A New Variant Anaplastic Lymphoma Kinase (ALK)-Fusion Protein (ATIC-ALK) in a Case of ALK-positive Anaplastic Large Cell Lymphoma

Mirella Trinéi, Luisa Lanfrancone, Elias Campo, Karen Pulford, David Y. Mason, Pier-Giuseppe Pelicci, and Brunangelo Falini

Department of Experimental Oncology, Istituto Europeo di Oncologia, 20141 Milan, Italy [M. T., L. L., P.-G. P.]; Department of Pathology, University of Barcelona, Barcelona, Spain [E. C.]; LRF Immunodiagnostics Unit, John Radcliffe Hospital, Oxford, United Kingdom [K. P., D. Y. M.]; and Institute of Hematology, University of Perugia, 06100 Perugia, Italy [B. F.]

Abstract

Anaplastic lymphoma kinase (ALK)-positive lymphomas ("ALKomas") constitute a distinct molecular and clinicopathological entity within the heterogeneous group of CD30-positive large cell lymphomas. In 80–85% of cases tumor cells express a M, 80,000 hybrid protein comprising the nucleolar phosphoprotein nucleophosmin (NPM) and the ALK. We report here the cloning and expression of a novel ALK-fusion protein from an ALK-positive lymphoma. This case was selected for molecular investigation because of (a) the absence of NPM-ALK transcripts; (b) the atypical staining patterns for ALK (cytoplasm-restricted) and for NPM (nucleus-restricted); and (c) the presence of a M, 96,000 ALK-protein differing in size from NPM-ALK. Nucleotide sequence analysis of ALK transcripts isolated by 5'-rapid amplification of cDNA ends revealed a chimeric mRNA corresponding to an ATIC-ALK in-frame fusion. ATIC is a bifunctional enzyme (5-aminomimidazole-4-carboxamid ribonucleotide transformylase and IMP cyclohydrolase enzymatic activities) that catalyzes the penultimate and final enzymatic activities of the purine nucleotide synthesis pathway. Expression of full-length ATIC-ALK cDNA in mouse fibroblasts revealed that the fusion protein (a) possesses constitutive tyrosine kinase activity; (b) forms stable complexes with the signaling proteins Grb2 and Shc; (c) induces tyrosine-phosphorylation of Shc; and (d) provokes oncogenic transformation. These findings point to fusion with ATIC as an alternative mechanism of ALK activation.

Introduction

Inappropriate expression of the neural-associated receptor tyrosine kinase ALK (1, 2) defines a distinct clinicopathological entity (ALK-positive lymphomas or "ALKomas"); Refs. (ref3, 4) within the heterogeneous group of ALCLs (5–9). In 80–85% of the cases, ALK expression is the consequence of the t(2;5)(p23;q35) chromosomal translocation (10), whose breakpoints are located in the NPM and ALK genes (10, 11). The resulting NPM/ALK chimeric gene encodes a M, 80,000 fusion protein containing the NH2-terminal portion of NPM and the cytoplasmic domain of ALK (11–13). NPM is ubiquitously expressed and encodes a nucleolar protein involved in nucleocytoplasmic trafficking (14). Similarly to other receptor tyrosine kinase (RTK) oncogenes, fusion with NPM results in constitutive dimerization and activation of the ALK tyrosine kinase (15).

In about 15% of ALK-positive lymphomas, immunostaining for ALK and for NPM shows atypical intracellular localization patterns, and Western blotting analysis of these cases has revealed the presence of ALK proteins with molecular weights differing from those of ALK and NPM-ALK (16). This correlates with the finding that, in about 15–20% of ALK-lymphomas, the t(2,5) is undetectable, and ALK expression is probably due to the fusion of ALK with other partner genes. Indeed, rearrangements involving chromosome 2p23 in ALCLs have been recently found in the t(1,2), t(2,3), and inv (2) (17–19); and the non-muscle tropomyosin TPM3 gene (20) and the TFG (21) have been cloned as partners of ALK in cases of ALCL carrying, respectively, the (1,2) (q25;p23) and (2,3) (p23;q21) translocations.

The cloning of novel ALK partners in ALCL is crucial to the understanding of the genetics of ALCLs and the functioning of ALK-fusion proteins. We report here the identification of the ATIC (Put H) gene as a novel ALK partner in one case of ALCL.

