Anaplastic large cell lymphoma (ALCL) is associated with the t(2;5)(p23;q35), which generates the NPM-ALK fusion gene encoding an 80-kD protein. Several studies have suggested that genes other than NPM may be fused to the ALK gene. Here we have identified TRK-fused gene (TFG) as a new ALK partner in 2 ALCL, 1 of which exhibited a t(2;3)(p23;q21). In these cases, TFG was involved in 2 different fusion genes, TFG-ALK\(\alpha\) and TFG-ALK\(\beta\), coding respectively 85-kD and 97-kD chimeric proteins. The ALK breakpoint in these translocations was the same as in the classic t(2;5) translocation. These 2 proteins were both active in an in vitro tyrosine kinase assay showing that the new cloned cDNA sequences are translated into chimeric proteins with functional activity. These findings indicate that TFG can provide an alternative to NPM as a fusion partner responsible for activation of the ALK and the pathogenesis of ALCL.

BRIEF REPORT

TRK-Fused Gene (TFG) Is a New Partner of ALK in Anaplastic Large Cell Lymphoma Producing Two Structurally Different TFG-ALK Translocations

By Luis Hernández, Magda Pinyol, Silvia Hernández, Silvia Beà, Karen Pulford, Andreas Rosenwald, Laurence Lamant, Brunangelo Falini, German Ott, David Y. Mason, Georges Delsol, and Elias Campo

Materials. The material used for the initial cloning experiments was a diagnostic lymph node biopsy from a 19-year-old man (case #789), which showed the typical features of “common”-type ALCL. However, reverse transcription-polymerase chain reaction (RT-PCR) analysis provided no evidence for the NPM-ALK fusion gene, and ALK protein was expressed with a cytoplasmic restricted pattern.

After identification of the TFG-ALK chimeric gene (see below), 6 additional cases of ALCL that lacked NPM-ALK and TPM3-ALK were examined by RT-PCR. One of these later cases (case #862) exhibited the t(2;3)(p23;q21) described previously. Expression of normal TFG transcripts was also examined by RT-PCR (see below) in 3 non-neoplastic lymphoid tissues, 14 non-Hodgkin’s lymphomas (NHLs), and 11 malignant hematopoietic cancer cell lines (8402, CEM, U937, HL-60, Karpas 299, MOLT-4, K562, Raji, Ramos, Namalwa, and HS2B). The SU-DHL-1 and Karpas 299 cell lines (both of which carry the (2;5) translocation) were used as positive controls.

Immunohistochemistry and antibodies. Immunohistological staining was performed using a panel of monoclonal antibodies for B- and T-cell antigens, together with antibodies to CD30 (Dako-BerH2), and HSB2. The SU-DHL-1 and Karpas 299 cell lines (both of which carry the (2;5) translocation) were used as positive controls.

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Western blotting and in vitro kinase assay. Cryostat sections (6 µm) were cut from frozen samples of normal tonsil and the biopsy specimens from cases no. 789 and 862. Cytocentrifuge preparations were made of the cultured SU-DHL-1 cell line. Western blotting using the monoclonal anti-ALK (ALKc) or anti-NPM (NA24) and an in vitro kinase assay were performed as previously described.19

RNA extraction and 5’ RACE reaction. Total RNA was isolated from a frozen sample of the diagnostic lymph node using guanidine isothiocyanate extraction and cesium chloride gradient centrifugation. cDNA was obtained from 1 µg of total RNA using 2.5 pmol/L of GSP1 ALK-specific primer (5’-ACCCCAATGCAAGGACAA-3’) and Super Script II Reverse Transcriptase (Life Technologies Inc, Paisley, UK). The RACE technique was used to obtain the 5’ sequence fused with the ALK gene following the manufacturer’s recommendations. The PCR primers used for 5’ RACE reaction were AAP primer (included in the kit) and GSP2 ALK primer (5’-CTGGTGGTGAATTTGCTGAT-3’) for the first round and AUAP primer (included in the kit) together with GSP3 ALK primer (5’-CTTGCGGCTGGGCAATC-3’) for the second nested round of PCR amplification. The specificity of the PCR fragments obtained were confirmed by hybridization with the fluorescein-labeled internal oligonucleotide ALK 3 (5’-GTGGAGGTGGAGCCTTCAGC-3’). DNA sequencing. The PCR products were purified from the gel and sequenced in ABI PRISM automated sequencer (Applied Biosystems, Foster City, CA). Both DNA strands were completely sequenced using different upstream and downstream primers by primer walking.

