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Molecular Characterization of a New *ALK* Translocation Involving *Moesin* (*MSN-ALK*) in Anaplastic Large Cell Lymphoma

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SUMMARY: The majority of anaplastic large cell lymphomas (ALCL) are associated with chromosomal abnormalities affecting the anaplastic lymphoma kinase (*ALK*) gene which result in the expression of hybrid ALK fusion proteins in the tumor cells. In most of these tumors, the hybrid gene comprises the 5' region of nucleophosmin (*NPM*) fused in frame to the 3' portion of *ALK*, resulting in the expression of the chimeric oncogenic tyrosine kinase NPM-ALK. However, other variant rearrangements have been described in which ALK fuses to a partner other than NPM. Here we have identified the moesin (*MSN*) gene at Xq11–12 as a new partner of ALK in a case of ALCL which exhibited a distinctive membrane-restricted pattern of ALK labeling. The hybrid MSN-ALK protein had a molecular weight of 125 kd and contained an active tyrosine kinase domain. The unique membrane staining pattern of ALK is presumed to reflect association of moesin with cell membrane proteins. In contrast to other translocations involving the *ALK* gene, the *ALK* breakpoint in this case occurred within the exonic sequence coding for the juxtamembrane portion of *ALK*. Identification of the genomic breakpoint confirmed the in-frame fusion of the whole *MSN* intron 10 to a 17 bp shorter juxtamembrane exon of *ALK*. The breakpoint in der(2) chromosome showed a deletion, including 30 bp of *ALK* and 36 bp of *MSN* genes. These findings indicate that *MSN* may act as an alternative fusion partner for activation of ALK in ALCL and provide further evidence that oncogenic activation of ALK may occur at different intracellular locations. (*Lab Invest 2001, 81:419–426*).

Anaplastic large cell lymphoma (ALCL) is associated with chromosomal translocations affecting the anaplastic lymphoma kinase (*ALK*) gene at 2p23. These translocations lead to the synthesis of novel chimeric ALK proteins with transforming properties. In most of these tumors, the (2;5)(p23;q35) translocation causes fusion of the gene to the region of the nucleophosmin (*NPM*) gene (Bitter et al, 1990; Mason et al, 1990; Morris et al, 1994). This rearrangement generates a novel fusion protein NPM-ALK of 80 kd molecular weight, which contains the N-terminal region of NPM fused to the C-terminal region of ALK (Bischof et al, 1997; Fujimoto et al, 1996; Mason et al, 1998; Morris et al, 1994). The *ALK* gene encodes a tyrosine kinase receptor that seems to play a role in the development of the nervous system. Recent studies showed that *ALK* mRNA and/or protein is only detected in neural cells, neuroblastomas, and neural cell lines, whereas it is not found in any normal or neoplastic hematopoietic tissues (Iwahara et al, 1997; Lamant et al, 2000; Morris et al, 1997; Pulford et al, 1997). In contrast, NPM protein is ubiquitously expressed in normal cells, being involved in shuttling ribonucleoproteins from the cytoplasm to the nucleus (Chan et al, 1989, 1997). As found for other proteins implicated in oncogenic tyrosine kinases, a dimerization motif within the NPM protein moiety mimics ligand binding and results in the constitutive activation of the tyrosine kinase (Bischof et al, 1997; Chan et al, 1989, 1997).

Several cytogenetic and molecular studies have now demonstrated that chromosome aberration other than t(2;5)(p23;q35) may give rise to novel ALK fusion genes in ALCL (Falini et al, 1998; Lamant et al, 1996; Mitev et al, 1998; Park et al, 1997; Pittaluga et al, 1997; Pulford et al, 1997; Rosenwald et al, 1999; Sainati et al, 1990; Wlodarska et al, 1998). Four different genes,

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nonmuscle tropomyosin (*TPM3*), TRK-fused gene (*TFG*), 5'aminoimidazole-4-carboxamide ribonucleotide formyltranferase/IMP cyclohydrolase (*ATIC*), and clathrin chain polypeptide-like gene (*CLTCL*), have been cloned as alternative partners to *NPM* (Colleoni et al, 2000; Hernandez et al, 1999; Lamant et al, 1999; Ma et al, 2000; Touriol et al, 2000; Trinei et al, 2000). These variant translocations encode ALK fusion tyrosine kinases with molecular weights ranging from 85 to 245 kd. Contrary to the nuclear and cytoplasmic distribution of the NPM-ALK protein, variant fusion proteins show a cytoplasmic restricted pattern (Colleoni et al, 2000; Hernandez et al, 1999; Lamant et al, 1999; Ma et al, 2000; Touriol et al, 2000; Trinei et al, 2000).

