The *EML4*-ALK transcript but not the fusion protein can be expressed in reactive and neoplastic lymphoid tissues

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ABSTRACT

Rearrangements involving the *ALK* gene define two distinct entities in the new 2008 WHO classification of lymphoid neoplasms, i.e. ALK+ anaplastic large cell lymphoma and a rare subset of ALK+ diffuse large B-cell lymphoma. Recently, rearrangements involving *ALK* and the echinoderm microtubule associated protein-like 4 (*EML4*) gene were described as a specific genetic alteration in about 6% of non-small cell lung cancer (NSCLC). We investigated the expression of EML4-ALK mRNA and protein in 51 reactive and 58 neoplastic lymphoid tissues. *EML4-ALK* transcripts were detected in 3/51 (5.9%) of reactive lymphoid tissues and 12/58 (20.7%) of lymphomas of different categories, including follicular lymphoma, diffuse large B-cell lymphoma and Hodgkin's disease. Notably, none of these cases expressed the EML4-ALK fusion protein at Western blotting samples and immunohistochemistry. These results indicate that *EML4-ALK* rearrangements are not specific of NSCLC and raise yet unsolved questions about their role in promoting human neoplasms.

Key words: lung cancer, anaplastic lymphoma kinase (ALK), EML4, fusion transcripts, lymphoma, fusion protein, kinase inhibitors.

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Introduction

The 2008 World Health Organization (WHO) classification of tumors of lymphoid and hemopoietic tissues recognizes two distinct lymphoma entities characterized by rearrangements of the anaplastic lymphoma kinase (*ALK*) gene.[1] They include ALK-positive anaplastic large cell lymphoma (ALCL) carrying the t(2;5)/*NPM-ALK* fusion gene or its molecular variants,[2] and a rare subset of diffuse large B-cell lymphoma.[3]

Recombinations involving the *ALK* gene may also occur in extra-hemopoietic neoplasms, such as in inflammatory myofibroblastic tumors.[4] More recently, *ALK* rearrangements have been reported in a small subset (6.7%) of non-small cell lung cancer (NSCLC).[5] In these cases, a small inversion within the short arm of chromosome 2 was found to cause the N-terminal portion (residues 1-496) of human echinoderm microtubule associated protein-like 4 (EML4) to fuse to the intracellular domain of human ALK (residues 497-1059).[5]

The same group also reported that, among human tumors, *EML4-ALK* transcripts were specifically detected only in NSCLC, being absent in 69 non-Hodgkin's lymphomas and in 313 other solid tumors analyzed by RT-PCR[5], thus supporting a pathogenetic role of *EML4-ALK* in lung cancer and its possible use as selective molecular marker for early diagnosis of NSCLC.[6] On the other hand, we recently found that *EML4-ALK* transcript are not tumor-specific since they are detectable by RT-PCR in about 15% of distant non-tumor lung tissues.[7] In this paper, we extended our analysis on the specificity of *EML4-ALK* transcripts expression to a large series of reactive and neoplastic lymphoid tissues and compared the results with the expression of EML4-ALK fusion protein.

Design and Methods

Tissue specimens

Snap frozen specimens for RT-PCR and Western blotting/immunoprecipitation studies were available from 51 reactive lymph nodes and 58 lymphoma patients. Lymphoma samples included: B lymphoblastic lymphoma (n=2), chronic lymphocytic leukemia (n=5), follicular lymphoma (n=9), mantle cell lymphoma (n=1), marginal zone lymphoma (n=2),

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Burkitt's lymphoma (n=1), diffuse large B-cell lymphoma (n=12), Hodgkin's lymphoma (n=22) and peripheral T-cell lymphoma (n=4). All tissues were freshly collected during surgery, snap-frozen in liquid nitrogen, and stored at -80°C. As control for the molecular procedures, we used 10 previously reported NSCLC (5 positive and 5 negative for *EML4-ALK* transcripts).[7] Paraffin-embedded material from all above pathological specimens was available for detection of ALK protein by immunohistochemistry (*see below*).

