Case Study

Variant Translocation Partners of the Anaplastic Lymphoma Kinase (ALK) Gene in Two Cases of Anaplastic Large Cell Lymphoma, Identified by Inverse cDNA Polymerase Chain Reaction

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In anaplastic large cell lymphoma (ALCL), the anaplastic lymphoma kinase (ALK) gene is rearranged with diverse partners due to variant translocations/inversions. Case 1 was a 39-year-old man who developed multiple tumors in the mediastinum, psoas muscle, lung, and lymph nodes. A biopsy specimen of the inguinal node was effaced by large tumor cells expressing CD30, epithelial membrane antigen, and cytoplasmic ALK, which led to a diagnosis of ALK+ ALCL. Case 2 was a 51-year-old man who was initially diagnosed with undifferentiated carcinoma. He developed multiple skin tumors eight years after his initial presentation, and was finally diagnosed with ALK+ ALCL. He died of therapy-related acute myeloid leukemia. G-banding and fluorescence in situ hybridization using an ALK break-apart probe revealed the rearrangement of ALK and suggested variant translocation in both cases. We applied an inverse cDNA polymerase chain reaction (PCR) strategy to identify the partner of ALK. Nucleotide sequencing of the PCR products and a database search revealed that the sequences of ATIC in case 1 and TRAF1 in case 2 appeared to follow those of ALK. We subsequently confirmed ATIC-ALK and TRAF1-ALK fusions by reverse transcriptase PCR and nucleotide sequencing. We successfully determined the partner gene of ALK in two cases of ALK+ ALCL. ATIC is the second most common partner of variant ALK rearrangements, while the TRAF1-ALK fusion gene was first reported in 2013, and this is the second reported case of ALK+ ALCL carrying TRAF1-ALK. [J Clin Exp Hematop 54(3) : 225-235, 2014]

Keywords: anaplastic large cell lymphoma, FISH, inverse cDNA PCR, ATIC gene, TRAF1 gene

INTRODUCTION

Anaplastic large cell lymphoma (ALCL) that is positive for the expression of ALK (ALK+) is a well-defined subtype of peripheral T-cell lymphoma.1,2 The disease is characterized by the anaplastic morphology of lymphoma cells, expression of CD30 on their cell surface, and chromosomal rearrangement involving the ALK gene locus at 2p23. The majority of cases carry t(2;5)(p23;q35), which leads to the generation of a fusion gene with the nucleophosmin (NPM) gene at 5q35, encoding the oncogenic kinase NPM-ALK chimeric protein. However, rearrangements involve diverse partner genes and/or chromosomal loci in the minority of cases.1,3 Over 20 genes and/or recurrent chromosomal rearrangements have been identified as partners of ALK.3 In contrast to the NPM-ALK fusion protein, which shows both the nuclear and the cytoplasmic expression of ALK, the subcellular localization of other fusion proteins varies depending upon each rearrangement.1,2

The 5' RACE (rapid amplification of cDNA ends) protocol has been used to clone an unknown partner that fuses to a known gene from an mRNA template.4 In a very recent case report on ALK- ALCL, deep RNA sequencing successfully identified the gene for tumor necrosis factor (TNF) receptor-associated factor 1 (TRAF1) as the partner of ALK.5 We herein present the clinical, histopathological, and cytogenetic features of two cases of ALK+ ALCL. To identify the partner genes of ALK, we applied an inverse cDNA polymerase chain reaction (PCR)-based technique, which allowed the amplification of unknown sequences enclosed by known ALK sequences.
CASE REPORT

Case 1

A 39-year-old man presented with progressive back pain and a right inguinal tumor. He had lost 20 kg during the previous month. Computed tomography scans of his body revealed multiple tumors; a soft tissue mass in the posterior mediastinum surrounding the esophagus, a mass in the psoas muscle infiltrating the lumbar spine, and a tumor in the right lung parenchyma; the inguinal tumor was 7 cm in diameter. His hemoglobin level was 13.5 g/dL, white blood cell count 17.0 × 10³/µL with a marked left shift, and platelet count 263 × 10³/µL. Blood chemistry values were: total protein, 5.8 g/dL; albumin, 2.6 g/dL; lactate dehydrogenase, 171 IU/L; aspartate aminotransferase, 41 IU/L; alanine aminotransferase, 31 IU/L; and alkaline phosphatase, 1,289 IU/L. His serum soluble interleukin-2 level was 24,280 U/mL and C-reactive protein 21.6 mg/dL.

