Diversity of Genomic Breakpoints in *TFG-ALK* Translocations in Anaplastic Large Cell Lymphomas

Identification of a New *TFG-ALK_{XL}* Chimeric Gene with Transforming Activity

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Anaplastic large cell lymphomas are associated with chromosomal aberrations involving the anaplastic lymphoma kinase (*ALK*) gene at 2p23 that result in the expression of novel chimeric *ALK* proteins with transforming properties. In most of these tumors, the t(2;5)(p23;q35) generates the *NPM-ALK* fusion gene. However, several studies have now demonstrated that genes other than *NPM* may be fused to the *ALK* gene. We have recently described two different *ALK* rearrangements involving the *TRK-fused gene (TFG)* in which the same portion of *ALK* was fused to different length fragments of the 5' *TFG* region. These two rearrangements encoded chimeric proteins of 85 kd (*TFG-ALK_S*) and 97 kd (*TFG-ALK_L*), respectively. In this study, we have identified a new *ALK* rearrangement in which the catalytic domain of *ALK* was fused to a larger fragment of the *TFG* gene (*TFG-ALK_{XL}*), encoding for a fusion protein of 113 kd. Genomic analysis of these three *TFG-ALK* rearrangements revealed that the *TFG* breakpoints occur at introns 3, 4,

and 5, respectively, whereas the *ALK* breakpoints always occur in the same intron. No homologous regions or known recombination sequences were found in these regions. Transfection experiments using NIH-3T3 fibroblasts showed a similar transforming efficiency of *TFG-ALK* variants compared with *NPM-ALK*. In addition, in common with *NPM-ALK*, the *TFG-ALK* proteins formed stable complexes with the signaling proteins Grb2, Shc, and PLC- γ . In conclusion, these findings indicate that the *TFG* may use a variety of intronic breakpoints in *ALK* rearrangements generating fusion proteins of different molecular weights, but with similar transforming potential than *NPM-ALK*. (*Am J Pathol* 2002, 160:1487-1494)

Anaplastic large cell lymphoma (ALCL) expressing *ALK* (anaplastic lymphoma kinase) (*ALK*-positive lymphoma or *ALKoma*) is a distinct clinicopathological and molecular entity characterized by a proliferation of T/null lymphoid cells showing a diverse immunostaining pattern for *ALK* protein.¹⁻³ This aberrant *ALK* expression is associated with chromosomal translocations involving the *ALK* gene at 2p23.⁴ These translocations lead to the synthesis of novel chimeric *ALK* proteins that possess transforming properties. In a high proportion of these tumors, the t(2;5)(p23;q35) translocation causes fusion of the *ALK* gene to the region of the nucleophosmin (*NPM*) gene.⁵ This rearrangement generates a novel fusion protein, *NPM-ALK*, of 80-kd molecular weight, that contains the N-terminal region of *NPM* fused to the C-terminal

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region of ALK.⁶ The *ALK* gene encodes a tyrosine kinase receptor that seems to play a role in the development of the nervous system and is not normally expressed in lymphoid cells. As found in other chimeric oncogenic tyrosine kinases, a dimerization motif within the NPM protein mimics ligand binding and results in the constitutive activation of the tyrosine kinase.⁷ Several cytogenetic and molecular studies have now demonstrated that chromosomal aberrations other than the t(2;5)(p23;q35) may give rise to novel *ALK* fusion genes in ALCL. The possible genomic mechanisms involved in these rearrangements are poorly understood. So far, five different genes, nonmuscle tropomyosin (*TPM3*), TRK-fused gene (*TFG*), 5'-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (*ATIC*), clathrin heavy chain gene (*CLTC*), and moesin (*MSN*), have been cloned as alternative partners to *NPM* in ALCL.⁸⁻¹⁴ These variant translocations encode *ALK* fusion tyrosine kinases with molecular weights ranging from 85 to 250 kd. Contrary to the nuclear and cytoplasmic distribution of the NPM-*ALK* protein, variant *ALK* fusion proteins show a variable subcellular localization depending on the case variant *ALK* protein present, indicating that *ALK* oncogenic activation may occur in different cytoplasmic compartments.

