Short Communication

Biochemical Detection of Novel Anaplastic Lymphoma Kinase Proteins in Tissue Sections of Anaplastic Large Cell Lymphoma

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The (2;5) translocation, found in many T-cell and null cell anaplastic large cell lymphomas (ALCLs), creates a hybrid gene encoding the 80-kd NPM-ALK protein. Typically neoplastic cells show labeling of both nucleus and cytoplasm for anaplastic lymphoma kinase (ALK) and for the N-terminus of nucleophosmin (NPM). However, 10–20% of cases exhibit cytoplasmic labeling only for ALK, indicating the probable presence of variants of the classical (2;5) translocation that do not involve the *NPM* gene. We report the detection (using Western blotting and an *in vitro* kinase assay) in seven such ALCL cases, of ALK proteins with molecular masses of 85 kd, 97 kd (one case exhibiting a (2;3)(p23;q21) translocation), 104 kd (one case carried a (1;2)(q21;p23) translocation), and 113 kd. Tyrosine kinase activity was detected in four of these proteins, but the N-terminal portion of NPM could not be detected. These results show how ALCL cases that express ALK proteins other than NPM-ALK can be detected by sensitive biochemical techniques using routine cryostat sections. (*Am J Pathol* 1999, 154:1657–1663)

The (2;5) chromosomal translocation, found in CD30-positive ALCL of T-cell or null cell phenotype, juxtaposes the nucleophosmin (*NPM*) gene at 5q35 with the anaplastic lymphoma kinase (*ALK*) gene at 2p23.¹ Nucleophosmin is a ubiquitous 38-kd protein involved in shuttling ribosomal components between the nucleus and cytoplasm.² *ALK* is a 200-kd receptor tyrosine kinase of unknown function, but its expression in scattered cells within the human central nervous system³ and the presence of *ALK* mRNA in fetal murine brain^{4,5} suggest a role in neural development.

The fusion gene created by the (2;5) translocation encodes an 80-kd chimeric NPM-*ALK* kinase, in which 40% of the N-terminal of NPM is linked to the entire intracytoplasmic region of *ALK*.^{1,6–8} This NPM-*ALK* kinase is capable of transforming cells both *in vitro* and *in vivo*.^{9,10}

ALK protein is absent from normal lymphoid tissue and, consequently, immunohistological labeling with polyclonal and monoclonal antibodies to *ALK* has been used to identify cases of ALCL that express the hybrid NPM-*ALK* kinase as a result of the (2;5) translocation.^{3,11–13} Initial estimates of the frequency of the (2;5) translocation in this tumor type varied widely, probably reflecting differing diagnostic criteria for the tumor (eg, whether or not B-cell lymphomas were included). However, immunohistological studies of *ALK* expression suggest that *ALK*-positive lymphomas constitute a clinicopathological entity (“*ALK*-omas”), associated with features such as young age, a cytotoxic T tumor cell phenotype, and the presence of characteristic “hallmark” neoplastic cells on histological examination.^{13,14}

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It has also become apparent that ALK-positive lymphomas may occasionally carry variants of the classical (2;5) translocation. For example, cases of ALCL exhibiting a (1;2)(q25;p23) translocation,^{3,12} a (1;2)(q21;p23) translocation, a (2;3)(p23;q21) translocation,¹⁵ and an inv(2)(p23q35) anomaly^{16,17} have been reported. It is assumed that in such cases the *ALK* gene fuses to a partner other than *NPM*. Additional chromosomal translocations involving the site of the *ALK* gene (2p23) have been reported, but in these cases no immunostaining data are available on ALK expression.^{18–20}

It is not clear (because of the rarity of cases studied by both cytogenetics and immunohistology) how frequently cases of ALCL exhibit variants of the classical (2;5) translocation. However, an indirect estimate can be made from the fact that in each of two large series of cases of ALK-positive lymphomas, approximately 15% of cases showed ALK labeling that was restricted to the cytoplasm and absent from the nucleus.^{13,14} It is known that the NPM-ALK protein tends to accumulate within cell nuclei, whereas when ALK fuses to another protein (either *in vitro* or *in vivo*) it remains within the cytoplasm.^{3,8,10,15} Consequently, between 10% and 20% of ALK-positive lymphomas are likely to carry variants of the classical (2;5) translocation, in which genes other than *NPM* are involved.