In the present report, we study a case ALCL with a unique ALK staining pattern restricted to the cell membrane and show that the gene fused to ALK is moesin (*MSN*). Biochemical analysis showed that the new fusion protein had a molecular weight of about 125 kd and tyrosine kinase activity in vitro. The breakpoint within the *ALK* gene was different from that observed in NPM-ALK gene and all of its reported variants.

Results

Immunomorphologic Characteristics

The lymph node biopsy showed the typical ALCL features of "common" morphology expressing CD4, CD30, and epithelial membrane antigen (EMA). The tumor cells were negative for CD2, CD3, CD43, CD8, CD15, CD19, and CD22. Immunostaining with ALKc and ALK-1 antibodies was positive in all tumor cells. However, contrary to previously described patterns, this case revealed a peculiar membrane distribution of ALK protein with strong accentuation of the cellular contour without cytoplasmic or nuclear staining. A similar pattern of labeling for phosphotyrosine was also observed with all of the ALK-positive cells expressing phosphotyrosine (Fig. 1). RT-PCR studies for

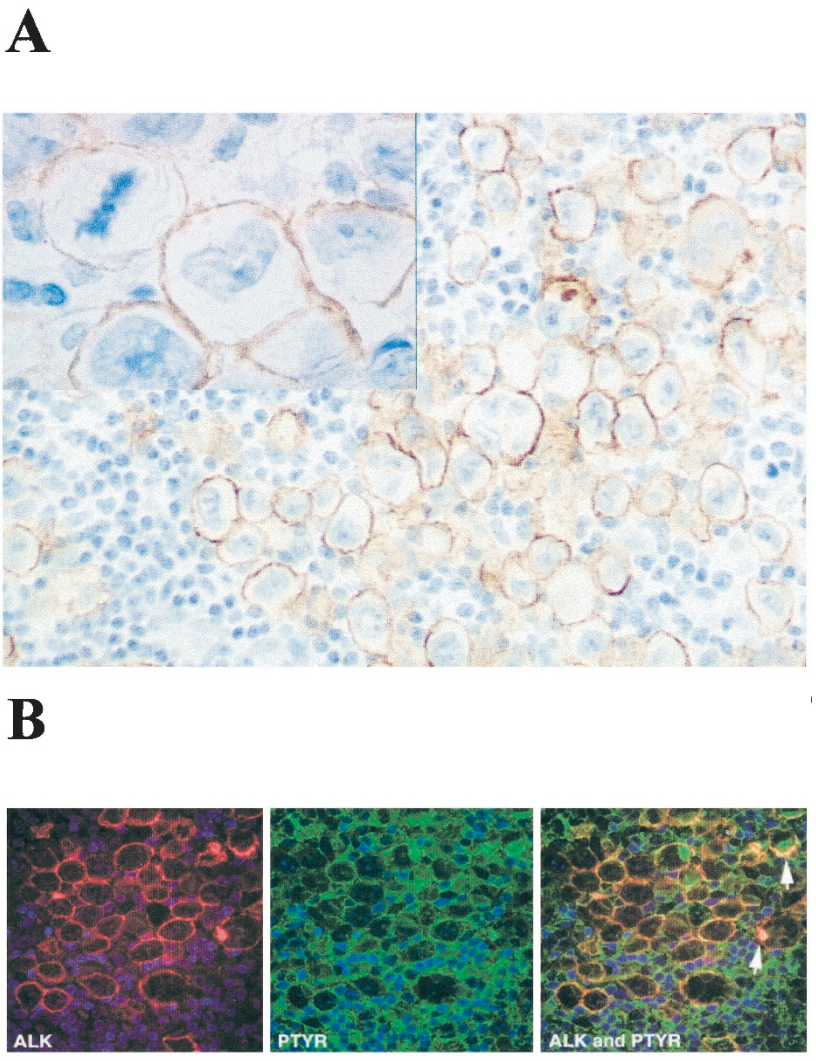

Figure 1.
Immunohistochemical analysis. A, The immunohistochemical staining of ALK in the present case shows a cell membrane-restricted pattern. B, Double immunofluorescent labeling for ALK (red) and phosphotyrosine (green) showing coexpression in the same cells and the same sites. Examples are indicated by *arrows*.

the previously described *NPM-ALK*, *TPM3-ALK*, *TFG-ALK*, *ATIC-ALK*, and *CLTCL-ALK* chimeric products were negative.

Molecular Characterization of Moesin *as a New Partner of* ALK