The *TFG* gene at 3q11-12¹⁵ has been found to be a fusion partner of *ALK* in two cases of ALCL.⁹ Protein analysis of these tumors showed chimeric *ALK* proteins of 85-kd and 97-kd molecular weight, with a cytoplasmic-restricted localization. Molecular cDNA analysis of these cases showed that the *TFG* segment included in both translocations encoded the full predicted coiled-coil oligomerization domain of the protein. However, the chimeric gene coding for the 97-kd protein contained an additional 165-bp fragment of the *TFG* gene that was not included in the *TFG-ALK* gene coding for the 85-kd protein. Because of the different lengths of the chimeric products, these two fused genes were named *TFG-ALK_S* (short) and *TFG-ALK_L* (long), respectively. The larger *TFG* portion present in the *TFG-ALK_L* chimeric gene was the same as in the *TFG-NTRK1* translocation that occurs in thyroid carcinomas.¹⁶ However, the genomic breakpoints of these translocations were not characterized.

In the present study, we examine the genomic breakpoints of the two previously described *TFG-ALK* rearrangements and identify a third *TFG-ALK* variant involving a new breakpoint located in *TFG* intron 5. We also demonstrate that *TFG-ALK* chimeric genes have *in vitro* transforming potential involving similar transduction pathway elements (*Grb2*, *Shc*, and *PLC-γ*) as the *NPM-ALK* gene.

Materials and Methods

Materials

The specimen used for the initial cloning studies was a diagnostic lymph node biopsy (case 893) from an 11-year-old female diagnosed with CD30-positive ALCL of null/T-cell phenotype (clinical stage IB) that was treated in 1991 according to the LSA2/L2 protocol and is at

present in complete remission. *ALK* protein was expressed with a cytoplasmic-restricted pattern and a reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was negative for the *NPM-ALK* fusion gene. Additional frozen material from the ALCLs with the two *TFG-ALK* fusion genes previously described (cases 789 and 862),⁹ were also used to determine the genomic breakpoint of these translocations.

Immunohistochemistry and Antibodies

Immunohistological staining of formalin-fixed paraffin-embedded tissues was performed using a panel of monoclonal antibodies for B- and T-cell antigens, together with antibodies to CD30 (DAKO-BerH2; DAKO, Copenhagen, Denmark) and EMA (DAKO-EMA/E29). Antibodies against the *ALK* protein (*ALK1* and *ALKc*) and the N-terminal region of nucleophosmin used in the immunohistochemical and Western blot analyses were produced in the authors' laboratories.^{1,17,18} Immunohistochemical studies were also performed on NIH-3T3 fibroblasts transformed with *NPM-ALK*, *TFG-ALK_S*, and *TFG-ALK_L*. Serial dilutions of these cells were seeded onto coverslips for 24 hours, washed twice with phosphate-buffered saline (PBS), then fixed with 95% ethanol/5% acetic acid at -20°C for 10 minutes. The cells were washed twice for 5 minutes each in PBS, and blocked with 10% heat-inactivated goat serum for 30 minutes at room temperature, then incubated overnight in a humidified chamber with polyclonal antibody *ALK-11* (1:100 in 0.2% heat-inactivated goat serum/0.05% Triton X-100 PBS), washed again four times with PBS, then incubated for 45 minutes with a Cy3-conjugated affinity-purified goat anti-rabbit IgG secondary antibody (1:1000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The cells were washed again, and the nuclei counterstained with propidium iodide. The immunofluorescent staining was examined with an Axiovert 135 TV microscope and Hamamatsu XC77 digital camera.

RNA Extraction and RT-PCR of the *TFG-ALK_{XL}* Fusion Gene

Total RNA was isolated from a frozen sample of case 893 using the Ultraspec RNA extraction kit (Biotech, Houston, TX), following the manufacturer's recommendations. Reverse transcription was performed using Super Script II Reverse Transcriptase (Life Technologies, Inc., Paisley, UK) with random hexamer priming from 1 μg of total RNA. PCR amplification including the cDNA breakpoint region in case 893 was performed using the same *TFG1* (5'-AGCTTGGAACCACTGGAGAACC-3') and *ALK3* (5'-GTGAGGTGCGGAGCTTGCTCAGC-3') primers and reaction conditions described previously for the short and long *TFG-ALK* variants.⁹ The PCR product was purified and sequenced as previously described.⁹ RT-PCR analysis of other *ALK* fused genes (*TPM-ALK*, *ATIC-ALK*, and *CLTC-ALK*) was also performed using previously described primers.^{8,10,14}

