RAPID COMMUNICATION

Adenoviral E1A-Associated Protein p300 Is Involved in Acute Myeloid Leukemia With t(11;22)(q23;q13)

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p300, which was originally cloned as a nuclear binding target of the adenovirus E1A oncoprotein, forms a family with cyclic-AMP response element binding protein (CREB)-binding protein (CBP). p300/CBP are considered to be transcriptional coactivators that connect the basal transcriptional machinery to various DNA-binding transcriptional factors. p300/CBP are implicated in both cell differentiation and regulation of cell-cycle. We identify here that the p300 gene is fused to the MLL gene and that in-frame MLL-p300 fusion protein is generated in acute myeloid leukemia (AML) with t(11;22)(q23;q13). These findings suggest that the basis for the leukemogenesis of t(11;22)-AML is the inability of p300 to regulate cell-cycle and cell differentiation after fusion with MLL.

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MATERIALS AND METHODS

Patient. A 4-year-old boy was initially diagnosed as having non-Hodgkin lymphoma. A complete remission was achieved by the conventional chemotherapy including etoposide (total dose of etoposide, 5,200 mg/m²). Sixty-seven months after diagnosis, he developed secondary AML, which was cytogenetically characterized as t(11;22)(q23;q13). He obtained a complete remission, but relapsed 20 months later. The leukemic cells in this study were obtained from peripheral blood at relapse.

Cytogenetic studies. The chromosomes of bone marrow samples were analyzed using the regular trypsin-Giemsa- or Q-banding method, as described previously. Isolation of genomic p300 clones. The human genomic library was screened using human p300 cDNA as a probe and 11 clones were isolated. Two of the clones (hp300-2 and hp300-9) were hybridized to the 1.4-kb BamHI fragment of 5′-region of p300 cDNA and were used for fluorescence in situ hybridization (FISH) analysis. FISH analysis. Chromosomal mapping of the genomic clones (hp300-2 and hp300-9) was performed using the FISH method. The phage clones were labeled by the standard nick translation method using biotin-16-DUTP (Boehringer Mannheim, Mannheim, Germany). To confirm the origin of cloned DNAs, we also mapped these genomic clones to leukemic cells together with whole chromosome painting probe for chromosome 22 (WCP22; Coatasome 22, digoxigenin-labeled; Oncor, Gaithersburg, MD).

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Southern and Northern blot analyses. Southern and Northern blot analyses were performed as described previously with some modifications. Briefly, high molecular weight DNA was extracted from frozen mononuclear cells of the patient carrying t(11;22)-leukemic cells and normal peripheral blood as a control by proteinase K digestion and phenol/chloroform extraction. Ten micrograms of DNA was digested with BamHI or HindIII, subjected to electrophoresis on 0.8% agarose gels, and transferred onto charged nylon filters (Pall BioSupport, Tokyo, Japan) in 20× SSC. The filters were hybridized with \(^{32}\)P-labeled random-primer probes under the described conditions. Total RNA from frozen cells was extracted using the acid guanidine isothiocyanate-phenol-chloroform method. Ten micrograms of total RNA was electrophoresed and transferred onto nylon filters. The filters were hybridized with \(^{32}\)P-labeled random-primer probes. A 1.5-kb BamHI fragment of p300 cDNA and a 0.9-kb BamHI fragment (designated probe x) that spans exons 5 through 11 of MLL cDNA were used.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Four micrograms of total RNA was reverse transcribed to cDNA in a total volume of 20 μL with random hexamers and 20 U of reverse transcriptase (AMV; Boehringer Mannheim). One microliter of cDNA was amplified by PCR in a total volume of 100 μL with 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 10 mmol/L Tris-HCl (pH 9.0 at room temperature), 25 μmol/L of each primer, 75 μmol/L of each dNTP, and 2.5 U of Taq polymerase (Boehringer Mannheim). Samples were overlaid with 100 μL of mineral oil (Sigma, St Louis, MO). After 30 rounds of PCR (30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C), 8 μL of PCR product was electrophoresed in a 1% agarose gel. Primers used were as follows: MLL-7S, 5′-TCCTCAGACTCTCTCCAAT-3′; MLL-9S, 5′-GGTTGTTGT-GTGTCGCCAA-3′; MLL-11A, 5′-TTTGCGGGATGCG-ATC-3′; p300-2S, 5′-CATCAGATTACCCCGGCAAA-3′; p300-7.5A, 5′-GCATGAATTCTGGCTGGTC-3′; p300-8A, 5′-GGCTCCT-GATACTGTCAGT-3′; and p300-11A, 5′-ACCTGTCCTCTCA-ATGCTTAC-3′.