To identify the gene involved in this new *ALK* translocation, we used a 5' rapid amplification of the cDNA end (RACE) technique with *ALK* primers close to the known breakpoint in other translocations. A 321 bp PCR product was amplified with this strategy, and its specificity was confirmed by Southern blot using an internal *ALK* oligonucleotide probe. The sequence of this product revealed a known ALK sequence fused in frame to a portion of the moesin (*MSN*) gene that was mapped to chromosome Xq11–12. The *ALK* breakpoint in this chimeric *MSN-ALK* cDNA was at nucleotide 4100, which is 17 bp downstream of the breakpoint identified in all other *ALK* translocations previously described (Fig. 2A). The *MSN* breakpoint was located at nucleotide 1444, close to its 3' end (GenBank accession number, M69066). Using primers from the 5' untranslated region of *MSN* and *ALK*, we could amplify the whole *MSN* fragment present in this translocation. Sequencing analysis of this product confirmed that it was identical to the *MSN* sequence previously described and included the complete N-terminal domain responsible for the interactions of MSN protein with cell membrane proteins.

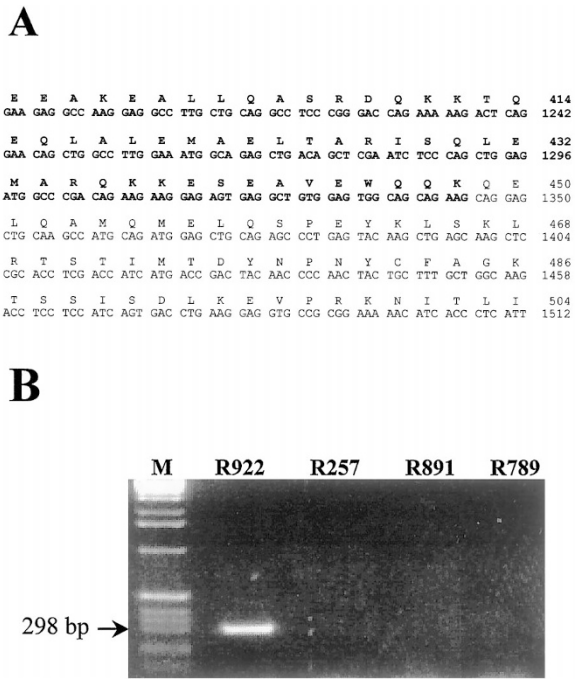

Figure 2.

RT-PCR detection of *MSN-ALK* transcript and cDNA breakpoint sequence. A, Nucleotide and amino acid sequence of the *MSN-ALK* cDNA junction. Nucleotides and amino acids are numbered from the beginning of the predicted *MSN-ALK* open reading frame. *MSN* sequence is in bold letters (Genbank accession number: AF295356). B, Only sample R922 showed the predicted MSN-ALK 298 bp band by RT-PCR analysis. RNA from ALCLs carrying the *NPM-ALK* (R257), *TPM3-ALK* (R891), and *TFG-ALK* (R789) translocations was used as a negative control and showed no evidence of the *MSN-ALK* transcript.