Such cases are obviously of interest because, by detecting new fusion partners for the *ALK* gene, it may be possible to identify molecules involved in oncogenesis in ALCL and other tumors. Variants of the (2;5) translocation can be studied by techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR), fluorescent *in situ* hybridization (FISH), and chromosome karyotyping, but these methods are time consuming and may not always be technically possible. In this paper we report the biochemical analysis, using Western blotting and an *in vitro* kinase assay, of ALK proteins extracted from routine cryostat sections. This has allowed us to identify four different ALK proteins whose molecular masses were clearly greater than that of NPM-ALK (80 kd) and which were not recognized by an anti-NPM antibody. They are likely to represent hybrid kinases containing ALK linked to a partner other than NPM.

The biochemical techniques described here could be applied to minimal amounts of tissue and could provide information on protein expression that would not be available from molecular biological analysis (eg, Southern blotting, PCR). Their use, in combination with immunocytochemical staining, should therefore greatly assist in the identification and characterization of ALK-positive ALCL cases that carry variants of the (2;5) translocation.

Materials and Methods

Tissues

Normal tonsil was obtained from the E.N.T. Department of the Radcliffe Infirmary, Oxford. Frozen and routinely fixed and embedded samples of ALCL were obtained from the following sources: Institute of Hematology, Perugia, and

Institute of Pathology, Aviano (four cases); Institute of Pathology, Würzburg (two cases); St. James's Hospital, Leeds (one case); Hospital/University Center, Purpan, Toulouse (one case); and the Department of Pathology, Barcelona (two cases). Three cases have been included in previous reports.^{15–17} The diagnosis of ALCL was based on previously described immunomorphological criteria.²¹

Cell Line

The SU-DHL-1 cell line (ALCL of T-cell phenotype carrying the 2;5 translocation) was obtained from Dr. M. L. Cleary (Stanford, CA). These cells were cultured³ and were also used to produce a solid tumor in SCID mice, as previously described.¹²

Preparation of Cells and Tissue Samples

Tissue samples were either snap frozen in liquid nitrogen or fixed in routine histological fixatives as previously described.³ Cryostat sections (6 μ m) were cut from frozen tissues, and cytocentrifuge preparations were made of the cultured SU-DHL-1 cell line. Sections and cytocentrifuge preparations were immediately fixed in acetone for 10 minutes at room temperature, air-dried for 5 minutes, wrapped in aluminum foil, and stored at -70°C until needed. Some tissue sections and cell preparations were stored immediately at -70°C without prior fixation.

Antibodies

Monoclonal antibodies recognizing ALK protein (ALK1 and ALKc), the N-terminal region of nucleophosmin (NA24), and mouse Ig (MR12) were produced in the authors' laboratories,^{3,13,22} as were APAAP complexes.²³ A monoclonal anti-phosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-mouse Ig (Z259) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (P0447) were obtained from DAKO A/S (Glostrup, Denmark).

Immunocytochemical Labeling

The APAAP technique was performed as described previously.^{13,23} Paraffin sections were subjected to microwave pretreatment^{12,24} before immunostaining.

Western Blotting Technique

Fifty-microliter aliquots of sample buffer²⁵ containing dithiothreitol (Sigma Chemical Co., Poole, England) were added to each tissue section or cytocentrifuge preparation immediately after their removal from -70°C storage. After 5 minutes at room temperature, the buffer was aspirated from the slides, heated to 95°C for 4 minutes, and loaded onto 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.²⁵ Western blot-