Characterization of the Genomic Breakpoints in TFG-ALK Variants

Genomic DNA from the three tumors with different variants of the *TFG-ALK* fusion gene was prepared using Proteinase K treatment and phenol-chloroform extraction.¹⁹ Amplification of the genomic breakpoints was performed using a forward primer of the flanking *TFG* exon together with a common reverse *ALK3* primer. *TFG* primers used were *TFG1* for *TFG-ALK_S* and *TFGLU* (5'-GCAG-CAAGTATGTCTGCTTTTATCC-3') for *TFG-ALK_L*. Different *TFG* primers from the flanking exon and intronic region were designed to amplify the *TFG-ALK_{XL}* genomic breakpoint but all of these amplifications were unsuccessful. Amplification conditions were 40 cycles consisting of 45 seconds at 94°C, 45 seconds at 65°C, and 1 minute 30 seconds at 72°C. The PCR mixture contained 1 U of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany), 0.8 mmol/L of each primer, 100 mmol/L dNTPs, and PCR buffer in a final volume of 25 μ L. Normal *TFG* introns involved in the *TFG-ALK* rearrangements were amplified using Pfx polymerase (Life Technologies, Inc.), and following the manufacturer's specifications. The primers used were forward *TGEN1U* (5'-TGGGTGAT-TCTTTCGCAACTAGTC-3') and reverse *TFG128D* (5'-CT-GATCATCTGTAAAGCCAAACGC-3') for the normal *TFG* intron involved in the short variant, and forward *TGEN9U* (5'-GCTCTCCCAGCCCTATAGTCAG-3') and reverse *XLDGENR* (5'-AGGAGGAGGAAGCAATGCTGTC-3') for the normal *TFG* intron involved in the long variant. PCR products were purified and sequenced as previously described,⁹ using different upstream and downstream primers for primer walking. The computer program CENSOR, designed by Jurka and colleagues²⁰ was used by E-mail procedure (censor@charon.lpi.org) for repeat sequence identification. Other sequence features that were searched included interchromosomal homologies and positions of candidate recombination sequences (topoisomerase I and II, translin, heptamer/nonamer, chi consensus, alternating purine/pyrimidine, polypurine, and polypyrimidine sequences).²¹⁻²⁸

cDNA Cloning and DNA Constructs of the ALK Fusion Genes

TFG-ALK_{XL}, *TFG-ALK_S*, and *NPM-ALK* constructs were prepared by PCR-based methods and were expressed with the pMEX and pLTR2 plasmids.²⁹ The *PsralphaMSVtkneo-NPM-ALK* plasmid (trimmed of most of its 5' and 3' UTR sequences) was previously described.⁷ The *NPM-ALK* insert was released with *HindIII* and *XbaI* restriction enzymes, and then subcloned into the pMEX plasmid between *EcoRI* and *Sall*, and also subcloned into the pLTR2 plasmid between *XhoI* and *Clal* sites. The *TFG-ALK* short and extra long cDNAs were cloned into the pMEX between *XhoI* and *Sall* restriction enzyme sites and also into the pLTR2 plasmid between *XhoI* and *Clal* sites.

Focus Formation Assay

Mouse NIH-3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% calf serum. Cells were seeded onto plastic dishes (1.5×10^5 cells per dish) and transfected by the calcium-phosphate precipitation method as previously described,³⁰ using 0.2 to 2 μ g of plasmid DNA (*NPM-ALK*, *TFG-ALK_S*, *TFG-ALK_{XL}*) together with 40 μ g of high-molecular weight calf thymus carrier DNA. Each transfection was done in duplicate, and carrier DNA and empty plasmids were used as negative controls. *H-RasK12* cloned into the pLTR2 was used as positive control. Transformed foci were selected in Dulbecco's modified Eagle's medium containing 5% calf serum and G418-resistant colonies were selected in Dulbecco's modified Eagle's medium supplemented with 10% serum and G418 antibiotic (750 μ g/ml). Transformed foci and drug-resistant colonies were either stained with Giemsa solution or isolated for further studies 2 or 3 weeks after transfection.