Materials and Methods

Patient. An 18-year old male patient presented in July 1997 with a 4-week history of fever and prominent lymphadenopathy in the neck and the right inguinal regions. Biopsy of the inguinal lymph node was diagnostic of CD30-positive ALCL of T type (CD3+, CD20-, CD79a-, EMA+, CD68–, high fraction of Ki-67+ tumor cells; Ref. 6). An attempt to perform cytogenetic analysis on lymph node cell suspension failed because of the low number and poor viability of the recovered tumor cells. The disease was staged as IIIB and the patient was treated with combined chemotherapy (MACOP-B regimen). At the last follow-up (June 1999), the patient was in complete remission.

Immunohistochemical Detection of ALK and NPM Proteins. Immunohistological detection of ALK and NPM protein was performed on lymph node paraffin sections using monoclonal antibodies directed against fixative-resistant epitopes of the cytoplasmic portion of ALK (antibodies ALK1 and ALK2; Refs. 4, 19), and against the NPM NH1 terminal (antibodies NA24 and NPMa) and COOH-terminal (antibody NPMc; Ref. 22). Before immunostaining, paraffin sections were subjected to antigen retrieval by microwave heating (750 w × 3 cycles of 5 min each) in 1 ml EDTA buffer (pH 8.0; Ref. 4, 22). The immuno-alkaline phosphatase (APAAP) technique was used as the immunodetection system (4, 22).

RNA Extraction and RACE. Total RNA was prepared from frozen lymph node samples by a single-step RNA isolation method (TRIZol Reagent, Life Technologies). Poly(A)+ RNA was purified from total RNA using oligo (dT) cellulose (MessageMaker Reagent Assembly, Life Technologies). The ATIC-ALK fusion transcript was isolated by 5'-RACE, a PCR-based method that allows rapid amplification of a given cDNA using 3'-specific primers (23). The 5'-RACE from poly(A)+ RNA was performed using the Marathon cDNA Amplification kit (Clonetech). The following oligo-primers were used: (a) ALK1 (5'-TCTTGGGCTTCAAGCAGGCCTTTT'; (b) ALK2 (5'-GGC-TCTCGAGGAGAGTCTGAGGGTC-3'); (c) ALK3 (5'-TCTTCCTGTT-GCTTCCGGCCTTTA-3'); (d) ATIC1 (5'-AACTCAGCGTGAAGCTGTTGTTG-3'); and (e) AP1 and AP2 (from the RACE kit). The 750-bp DNA fragment obtained by nested PCR (see "Results") was cloned into the pTAs-dv vector (Clontech) and sequenced by the di-deoxo method. Nucleotide and deduced aa sequences were subjected to homology search with GenBank using...
the BLAST search program. The NPM-ALK (22) and ATIC-ALK cDNAs were subcloned into the pCDNA3 expression vectors.

**Immunoblotting and Immunoprecipitation.** For the preparation of cellular lysates from patient samples, cryostat sections (6-um) were cut from a portion of the patient’s lymph node biopsy that had been previously snap-frozen in liquid nitrogen. Sections were air-dried for 5 min, wrapped in aluminum foil, and stored at -70°C. Aliquots (50-μl) of sample buffer containing DTT (Sigma Chemical Co.), were added to each tissue section after their removal from -70°C storage. After 5 min at room temperature, the buffer was aspirated from the slides, heated at 95°C for 4 min, and loaded onto 7.5% SDS-PAGE gels. Cultured cells were lysed in PLC lysis buffer, 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 500 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin and 10 mg/ml leupeptin. Immune complexes were absorbed on protein G-Sepharose beads (Pharmacia) and washed extensively with PLC buffer. For immunoblotting, the proteins were subjected to PAGE-SDS and then transferred to PVDF membrane (Pall). The blotted membrane was probed with appropriate antibodies followed by chemiluminescent detection (Amersham). Monoclonal anti-Alk and anti-Npm antibodies and polyclonal anti-Shc antibodies were prepared in the authors’ laboratories (4, 19, 22, 24). Polyclonal anti-Grb2 and monoclonal antiphosphotyrosine antibodies were purchased from Santa Cruz Biotechnology and Upstate Biotechnology, respectively.