Identification of the MSN-ALK *and Reciprocal* ALK-MSN *Genomic Breakpoints*

To determine the genomic breakpoint of this translocation, we amplified the region between the 3' end of *MSN* exon 10 and the 5' end of the juxtamembrane portion of *ALK* included in the chimeric *MSN-ALK*. Sequencing demonstrated the *MSN* genomic breakpoint in this case to be located exactly at the end of intron 10, including the consensus acceptor-donor splicing sequences. This *MSN* intronic sequence was directly fused to the juxtamembrane exon of *ALK*, 17 bp downstream of the beginning of the exon (Fig. 3).

The genomic breakpoint of the derivative translocation on chromosome 2 was amplified using primers of the *MSN* exon 11 and the intronic region of *ALK* between the transmembrane and juxtamembrane exons. The amplified product was a 155 bp fragment, 66 bp shorter than the expected size. Sequencing analysis revealed that the derivative breakpoint of *ALK* was located 13 bp upstream of the 5' end of the juxtamembrane exon. This *ALK* intronic sequence was directly fused to the exon 11 of the *MSN* gene, 36 bp downstream of the beginning of the exon. Therefore, the breakpoint in the derivative 2 translocation had a 66 bp deletion, including 13 bp of intronic and 17 bp of exonic *ALK* sequence together with the first 36 bp of MSN exon 11 (Fig. 3).

Expression of the MSN-ALK *Transcript*

To confirm the existence of the *MSN-ALK* fusion transcript in the original lymph node biopsy, an RT-PCR technique was performed using *MSN*- and *ALK*-specific primers. This amplification yielded the expected 298 bp band, whose specificity was confirmed by hybridization with an *ALK*-specific oligonucleotide probe and subsequent sequencing analysis. No band was detected in any of the other ALCL that were analyzed (Fig. 2B). Expression of the reciprocal *ALK-MSN* fusion cDNA was not detectable by RT-PCR.

MSN-ALK Fusion Protein

The predicted chimeric MSN-ALK protein was composed of 1005 amino acids with an estimated molecular weight of 115 kd. The N-terminal region of the fusion protein corresponded to the first 448 amino acids of MSN, whereas the other 557 amino acids were encoded by ALK and contained the tyrosine kinase domain. Western blot analysis of the ALK

Figure 3.

Sequence analysis of the genomic junction for both derivatives of the *MSN-ALK* translocation. Exons and introns are represented by upper- and lower-case letters, respectively. *MSN* gene is indicated by bold characters. *Arrowheads* show the breakpoints in der(X) whereas *arrows* indicate the breakpoints in der(2). Sequences between the arrows were deleted in the translocation (Genbank accession numbers: AF295078 and AF295079, respectively).

protein present in the ALCL tissue confirmed the presence of a protein with a molecular weight of 125 kd, which was consistent with the predicted size of the MSN-ALK fusion protein (Fig. 4A).

The in vitro kinase assay of proteins immunoprecipitated with anti-ALK and anti-phosphotyrosine from cryostat sections of the ALCL case demonstrated the presence of a 125 kd phosphorylated protein corresponding in size to MSN-ALK (Fig. 4B). These results indicate that the tyrosine kinase domain in the MSN-ALK protein is active, resulting in autophosphorylation in vitro.

