Identification of a melanoma antigen, PRAME, as a BCR/ABL-inducible gene

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Abstract In order to elucidate molecular events in BCR/ABL-induced transformation, we adopted a polymerase chain reaction (PCR)-based technique of differential display and compared mRNA expression in human factor-dependent cells, TF-1, with that in factor-independent cells, ID-1, which were established from TF-1 cells by transfection of BCR/ABL. Cloning and sequencing of a gene which was upregulated in ID-1 cells revealed that the gene was identical to a melanoma antigen, PRAME. Our present study demonstrated that PRAME was markedly expressed in primary leukemic cells with chronic myeloid leukemia (CML) in blastic crisis and Philadelphia (Ph)$^+$-acute lymphoblastic leukemia (ALL), in which BCR/ABL played an important role as a pathogenic gene. Moreover, comparison of PRAME expression among CD34$^+$ cells with CML in blastic, accelerated, and chronic phases revealed a higher expression in CML in advanced phases. Thus PRAME was considered to be a good candidate for a marker of Ph$^+$-leukemic blast cells as well as a new target antigen of leukemic blast cells that cytotoxic T cells can recognize.

Key words: Differential display; PRAME; BCR/ABL; Chronic myeloid leukemia; Blastic crisis; Philadelphia$^+$-acute lymphoblastic leukemia

1. Introduction

BCR/ABL is a fusion gene resulting from Philadelphia (Ph) chromosome, a hallmark of chronic myeloid leukemia (CML). It encodes an active tyrosine kinase and plays a crucial role in the pathogenesis of CML [1]. To isolate genes which are closely related to BCR/ABL-induced transformation, we used a differential display approach based on polymerase chain reaction (PCR) and compared the expression of many sets of genes between human factor-dependent TF-1 cells [2] and ID-1 cells which became factor-independent after transfection of TF-1 cells with BCR/ABL [3]. ID-1 cells were less sensitive to a growth inhibitory effect of interferon-α than parent TF-1 cells. The basal expression level of IRF-1 and ISG15 genes, which were known as interferon-α-inducible genes, was lower in ID-1 cells than parent TF-1 cells [3]. These results suggested the feasibility of using ID-1 cells as a model of human CML cells in accelerated or blastic phase and isolating BCR/ABL-inducible genes. In the present study, we successfully identified mRNA of a tumor antigen, PRAME, and also studied expression of PRAME gene in primary leukemic cells.

2. Materials and methods

2.1. Cells

ID-1 was established in our laboratory and reported elsewhere [3]. Briefly, TF-1 cells [2] were prepared at a concentration of 10^7 cells/ml in phosphate-buffered saline (PBS). 10 μg of pGD210, which was kindly provided by Dr. George Q. Daley at the Whitehead Institute for Biomedical Research, was electroporated with a condition of 500 mFD of capacitance and 0.3 V of voltage. The transfected TF-1 cells were positively selected in 1 mg/ml of G-418 for 7 days and washed twice with Iscove's modified Dulbecco's medium (IMDM; GIBCO-BRL, Gaitherburg, MD, USA) containing 10% fetal bovine serum (FBS) (GIBCO-BRL). The cells were cultured in IMDM supplemented with 10% FBS and 5 ng/ml of granulocyte/macrophage colony-stimulating factor (GM-CSF) for 7 days, then split to a single cell per well and cultured in the same condition. Clones which had been grown well were selected and a clone was designated as ID-1. ID-1 cells could grow independent of GM-CSF.

2.2. Primary samples

Primary cells or mononuclear cells were obtained from peripheral blood or aspirated bone marrow cells of normal volunteers and patients with CML or ALL by density centrifugation using Ficoll-metrizoate (Lymphoprep; Nycomed, Oslo, Norway). Microscopically, more than 80% of nucleated cells were leukemic blast cells in specimens with CML in blastic phase or crisis (CML-BC) and ALL. Clinical stage of CML was classified into chronic, accelerated, and blastic phases according to the criteria of international bone marrow transplant registry (IBMTR) [4]. Surface marker analysis of the leukemic blast cells in CML-BC revealed that they were all positive for CD13, CD33, and CD34, denoting their myeloid character.