Western Blot Analysis

Protein was extracted from cryostat frozen sections using a previously described method.³¹ Protein was also extracted from cells using a lysis buffer composed of 20 mmol/L HEPES, pH 7.5, 10 mmol/L EGTA, 40 mmol/L β -glycerophosphate, 1% Nonidet P-40, 25 mmol/L $MgCl_2$, 2 mmol/L sodium orthovanadate, 2 mmol/L dithiothreitol, 1 mmol/L phenylmethyl sulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Equal amounts of protein were separated by electrophoresis on SDS-10% polyacrylamide gels and transferred to Immobilon-P (Millipore, Bedford, MA) membranes. The membranes were incubated with monoclonal antibodies against ALK (ALK-1 and ALKc) or anti-NPM. Antibody binding was detected using a secondary antibody (anti-rabbit or anti-mouse immunoglobulin; Amersham, Buckinghamshire, UK) conjugated to horseradish peroxidase and an enhanced chemiluminescence detection kit (Amersham).

Immunoprecipitations

Cell lysates (1 mg) were incubated overnight with 10 μ L of antibodies to one of the following: ALK, Shc (Transduction Laboratories, Lexington, KY), Grb2 (Transduction Laboratories), or PLC- γ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies at 4°C. Protein A-Sepharose was then added to the samples for 1 hour at 4°C. Immunoprecipitates were washed three times with lysis buffer and electrophoresed in 10 to 20% polyacrylamide gradient gels (Bio-Rad). The proteins were then transferred to Immobilon-P and detected using anti-ALK as described above.

Figure 1. Western blotting of protein extracted from sections of the anaplastic large-cell lymphoma 893 using anti-ALKE demonstrates the presence of a 113-kd TFG-ALK_{XL} fusion protein in tumor cells.

Results

Characterization of the TFG-ALK_{XL} Variant

The lymph node biopsy of case 893 showed a typical ALCL of common morphology expressing CD30 and epithelial membrane antigen. ALK protein was detected in all tumor cells and exhibited a cytoplasmic-restricted expression pattern. Immunohistochemical staining using monoclonal antibodies against the N-terminal portion of NPM detected only nuclear NPM protein. Western blot analysis using anti-ALK and anti-NPM (N-terminus) antibodies detected an abnormal ALK protein with an apparent molecular weight of 113 kd, and only the wild-type 38-kd NPM protein (Figure 1). These findings indicated that a partner other than NPM was fused to ALK in this tumor.³²

RT-PCR studies using primers for different ALK chimeric products (*NPM*, *TPM3*, *TFG*, *AT1C*, and *CLTC*) detected a TFG-ALK transcript. However, the amplified product showed an unexpectedly larger size (447 bp) than that obtained in our two previously described cases (141-bp segment in TFG-ALK_S and 306 bp in TFG-ALK_L) (Figure 2). Sequencing analysis of this product confirmed a TFG-ALK rearrangement in which the ALK fragment was the same as in other ALK translocations. However, the TFG fragment contained an additional 141-bp segment from the TFG gene not present in the TFG-ALK_L sequence. In this new rearrangement creating a larger TFG-ALK (TFG-ALK_{XL}) gene, the additional 141-bp TFG sequence was also in frame with the 5' TFG portion of our previous TFG-ALK long variant and with the distal 3' ALK sequence (Figure 2). The predicted molecular weight of this new chimeric TFG-ALK_{XL} protein was 108 kd, consistent with the value of 113 kd determined by Western blot.³¹

Identification of TFG-ALK Genomic Breakpoints

The TFG-ALK_{XL} genomic breakpoint could not be amplified despite the use of numerous different primers. Nevertheless, the human TFG genomic organization could be

Figure 2. A: PCR amplification of the cDNA breakpoint region using primers from the TFG and ALK genes. Cases with the TFG-ALK_S, _L, and _{XL} variants showed amplification products of different sizes (141, 306, and 447 bp, respectively). C- represents the negative PCR control without template. B: Nucleotide and amino acid sequence of the TFG-ALK_{XL} cDNA junction. Underlined sequence corresponds to the 141 bp of TFG exon 5 present only in the TFG-ALK_{XL} variant. The translocation breakpoint in the cDNA is marked with an arrow. C: Automated sequence profile of the breakpoint region in the TFG-ALK_{XL} variant.