**Focus-forming Assay for Transformation.** NIH-3T3 cells were cultured in DMEM/5% bovine calf serum. Cells were seeded onto plastic dishes (1.3 × 10⁶ cells per dish) and transfected with 10 μg of plasmid DNA. After 24 h, DMEM/5% bovine calf serum was added and scored for focus formation after 10–14 days by staining with GIEMSA solution.

**Results**

**Identification of ALK-Lymphoma Showing Atypical NPM and ALK Localization.** The typical intracellular localization pattern of NPM-ALK in lymphomas carrying the t(2;5), as revealed by the available anti-Alk antibodies, is nuclear, with or without cytoplasmic staining. Immunolabeling with antibodies directed against the NH₂-region of NPM, which is retained within the fusion protein, reveals similar staining patterns (22, 25). We have screened a large panel of ALCLs using monoclonal anti-Alk and anti-Npm antibodies for atypical staining patterns and identified a case in which lymph node paraffin sections showed a cytoplasm-restricted expression of ALK and a nucleus-restricted positivity for NPM (Fig. 1). This immunohistological finding suggested the presence of an ALK-fusion protein other than NPM-ALK (25).

**Cloning of the ATIC-ALK Fusion Transcript.** To clone the 5’ end of the putative novel ALK-fusion cDNA, 5’-RACE was performed using poly(A)⁺ RNA from diagnostic and normal lymph nodes. The first PCR, using primers AP1 and ALK1 (Fig. 2A, upper diagram), enriched for DNA fragments in the diagnostic sample that were absent from the control sample (Fig. 2B, Lanes 4–5 and 6–7, respectively). Southern blot analysis using the internal primer ALK3 as probe (Fig. 2A, upper diagram) confirmed the specificity of those fragments (data not shown). A 1.3-kb ALK–hybridizing fragment was gel-purified and used as template in a nested PCR reaction using the AP2 and ALK2 primers (Fig. 2A, upper diagram). The resulting 750-bp DNA fragment (Fig. 2C, Lane 2) was purified and cloned into the pT-Adv vector. DNA sequence analysis revealed a single open reading frame from nucleotides 1 to 735 (245 aas; Fig. 2E). A database search revealed identity (at both nucleotide and protein levels) as ATIC (from nucleotides 1 to 274; 26–27) and ALK (from nucleotides 275 to 735; Refs. 1, 2; Fig. 2E). To confirm the existence of the ATIC-ALK fusion transcript in the original RNA sample from the biopsy, a PCR reaction was performed using ATIC- and ALK-specific primers (ATIC1 and ALK2 primers; Fig. 2A, lower diagram), which revealed the expected 750-bp chimeric DNA fragment (Fig. 2D). In contrast, NPM and ALK primers showed no evidence for the NPM-ALK fusion transcript (data not shown). Full-length ATIC-ALK cDNA was PCR-cloned from the patient’s RNA using specific 5’-ATIC and 3’-ALK primers. The ATIC-ALK fusion incorporates the first 229 aas of ATIC fused to the COOH-terminal 563 aas of ALK (not shown). Together, these data confirmed that RNA from the biopsy contained a novel chimeric transcript corresponding to the ATIC-ALK fusion protein.