Transfected cells and cell lines

Either NIH 3T3 or Phoenix cell lines transfected with pcDNA3_EML4-ALK[7], and the NSCLC human cell line H2228 (American Type Culture Collection, ATCC, Rockville, MD, USA) served as positive controls for the variant 1 and the shorter variant 3 of *EML4-ALK* transcript,[7] respectively. The ALCL (Karpas 299) cell line was used as control for expression of the NPM-ALK fusion protein.[8]

Reverse transcription polymerase chain (RT-PCR) analysis of EML4-ALK transcripts

Total RNA was extracted from cells or frozen tissues using RNA isolation TRIZOL® Gibco 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, 1 μg of total RNA was retrotranscribed using Random Primer and 200 U of Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) followed by a PCR with the primers Fusion-RT-S 5'-GTG CAG TGT TTA GCA TTC TTG GGG-3' and Fusion-RT-AS 5'-TCT TGC CAG CAA AGC AGT AGT TGG-3'. These primers could detect both variant 1 and variant 2, as previously described.[5] To analyze the shorter variant of EML4-ALK transcript,[9] the *ALK* Fusion-RT-AS primer was combined with a forward primer located in exon 6 of *EML4*: *EML4-ex6F* 5'-GCA TAA AGA TGT CAT CAT CAA CCA AG-3'.

PCR primers GAPDH-S 5'- ACC ACA GTC CAT GCC ATC AC –3' and GAPDH-AS 5'- TCC ACC ACC CTG TTG CTG TA –3' for glyceraldehyde-3-phosphate dehydrogenase cDNA (452bp) were used as control for cDNA integrity. Samples were processed in a Gene-Amp PCR system 9700 thermal cycler through 25 cycles for *GAPDH* (Ta 58°C, 30" elongation) and 40 cycles for *EML4-ALK* (Ta 60°C, 1' elongation) and *ALK* wild type (Ta 58°C, 30" elongation). Nucleotide sequencing of PCR products was performed to confirm identity of amplified fragments.

Expression of EML4-ALK protein

The expression of EML4-ALK protein was investigated by Western blotting and immunoprecipitation with anti-ALK antibodies on lysates from 2 reactive lymphoid specimens and 7 lymphomas harboring *EML4-ALK* transcripts. Full details of the procedures are given in the *Online Supplementary Appendix*.

Expression of ALK protein was also searched by immunohistochemistry in paraffin sections from all lymphoid samples (51 reactive, 58 neoplastic). Sections were microwave-heated (750-W, three 5' cycles) in 0.01 mol/L citrate, pH 6.0, or 1-mmol/L EDTA, pH 8.0, and immunostained with three different anti-ALK monoclonal antibodies: ALK1[10], ALKc[8], and Clone 5A4 (Thermo Fisher Scientific, Fremont CA, USA). Monoclonal antibody against CD34 was used as unrelated antibody of the same subclass. The antibody:antigen reaction was revealed using both the sensitive Dako-REAL™, Alkaline-Phosphatase/RED detection system (Dako, Glostrup, Denmark) and immunoperoxidase technique. Positive controls for ALK expression included sections from: i) paraffin-embedded pellets of EML4-ALK transfected Phoenix cells; and ii) a paraffin-embedded biopsy from a case of ALCL carrying NPM-ALK.

Results and Discussion

EML4-ALK transcripts can be expressed in reactive and neoplastic lymphoid tissues

EML4-ALK fusion transcripts were detected in 3/51 (5.9%) of reactive lymphoid tissues and 12/58 (20.7%) of lymphoma samples examined (Table 1 and Figure 1). Sequencing of the PCR products amplified from the 3 reactive tissues confirmed the presence of *EML4-ALK* variant 1 cDNA (*EML4* exon 13 – *ALK* exon 20) in one case and of *EML4-ALK* variant 3 (*EML4* exon 6 – *ALK* exon 20) in 2 cases (Figure 1A). At morphological examination, these cases showed a reactive hyperplasia of B-cell follicles associated with slight expansion of the T-cell area.