2.3. Separation of CD34-positive progenitor cells from bone marrow of patients with CML

CD34-positive cells were isolated from bone marrow of patients with CML by using a MACS system [5] and CD34 progenitor cell isolation kit (QBEND/10) (Miltenyi Biotec Inc., Sunnyvale, CA, USA) as recommended by the manufacturer. Briefly, heparinized bone marrow cells were incubated with 1:10 (vol/vol) silica (IBL Co., Fujioka, Japan) at 37°C for 60 min to remove phagocytic cells efficiently by density centrifugation. Mononuclear cells were separated by using Lymphoprep. 10^8 mononuclear cells were successively incubated for 15 min at 4°C with a blocking reagent (human IgG) and mouse monoclonal anti-CD34 antibody followed by washing once and resuspended. Colloidal superparamagnetic MACS microbeads recognizing mouse IgG were added, incubated for 15 min at 6°C.

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Washed cells were then applied onto buffer-prefilled MiniMACS column. After the column was washed, enriched CD34-positive cells were collected. The cells were stained with fluorochromated anti-mouse reagent and their purity determined flowcytometrically.

2.4. Isolation of total RNA and polyadenylated RNA

Total RNAs were isolated by a modified acid-guanidine thiocyanate-phenol-chloroform method using ISOGEN (Nippon Gene, Tokyo, Japan) reagents according to the manufacturer's instructions. Polyadenylated RNAs were prepared by using FastTrack 2.0 Kit (Invitrogen, San Diego, CA, USA) as recommended by the manufacturer. RNAs were resuspended in RNase-free water and stored at −80°C until use.

2.5. Removal of contaminating chromosomal DNA

100 μg of total RNA was incubated for 60 min at 37°C with 50 units of human placental ribonuclease inhibitor (GIBCO-BRL), 3 units of DNase I (Promega, Madison, WI, USA) in 40 mM Tris–Cl, pH 7.4, 0.6 mM $MgCl_2$, 10 mM DTT. After extraction with phenol/$CHCl_3$/isoamyl alcohol (25:24:1), the supernatant was precipitated by ethanol in the presence of 0.3 M sodium acetate and RNA was redissolved in RNase-free water.

2.6. Differential display of mRNAs by PCR [6,7]

Reverse transcription (RT) was performed by incubation of 2.5 μg of DNase-treated total RNA at 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min in a final volume of 20 μl with 200 U of Superscript II (GIBCO-BRL), 10 mM DTT, 1:10 (vol/vol) 10×PCR buffer (GIBCO-BRL preamplification kit), 50 pmol of anchor primer [$G(T)_{15}A$] and 0.5 mM of each dNTP (Pharmacia, Piscataway, NJ, USA). After incubation, RT product was diluted by adding 80 μl of TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). The PCR was performed by mixing 2 μl of the diluted RT product to a final volume of 20 μl with 0.5 U of a Taq DNA polymerase (GeneTaq) (Nippon Gene) and 0.5 U of another Taq DNA polymerase (Promega), 0.2 mM dNTP, 0.5 μM of the anchor primer [$G(T)_{15}A$], 0.5 μM of an arbitrary primer (AATCGGGCTG) (Operon Technologies, Alameda, CA, USA), and 1:10 (vol/vol) 10×GeneTaq PCR buffer (Nippon Gene). The durations and temperatures used were 3 min at 94°C, 5 min at 40°C, and 1 min at 72°C for the initial cycle and 15 s at 94°C, 2 min at 40°C, and 1 min at 72°C for subsequent 25 cycles. 12 μl of each PCR product was loaded on a 8% polyacrylamide gel and analyzed by electrophoresis. The gel was stained by ethidium bromide staining and differentially expressed bands were identified. The gel containing bands which were overexpressed in ID-1 cells were cut out and washed in distilled water three times. A half of gel slice was applied into the same PCR solution as above and reamplified as follows: 3 min at 94°C and 15 s at 95°C, 2 min at 40°C, and 1 min at 72°C for 20 cycles and 72°C for 5 min. The PCR products were electrophoresed. The amplified band of interest was cut out, purified by EASYTRAP (TaKaRa Biomedicals, Shiga, Japan), and cloned into pCR2.1 by using the TA cloning system (Invitrogen). To confirm that the clone was derived from the band of interest, ECL-Southern blotting of the initial samples was performed by using the cloned cDNA probes. Briefly, 10 μl of the original samples for differential display from one of which the clone was derived were electrophoresed again in a 1% agarose gel, transferred onto Hybond N+ (Amersham Life Science, Buckinghamshire, England), and hybridized with the cloned DNA probes that were labeled by an ECL system (Amersham Life Science) as recommended by the manufacturer. Once the clone was confirmed to be of interest, the insert was sequenced by the dideoxy technique using AutoRead Sequencing Kit (Amersham Pharmacia Biotech, Tokyo, Japan) and A.L.F. DNA Sequencer II (Amersham Pharmacia Biotech).

