifics for each case shown here. Fifty-nucleotide fragment ladder (lane L) and sizes are shown to the left.

School, Ann Arbor, MI), Martina Storz (Pathology, University of Zurich, Zurich, Switzerland), and Joelle Tchinda (Section of Tumor-cytogenetics of the Children's Hospital of the University of Zurich, Zurich, Switzerland) for critical review and excellent technical support pertinent to this study.

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