In the 12 lymphomas displaying *EML4-ALK* transcripts, 5 cases (8.6%) harbored variant 1 (247-bp) and 7 cases (12.1%) variant 3 (155/188 bp) (Table 1); expression of variant 1 and 3 was mutually exclusive; lymphoma subtypes expressing the *EML4-ALK* transcripts are indicated in Table 1. Variant 3 presents two isoforms of the fusion transcript, the long one including 33bp

Table 1. Type and frequency of the *EML4-ALK* transcripts in lymphoid tissues.

Lymphoid tissues	*EML4-ALK* V1* (247 bp) n (%)	*EML4-ALK* V3• (188-155 bp) n (%)
MZL (n=2)	0/2	0/2
MCL (n=1)	0/1	0/1
FL (n=9)	1/9	0/9
PTCL (n=4)	0/4	1/4
B-CLL (n=5)	1/5	0/5
DLBCL (n=12)	2/12	0/12
B-ALL (n=2)	1/2	0/2
HL (n=22)	0/22	6/22
Total (neoplastic) (n=58)	5/58 (8.6%)	7/58 (12.1%)
Reactive (n=51)	1/51 (2.0%)	2/51 (3.9%)

**V1: EML4-ALK variant 1 [EML4 exon 13-ALK exon 20; •V3: EML4-ALK variant 3 [EML4 exon 6-ALK exon 20 with or without the insertion of 33 nucleotides from intron 6 as alternative splicing variant]; MZL: marginal zone lymphoma; MCL: mantle cell lymphoma; FL: follicular lymphoma; PTCL: peripheral T-cell lymphoma; B-CLL: B-chronic lymphocytic leukemia; DLBCL: diffuse large B-cell lymphoma; B-ALL: B-acute lymphoblastic leukemia; HL: Hodgkin's lymphoma.*