2.7. RT for primary samples

RT was performed by incubation of 1∼5 μg of total RNA derived from primary samples at 37°C for 60 min in 20 μl of final volume with 200 U of Moloney murine leukemia virus-reverse transcriptase (GIBCO-BRL), 10 mM DTT, 1:5 (vol/vol) 5×RT buffer (GIBCO-BRL), 5 μM of random hexamer [$pd(N)_6$; Pharmacia], and 0.5 mM of each dNTP (Pharmacia).

2.8. PCR for PRAME or β-actin and sample analysis

The PCR was performed by mixing an aliquot of RT product, which corresponded to 1 μg total RNA, to a final volume of 50 μl with 1.25 U of *Taq* DNA polymerase (Promega), 0.25 mM dNTP, 1 μM of 5′ sense and 3′ antisense primer, 1 mM of $MgCl_2$, and 1:10 (vol/vol) 10×PCR buffer (Promega). The mixture was overlaid by 50 μl of mineral oil and then amplified for 40 cycles by the Takara PCR thermal cycler MP (Takara Biomedicals, Osaka, Japan). The durations and temperatures used in the first cycle were 30 s at 94°C for denaturation, 1 min at 56°C for annealing, and 2 min at 72°C for polymerization. The polymerizing phase was extended 5 s per cycle. In some experiments, the RT product corresponding to 1 μg total RNA was amplified with primers for PRAME and β-actin mixed together because T_m of primers were close to each other (Fig. 4B). 10 μl of the PCR product was loaded onto 1% agarose gel containing 0.5 μg/ml of ethidium bromide and electrophoresed at 100 V for 30 min before taking picture.

2.9. Primers

Primers used for PCR amplification and the expected size of products were as follows, in order, (i) 5′ sense primer (with the expected size of PCR product) and (ii) 3′ antisense primer. PRAME: (i) 5′-GCCCAGTTCACCTCTCAGTT-3′ (764 bp), (ii) 5′-TGTGGCTGCTTTGTTGCTTC-3′, β-actin [8]: (i) 5′-GGCATCGTCACCAACTGGGACGAC-3′ (878 bp), (ii) 5′-ATTTGCGGTGGACGATGGAGGGGC-3′.

3. Results

3.1. Differential display between TF-1 cells and ID-1 cells

Differential display technique using PCR was applied to find unique genes that were upregulated in ID-1 cells. Comparison of gene expression between TF-1 and ID-1 cells by differential display using more than 20 primer pairs resulted in identifying several bands which were upregulated in ID-1 cells. Fig. 1 showed a result in which a gene was found to be overexpressed in ID-1 cells. To exclude a possible contamination of genomic DNA in the original RNA samples, we treated samples with DNase prior to reverse transcription of the RNA. Fig. 1A showed no amplification of bands from samples without reverse transcription, denoting no contamination of the RNA samples by genomic DNA. Those bands that were upregulated in ID-1 cells were cloned into pCR2.1

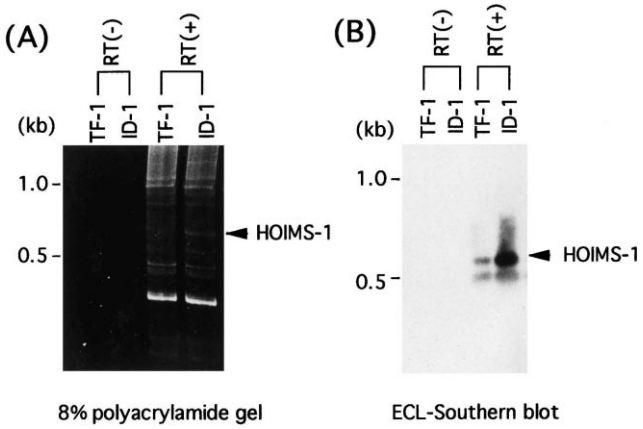

Fig. 1. Electrophoresis of PCR products using an anchor primer [$G(T)_{15}A$] and an arbitrary primer (AATCGGGCTG). In order to deny contamination of genomic DNA, 1.25 μg of total RNA of a sample was amplified without reverse transcription (RT) (A). Note that no bands were obtained from samples with no RT. A band which was overexpressed in ID-1 cells was cloned and designated as HOIMS-1. To confirm the clone was derived from the band of interest, ECL-Southern blotting of the initial samples was performed by using the HOIMS-1 cDNA probes (B).