Sequencing of PCR products. PCR products were cloned into the TA cloning vector (Invitrogen, Carlsbad, CA). Nucleotide sequences were determined by the fluorometric method (Dye Terminator Cycle Sequencing Kit; Applied Biosystems, Urayasu, Japan).

Western blot analysis. Protein extraction and Western blotting were performed as described. Proteins were resolved on sodium dodecyl sulfate-5% polyacrylamide gels and transferred to filters (Hybond-ECL; Amersham, Buckinghamshire, UK) by electrophoresing as described elsewhere. Filters were blocked in 5% nonfat dried milk, dissolved in phosphate-buffered saline plus 0.1% Tween-20 (PBS-T). After extensive washing in PBS-T, the filters were incubated for 1 hour at room temperature with p300-specific monoclonal antibody (RW105; UBI, Lake Placid, NY). After further washes in PBS-T, the filters were incubated with horseradish peroxidase-conjugated antibodies raised in goat against rabbit IgG (Amersham) and then washed with PBS-T. The immune complexes were visualized with the ECL detection system (Amersham).

RESULTS

Rearrangement of the p300 gene in t(11;22)-AML. FISH analysis using human genomic DNA clones spanning the p300 gene showed that two of the clones (Ahp300-2 and

![Fig 1. FISH analysis of the leukemic metaphase chromosomes. Chromosome 22 and a phage clone Ahp300-2 were detected with TRITC and FITC, respectively. Split signals of Ahp300-2 are observed on the boundary between WCP22-painted and unpainted regions of der(11)t(11;22) and der(22)t(11;22) (arrows). The intact signal of Ahp300-2 was observed on the normal chromosome 22 (arrowhead).](attachment:fig1.png)
analysis showed a 9.5-kb germline band of p300 and one abnormally sized band (11.5 kb; Fig 2C). On the other hand, the transcript of 11.5 kb in size was also detected by the MLL probe in leukemic cells of the patient, but the normal MLL transcript was not detected, possibly because the quality of mRNAs was poor (Fig 2D).

Rearrangement of the MLL gene in t(11; 22)-AML. The breakpoint in the 11q23 region was identified within the breakpoint cluster region of the MLL gene by Southern blotting using an MLL cDNA probe (probe x; Fig 2B). We detected additional bands, the same size as the bands detected with the BamHI-fragment of p300 cDNA, suggesting that the p300 gene is fused to the MLL gene.

Isolation of the MLL-p300 fusion transcript. We performed RT-PCR and sequence analysis to confirm the fusion transcript. First, we set an antisense primer on the p300 gene in reference to the breakpoint on the CBP gene in t(8;16)(p11; p13) and t(11;16)(q23; p13). However, we could not detect the PCR product using sense primer sets for various positions in the breakpoint cluster region of the MLL gene. Using a sense primer on MLL exon 7 (MLL-7S) and an antisense primer (p300-8A) on the 5*-region of bromodomain of p300, we obtained PCR products of 868 bp (Fig 3). Furthermore, we performed RT-PCR assay by using inner sets of primers (sense primer, MLL-9S; antisense primer, p300-7.5A) and detected an amplified fragment of an expected size (Fig 3). Sequence analysis of the amplified fragments showed that exon 9 on the MLL gene was juxtaposed to the p300 cDNA sequence with an in-frame junction (Fig 4). The breakpoint on the MLL gene was expected to