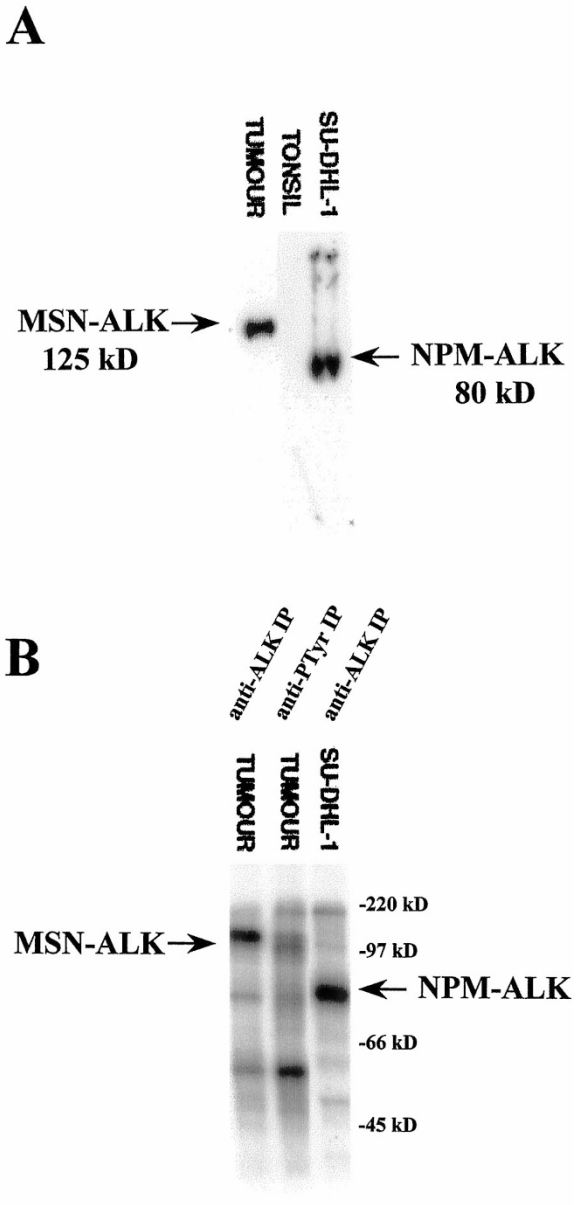

Figure 4.

Biochemical assays of proteins extracted from cryostat tissue sections and cytocentrifuge preparations. A, Western blotting of proteins extracted from sections of the anaplastic large cell lymphoma (ALCL) case using anti-ALK (ALKc) demonstrated the presence of a 125 kd protein (MSN-ALK) in the tumor cells. This was in contrast to the 80 kd NPM-ALK protein detected in the t(2;5)-positive SU-DHL-1 cell line. No ALK proteins were detected in normal tonsil lysates used as a negative control. B, An in vitro kinase assay of proteins immunoprecipitated with anti-ALK from the ALCL tumor demonstrated the presence of a 125 kd phosphorylated protein. A weaker band of comparable size was also observed in proteins immunoprecipitated by antiphosphotyrosine. In the ALK immunoprecipitates from the SU-DHL-1 cell line, the only band seen represents the 80 kd NPM-ALK.

Discussion

In the present study, we have characterized a new chimeric *ALK* translocation in a case of ALCL in which ALK protein showed a unique cell membrane-associated distribution. Using a 5' RACE strategy, we identified an in-frame fusion of the 5' region of moesin (*MSN*) gene to the 3' portion of *ALK* containing the catalytic domain. This gene has been mapped to chromosome Xq11–12, and demonstrates that the chimeric *MSN-ALK* gene is the result of a (X;2)(q11–12;p23) translocation, a chromosomal abnormality not previously described in ALK-positive ALCL.

Moesin is a 75 kd protein that belongs to the highly homologous ERM (ezrin/radixin/moesin) protein family. These proteins have been shown to localize beneath the cell membrane and are considered to act as cross-linkers between the plasma membrane and the cortical actin cytoskeleton, playing a major role in cell adhesion and motility (Bretscher, 1999; Tsukita and Yonemura, 1997a, 1997b, 1999). As is the case for other fusion partners of ALK, moesin is widely expressed in normal cells, including B and T lymphocytes (Berryman et al, 1993; Masumoto et al, 1998). Structurally, moesin is characterized by a globular N-terminal membrane-binding domain, followed by a large α-helix domain, and a C-terminal region with a consensus sequence for actin binding. In normal steady-state situations, the amino-terminal and C-terminal domains of a single moesin molecule may bind to each other in a head-to-tail manner, resulting in the inactivation of the protein by preventing its interaction with both cell membrane proteins and the actin cytoskeleton (Bretscher, 1999; Tsukita and Yonemura, 1997a, 1997b, 1999). Phosphorylation of the N- and C-terminal domains seems to act as an activation signal inhibiting this intramolecular interaction, thereby permitting the simultaneous binding of the N-terminus of MSN to membrane proteins (eg, CD43, CD44, CD50 and proteins containing the PDZ dimerization) and binding of the C-terminus to actin (Bretscher, 1999; Fouassier et al, 2000; Reczek et al, 1997; Tsukita and Yonemura, 1997a, 1997b, 1999).