RT-PCR expression analysis of NPM-ALK, TPM3-ALK, TFG and TFG-ALK genes. cDNA from all the analyzed samples was obtained from 1 µg of total RNA, random hexamer priming and SuperScriptTM II Reverse Transcriptase (Life Technologies Inc) following the manufacturer’s recommendations. Amplification of the RPS14 ribosomal mRNA was performed as control. NPM-ALK and TPM-3-ALK expression was examined using previously described methods.3,19 Expression analysis of wild-type TFG gene was performed using TFG2 (5’-AACATCCTGGAGTCCACCAGT-3’) and TFG4D (5’-GCCCTGAACCTGAT-CATCCTG-3’) primers to amplify a 601 bp fragment from 2 µL of cDNA. The PCR conditions were 35 cycles consisting of 45 seconds at 94°C, 45 seconds at 65°C and 45 seconds at 72°C, followed by a final extension of 20 minutes at 72°C. The PCR mixture contained 1 U of Taq (Boehringer Mannheim, Mannheim, Germany), 0.8 mmol/L each primer, 100 mmol/L dNTPs, and PCR buffer in a final volume of 25 µL. TFG-ALK expression analysis was assessed using a set of primers containing the translocation breakpoint described in this study. The primers used were TFG1 (5’-AGCTTGGACCCACCTGGAGAACC-3’)/ALK3.

RESULTS AND DISCUSSION

The lymph node biopsy specimen of case no. 789 showed a typical ALCL of “common” morphology with a T-cell phenotype (CD3-positive, CD5-positive) coexpressing CD30 and EMA. ALK protein was detected in all tumor cells but was restricted to the cytoplasm. RT-PCR studies for NPM-ALK and TPM-ALK chimeric products were negative. Western blot analysis (using ALKc antibody) detected an ALK protein with an apparent molecular weight of 85 kD, higher than the 80 kD of the classic NPM-ALK fusion protein.19 Immunohistochemical studies using a monoclonal antibody against the N-terminal portion of NPM only detected nuclear NPM. This was in contrast to the cytoplasm and nuclear distribution of NPM observed in cases of t(2;5)-positive NPM-ALK fusion protein.19 Furthermore, Western blotting studies only detected the presence of wild-type 38-kD NPM (Fig 1 A). These observations confirmed at the protein level that NPM was not fused to ALK in this tumor. However, an in vitro kinase assay showed
that the ALK portion of the novel protein present in this tumor possessed tyrosine kinase activity (Fig 1B).

To identify the gene involved in this new ALK translocation, we used a 5'RACE strategy. The primers for this technique were designed within the known 3' catalytic domain of the ALK. The result of the 5' amplification yielded a major product of approximately 800 bp. Hybridization with an internal ALK primer confirmed the specificity of this product, which was subsequently purified, cloned, and sequenced.

The sequence of this fragment confirmed that a gene other than NPM was fused to the 5' region of ALK. Comparison of this sequence with the Genbank database showed 99.7% homology with the 5' region of the TFG gene (TRK-fused gene), which has been mapped to chromosome 3q11-12. This gene was initially identified as the rearranged partner of the NTRK1 gene in a thyroid papillary carcinoma generating the TRK-T3 fusion gene that has transforming activity in different models. Interestingly, the TFG gene, in common with other genes which can cause oncogenic activation of receptor tyrosine kinase genes, is constitutively expressed in many different tissues. To determine whether the TFG gene is also expressed in lymphoid cells, we analyzed different lymphoid samples by RT-PCR and could show that wild-type TFG was constitutively expressed in all non-neoplastic lymphoid tissues, in 14 NHLs, and in 11 neoplastic hematopoietic cell lines.

Using primers from the 5' TFG and 3' ALK distal regions we could amplify by RT-PCR a 2.5-Kb fragment containing the full-length TFG-ALK coding sequence. The 5' TFG segment was composed of 459 bp containing 44 bp of the 5' untranslated region and 415 bp of the coding sequence. This region included the predicted coiled-coil oligomerization domain of the protein (Fig 2A). The breakpoint was located at 415 bp from the first methionine. The only difference between the TFG coding sequence obtained in our case and that previously described (GenBank accession number Y07968) was a single nucleotide polymorphism (gtc→atc; Val→Ile). This change seems to be a polymorphic variant because it was also present in genomic DNA obtained from normal epithelial cells of the patient.