\[ \text{hp300-9) containing the 5' end of the coding region of the} \]
\[ \text{p300 gene hybridized to both der(11) and der(22) as well as} \]
\[ \text{to normal chromosome 22 in metaphase chromosomes of} \]
\[ \text{the leukemic cells (Fig 1). These results suggest that the} \]
\[ \text{p300 gene is disrupted in t(11;22) and that the breakpoint} \]
\[ \text{is located in the 5' region of the p300 gene. To determine} \]
\[ \text{the breakpoint in the p300 gene, we performed Southern blot} \]
\[ \text{analysis using several fragments of the p300 cDNA as} \]
\[ \text{probes. Using a 1.4-kb BamHI-fragment spanning nucleo-} \]
\[ \text{tides 2987 to 4405 in the 5'-region of p300 bromodomain,} \]
\[ \text{we detected aberrant bands in the 5'-region of the} \]
\[ \text{p300 gene (Fig 2A). Northern} \]
Fig 4. Schematic representation of the MLL, p300, and putative MLL-p300 chimeric proteins. The functional domains of MLL and p300 are shown beneath the figure. The nt and single-letter amino acid sequences surrounding the MLL-p300 breakpoint are shown at the bottom of the figure. NLS, nuclear localization signal; C/H, cysteine/histidine-rich region; Q rich, glutamine-rich region; ZF, Zinc finger domain; CREB, CREB-binding site; E1A, E1A binding site. Arrows indicate the breakpoint of each gene and the fusion point of chimeric protein. MLL-p300 fusion junction is in-frame, and putative chimeric protein loses part of the C/H-rich region and the CREB-binding site located in p300.

be located on intron 9. The reciprocal PCR product of p300-MLL fusion transcript was detected by RT-PCR assay using a sense primer on p300 (p300-2S) and an antisense primer on MLL exon 11 (MLL-11A; Fig 3). We also performed genomic PCR assay by using a set of primers (sense primer, MLL-9S; antisense primer, p300-11A) and detected a 1.6-kb fragment containing MLL and p300 genomic breakpoints (Fig 5).

Expression of the MLL-p300 fusion protein. To examine the expression of the fusion protein that resulted from t(11;22), Western blot analysis was performed with a p300-specific monoclonal antibody. As shown in Fig 2E, a band (~350 kD) was observed in addition to the germline band (~270 kD). Because the antibody specifically recognizes the C-terminal region of p300, this extra band probably corresponds to MLL-p300 fusion protein. Further study using MLL-specific antibody is required to substantiate the identity of the extra band.

DISCUSSION

To date, the t(11;22) has been so far reported in patients with de novo acute myelomonocytic leukemia (AMoL). A chromosomal breakpoint in 11q23 was detected within the MLL breakpoint cluster region by Southern blotting and the partner gene was shown to be localized in a region centromeric to BCR gene on 22q11. Therefore, the partner gene may differ from the p300 gene. In the present study, we found that the p300 gene was involved in leukemia and that the MLL-p300 fusion protein was generated in a secondary AML patient with t(11;22).

We detected transcribed chimeric mRNAs from both derivative chromosomes by RT-PCR assay. Previous studies of variant 11q23 translocations involving three-way rearrangement showed that the conserved junction arises from translocation of chromosomal material to the der(11) chromosome. Furthermore, in a case with t(4;11), expression of der(4) product was not detectable. These results suggest that the der(11) MLL-p300 fusion protein is a pathogenetically important fusion product.

A schematic representation of the predicted MLL-p300 fusion protein is shown in Fig 4. The predicted MLL-p300 fusion transcript encodes a protein of 3,006 amino acids, 1,531 amino acids from MLL (amino acids 1 to 1,531) and 1,475 amino acids from p300 (amino acids 940 to 2,414). The AT-hook domains, putative methyltransferase, and a transcriptional repression domain of MLL are retained in the MLL-p300 chimeric protein. On the other hand, the chimeric protein retains the acetyltransferase domain and the TFIIB-binding domain of p300, but it lacks most of the N-terminal C/H-rich region and the putative CREB-binding region of p300. However, almost all of the parts of CBP are retained in the MOZ-CBP and MLL-CBP fusion proteins, which are created by t(8;16) and t(11;16), respectively. These findings suggest that a simple truncation of the region of p300 is unlikely to be sufficient to promote leukemogenesis and that the basis for the leukemogenesis of t(11;22)-AML may
be altered function of p300 by structural changes through fusion with MLL.

It is noted that the MLL gene is fused with various partner genes by 11q23 chromosome translocations. To date, 12 partner genes for MLL have been cloned from leukemia cells with various types of reciprocal translocations. However, the normal functions of MLL and the fusion protein remain unknown. We identified here one of the partner genes of MLL involved in both cell differentiation and regulation of cell cycle. Our results suggest that the basis for the leukemogeneses induced by MLL gene translocations may be altered function of the chimeric partner gene product. Functional analysis of the MLL-p300 fusion protein will provide new insights into leukemogenesis.

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