obtained from alignment analysis of the TFG cDNA sequence with the corresponding genomic sequence of human chromosome 3 (GenBank accession NT.005863). This information revealed that the extra sequence of TFG present in the cDNA of the TFG-ALK_{XL} variant corresponded to a complete exon 5; thus, the genomic breakpoint in this case must be located within TFG intron 5 (Figure 3). Considering the size of the introns involved in this rearrangement (8116 bp for TFG and 1935 bp for ALK), the rearranged intron of the resulting TFG-ALK_{XL} fusion gene may be around 10 kb long, helping to explain our difficulty in amplifying this fragment.

Figure 3. Genomic organization of TFG-ALK_S, TFG-ALK_L, and TFG-ALK_{XL} variants. Intronic organization is detailed only for the TFG sequence. The striped box in the ALK sequence represents the kinase domain.

PCR analysis of the intronic breakpoint regions of the *TFG-ALK_S* and *TFG-ALK_L* rearrangements amplified fragments of 2117 bp and 3500 bp, respectively (Figure 3). In the *TFG-ALK_S* rearrangement, the sequencing analysis showed that the first 430 bp belonged to *TFG* intron 3 whereas the remaining sequence corresponded to the *ALK* intron involved in the *NPM-ALK* rearrangement. Comparison with the normal *TFG* and *ALK* intronic sequences indicated that the translocation breakpoint had no gain or loss of genetic material. The translocated sequences were identical to the normal intronic regions with the exception of seven single nucleotide changes in the *ALK* sequence that probably correspond to undescribed polymorphisms. Sequencing analysis of the *TFG-ALK_L* rearrangement showed that the first 2559 bp were derived from *TFG* intron 4 whereas the remaining sequence corresponded to the same *ALK* intron present in *NPM-ALK* (Figure 3). The comparison with the normal *TFG* and *ALK* intronic sequences showed that the translocation breakpoint had a gain of a single thymidine. The remaining *TFG* and *ALK* intronic sequences were similar to that previously described, and possessed a single nucleotide change in the *ALK* intron that was also present in the *TFG-ALK_S* case, corresponding to position 1629 in the normal *ALK* intron with a change from the normal guanine to adenine. The genomic breakpoints of the derivative chromosome 2 translocations could not be amplified in any of the three translocations.

Several possible mechanisms leading to the genomic breakpoints involving *TFG* and *ALK* were explored by analyzing for the presence of homologous regions and candidate recombination sequences. However, no significant homologous, repeated, or consensus sequences were found in the involved intronic regions (all of the homologies found scored below 50%).

Oncogenic Potential of TFG-ALK Fusion Transcripts

To assess the oncogenic activity of the *TFG-ALK* variants, we cloned the full-length *TFG-ALK_S* and *TFG-ALK_L* cDNAs into the pMex and pLTR2 expression vectors. These constructs were transfected into NIH-3T3 fibroblasts and the transforming activity was analyzed in focus formation assays. Cells transfected with *NPM-ALK*, *TFG-ALK_S*, and *TFG-ALK_L* produced similar numbers of foci/ μ g of cDNA, with slightly higher transforming efficiency observed for *TFG-ALK_S*. No foci were observed after transfection with empty vectors or carrier DNA (Table 1). Cells picked from the foci showed the typical morphology and lack of contact inhibition of transformed cells.

Protein lysates were obtained from each transfection and expression of the TFG-ALK variants was confirmed by Western blotting. No protein reactive with ALK antibody was observed in cells transfected with vector alone, whereas proteins of 113 kd, 85 kd, and 80 kd were observed in cells transfected with *TFG-ALK_L*, *TFG-ALK_S*, and *NPM-ALK*, respectively. To analyze the subcellular localization of the variant TFG-ALK proteins, we performed an immunocytochemical analysis of the NIH-3T3-

Table 1. Focus Formation Assay in NIH-3T3 Cells Transfected with *NPM-ALK*, *TFG-ALK_S*, and *TFG-ALK_L* Expression Vectors