**Expression of the ATIC-ALK Fusion Protein.** The full-length ATIC-ALK cDNA was then cloned into the pCDNA3 expression vector and transiently transfected into NIH-3T3 cells. Western blotting analysis using anti-Alk antibodies revealed a Mr 96,000 anti-Alk immunoreactive polypeptide (Fig. 3A, panel 2), that was not seen in cells transfected with the vector alone. The difference between the predicted ATIC-ALK MW (Mr 88,000) and that observed by Western blotting (Mr 96,000) may be due to posttranslation modifications, including tyrosine-phosphorylation (Fig. 3A, panel 4). Side-by-side comparison of lysates from the patient’s lymph node and from ATIC-ALK transfected NIH-3T3 cells revealed comigration of the two ALK-proteins (Fig. 3A, panel 1). Together, these data indicate that the ATIC-ALK cDNA cloned in this case corresponds to the ATIC-ALK fusion protein formed in the patient’s lymph node.
The ATIC-ALK cDNA Encodes a Constitutively Active Tyrosine-Kinase That Associates with Grb2 and Shc Adaptor Proteins. The NPM-ALK fusion protein possesses constitutive tyrosine kinase activity and forms stable complexes with adaptor proteins, such as Grb2 and Shc (28). Its potential to activate downstream signal transduction pathways is demonstrated by its ability to induce constitutive tyrosine-phosphorylation of Shc (28). To investigate whether ATIC-ALK has a similar activation mechanism, we compared the status of kinase activity, the association with Grb2 and Shc, and the ability to induce Shc tyrosine-phosphorylation of ALK-proteins in lysates from NIH-3T3 cells transiently transfected with ATIC-ALK and NPM-ALK cDNA. Anti-ALK immunoprecipitates showed comparable amounts of ATIC-ALK and NPM-ALK fusion proteins (Fig. 3A, panel 3) with similar content of phosphotyrosine residues (Fig. 3A, panel 4). Similarly, antiphosphotyrosine immunoprecipitates from the same cellular lysates revealed comparable amounts of ATIC-ALK and NPM-ALK proteins (Fig. 3A, panel 6). Kinase assays of ALK-immunoprecipitates from cellular lysates of ATIC-ALK and NPM-ALK...
NIH3T3-transfected cells showed the presence of a $M_r$ 96,000 and a $M_r$ 80,000 polypeptide respectively, representing autophosphorylated fusion proteins (data not shown). The p52 and p46 Shc proteins coprecipitated with both fusion proteins (though to a lesser extent in the case of the ATIC-ALK fusion protein (Fig. 3A, panel 5).

To evaluate the association of Grb2 with the two ALK-fusion proteins, anti-Grb2 immunoprecipitates from the same lysates were blotted with anti-Alk antibodies (Fig. 3A, panel 7). Anti-Grb2 blots served as controls for the efficiency of the Grb2 immunopurification (Fig. 3A, panel 8). The results showed that Grb2 coprecipitated with both NPM-ALK and ATIC-ALK, although again the coprecipitation showed less Grb2-associated with ATIC-ALK than that found in the NPM-ALK-Grb2 complex. Finally, antiphosphotyrosine blots of anti-Shc immunoprecipitates revealed constitutive tyrosine-phosphorylation of Shc in both NPM-ALK and ATIC-ALK expressing cells (Fig. 3A, panel 10). Anti-Alk blots of the same immunoprecipitates confirmed the existence of a stable Shc-ALK-fusion protein complex (Fig. 3A, panel 9).

It appears, therefore, that, like NPM-ALK, the ATIC-ALK fusion protein is a constitutively active tyrosine kinase with the potential to deliver intracytoplasmic activating signals.

**ATIC-ALK Expression Induces Neoplastic Transformation of NIH-3T3 Cells.** NPM-ALK is known to transform NIH-3T3 cells (28, 29), and the ATIC-ALK expression vector induced foci of transformation 10–14 days after transfection. Morphology of NPM-ALK and ATIC-ALK transformed cells (lower panel) derived from one typical focus compared with control cells derived from vector-only transfected cells, as indicated.
formed cells with comparable or higher efficiency (NPM-ALK and ATIC-ALK yielded a comparable amount of resistant colonies in parallel dishes treated with G418 for selection of the expression vectors; data not shown; Fig. 3B, upper panel). Cells picked from the NPM-ALK and ATIC-ALK foci showed the same morphology of transformed fibroblasts (refractile, spindle-shaped, and elongated cells; Fig. 3B, lower panel).

Discussion

We have reported here the cloning and expression of a novel ALK fusion protein from a case of ALK-positive ALC. This case was initially suspected as genetically distinct from classic NPM-ALK-positive ALCs when anti-Npm and anti-ALK staining of paraffin sections revealed atypical localization patterns. Indeed, anti-ALK staining revealed a “cytoplasmic-only” pattern, whereas monoclonal antibodies directed against the NH2-terminal portion of NPM stained the nuclei of tumor cells (reflecting reactivity with wild-type NPM). This study underscores the usefulness of anti-ALK and anti-Npm antibodies for detecting cases containing new genetic abnormalities in ALCs. Because the products of the genes involved in the generation of other tumor-associated fusion proteins are frequently localized to specific subcellular compartments, screening of hemopoietic tumors with antibodies against the known translocation partners may lead to the identification of other novel genetic abnormalities.

