Novel SH3 protein encoded by the *AF3p21* gene is fused to the mixed lineage leukemia protein in a therapy-related leukemia with t(3;11) (p21;q23)

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The *mixed lineage leukemia* (*MLL*) gene located at chromosome band 11q23 is frequently rearranged in patients with therapy-related acute monocytic leukemia who received topoisomerase II inhibitors. We have identified a novel fusion partner of *MLL* (FAB M5b) in a patient who developed t-AML 9 years after treatment for acute lymphoblastic leukemia (ALL). The leukemic cells had a sole karyotypic abnormality of t(3;11) (p21;q23). Screening of a genomic DNA library, prepared from leukemic cell DNA, identified rearranged clones composed of *MLL* and a novel gene on chromosome 3p21 (*AF3p21*). The *AF3p21* gene encodes a protein of 722 amino acids, which contains an Src homology 3 (SH3) domain, a proline-rich domain, and a bipartite nuclear localizing signal (NLS). RNA analysis demonstrated that exon 6 of the *MLL* gene fused to exon 2 of the *AF3p21* gene. The resulting chimeric protein consists of AT-hooks, methyltransferase, and transcription repressor domains of MLL in addition to the AF3p21 proline-rich domain and NLS but not the AF3p21 SH3 domain. (Blood. 2000;95:1066-1068)

Introduction

Therapy-related acute myelogenous leukemia (t-AML) is a complication of effective cancer chemotherapy. The mixed lineage leukemia (*MLL*) gene (also called the *acute lymphoblastic leukemia-1* [*ALL-1*] gene or the *human trithorax* [*HRX*] gene) is localized on chromosome 11q23[1-3] and is involved in 11q23 abnormalities of t-AML.[4] The MLL protein contains 2 potential DNA-binding motifs (AT-hooks and zinc fingers), a transcriptional activation domain in the α-carboxyl (C) terminus, and a repression domain in the N-terminal portion. MLL possesses a highly conserved SET (Su[var]3-9, enhancer of zeste, and trithorax) domain that is found in *Drosophila trithorax* and *Polycomb* genes, which are known to regulate the expression of the homeotic genes in *Drosophila*. Defects in yolk sac hematopoiesis in *MLL*-null mouse embryos and the differentiation block in *MLL*-null embryonic stem (ES) cells support the notion of *MLL* playing a major role in hematopoietic differentiation.[5,6]

To date, 19 partner genes of *MLL* have been cloned from leukemia cells with various types of reciprocal translocations, and they form in-frame fusion products of the N-terminal portion of MLL with partner proteins.[4] A mouse knock-in study showed that *MLL-AF9* chimeras develop AML, whereas *MLL-myc* chimeras do not.[7] Moreover, retrovirus-mediated gene transfer of *MLL-ENL* into murine hematopoietic progenitors led to their immortalization, and this activity required the presence of the ENL moiety of the fusion protein.[8] These results suggest that the partner genes of *MLL* are critical in leukemogenesis. The detection of t(3;11)(p21;q23) in a patient with t-AML prompted us to identify the resulting molecular rearrangement.

Patient and methods

Patient

A 14-year-old female was diagnosed as having T-cell ALL in 1988. The karyotype of the leukemic blasts was 46/XX. She was treated according to a protocol for high-risk ALL of Osaka Children's Leukemia Study Group, followed by an autologous peripheral blood stem cell transplantation. The cumulated doses of antileukemia agents used were: 300 mg/m^2 daunorubicin, 2600 mg/m^2 etoposide, 7500 mg/m^2 cyclophosphamide, and 460 mg/m^2 ranimustine. The patient had been well until she developed AML (FAB M5b) in November 1997. The karyotype of the t-AML blasts was t(3;11) (p21;q23). Complete remission was achieved with a Koseisho protocol for acute nonlymphoblastic leukemia (ANLL-91), and in September 1998, she underwent bone marrow transplantation with cells donated from a human lymphocyte antigen–identical unrelated donor. Since that time, she has been in remission with chronic graft-versus-host disease.

Molecular cloning of the chimeric DNA

A genomic library from the patient's leukemic cell DNA was constructed by the complete digestion of genomic DNA with BamH I and ligation into a phage vector (λZAP Express; Stratagene, La Jolla, CA). Rearranged BamH-I fragments were isolated by screening with the *MLL* complementary DNA (cDNA) probe.

Total RNA extracted from the leukemic cells was subjected to reverse transcriptase–polymerase chain reaction (RT-PCR) using *MLL* exon 5 (forward) and *AF3p21* (reverse) primers for amplification of *MLL-AF3p21* fusion transcripts. The sequences of the primers were: forward, 5′-AAGCAGCCTCCACCACCAGA, and reverse, 5′-GCTGGAGCCTG-

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Submitted August 30, 1999; accepted September 30, 1999.