Transfected cDNA	Dose (ng)	Foci (N/ μ g DNA $\times 10^3$) 2 weeks
<i>TFG-ALK_S</i>	200	115
<i>TFG-ALK_L</i>	200	65
<i>NPM-ALK</i>	200	80
<i>KRask12</i>	100	820
Empty vector	—	<0.001
Carrier DNA	40,000	<0.001

transfected cells. This study showed a cytoplasmic staining pattern, with reinforcement of the perinuclear region, in fibroblasts transfected with *TFG-ALK_S* and *TFG-ALK_L* but the nucleus was always negative. As expected, the vast majority of *NPM-ALK*-transformed fibroblasts showed a diffuse cytoplasmic pattern associated also with strong nuclear anti-ALK staining. These results are concordant with the previously analyzed patterns of ALK protein expression in the corresponding ALCL tumors (data not shown).

Association of ALK Fusion Proteins with Grb2, Shc, and PLC- γ

We next analyzed whether these TFG-ALK fusion proteins had similar activation mechanisms to those that have been previously described for the *NPM-ALK* and *ATIC-ALK* fusion proteins. Cell lysates from NIH-3T3 clones expressing *NPM-ALK*, *TFG-ALK_S*, or *TFG-ALK_L* were immunoprecipitated with anti-Shc, anti-Grb2, and anti-PLC- γ antibodies, then blotted with anti-ALK antibody. As seen in Figure 4, *TFG-ALK_S* was co-immunoprecipitated with Shc, Grb2, and PLC- γ , indicating an interaction between these proteins. Similar results were observed with *TFG-ALK_L*, although a slightly smaller amount of this protein was co-immunoprecipitated with Grb2. These results indicate that TFG-ALK fusion proteins are associ-

Figure 4. Interaction of TFG-ALK variants with PLC- γ , Grb2, and Shc. Cell lysates from NIH-3T3 clones expressing *NPM-ALK*, *TFG-ALK_S*, or *TFG-ALK_L* were immunoprecipitated with the indicated antibodies, then analyzed by Western blot using anti-ALK. IP, immunoprecipitated; SN, supernatant.

ated in cells with the same signaling intermediates used by NPM-ALK for signal transduction, suggesting that different ALK chimeric products likely use similar transforming pathways.

Discussion

The *TFG* gene was initially identified as the rearranged partner of the *NTRK1* gene in thyroid papillary carcinoma, generating the *TRK-T3* fusion gene that has transforming activity in different models.¹⁶ *TFG* is constitutively expressed in many different normal tissues including lymphoid cells but the physiological role of this protein has not yet been determined.⁹ Noticeably, in *TRK-T3* oncogene, the breakpoints involved an exonic region of *NTRK1* and *TFG* intron 4, the same as used in *TFG-ALK_L*. This chimeric gene generated two different fusion transcripts.¹⁶ One resulting from the fusion of the whole *TFG* exon 4 with the 3' *NTRK1* exon, and a second one incorporating an additional 9-bp intronic *TFG* sequence between *TFG* exon 4 and *NTRK1* exon. This short intronic *TFG* sequence was flanked at 3' by the breakpoint and a 5' cryptic splicing signal.

In the present work we described three different *TFG* intronic breakpoints involving in all of the cases a rearrangement with the *ALK* gene, thus indicating that the *TFG* gene structure may facilitate multiple rearrangements with different genomic sequences. This diversity in the number of breakpoints may be because in part of the fact that the three *TFG* intronic regions described in this work and an additional 3' intron of *TFG* may fuse with *ALK* generating in-frame transcripts that also maintain the oligomerization domain of the protein. On the contrary, *NPM*, *TPM3*, and *CLTC* genes have only one possible intron that would allow a rearrangement with *ALK* resulting in an in-frame sequence including the oligomerization domain of the partner. The other two *ALK* fused genes, *MSN* and *ATIC*, have two and three potential introns, respectively, that may fuse in-frame with *ALK*, but, so far only one type of chimeric gene has been described.

The sequence retained in all of the *TFG* translocations contained the predicted coiled-coil oligomerization domain of the protein, a region that, like the dimerization motif of NPM in the NPM-ALK fusion, is absolutely required for oligomerization and the transforming activity of the TFG-NTRK (TRK-T3) oncoprotein.³³ Therefore, it is also likely that TFG plays a similar role in the activation of ALK in ALCL. However, in contrast to NPM, TFG lacks nuclear localization-signaling domains, a finding that is in keeping not only with the cytoplasmic-only pattern of ALK expression observed in our immunohistochemical analysis of the three variants in ALCL specimens, but also with the immunocytochemical localization of the TFG-ALK_S and TFG-ALK_L fusion proteins in NIH-3T3-transformed cells.