A biopsy specimen of the inguinal node was effaced by cohesive sheets of pleomorphic large cells, and many mitotic figures were detected (Fig. 1). Immunohistochemistry revealed that the tumor cells expressed CD30, epithelial membrane antigen, and cytoplasmic ALK, leading to a diagnosis of ALK⁺ ALCL (Fig. 1). Additional immunohistochemistry included CD3⁺, CD4⁺, CD8⁺, and granzyme B⁺ (Fig. 1), and Epstein-Barr virus-encoded RNAs (EBERs) were negative. The patient was treated with cyclophosphamide, doxorubicin, and vincristine, leading to a transient response. However, he developed a severe pulmonary infection and septicemia due to Streptococcus oralis; a bronchoscopic examination showed a tracheoesophageal fistula. The patient died on the 41st hospital day.

Case 2

A 51-year-old man first presented to our institution with left axillary tumors associated with infiltration of the overlying skin and a biopsy sample suggested undifferentiated carcinoma; the patient was treated with regional radiotherapy and the intra-arterial infusion of mitomycin C and doxorubicin. He developed multiple cutaneous tumors of the right lower leg and left thigh eight years after his initial presentation. A biopsy of the skin tumor revealed the diffuse infiltration of...
large cells in the dermis that expressed CD30, epithelial membrane antigen, and cytoplasmic ALK, which finally established a diagnosis of ALK+ ALCL (Fig. 2). The lymphoma cells were CD3-, CD4+, CD8-, CD10-, and CD20-, and EBERs were negative (Fig. 2). A pathological review of the first biopsy specimen by ALK immunohistochemistry confirmed ALK+ ALCL of the first tumor. The patient was treated with lymphoma-directing chemotherapy, including CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone), DeVIC (dexamethasone, etoposide, ifosfamide, and carboplatin), and DHAP (dexamethasone, high-dose cytarabine, and cisplatin), as well as radiotherapy; however, the disease showed multiple relapses involving the skin, subcutaneous tissues, and lymph nodes. He finally developed therapy-related acute myeloid leukemia (t-AML) (Fig. 3) and died of disease progression at the age of 66.

MATERIALS AND METHODS

G-banding

Tumor specimens were aseptically minced to prepare a cell suspension in culture medium. The cells were incubated overnight at 37°C under a CO₂ concentration of 5%, and then cultured in the presence of 0.1 µg/mL colcemid for 2 hr. After harvesting, the cells were treated with hypotonic solution and fixed with methanol: acetic acid (3:1). Chromosomes were banded by trypsin-Giemsa staining and the results of chromosome analysis were described according to the ISCN.⁶

Fluorescence in situ hybridization (FISH)

Three FISH probes, namely, the Vysis ALK dual-color break-apart probe, Vysis LSI EGR1/D5S23, D5S721 dual-color probe, and Vysis D7S486/CEP7 FISH probe, were purchased from Abbott Laboratories, Abbott Park, IL, USA. Denaturing of the chromosome/probe, hybridization, and washing conditions were as recommended by the manufacturer. FISH results were analyzed using a fluorescence microscope (Nikon Corporation, Tokyo, Japan) equipped with DAPI, fluorescein isothiocyanate (FITC), and tetramethylrhodamine B isothiocyanate (TRITC) fluorescence filters, as well as a DAPI/FITC/TRITC triple band-pass filter (Nikon