The predicted chimeric MSN-ALK protein in this tumor comprises the first 448 residues of MSN, including the complete N-terminal globular domain and almost all the α-helix region. The rest of the moesin protein is replaced by the C-terminal portion of ALK containing the kinase catalytic domain. The unique labeling pattern of ALK found in the tumor cells of this case presumably reflects binding to the cell surface membrane of MSN-ALK through the intact MSN N-terminus. Experimental deletion of the C-terminal end of the moesin-homolog erzin activates this protein, allowing its direct interaction with cell membrane proteins (Reczek and Bretscher, 1998). Thus, in the hybrid MSN-ALK protein, substitution of the moesin

C-end sequence by ALK may represent a comparable mechanism for the activation of the N-terminal domain. It is also possible that the binding properties of moesin to cell membrane proteins may lead to dimerization of the MSN-ALK proteins, permitting the constitutive phosphorylation of the chimeric MSN-ALK protein through its intact kinase domain. Additional evidence was obtained from the comparable patterns of immuno-labeling of tumor cells obtained using both the anti-ALK and anti-phosphotyrosine antibodies because a previous study has demonstrated that the immunohistological detection of increased levels of phosphotyrosine provides evidence for the presence of activated tyrosine kinases (Pulford et al, 1999a).

In contrast to other rearrangements involving the *ALK* gene in which the *ALK* breakpoint consistently occurs in the same intron, between the transmembrane and juxtamembrane exonic sequences (Downing et al, 1995; Hernandez et al, 1999; Lamant et al, 1999; Ma et al, 2000; Sarris et al, 1998; Touriol et al, 2000; Trinei et al, 2000), the *ALK* breakpoint in the *MSN-ALK* translocation is localized in an exonic sequence, 17 bp downstream of the 5' end of the first *ALK* exon present in other translocations. Characterization of the genomic derivative chromosome X breakpoint confirmed that the whole *MSN* intron 10 was fused in frame to this *ALK* exonic sequence, maintaining the consensus splicing signal. Amplification and sequencing of the reciprocal *ALK-MSN* breakpoint on derivative chromosome 2 showed a 66 bp deletion, including both *ALK* and *MSN* sequences, as has been described for reciprocal translocations (Downing et al, 1995; Zucman-Rossi et al, 1998). Although the genomic breakpoint of the NPM-ALK translocation has been characterized in several ALCL, the sequence of derivative ALK chromosome has been examined in only a limited number of cases (Beylot-Barry et al, 1998). Similarly to these studies, we have not been able to identify a possible mechanism for the generation of this translocation.

All of the different protein partners in the ALK fusion proteins identified thus far are functionally important because they induce the activation of the ALK kinase domain (Bischof et al, 1997; Colleoni et al, 2000; Fujimoto et al, 1996; Hernandez et al, 1999; Lamant et al, 1999; Ma et al, 2000; Mason et al, 1998; Morris et al, 1994; Touriol et al, 2000; Trinei et al, 2000). Furthermore, the partner protein determines the subcellular localization of the chimeric ALK protein. Thus, the classic NPM-ALK fusion protein is localized in both the nucleus and the cytoplasm, whereas TFG-ALK and ATIC-ALK show a diffuse staining restricted to the cytoplasm (Colleoni et al, 2000; Hernandez et al, 1999; Trinei et al, 2000), TPM3-ALK shows a diffuse cytoplasmic distribution with peripheral intensification (Lamant et al, 1999), and CLTC-ALK shows a finely granular intracytoplasmic pattern (Touriol et al, 2000). The MSN-ALK translocation described in this study is associated with a distinctive ALK staining restricted to the cell membrane. All of these observations indicate that the oncogenic activation of ALK may occur in different cytoplasmic compartments. In addition, the different ALK immunostaining patterns may be a relatively good indicator of the underlying cytogenetic alteration present in the tumor.