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gcttcagggt acagctcccc cgcagccaga agccgggcct gcagcccctc agcaccgctc   60
cgggacaccc cacccgcttc ccaggcgtga cctgtcaaca gcaacttcgc ggtgtggtga  120
actctctgag gaaaaaccat tttgattatt actctcagac gtgcgtggca acaagtgact  180
gagacctaga aatccaagcg ttggaggtcc tgaggccagc ctaagtcgct tcaaaatgga  240
acgaaggcgt ttgtggggtt ccattcagag ccgatacatc agcatgagtg tgtggacaag  300
cccacggaga cttgtggagc tggcagggca gagcctgctg aaggatgagg ccctggccat  360
tgccgccctg gagttgctgc ccagggagct cttcccgcca ctcttcatgg cagcctttga  420
cgggagacac agccagaccc tgaaggcaat ggtgcaggcc tggcccttca cctgcctccc  480
tctgggagtg ctgatgaagg gacaacatct tcacctggag accttcaaag ctgtgcttga  540
tggacttgat gtgctccttg cccaggaggt tcgccccagg aggtggaaac ttcaagtgct  600
ggatttacgg aagaactctc atcaggactt ctggactgta tggtctggaa acagggccag  660
tctgtactca tttccagagc cagaagcagc tcagcccatg acaaagaagc gaaaagtaga  720
tggtttgagc acagaggcag agcagccctt cattccagta gaggtgctcg tagacctgtt  780
cctcaaggaa ggtgcctgtg atgaattgtt ctcctacctc attgagaaag tgaagcgaaa  840
gaaaaatgta ctacgcctgt gctgtaagaa gctgaagatt tttgcaatgc ccatgcagga  900
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gtgatctttg gggagataca tcttatagag ttagaaatag aatctgaatt tctaaaggga 2040
gattctggct tgggaagtac atgtaggagt taatccctgt gtagactgtt gtaaagaaac 2100
tgttgaaaat aaagagaagc aatgtgaagc aaaaaaaaaa aaaaaaaa              2148
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Fig. 2. PRAME cDNA and HOIMS-1. PRAME cDNA is shown from 5′ to 3′ end. HOIMS-1 was revealed to be a 1017 bp fragment of 3′ end of PRAME cDNA as indicated in a rectangle.

at *Eco*RI site. To confirm that a cloned band was the one of interest, ECL-Southern blotting of the initial RT-PCR products was performed using the cloned sequences as probes. As shown in Fig. 1B, a gene was confirmed to be highly expressed in ID-1 cells and was temporarily designated as HOIMS-1.

3.2. Genetic analysis of HOIMS-1 gene

Sequencing of HOIMS-1 gene and the initial search for homology in data banks revealed that it was 99.3% homologous to an anonymous sequence, HTGS phase 3, complete sequence (GenBank locus: HSAC000024), 96.7% to testis 2 Homosapiens cDNA clone (T19428), 98.4% to human HL60 3′ directed *Mbo*I cDNA (HUMGS 01429), and 100% to 104 bp of a human erythroleukemic expressed sequence tag (EST) mRNA fragment (HUMRBCESTC). A couple of months later, the second computer search for homology revealed that HOIMS-1, which was further sequenced to 1017 bp, was exactly identical to a 3′ end of the cDNA of a melanoma antigen, PRAME (Fig. 2) [9]. Northern blotting of poly(A) RNA of TF-1 and ID-1 cells by using PRAME cDNA as probes showed marked expression of PRAME mRNA in ID-1 cells (Fig. 3). These results strongly suggested that overexpression of BCR/ABL might induce PRAME expression in ID-1 cells.