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Fig. 2. Histopathology of the cutaneous tumor biopsy specimen of case 2. (2A) H&E stain, ×2 objective lens; (2B) H&E stain, ×20; (2C) anti-CD3, ×20; (2D) anti-CD4, ×20; (2E) anti-CD8, ×20; (2F) anti-CD30, ×20; (2G) anti-epithelial membrane antigen, ×20; (2H) anti-ALK, ×20; and (2I) in situ hybridization for EBERs (Epstein-Barr Virus [EBER] PNA Probe/Fluorescein and DakoCytomation PNA ISH Detection Kit, Dako), ×20.
Isolation of RNA

Total RNA was prepared from cryopreserved samples embedded in Tissue-Tek® O. C. T.™ Compound (Sakura Finetek Japan, Tokyo, Japan) using an RNeasy Mini Kit (QIAGEN, Hilden, Germany).

Inverse cDNA PCR

Double-strand cDNA was synthesized from 1 µg of total RNA using the ALKREVex22-23 primer, 5′-TGGTTGAATTCTGATGATC-3′, and a cDNA Synthesis Kit (M-MLV Version) (code 6130, Takara Bio, Otsu, Shiga, Japan). The double-strand cDNA was self-ligated by incubating at 12°C for 15 hrs with T4 DNA ligase (code 6022, Takara Bio) in a volume of 20 µL. The resulting circular cDNA was subjected to nested PCR using primer combinations designed for the known ALK sequences in a divergent orientation. First-round PCR (35 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 2 min) was performed with the primers ALKREV3T, 5′-CTGATGGAGGAGGTCTTGCC-3′, and ALKFWDex20-21, 5′-ATTCGGGGTCTGGGCCAT-3′, in a volume of 20 µL. One microliter of the 1:100 diluted reaction products from first-round PCR was then subjected to second-round PCR (35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min) with the primers ALKREV4T, 5′-GGTGCTAGTCGGTCATGATGGTC-3′, and ALKREV3T, 5′-CTGATGGAGGAGGTCTTGCC-3′, in a volume of 20 µL. The second-round PCR products were visualized by ethidium bromide-stained gel electrophoresis and then directly sequenced in both directions using the primers ALKFWDex21-22 and ALKREV4T and an ABI 310 automated sequencer (Applied Biosystems, Inc., Foster City, CA).

Reverse transcriptase (RT)-PCR

First-strand cDNA was synthesized from 2 µg of total RNA in the reaction mixture containing random hexamer primers (Roche Applied Science, Penzberg, Germany) and SuperScript® reverse transcriptase (Invitrogen, Carlsbad, CA). PCR primers and the size of the PCR products were as follows: ALK reverse, 5′-CGAGGTTGGAGCTTGCTCAGC-3′, and ATIC (5-aminomidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase) forward, 5′-GTGTCCACCGAGATGCAGAG-3′, for the ATIC-ALK fusion mRNA (315-bp); ALK reverse, 5′-AAAGCAGTAGTTGGGGTTGT-3′, and TRAF1 forward, 5′-TGTTGAAGATCAACATGC-3′, for the TRAF1-ALK fusion mRNA (196-bp); and ALK-III-R, 5′-GTGTTGAATGGTCTGCATATGTC-3′, and ALK-III-F, 5′-GGCCCATGCGCCTTTGAGGAGT-3′, for the tyrosine kinase domain of ALK (760 bp).

RESULTS

G-banding and FISH

Case 1: Cytogenetic specimens were prepared from the inguinal lymph node biopsy. G-banding showed a complex karyotype of tetraploidy or octaploidy. FISH using the Vysis ALK dual-color break-apart probe to the metaphase spreads revealed multiple copies of a submetacentric marker chromosome labeled solely by the red signal representing 3′ ALK, in
addition to normal chromosome 2 labeled by both the red and the green (representing 5′ ALK signals on 2p23 (Fig. 4A & 5, top). These results suggest that the rearrangement may have occurred within the ALK gene locus and the rearranged gene was amplified. However, karyotyping was not completed due to the complexity.