The ALK sequence in our case was identical to that previously described, and it was in frame with the upstream TFG open reading frame. Noticeably, the ALK breakpoint in this TFG-ALK cDNA was the same as that found in the NPM-ALK fusion gene. The predicted chimeric TFG-ALK protein was composed of 701 aminoacids, 138 of which were encoded by the TFG gene and 562 by the ALK gene. The valine in position 139 is encoded by a new codon created by the rearrangement. The predicted molecular weight of the new TFG-ALK fused protein is 83 kD, which is very similar to the value obtained by Western blot analysis (85 kD).

To determine if this new TFG-ALK translocation was expressed in other ALCLs, we analyzed 6 additional tumors with ALK protein expression restricted to the cytoplasm and which were negative for NPM-ALK and TPM3-ALK translocations. In addition, 3 NPM-ALK-positive and 1 TPM3-ALK–positive ALCL, and 5 thyroid carcinomas were also analyzed. Expression of the TFG-ALK chimeric product was examined with a set of primers from TFG and ALK genes spanning 141 bp of the breakpoint region. The TFG-ALK chimeric transcript could be amplified only in our control tumor and also in 1 ALCL (case #862) that was negative for NPM and TPM3-ALK translocations. The cytogenetic analysis of this tumor had shown the presence of a (2;3)(p23;q21) translocation. Interestingly, however, the amplified product in this case showed an unexpected larger size (306 bp) than that obtained (141 bp) in our control case. Sequencing of this product confirmed a TFG-ALK rearrangement in which the ALK breakpoint was the same as in the other ALK translocations. However, the TFG fragment contained an additional 165 bp sequence from the TFG gene not present in the hybrid gene in our previous case (Fig 2B).

The TFG breakpoint region in this second case was the same as in the TFG-NTRK1 translocation in thyroid carcinomas. In this new rearrangement creating a larger TFG-ALK (TFG-ALKL) gene, the additional 165 bp TFG sequence was also in frame with the 5' TFG fragment of our previous shorter

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A

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Fig 2. Nucleotide and deduced amino acid sequences of TFG-ALK cDNA in short TFG-ALK\(_L\) (A) and long TFG-ALK\(_L\) (B) forms. (A) Nucleotide sequence was numbered according to the previously described sequences. The putative coiled-coil domain of the TFG sequence is underlined. The asterisk marks a previously undescribed polymorphism (gtc→atc; Val→Ile). The translocation breakpoint in the cDNA is marked with an arrow. (B) The additional 165 bp TFG sequence is underlined. The translocation breakpoint in the cDNA is marked with an arrow.
TFG-ALK (TFG-ALK<sub>L</sub>) translocation, and with the distal 3′ ALK sequence. The predicted molecular weight of this new chimeric TFG-ALK<sub>L</sub> protein was 96 kD, in keeping with the value of 97 kD determined by Western blot and in vitro kinase analysis of this case (Fig 1).

These findings indicate that TFG is an alternative to NPM as a partner for ALK in ALCCL, and that it can be involved in 2 structurally different translocations, TFG-ALK<sub>L</sub>, encoding 85-kD and 97-kD chimeric proteins, respectively. An in vitro kinase assay showed that these proteins also had tyrosine kinase activity (Fig 1) indicating that the oncogenic expression observed in our tumors. These findings support the TFG<sub>L</sub> segment included in this translocation contains the full predicted coiled-coil oligomerization domain of the gene. Similarly to the 5′ region of NPM in the NPM-ALK translocation, this TFG domain is absolutely required for oligomerization and transforming activity of TFG-NTRK (TRK-T) oncogene. Therefore, it is also possible that TFG may play a similar role in the activation of ALK in ALCCL. However, in distinction to NPM, the TFG gene lacks nuclear-signaling domains, which is in keeping with the restricted cytoplasmatic pattern of ALK expression observed in our tumors. These findings support previous in vitro experiments, indicating that the oncogenic rearrangement of the TFG<sub>L</sub> sequence is independent of the nuclear localization.

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