Materials and Methods

Patient

An 18-year-old man presented with cervical lymphadenopathy and a paragastric mass. Biopsy of a cervical lymph node was diagnostic of CD30-positive ALCL of null/T-cell phenotype expressing ALK protein. Staging of the patient, including CD30 immunohistochemical analysis of the bone marrow biopsy, revealed stage IIIb disease. He was treated according to one of both arms of the EORTC 20901 trial, consisting of 6 cycles of CHVmP/BV (doxorubicin, teniposide, cyclophosphamide, prednisone, vincristine, and bleomycin) followed by autologous bone marrow transplant. The patient achieved complete remission but died in a car accident 14 months after diagnosis.

Preparation of Frozen Tissue Sections and Cells

Fresh and routinely processed paraffin-embedded samples from the ALCL were obtained from the Department of Pathology (Leiden University Medical Center, Leiden, The Netherlands). Fresh tonsil was obtained from the Ear, Nose and Throat Department (Radcliffe Infirmary, Oxford, United Kingdom). Cryostat sections (6-μm thickness) were cut from both the ALCL case and from fresh tonsil. The ALCL-derived t(2;5)-positive SU-DHL-1 cell line was maintained in culture and cytocentrifuge preparations made as previously described (Pulford et al, 1997, 1999b). All tissue sections and cytocentrifuge preparations were fixed in acetone for 10 minutes and stored at $-70°$ C.

Immunolabeling

Immunohistological staining was performed using the following panel of antibodies: polyclonal anti-CD3, monoclonal anti-CD2 (T9–10), anti-CD8 (C8/144), anti-CD15 (By87), anti-CD19 (Leu12), anti-CD30 (BerH2), anti-CD43 (DFT1), and EMA (E29). Sections were also stained with two anti-ALK monoclonal antibodies (ALK1 and ALKc) and with monoclonal antiphosphotyrosine (PY72.10.5) donated by Dr. N. Hogg (Imperial Cancer Research Fund, London, United Kingdom) (Falini et al, 1998; Pulford et al, 1997).

Double immunofluorescent labeling was performed using anti-ALK (ALK1-IgG3 isotype) and antiphosphotyrosine (IgG1 isotype), followed by goat antimouse subclass-specific antibodies conjugated to either fluorescein isothiocyanate (FITC) or Texas Red™ (Eurogenetics, Middlesex, United Kingdom) as previously described. Slides were viewed on a conventional fluorescent microscope equipped with a cooled CCD camera (Mason et al, 2000; Pulford et al, 1999a).

Biochemical Studies

Western blotting of proteins extracted from cryostat sections and cytocentrifuge preparations of the cultured SU-DHL-1 cell lines was performed as previously described (Pulford et al, 1999b). Briefly, the samples were incubated with 50 μl of sample buffer for 5 minutes at room temperature. The buffer was removed, heated to 95° C for 4 minutes, and subjected to SDS-PAGE. After transfer to Immobilon (Serva, Heidelberg, Germany), the separated proteins were detected using a monoclonal anti-ALK (ALKc) reagent.

An in vitro kinase assay using tissue sections and cytocentrifuge preparations was performed as previously described (Pulford et al, 1999b). Briefly, cryostat tissue sections of ALCL and tissue sections and cytocentrifuge preparations were lysed in buffer containing 1% Brij 97, 140 mM NaCl, 25 mM Tris pH 7.6, 10 mM NaF, 1 mM $Na_3(VO)_4$, 1 mg/ml bovine serum albumin, and metabolic inhibitors (1 mM leupeptin, 1 mM pepstatin, 1 mM Pefabloc and 20 mM tosyl-L-phenylalanine chloromethyl ketone) (Boehringer-Mannheim, Lewes, United Kingdom). The samples were precleared for 2 hours with 200 μl of 20% (vol/vol) of Protein G (Sepharose; Pharmacia, Uppsala, Sweden). ALK proteins from the precleared lysates were then immunoprecipitated using 50 μl of Protein G Sepharose preloaded with either monoclonal anti-ALK antibody (ALK1) or antiphosphotyrosine (4G10; Upstate Biotechnology Inc., Lake Placid, New York). After washing, the samples were added to 5 μCi ^{32}P ATP in 20 μl of freshly prepared kinase buffer containing 10 mM NaF, 1 mM $Na_3(VO)_4$, and 10 mM $MnCl_2$ for 15 minutes at 25° C. Proteins were then separated by SDS-PAGE on a 10% gel, and dried gels were subjected to autoradiography.