**Case 2**: A lymph node biopsy specimen obtained on relapse was subjected to cytogenetic and FISH studies. G-banding revealed multiple numerical and structural abnormalities, including a der(2) [add(2)(p1?3)] and der(5) [add(5)(q31)] chromosome (Fig. 4B). FISH using the ALK break-apart probe showed that the 5′ ALK green signal was localized on the long arm of der(5) and the 3′ ALK red signal was on the end of the long arm of chromosome 12; the der(2) chromosome had lost the ALK signal (Fig. 5, middle).

On examination of the bone marrow smear slide at the time of the development of t-AML, ALCL tumor cells comprised 3.7% of the nucleated cells (Fig. 3). G-banding revealed metaphase spreads of hypodiploid and tetraploid karyotypes. The former karyotype represented the t-AML clone, carrying del(5q) and del(7q), both of which were confirmed by FISH using each probe (Supplementary Fig. S1 & S2); the del(7q)-carrying cells comprised 88.0% of the nucleated cells. On the other hand, the tetraploid karyotype included marker chromosomes identical to those identified in the karyotype of the lymph node biopsy specimen, which was consistent with bone marrow infiltration by ALCL. ALK-FISH applied to the tetraploid metaphases confirmed two copies of der(5) labeled by the 5′ ALK green signal and two copies of chromosome 12 labeled by the 3′ ALK red signal (Fig. 5, bottom). Additional FISH using the Vysis LSI EGR1/D5S23, D5S721 dual-color probe, which was designed to detect del(5q), showed that the short arm of der(2) contained the EGR1 locus normally localized on 5q31, indicating that chromosomal materials including ALK on 2p23 and EGR1 on 5q31 had been reciprocally exchanged (Supplementary Fig. S3), presumably unrelated to the ALK rearrangement.

**Identification of translocation partners of ALK**

The cytoplasm-only ALK positivity on immunohistochemistry and absence of t(2;5)(p23;q35) on metaphase FISH analysis suggested that ALCL of the present two cases carried variant ALK translocations.1,2,10 We employed an inverse
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Fig. 5. Fluorescence in situ hybridization (FISH) using the Vysis ALK dual-color break-apart probe. The probe consisted of a red signal representing 3′ ALK and a green signal representing 5′ ALK. Top, a metaphase spread of octaploidy obtained from the lymph node biopsy sample of case 1. Pictures with DAPI, FITC, and TRITC fluorescence filters are arranged from left to right. Submetacentric marker chromosomes labeled solely by a red signal are indicated by closed arrows and normal chromosome 2 labeled by both red and green signals is indicated by open arrows. G-banded pictures of these chromosomes are shown in Fig. 4A. Middle, G-banding, FITC, and TRITC pictures of a metaphase spread obtained from the lymph node biopsy specimen of case 2. Red signals localized on chromosomes 2 and 12, and green signals on chromosomes 2 and der(5) [add(5)(q31)] are shown with arrows of each respective color. The der(2) [add(2)(p13)] chromosome lacked the ALK signal (white arrows). Additional FISH showed that the c-MYC, which normally localized on 8q24, had been translocated to the end of the long arm of der(5) (data not shown). Bottom, G-banding and triple band-pass filter pictures of a tetraploid metaphase spread obtained from the bone marrow aspirates of case 2, showing red signals on the two copies of chromosome 12 and green signals on the two copies of der(5). Two copies of chromosome 2 were labeled by the ALK fusion signal (yellow), while two copies of der(2) lacked ALK.
cDNA PCR strategy to identify the partner of ALK. As shown in Fig. 6A, single species of PCR products of approximately 0.6 and 0.5 kb in size were successfully amplified. Nucleotide sequencing of the PCR products and a database search revealed that the sequences of ATIC in case 1 and TRAF1 in case 2 appeared to follow that of ALK (Supplementary Fig. S4 & S5). We subsequently confirmed ATIC-ALK and TRAF1-ALK fusion mRNA by conventional RT-PCR using a primer combination designed for each gene (Figure 6B). Nucleotide sequencing of the RT-PCR products determined that exon 7 of ATIC and exon 6 of TRAF1 had fused in frame to exon 20 of ALK, encoding the ATIC-ALK and TRAF1-ALK chimeric protein (Fig. 6C).