Table 1. Details of ALK-Positive Lymphomas Reported in This Study

Case	Genetic alterations		Immunocytochemistry ALK protein distribution	Western blotting		In vitro kinase assay	
	Cytogenetic abnormality	RT-PCR for NPM-ALK		Anti-ALK (kd)	Anti-NPM (kd)	ALK1-immunoprecipitates (kd)	Ptyr immunoprecipitates (kd)
Classical ALK-positive ALCL							
SU-DHL-1 (cell line)	t(2;5)(p23;q35)	Pos	Nucleus/cytoplasm	80	80, 38	80	80
Case 1	ND	Pos	Nucleus/cytoplasm	80	80, 38	80	80
Case 2	ND	Pos	Nucleus/cytoplasm	80	80, 38	80	80
Case 3	ND	Pos	Nucleus/cytoplasm	80	80, 38	80	80 (wk)
Atypical ALK-positive ALCL							
Case 4	ND	Neg	Cytoplasm	85	38	85	85
Case 5	t(2;3)(p23;q21)	ND	Cytoplasm	97	38	97	ND
Case 6	No mitoses	Neg	Cytoplasm	97	38	Not detectable	ND
Case 7	ND	Neg	Cytoplasm	97	38	Not detectable	ND
Case 8	t(1;2)(q21;p23)	ND	Cytoplasm	104	38	104	104
Case 9	ND	Neg	Cytoplasm	104	38	104	104
Case 10	ND	ND	Cytoplasm	113	38	Not detectable	ND

All biochemical studies were performed on cryostat tissue sections of cytocentrifuge preparations.
 ND, Not determined.

ting was performed using a semidry transfer technique and chemiluminescent detection of the antigen/antibody complexes as described previously, but using a 1:250 dilution of horseradish peroxidase–goat anti-mouse Ig.³

In Vitro Kinase Assay

After removal of the slides from -70°C storage, 50–100- μl aliquots of lysis buffer (1% Brij 96 in 140 mM NaCl, 25 mM Tris (pH 7.6), 10 mM NaF, 1 mM $\text{Na}_3(\text{VO})_4$, and 1 mg/ml bovine serum albumin containing 1 mM leupeptin, 1 mM pepstatin, 1 mM Pefabloc, and 20 mM tosyl-L-phenylalanine chloromethyl ketone; Boehringer-Mannheim, U.K.) were added to the site of the cryostat tissue section or cytocentrifuged cells. After incubation at 4°C for 30 minutes, the cells and buffer were scraped off, placed in tubes, and rotated for 30 minutes at 4°C before centrifugation for 10 minutes at 13,000 rpm. The supernatants were removed, placed in clean tubes, and pre-cleared for 2 hours with 200 μl of 20% (v/v) Protein G Sepharose (Pharmacia, Uppsala, Sweden). After a brief spin the precleared lysates were added to 50 μl of Protein G Sepharose preloaded with either monoclonal anti-ALK antibody (ALK1) or anti-phosphotyrosine and rotated for 90 minutes at 4°C .

The samples were then washed three times in lysis buffer and twice in assay buffer (0.1% Brij 96, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5). Five microcuries of [^{32}P]ATP in 20 μl of freshly prepared kinase buffer containing 10 mM NaF, 1 mM $\text{Na}_3(\text{VO})_4$, and 10 mM MnCl_2 were added to each sample, and the kinase reaction was allowed to proceed for 15 minutes at 25°C . It was stopped by the addition of SDS sample buffer. Proteins were separated by SDS-PAGE on a 10% gel, and dried gels were subjected to autoradiography.

RT-PCR

RT-PCR using primers specific for NPM and ALK were performed as previously described.¹²

Cytogenetics

Cytogenetic analysis using unstimulated short-term cultures of tumor tissues was performed according to standard protocols as previously described.¹⁵

Results

Immunohistochemical Labeling for ALK

A summary of the results obtained is shown in Table 1.

Samples Carrying the (2;5) Translocation

Three cases of morphologically typical ALCL were identified as t(2;5)-positive because ALK immunoreactivity was seen in both the nucleus and cytoplasm (Figure 1a). This differential labeling is indicative of the presence of NPM-ALK.^{3,14} The t(2;5)-positive SU-DHL-1 cell line was used as the positive control, and nucleolar, nuclear, and cytoplasmic labeling for NPM-ALK was seen, as described previously.^{3,10}

Samples with Variants of the (2;5) Translocation

Seven otherwise typical ALCLs in which ALK staining was restricted to the cytoplasm were identified in the authors' laboratory (Figure 1b), suggesting that the ALK protein present was not classical NPM-ALK. This was further supported by the immunocytochemical demonstration of the nuclear restricted expression of the N-terminal portion of NPM in three of the seven cases investigated.²⁶