Nucleotide sequencing of the novel chimeric transcript revealed an in-frame 5′-3′ fusion of ATIC and ALK sequences. The ATIC gene (previously named as Pur H) encodes a bifunctional protein which catalyzes the pentulinate and the final steps of the de novo purine nucleotide biosynthetic pathway. It acts as a AICARFT and as an IMPCH, to catalyze the formation of FAICAR and IMP, respectively (26–27). Deletion mutant analysis of the ATIC cDNA has demonstrated that the IMPCH and the AICARFT enzymatic activities segregate within two nonoverlapping functional domains, with the IMPCH domain located at the NH2 terminus of the protein (30–32).

The crystal structure of the ATIC protein revealed a smaller NH2-terminal globular portion (approximately from aa 1 to 200), a 30-aahelical (linker) region, and a COOH-terminal globular region (31–32). The isolated NH2-terminal portion of the ATIC protein (aa 1–199) retains IMPCH activity in vitro, which suggests that the NH2-terminal globular region of ATIC has independent folding properties and contains the IMPCH enzymatic activity (31). The portion of the ATIC transcript retained within the ALK-hybrid mRNA corresponds to aa 1–229 (Fig. 2E). It is, therefore, possible that the ATIC-ALK transcript encodes a fusion protein with IMPCH activity. The de novo purine pathway is an important target for the chemotherapeutic agents; for example, methotrexate exerts a potent inhibitory effect on AICARFT activity (33). It will be of interest to determine whether the ATIC-ALK fusion protein alters purine metabolism in ALC cells and/or their sensitivity to purine-inhibitors.

We have not been able to investigate the chromosomal origin of the ATIC-ALK recombination because of the inadequacy of patient material for cytogenetic studies. The ATIC gene has been previously mapped between bands q34 and q35 of the long arm of chromosome 2 (31), a region that is rearranged in the cryptic inversion of chromosome 2 in cases of NPM-ALK-negative, ALK-positive ALCs (18). It is, therefore, possible that the ATIC-ALK fusion gene is the product of the cryptic inv(2) (p23q35). Notably, as in our case, in all of the ALC cases with inv(2) (p23q35), the ALK protein accumulates in the cytoplasm only (18).

The portion of ALK retained in the ATIC-ALK fusion protein is the same as in NPM-ALK as well as in the other two variant ALK-fusion proteins thus far identified (TPM3-ALK and TFG-ALK; Refs. 20–21). In common with NPM-ALK, ATIC-ALK possesses constitutive tyrosine kinase activity, activates cytoplasmic signal transduction pathways, and is able to transform rodent fibroblasts. The activation of the ALK kinase domain is the consequence of the constitutive homodimerization of the fusion protein, triggered by the NPM oligomerization domain present in NPM-ALK (15). Likewise, TPM3 and TFG contain coiled coil domains that may induce dimerization of their corresponding ALK-fusion proteins (34, 35). This could also occur with ATIC the crystal structure of which is a homodimer (31).

Fusion to an heterologous oligomerization domain is sufficient to activate the capacity of ALK sequences to transform immortal rodent fibroblasts, which suggests that the NPM portion of the molecule (or of the other ALK-partners) is responsible only for dimerization, with no apparent further function for the delivery of mitogenic stimuli (15, 36). However, the contribution of the various ALK-partners during lymphomagenesis in vivo remains to be established. It will be of interest to determine whether alteration of purine metabolism contributes to lymphomagenesis.

Acknowledgments

We thank Barbara Bigerna and Roberta Pacini for their skilful technical assistance and Sara Barozzi for important experimental contributions. We also thank Claudia Tibido` and Annalisa Ariesi for excellent secretarial assistance.

References


A New Variant Anaplastic Lymphoma Kinase (ALK)-Fusion Protein (ATIC-ALK) in a Case of ALK-positive Anaplastic Large Cell Lymphoma

Mirella Trinei, Luisa Lanfrancone, Elias Campo, et al.

*Cancer Res* 2000;60:793-798.

Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/4/793

Cited articles

This article cites 33 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/4/793.full#ref-list-1

Citing articles

This article has been cited by 22 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/4/793.full#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.