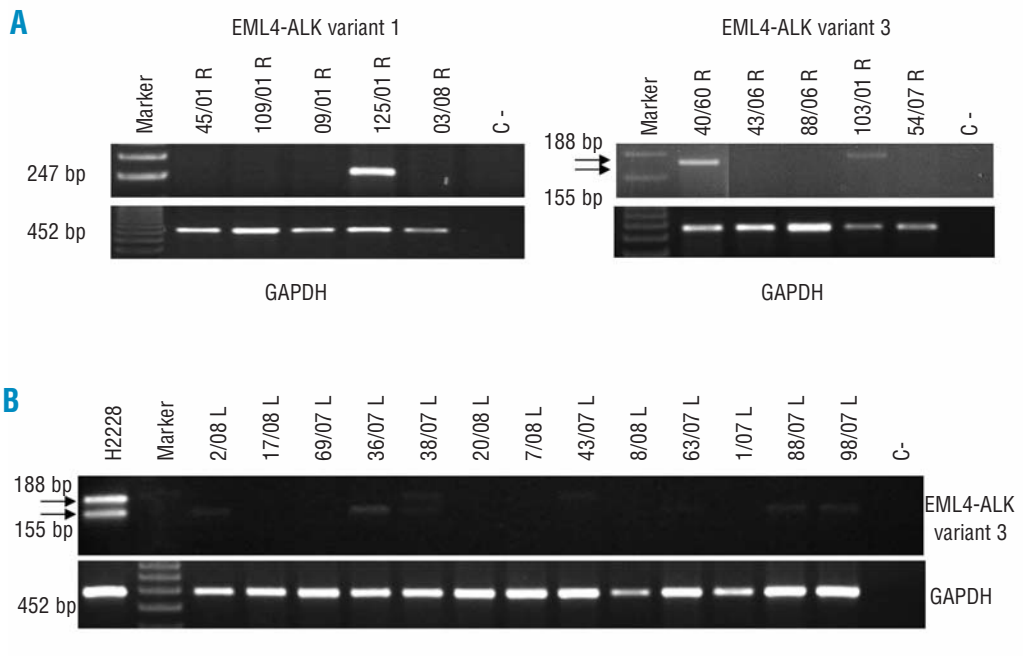

Figure 1. ***EML4-ALK*** **transcripts in reactive lymphoid tissues (A) and lymphoma samples (B). Genes analyzed are indicated. Amplicon size in base pairs (bp) is indicated on the left. Case IDs are reported on top; suffix "L" indicates lymphoma samples, while suffix "R" indicates reactive lymphoid samples. C- is the no-template negative control of PCR.**

from intron 6 of *EML4* gene. Lymphoma and reactive lymphoid tissue samples presented either the short (case 2/08L, 40/06R), the long (case 43/07L, 103/01R) or both (case 38/07L) isoforms whereas the H2228 cell line consistently showed an abundantly expressed variant 3 transcript with both isoforms of 155 and 188 bp (Figure 1B). The presence of *EML4-ALK* fusion transcripts in positive cases was confirmed in at least 2/3 independent PCR experiments. Representative transcripts of each variant were subjected to sequencing that confirmed identity of the amplified products.

Our findings demonstrate for the first time that a subset of reactive lymphoid tissues and lymphoma cases harbor the *EML4-ALK* transcripts.

The EML4-ALK protein is not expressed in lymphoid tissues

We next investigated the significance of *EML4-ALK* transcripts in our cases. To address this question, 9 lymphoid specimens (2 reactive: 40/06, 125/01; 7 neoplastic: 02/08, 14/07, 36/07, 68/07, 82/07, 98/07, 99/07) that at RT-PCR showed an *EML4-ALK* transcript, were analyzed by Western blot on either whole cell lysates or ALK-immunoprecipitates (to enrich for the target protein) to search for EML4-ALK fusion protein. Notably, no EML4-ALK fusion protein was detectable in any of the lymphoid samples investigated (Figure 2A). In contrast, the EML4-ALK fusion protein was clearly expressed in *EML4-ALK* transfected NIH 3T3 cells and the H2228 cell line that were used as controls, and strongly enriched in the ALK-immunoprecipitate (Figure 2A).

Inability to detect EML4-ALK by Western blotting and immunoprecipitation was also confirmed by immunostaining of paraffin sections with three different mAbs (ALK1, ALKc and clone 5A4) which did not reveal the presence of ALK-positive cells, not even at a low percentage, in any of the lymphoid specimens investigated (Figure 2B). In contrast, all positive controls showed the expected subcellular ALK expression: i) cytoplasmic-restricted in EML4-ALK transfected Phoenix cells[7] (Figure 2C); and ii) cytoplasmic plus nuclear in ALCL harboring t(2;5)/NPM-ALK[8,11-13] (*data not shown*). These findings indicate that lymphoid tissues harboring *EML4-ALK* transcripts lack ALK protein or express protein levels below the detection threshold of the above techniques.

Our results clearly demonstrate that, in addition to non-tumor lung,[7] also non-neoplastic lymphoid tissues can express *EML4-ALK* transcripts. This finding is in keeping with previous reports that reactive lymphoid tissues may express *NPM-ALK* and *ATIC-ALK* transcripts[14] and it has practical implications since it further questions the specificity of *EML4-ALK* as molecular marker for early diagnosis of NSCLC, as previously claimed.[5]

The present findings are in contrast to those reported by Soda *et al.*[5] that failed to show *EML4-ALK* transcripts in 69 cases of non-Hodgkin's lymphomas. Even though we have used the same primers and PCR conditions, it is still possible that the sensitivity of the PCR technology, whose robustness varies greatly in different laboratories, could be the reason for the discrepancy in results. Alternatively, it could be argued that similarly to other rearrangements, like *EGFR* mutations in NSCLC, the *EML4-ALK* occurrence is different among Caucasian and Japanese patient populations. Finally, it cannot be excluded that the results in our cases may be due to the different epidemiology of lymphomas in Japan as compared to Western countries. Unfortunately, no informa-