Supported by grants-in-aid for Scientific Research and for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, and Culture, Japan, and by a grant for Cancer Research from the Hyogo Health Foundation, Kobe, Japan.

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GTTCAGATG. The PCR products were subcloned into a plasmid vector (pGEM-T Easy vector; Promega, Madison, WI) and sequenced.

Results

Cloning and identification of a novel *MLL* fusion product

To analyze the involvement of the *MLL* gene in this translocation, we performed Southern blot analysis of DNA prepared from the leukemic cells of the patient. Hybridization of BamH-I digested DNA with the *MLL* cDNA probe revealed the presence of approximately a 4-kilobase (kb) rearranged band in addition to normal-size fragments (data not shown). To clone the rearranged DNA, we constructed a genomic DNA library prepared from BamH-I digested leukemic cell DNA. Screening of the library identified 2 clones composed of a rearranged *MLL* segment among 13 positive clones. Sequence comparison between these clones and the 8.3-kb *MLL* breakpoint cluster region (bcr) revealed that *MLL* sequences downstream of exon 6 were substituted with the non-*MLL* DNA segment.

Complete AF3p21 cDNA sequence

A BLAST database search for the novel sequence identified a highly homologous expressed sequence tag (EST) clone (GenBank accession no. AA227 226). Since only a portion of the 5′ terminus sequence of AA227 226 was deposited in the database, we determined the entire sequence of the EST clone. This clone contained a 3003–base pair (bp) insert, and a reading frame analysis suggested that this clone possibly contains an extraneous sequence in its 5′ region. Therefore, we performed 5′ RACE (rapid amplification of cDNA ends) to isolate the major spliced products and to determine the transcription start site of the novel gene using a cDNA of K562 leukemia cell line as a template. This analysis revealed that AA227 226 indeed contains a 29-bp intronic sequence at the exon-intron junction of exon 2 and lacks the 49-bp sequence at its 5′ terminus. The corrected sequence has 3023 bp with an open reading frame of 2166 bp encoding a polypeptide of 722 amino acids with a predicted molecular mass of 80 kd. In keeping with the nomenclature for *MLL* fusion partner genes, we designated this gene *AF3p21* (ALL-1 fused gene from chromosome 3p21). The complete sequence of AF3p21 has been deposited with the National Center for Biotechnology Information (NCBI) Data Library (accession no. AF 178432).

Sequence comparisons with the public database revealed that the N-terminus of the predicted AF3p21 protein (amino acid [aa] 1-58) contains an SH3 domain. The SH3 domain of AF3p21 is mostly similar to that of Fyn proto-oncogene tyrosine kinase. A database search for other functional domains revealed the presence of a bipartite NLS (aa 171-192) and a proline-rich domain (aa 170-249) downstream of the SH3 domain.

Genomic sequences at the breakpoint

To determine the nucleotide sequence around the breakpoint of the chromosome 3p21 region, bacterial artificial chromosome (BAC) DNA containing the *AF3p21* gene (clone 425p07; Genome Systems, St Louis, MO) was directly sequenced. A sequence comparison of the genomic segments of *MLL*, *AF3p21*, and *MLL-AF3p21* at the breakpoint is shown in Figure 1A. There is an identical stretch of 3 nucleotides (CCA) in the *MLL* and *AF3p21* genes at the rearranged site. Therefore, the breakpoint of the *MLL* gene is located between nucleotide positions 1587 and 1589 (numbering according to the 11q23-breakpoint sequence in GenBank accession no. HSU04 737). This is within an *Alu-Sb* repetitive sequence. The breakpoint on chromosome 3 is located at 61 bp upstream of the putative transcription start site of the *AF3p21* gene.