Recurrent chromosome translocations are characteristic of a number of human hematological tumors. It has been proposed that homologous recombination and/or site-specific recombinogenic sequences may be implicated in the generation of these reciprocal translocations.

The lymphoid-specific recombinogenic machinery could be involved in some translocation events through heptamer/nonamer or translin consensus sequences. In addition, other types of promoting sequences have been suggested to play a role in several translocations by using different recombinogenic mechanisms. Among these, topoisomerase I and II, chi consensus, Alu, palindromic, purine/pyrimidine, polypurine, or polypyrimidine sequences have been suggested as potential sequences involved in translocations.²¹⁻²⁸ In the present work, we have analyzed the genomic organization and intronic sequences of *ALK* translocations involving *TFG*. Only poor homologies with previously described recombinogenic sequences were found in the genomic regions surrounding the translocation breakpoints in both the *TFG-ALK_S* and *TFG-ALK_L* chimeric genes. Alternatively, an illegitimate recombination process may be involved in these translocations. This mechanism has been proposed for translocations occurring in different tumors and involves an independent generation of single strand DNA ends that are processed individually before interchromosomal joining.³⁴ This process frequently results in local sequence duplications, deletions, or inversions in the rearranged flanking sequences.³⁴ In keeping with this, we have recently described the *MSN-ALK* translocation to be associated with a 66-bp deletion in the derivative chromosome.¹³ However, these features were not found in the short and long *TFG-ALK* rearrangements described here, although the inserted thymidine found in the *TFG-ALK_L* variant could be interpreted as a duplication of an upstream *ALK* thymidine close to the breakpoint. Unfortunately, the amplification of the derivative chromosome 2 translocation was unsuccessful in both cases; thus, the presence of duplications, deletions, or inversions in these resulting regions could not be assessed. Recently, it was described that the illegitimate recombination process might be promoted by the proximity of the involved sequences in the nucleus.³⁵ It would be interesting to know the possible contribution of this mechanism to the generation of *ALK* translocations with its different partners.

The transforming potential of the NPM-ALK fusion gene has been previously demonstrated in *in vitro* experiments using rodent fibroblasts and *in vivo* in a murine model using retroviral transduction of bone marrow.^{7,36,37} Downstream targets of the resulting constitutive ALK tyrosine kinase activation by the NPM-ALK fusion protein have been characterized in different studies and include proliferation-related elements such as Shc, Grb2, PLC- γ , IRS-1, and STAT5,^{36,38-40} as well as the PI3K p85 subunit and PKB/AKT that result in an inhibition of apoptosis.^{38,40,41} However, whether all these mediators play a role in the pathogenesis of ALCL is not clear because experiments using NPM-ALK mutants suggest that neither IRS-1 nor Shc are essential for transformation.^{7,36} The possible transforming potential and signaling pathways of other ALK fusion proteins are not well known. In the *ATIC-ALK* variant, transformation potential was demonstrated *in vitro* using NIH-3T3 fibroblasts and the murine pro-B cell line BaF3.^{11,12} Similar to NPM-ALK, the *ATIC-ALK* protein seems also to form stable complexes with Grb2 and Shc. In the present work, the transforming

activity of TFG-ALK_S and TFG-ALK_{XL} could be analyzed *in vitro*, showing a transformation potential similar to NPM-ALK and also the binding with Grb2, Shc, and PLC-γ.

In conclusion, we have characterized a third variant of the TFG-ALK rearrangements (TFG-ALK_{XL}) involving TFG intron 5 and the same ALK intron found in other ALK translocations. This finding and the identification of TFG genomic breakpoints in introns 3 and 4 in previous TFG-ALK variants confirm the peculiar versatility of TFG as an activating partner in oncogenic translocations. We also demonstrate that TFG-ALK chimeric proteins have *in vitro* transforming potential and bind to some of the molecules that are also associated with the NPM-ALK fusion protein.

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