DISCUSSION

We described the clinical, histopathological, and cytogenetic findings of two cases of ALK+ ALCL, showing “cytoplasm-only” ALK immunopositivity. FISH using an ALK break-apart probe demonstrated the rearrangement of ALK, while the rearrangement did not represent the most common translocation, t(2;5)(p23;q35), but suggested a variant translocation in each case. We finally determined that the genes encoding ATIC and TRAF1 were the partners of ALK using an inverse cDNA PCR strategy.

The ATIC-ALK fusion mRNA was first identified by means of the 5′ RACE protocol, in which an anchor sequence is added to the 5′ end of the cDNA, followed by a series of
hemi-nested anchored PCRs. In principle, as only one gene-specific primer is used, 5' RACE often generates a high level of nonspecific amplification. On the other hand, we previously performed inverse PCR from genomic DNA templates to identify partner genes of BCL6 in B-cell tumors, leading to the isolation of a variety of non-immunoglobulin gene partners of BCL6. In the current study, we applied the inverse PCR strategy for cDNA synthesized from mRNA that theoretically contained the sequence of an unknown partner. In contrast to 5' RACE, as the nested PCR primer pairs were specifically designed for the known sequence of ALK, we successfully obtained the amplification products that were readily identifiable on ethidium bromide-stained gel electrophoresis (Fig. 6A).

ATIC is the second most common partner gene of variant ALK rearrangements. The ATIC-ALK fusion was previously shown to be generated by the pericentric inversion of chromosome 2 at the breakpoints of p23 and q35 [inv(2)(p23q35)], in which the ALK and ATIC genes are localized, respectively. However, in case 1, inv(2)(p23q35) was not recognized by either G-banding or metaphase FISH analysis. Since the inv(2)(p23q35) chromosome was reported to be affected by a secondary structural abnormality by us and others, chromosomal segments containing the reciprocal ALK-ATIC fusion, which was labeled by the 5' ALK-green signal, were most likely deleted. This generated the submetacentric marker chromosome, which theoretically contained the ATIC-ALK fusion gene alone; the marker chromosome may be designated as der(2)inv(2)(p23q35)del(2)(p?21) by ISCN (Fig. 4A). An increase in the copy number of the ATIC-ALK fusion gene may have enhanced the expression of chimeric proteins, potentially contributing to the aggressive clinical behavior of this particular case.

The TRAF1-ALK fusion gene was first reported by Feldman et al. in 2013, and case 2 is the second reported case of ALK-ALCL carrying this particular fusion gene. The chromosomal locus of the TRAF1 gene has been mapped at 9q33-34. Thus, owing to similarities to the NPM-ALK generated by t(2;5)(p23;q35), the TRAF1-ALK fusion gene is predicted to be generated by t(2;9)(p23;q33-34) chromosomal translocation. However, no chromosomal abnormality was visible in the three copies of chromosome 9, and chromosome 12, in which 3' ALK was located by FISH and the TRAF1-ALK fusion gene was theoretically localized, was cytogenetically normal (Fig. 4B). As the locus of TRAF1 is close to the telomeric end of the long arm of chromosome 9, the chromosomal materials involved in translocation may have been too small to be identified under a microscope. On the other hand, it currently remains unknown whether 5' ALK on der(5) represented reciprocal ALK-TRAF1 fusion. Unfortunately, we were unable to verify the fusion gene at the cytogenetic level because chromosomal specimens were no longer available.