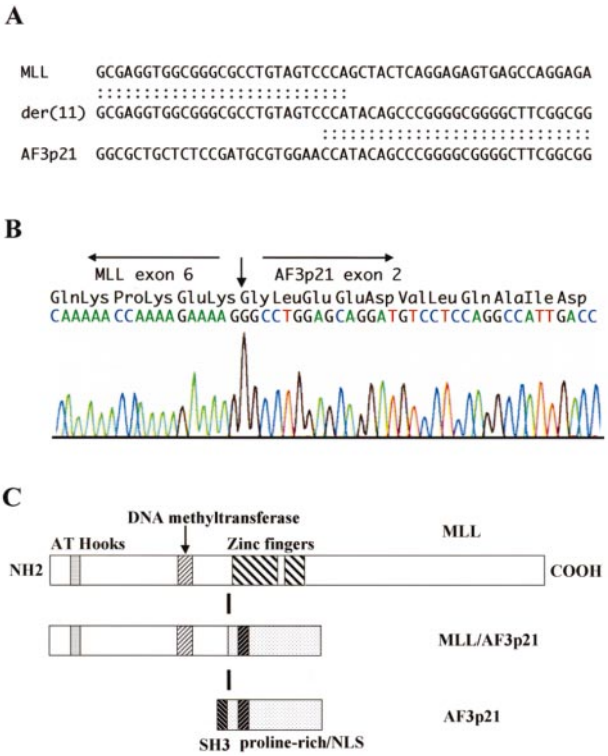

Figure 1. MLL is fused in-frame to the AF3p21 in the t(3,11) (A) Genomic sequences of the *MLL*, *MLL-AF3p21* (derived from the derivative chromosome 11[der(11)]), and *AF3p21* genes at the breakpoint region. (B) Sequence of the der[11] *MLL-AF3p21* fusion junction amplified by RT-PCR. (C) Schematic diagram of the consequences of the MLL, MLL-AF3p21, and AF3p21 proteins.

RT-PCR analysis of the patient material

To determine whether the translocation results in an in-frame fusion of *MLL* to *AF3p21*, RT-PCR was performed to amplify the cDNA junction of leukemic cells. Sequence analysis of the chimeric transcripts revealed an in-frame fusion between *MLL* exon 6 and *AF3p21* exon 2 (Figure 1B). The resulting MLL-AF3p21 fusion protein consists of AT-hooks, methyltransferase, and transcription repressor domains of MLL in addition to the AF3p21 proline-rich domain and bipartite NLS but not the AF3p21 SH3 domain (Figure 1C).

Discussion

Topoisomerase II inhibitor–associated t-AML is characterized by chromosomal translocations, most of which disrupt introns within an 8.3-kb bcr between exons 5 and 11 of the *MLL* gene. Several translocation breakpoints in *MLL* and in the partner DNAs are in intronic *Alu* repeats, suggesting that homologous repetitive sequences may be of importance in joining *MLL* with its translocation partners. The breakpoint in the *MLL* gene of our case occurs in an *Alu-Sb* sequence in intron 6. Although the breakpoint region of the *AF3p21* gene does not contain an *Alu* repetitive sequence, both *MLL* and *AF3p21* contained a homologous CCA sequence at the breakpoint (Figure 1A). These results suggest that the base pairing

of homologous DNA ends of *MLL* and *AF3p21* may be a first step in the translocation event in this case.

MLL fusion partner genes encode protein products of different types, and most of them are putative transcription factors.[4] There are 2 genes, including *EEN* on band 19p13[9] and *ABI-1* on 10p11.2,[10] that encode SH3 domain-containing protein. EEN is a human homologue of a member of a recently described murine SH3 domain-containing protein family.[11] The SH3 domain in EEN is most similar to that found in the GRB2 family proteins, suggesting that it acts as an adapter protein in signal transduction. ABI-1 is a human homologue of mouse Abi-1 (Abl-interactor-1) that suppresses *v-Abl* transforming activity by binding to the Abl protein.[12] The SH3 domain of Abi-1 is responsible for its binding to c-Abl. Both EEN and ABI-1 have an SH3 domain in their C-terminus, and fusion proteins with MLL retain their SH3 domains. However, the SH3 domain in AF3p21 is located at the N-terminus, and the resulting MLL-AF3p21 fusion protein does not retain the SH3 domain (Figure 1C).

Assuming that the MLL partner protein is required for leukemogenesis, the region downstream of the SH3 domain of AF3p21 may have functionally important domain(s). AF3p21 has a bipartite NLS downstream of the SH3 domain and a proline-rich domain. Since the N-terminus of MLL protein retains an NLS,[13] the bipartite NLS of AF3p21 may not be required for nuclear localization of the MLL-AF3p21 fusion protein. Proline-rich domain is found in a number of SH3-binding proteins, and this region is responsible for physical interaction between SH3 proteins and their ligands.[14] AF3p21 contains 1 class I SH3-binding motif (RXXPXXP) at aa 176-182 and 2 class II motifs (XPPXPXR) at aa 170-176 and aa 242-249. These domain structures suggest that AF3p21 might interact with other proteins in the nucleus. However, the precise intracellular localization and identification of interacting proteins (if any) of AF3p21 await further study.

Chromosomal abnormalities involving 3p21 without involvement of the 11q23 region have been reported in t-AML and t-MDS.[15] Moreover, the short arm of chromosome 3 is thought to contain multiple tumor suppressor genes because 1 copy of this chromosomal arm frequently is missing in carcinomas that have arisen in a variety of tissues, and region 3p21.3 is frequently deleted in lung cancers.[16] It will be interesting to examine whether rearrangements of the *AF3p21* gene occur in those malignancies.

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