Biochemical Analysis of Cryostat Section Extracts

Western Blotting

An 80-kd band could be detected by Western blotting, using anti-ALK monoclonal antibodies, in extracts of cry-

Figure 1. Immunocytochemical labeling of cases of ALCL (APAAP technique, paraffin-embedded sections, $\times 250$). **a:** The nuclear, nucleolar, and cytoplasmic labeling pattern for ALK protein, seen here for case 1, is indicative of the presence of the NPM-ALK protein in the tumor cells. **b:** An atypical case of ALK-positive ALCL (case 4) in which ALK labeling is confined to the cytoplasm (arrows). This suggests the presence of a hybrid protein in which ALK is linked to a partner other than NPM.

ostat sections from each of the three cases¹⁻³ of classical ALCL (Figure 2a). Western blotting with an antibody to the portion of nucleophosmin present in NPM-ALK (NA24) also recognized an 80-kd band in these ALCLs, confirming the identity of this protein as NPM-ALK (Figure 2b). In addition, antibody NA24 detected wild-type 38-kd NPM (also seen in tonsil lysates). Identical results were obtained with both tissue sections and cytocentrifuge preparations of the SU-DHL-1 cell line (used as the positive control).

Western blotting studies of each of the seven cases of ALCL in which immunostaining for ALK was restricted to the cytoplasm demonstrated the presence of ALK proteins differing in size from NPM-ALK. The results appeared to represent four different ALK proteins (Table 1), with molecular masses of 85 kd (case 4), 97 kd (cases 5-7), 104 kd (cases 8 and 9), and 113 kd (case 10) (Figure 2c). The use of anti-NPM revealed the presence of only wild-type NPM (38 kd) in blots of these cases (Figure 2d). This is in contrast to the results obtained for the typical cases of ALCL (Figure 2b).

In Vitro Kinase Assay

Kinase assays of ALK1-immunoprecipitates of cryostat sections from the three cases (1, 2, and 3) of classical ALCL showed the presence of an 80-kd band, representing autophosphorylated NPM-ALK (Figure 3a). This was also observed in protein extracts from the t(2;5)-positive control SU-DHL-1 cell line (Figure 3a). Comparable results, although weaker, were obtained using anti-phosphotyrosine immunoprecipitates of the tissue section extracts (Table 1).

When ALK1-immunoprecipitates of the cases of ALCL thought to carry variants of the classical (2;5) translocation were studied, phosphorylation was seen for bands corresponding in size to the 85-kd (case 4), 97-kd (case 5), and 104-kd (cases 8 and 9) ALK-containing proteins (Figure 3b), suggesting that they carried active kinase domains. Anti-phosphotyrosine immunoprecipitates from three of these cases also revealed phosphorylated bands of the expected size (85 kd and 104 kd) (Table 1). In three cases no interpretable results were obtained by the kinase assay.

RT-PCR and Cytogenetic Studies

Cases 1, 2, and 3 contained mRNA encoding for NPM-ALK. This correlated with the presence in the tumor cells of both a cytoplasmic and nuclear distribution of ALK protein. In contrast, cases 4, 6, 7, and 9, which exhibited cytoplasmic labeling only for ALK, were negative for the (2;5) translocation by RT-PCR.

Cytogenetic studies in two other cases of ALCL exhibiting cytoplasmic labeling only for ALK revealed the presence of a (2;3)(p23;q21) translocation in cells from case 5, whereas a (1;2)(q21;p23) translocation was present in case 8.¹⁵

Discussion

Monoclonal antibodies to the ALK kinase and to C- and N-terminal epitopes on the NPM molecule have been used previously to identify cases of ALCL-expressing ALK protein(s) and to provide evidence of variants of the classical (2;5) translocation.^{22,26} In the present paper we provide direct biochemical data on novel ALK proteins expressed in seven of these atypical cases of ALCL. An important technical point is that the sensitivity of the Western blotting and *in vitro* kinase assay procedures used was such that it was possible to perform these procedures on cryostat sections and cytocentrifuge preparations.