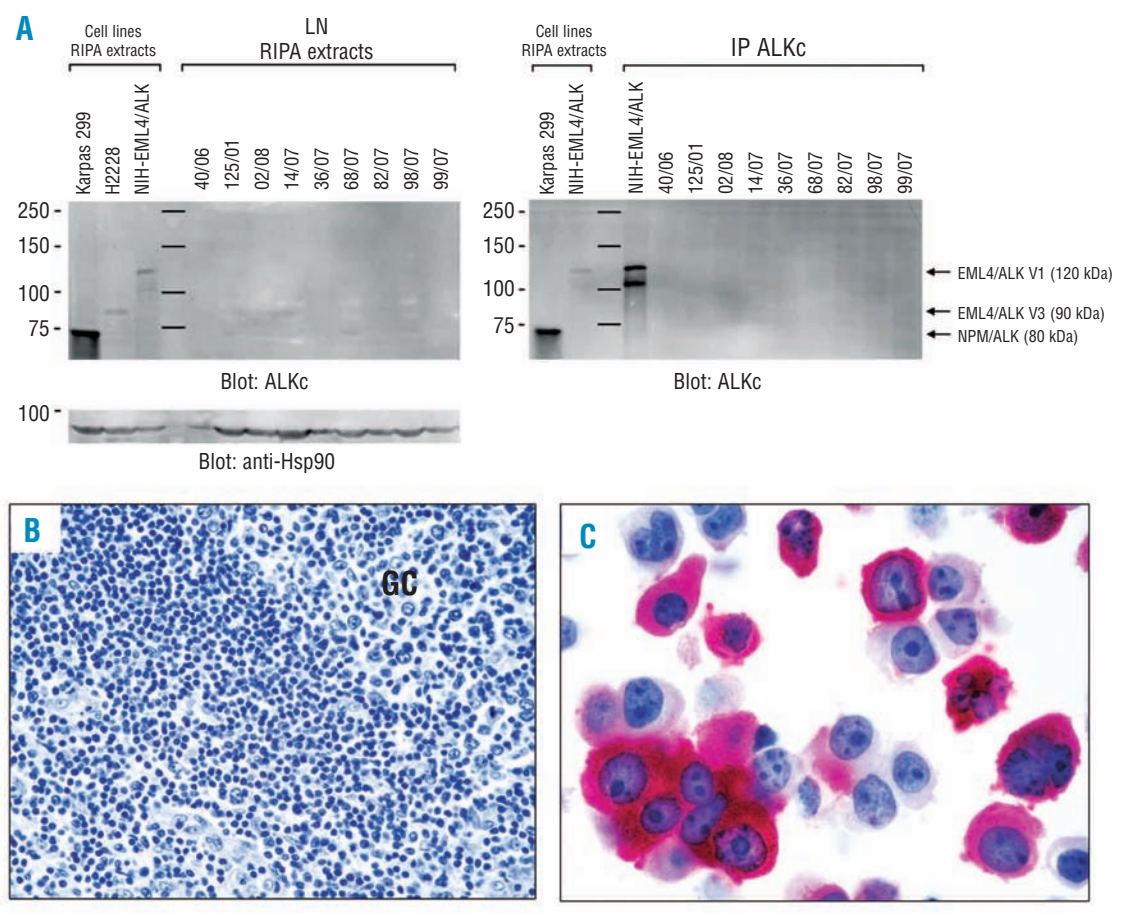

Figure 2. (A) **Western blotting and immunoprecipitation assays on lymphoid tissue specimens harboring *EML4-ALK* transcripts: a band corresponding to EML4-ALK fusion protein (variant 1, about 120 kDa; variant 3, about 90 kDa) is not detectable in either whole cell lysates (LN RIPA extracts) (left panel) or ALK-immunoprecipitates (IP ALKc) (right panel) from either lymphoma samples (cases 14/07, 68/07, 82/07, 99/07 for variant 1; 02/08, 36/07, 98/07 for variant 3) or non-neoplastic reactive lymphoid tissues (case 125/01 for variant 1; 40/06 for variant 3). Karpas 299 and H2228 cell lines, expressing NPM-ALK (80 kDa) and the short form of EML4-ALK fusion protein (about 90 kDa), respectively, and NIH 3T3 cells transfected with EML4-ALK fusion gene construct, variant 1 (NIH-EML4-ALK) are used here as positive controls for Western blotting and immunoprecipitation procedures. Arrows on the right indicate levels where corresponding protein bands should appear in the gel. Western blotting of the membrane with anti-Hsp90 rabbit polyclonal antibody (left, lower panel) indicates good quality of lymphoid tissue protein extracts and sample loading. (B) No ALK positive cells are observed in one of the reactive lymph nodes carrying the *EML4-ALK* transcript; GC indicates a germinal center (hematoxylin counterstaining; x400). (C) Phoenix cells transfected with *EML4-ALK* show strong cytoplasmic-restricted ALK positivity (section from paraffin-embedded transfected Phoenix cells; hematoxylin counterstaining; x800).**

tion was provided by Soda *et al.*[5] about WHO lymphoma categories in their 69 patients.

Overall, our findings indicate that *EML4-ALK* rearrangement might be very easily acquired and different cell lineages be involved, reinforcing the concern of whether this rearrangement is itself sufficient to induce tumor formation. In fact, these lymphomas belonged to categories other than those recognized by the 2008 WHO classification as pathogenetically related to rearrangements of *ALK* gene, i.e. ALK-positive ALCL[2] and a small subset of diffuse large B-cell lymphoma.