TRAF proteins mediate the transduction of signals from various receptors of the TNF receptor superfamily. TRAF1 in association with TRAF2 forms a heterodimeric complex, which is required for the TNFα-mediated activation of MAPK8/JNK and NF-κB. The theoretical TRAF1-ALK chimeric protein, in the N-terminus toward a C-terminus orientation, is composed of the coiled-coil domain of TRAF1, truncating the MATH/TRAFl domain that binds to the receptor cytoplasmic domain, and the intracellular tyrosine kinase domain of ALK (Supplementary Fig. S5). As the TRAF1 coiled-coil domain mediates homo- and hetero-oligomerization, the TRAF1-ALK chimeric protein appears to undergo homodimerization in the cytoplasm, similar to other non-nuclear ALK fusion chimeras, thereby enhancing ALK kinase activity.

ALK+ ALCL most frequently occurs in the first three decades of life. In reflection of this younger age distribution, the rate of a complete response following first-line chemotherapy was reported to be 86%, and the progression-free and overall survival rates at 8 years were as high as 72% and 82%, respectively. It remains to be clarified whether the clinical features and treatment outcomes of ALK+ ALCL with variant ALK rearrangements are similar to those of the majority of cases with t(2;5)(p23;q35)/NPM-ALK. In the Japanese literature, Matsubara et al. reported a pediatric case having the ATIC-ALK fusion gene, in which the disease corresponded to the standard risk category and responded well to ALCL 99 chemotherapy. In contrast, we previously reported an elderly patient with inv(2)(p23q35)/ATIC-ALK-carrying ALK+ ALCL who had disseminated disease at presentation and showed a rapidly deteriorating clinical course, similar to case 1 described herein. However, in case 2, the patient with TRAF1-ALK developed the disease in the fifth decade of life, and showed multiple relapses 8 years after the initial treatment in spite of intensive chemoradiotherapies. These features are common with those of Feldman's case, in which the patient developed ALK+ ALCL at the age of 41, relapsed after a long-term complete response induced by initial CHOP chemotherapy, and finally underwent salvage chemotherapies including high-dose therapy with autologous stem-cell transplantation. Thus, it is possible that the TRAF1-ALK fusion gene may define a unique subset within ALK+ ALCL, which preferentially develops in older individuals and shows multiple relapses. Further studies are required to determine whether individual variant translocations/inversions in ALK+ ALCL are related to particular clinical features and treatment outcomes.

REFERENCES

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Supplementary Figures

**Fig. S1.** G-banded karyotype of the t-AML clone of case 2. Numerical and structural abnormalities are indicated by arrows. The karyotype was: 44,XY,del(5)(q13q31),der(7;17)(p10;q10),-18[1]/43,idem,-20[7].

**Fig. S2.** Fluorescence in situ hybridization confirming del(5q) (top) and del(7q) (bottom) in hypodiploid metaphases representing the t-AML clone of case 2. Arrows indicate the del(5)(q13q31) and der(7;17)(p10;q10) chromosomes. The probes used were the Vysis LSI EGR1/D5S23, D5S721 dual color probe for del(5q) and Vysis D7S486/CEP7 FISH probe for del(7q).

**Fig. S3.** A G-banded metaphase spread of the tetraploid karyotype of case 2 (left) and FISH using the Vysis LSI EGR1/D5S23, D5S721 dual color probe (right). The EGR1 locus on 5q31 represented by the red signal was translocated to the two der(2) [add(2)(p1?3)] chromosomes (red arrows). Two copies of normal chromosome 5 and the der(5) [add(5)(q31)] chromosome are indicated by yellow and green arrows, respectively.
Variant translocation partners of ALK in ALCL

**Fig. S4.** Nucleotide sequences of the inverse cDNA product, representing the sequences of ALK and ATIC (case 1) and ALK and TRAF1 (case 2). The sequencing primer used was ALKREV4T.

**Fig. S5.** Schematic diagram of the TRAF1-ALK chimeric protein.

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MAM, MAM domain
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PK, protein kinase domain
CC, coiled-coil domain
MATH, MATH (TRAF) domain