The novel ALK proteins were of four different sizes: 85 kd (one case), 97 kd (three cases), 104 kd (two cases), and 113 kd (one case). Functional kinase domains were detected in the 85-, 97-, and 104-kd proteins, indicating that these proteins should be capable of the phosphorylation of other substrates *in vivo*. Western blotting studies showed no evidence of NPM in these proteins, and we assume that they represent the consequences of the ALK gene fusing to genes other than NPM. This suggests that

Figure 2. Western blotting of proteins extracted from cryostat sections of tissues. **a:** The classical 80 kd NPM-ALK fusion protein is detected by anti-ALK (antibody ALKc) and (more weakly) by (b) the anti-N-terminus NPM (antibody NA24) in lysates from SU-DHL-1 cells and from cases of typical ALCL (cases 1–3). Normal tonsil contains no ALK protein. Wild-type 38 kd NPM can be seen in all samples (including tonsil). The higher molecular weight proteins probably represent oligomers of NPM. **c:** Cases of ALK-positive lymphoma with the atypical immunolabeling pattern (suggestive of variant translocation affecting the ALK gene; see Figure 1b) contain ALK proteins larger than the 80 kd NPM-ALK. These ALK proteins can be divided into four groups, namely, 85 kd (case 4), 97 kd (cases 5–7), 104 kd (cases 8 and 9), and 113 kd (case 10). It can be seen from **d** that only wild-type 38 kd NPM is present in these cases, and there is no evidence for NPM involvement with the novel ALK proteins. (Molecular weight standards are shown.)

at least four partners other than *NPM* can link to *ALK* in cases of morphologically typical ALCL.

One of the two cases expressing the 104-kd ALK protein exhibited a (1;2)(q21;p23) translocation.¹⁵ A recent study by Lamant et al²⁷ has identified the partner gene involved in a (1;2) translocation described in a case of ALK-positive ALCL¹² as *TPM3* (nonmuscle tropomyosin). This gene has previously been implicated in rearrangements with the *NTRK1* and *TRKA* (tropomyosin receptor kinase A) genes. mRNA encoding for the resulting oncogenic tyrosine kinase, TPM-TRK, has been described in

colonic²⁸ and papillary thyroid²⁹ carcinomas. Although the break point on chromosome 1 in the present study was cytogenetically different from that in the previously reported case (q21 versus q25), it will nevertheless be of interest to see if this case (and/or the other case expressing a 104-kd ALK protein) also carries the *TPM-ALK* fusion gene.

The presence of a (2;3)(p23;q21) translocation¹⁵ in one of the three lymphomas expressing a 97-kd ALK protein suggests that this may be a recurrent genetic anomaly present in ALCL. Break points at 3q21 have

Figure 3. *In vitro* kinase assay using ALK immunoprecipitates prepared from cryostat sections. **a:** Autophosphorylation is observed for the 80 kd NPM-ALK protein present in the SU-DHL-1 cell line and in the three cases of typical ALCL (cases 1–3). No corresponding band was present in the tonsil used as negative control. **b:** Kinase activity was present in proteins corresponding in size to the 85 kd (case 4), 97 kd (case 5), and 104 kd (cases 8 and 9) ALK proteins present in these cases of atypical ALCL. The absence of autophosphorylation in cases 6, 7 (expressing 97 kd ALK), and 10 (113 kd ALK) is probably due to the preparation and storage of the cryostat sections of ALCL.

been reported in acute myeloid leukemia.^{30,31} No candidate gene that could be involved in the dysregulation of a tyrosine kinase has been identified at this break point, however.

In conclusion, this paper has demonstrated the feasibility of detecting ALK proteins by biochemical techniques in cryostat sections of ALCL. The use of such small amounts of tissue means that efficient use can be made of precious biopsy material. Furthermore, suggestive evidence was obtained for at least four genetic abnormalities involving the *ALK* gene other than the (2;5) translocation. The new partners for *ALK* involved in these rearrangements remain to be identified, but one can predict that they are likely to contain motifs that cause dimerization of the hybrid ALK proteins and thereby induce oncogenic tyrosine kinase activation. Of particular interest is the possibility that some of these proteins will not have been identified previously and will prove to be involved in other types of human malignancy.

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