[3] Moreover, unlike the latter categories in which *ALK* rearrangements always lead to constitutive expression of ALK-fusion proteins[13,15] (easily detectable by immunohistochemistry[13,15,16] or Western blotting[17]), our cases showed no expression of EML4-ALK fusion protein at Western blot, immunoprecipitation or immunohistochemistry. The discrepancy between mRNA and protein detection is probably due to the fact that cells harboring *EML4-ALK* transcripts produced only a low amount of fusion protein and/or that only a minority of cells carried the fusion gene. This may explain why 40 PCR cycles were required for detecting the transcript and why one out of 3 PCR experiments did not give any positive result. Whether, in lymphoma samples, cells carrying the *EML4-ALK* transcripts represent tumor or non-neoplastic bystander cells is unknown since no cells expressing ALK were detectable by immunohistochemistry.

Thus, the situation hereby described is comparable to that previously reported in lymphomas other than ALK-positive ALCL,[14,18-20] where *NPM-ALK* hybrid transcripts were detected without the corresponding fusion protein, and are currently not thought to play a pathogenet-

ic role. Interestingly, expression of *EML4-ALK* transcripts in the absence of EML4-ALK protein detectable by immunohistochemistry, immunoprecipitation and Western blotting has been also reported in NSCLC,[7] which raises yet unsolved questions on the pathogenetic role of *EML4-ALK* rearrangement even in lung cancer. The recently described mouse model of EML4-ALK positive lung cancer[21] does not provide a definitive answer to these questions. In fact, this model may not really mimic lung cancer in humans since the EML4-ALK protein in transgenic mice is expressed artificially at higher levels than in primary human NSCLC.[7]

Authorship and Disclosures

Sozzi G, co-ordinated the molecular studies and contributed to write the manuscript; MPM and VP were involved in Western blot, immunoprecipitation and immunohistochemical analysis of EML4-ALK protein; DC and PGM carried out the PCR analysis of lymphoid samples for the EML4-ALK transcript; SP provided lymphoma samples and contributed to the writing of the manuscript; BF had the original idea for the study and wrote the manuscript.

The authors reported no potential conflicts of interest.

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