RNA Extraction and 5'RACE Reaction

Total RNA was isolated from a frozen sample of the diagnostic lymph node biopsy using guanidine-isothiocyanate extraction and cesium chloride gradient centrifugation. cDNA was obtained from 1.5 μg of total RNA using SuperScript II reverse transcriptase (Life Technologies Inc, Paisley, United Kingdom) and 2.5 pmol/L of the *ALK*-specific primer 5'-ACCCCAA-TGGCAGCGAACAA -3'. The RACE technique was used following the manufacturer's recommendations to obtain the 5' sequence fused with the *ALK* gene. The *ALK* primers used for the amplification and to confirm the specificity of the PCR fragments obtained were previously described (Hernandez et al, 1999).

DNA Sequencing

The PCR products were extracted from 1% agarose gels and purified using the Concert Rapid Gel Extraction System (Life Technologies Inc.). These products were directly sequenced using the Dye Terminator Cycle Sequencing method (Applied Biosystems, Foster City, California) and the ABI PRISM 377 automated sequencer (Applied Biosystems).

Expression Analysis of ALK Chimeric Transcripts

cDNA was obtained from 1 μg of total RNA by random priming and SuperScriptTM II Reverse transcriptase (Life Technologies Inc.) following the manufacturer's recommendations. Detection of hybrid *MSN-ALK* transcript was performed using Moe3U (5'-CAGCTGGAGATGGCCCGACA-3') and GSP3 (5'-CTTGGGTCGTTGGGCATTC-3') primers, yielding an amplified product of 298 bp. The PCR conditions were 35 cycles consisting of 45 seconds at 94° C, 45 seconds at 60° C, and 1 minute at 72° C, with a final extension step of 10 minutes at 72° C. *NPM-ALK*, *TPM3-ALK*, *TFG-ALK*, *ATIC-ALK*, and *CLTCL-ALK* expression was analyzed as previously described (Hernandez et al, 1999; Ma et al, 2000; Morris et al, 1994; Pulford et al, 1999b; Touriol et al, 2000). To analyze the possible expression of the reciprocal *ALK-MSN* product, a heminested PCR strategy was used. The first PCR reaction was performed using the primers ALKD (5'-GGTGACCTCTGCCCTCGTGG-3') and Moe9D (5'-CAAGGACCGCAGTGAGGAGG-3'). The second round was performed using ALKD and Moe8D (5'-GCTGACCTACGGGCTGATGC-3') specific primers.

DNA Extraction and Identification of the Genomic Breakpoint

High-molecular-weight DNA was isolated from a frozen sample of the diagnostic lymph node by proteinase K treatment and phenol-chloroform extraction. Amplification of genomic breakpoint was performed using a semi-nested PCR strategy. The first round of PCR was performed using Moe3U and ALKGD (5'-GCCCTGAGTACAAGCTGAGCA-3') primers, yielding a 465 bp fragment. The PCR conditions were 35 cycles comprising a denaturing step at 94° C for 45 seconds, an annealing step of 45 seconds at 58° C, and an elongation step of 1 minute and 30 seconds at 72° C, followed by a final extension time of 7 minutes at 72° C. The second round was performed using ALKGD and MoeGU (5'-GAGTGAGGCTGTGGAGT-GGCA-3') primers, following the same conditions as in the first PCR reaction. This second PCR yielded an amplified product of 436 bp. The same semi-nested PCR strategy was used to analyze the derivative translocation. In this case, the first round of PCR was performed with ALKIU (5'-AGTGCTTCAAGGGCCA-GGCT-3') and Moe9D primers to amplify an expected 179 bp fragment. The second round of PCR was performed using ALKIU- and Moe8D-specific primers, obtaining an expected band of 155 bp. Both PCR reactions were performed following the same conditions as described above to amplify the genomic breakpoint. Finally, all of these PCR products were analyzed and sequenced as previously described.

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