3.3. PRAME mRNA expression in primary leukemic cells

RT-PCR analysis of primary leukemic cells demonstrated that leukemic blast cells from three cases with CML-BC (myeloid crisis) and three cases with Ph-positive ALL were all positive for PRAME mRNA, while bone marrow or peripheral mononuclear cells from three cases with chronic phase of CML (CML-CP), two cases with Ph-negative ALL, and two healthy volunteers were weakly positive or negative for the gene (Fig. 4A). Since the blast cells from the patients with CML-BC were positive for a surface antigen of CD34, which is characteristic for hematopoietic progenitor cells, the result of Fig. 4A might reflect merely quantity of CD34 cells which might preferentially express PRAME. Thus we analyzed the mRNA expression of PRAME in CD34-positive blast cells

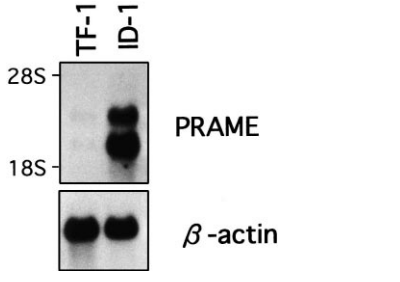

Fig. 3. Northern blot analysis of PRAME mRNA in TF-1 and ID-1 cells. 5 μg per lane of each poly(A) RNA was analyzed. PRAME cDNA was used as probe. As RNA loading control, the same blot was reprobed with β-actin cDNA. Note that PRAME mRNA was apparently upregulated in ID-1 cells.

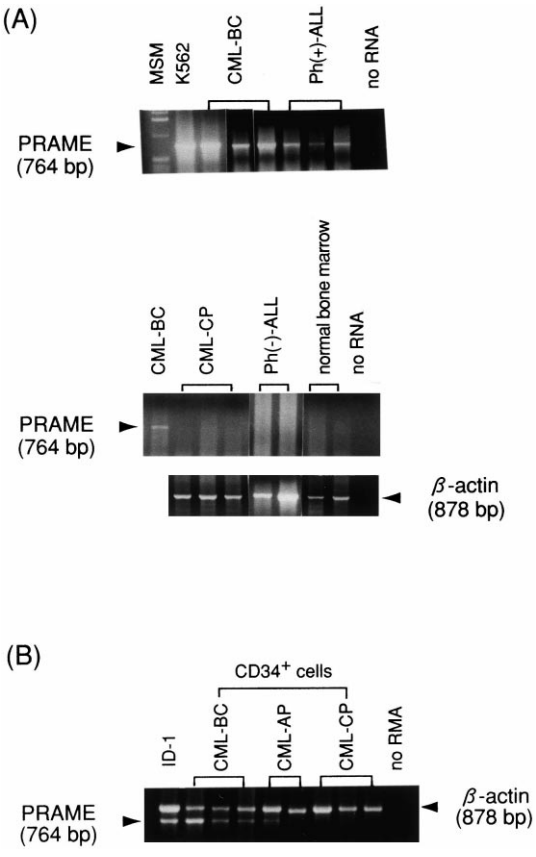

Fig. 4. A: Representative data of RT-PCR analysis for PRAME mRNA expression in human primary leukemic cells and normal bone marrow mononuclear cells. B: Expression of PRAME mRNA in primary CD34-positive cells from patients with CML in blastic crisis (BC), accelerated phase (AP), and chronic phase (CP).

from CML-BC and CD34-positive bone marrow cells from the patients with CML in accelerated phase (CML-AP) and CML-CP by RT-PCR. The purity of CD34-positive cells was more than 90%. The cells from CML-BC were all positive for PRAME mRNA by RT-PCR (Fig. 4B). The CD34-positive cells from a patient with CML-AP, who showed additional chromosomes, was positive for PRAME. However, cells from another patient with CML-AP, who had no additional chromosomes but had myelofibrosis and basophilia, was negative for PRAME mRNA. The CD34-positive cells from CML-CP were again all negative for PRAME expression by RT-PCR (Fig. 4B).

4. Discussion

BCR/ABL is well known as a gene that can transform hematopoietic cells [10] and induce CML in mice [11,12]. Hence the gene is believed to be crucial in the pathogenesis of CML and Ph-positive ALL. Knowing effectors downstream of BCR/ABL in signal transduction of the affected cells is useful to develop a new strategy to control the leukemic cells of CML and Ph-positive ALL. As BCR-ABL-inducible genes, kir/gem was reported although its function remains to be clarified [13]. In the present study, we performed a PCR-based differential display approach to identify a new BCR/ABL-inducible gene and compared sets of genes expressed between parent TF-1 cells and ID-1 cells that were BCR/ABL cDNA transformed, factor-independent, and interferon-α-resistant TF-1 cells. In our experiments, we modified original methods to use non-denaturing gels to avoid the complexity of the patterns obtained in the denaturing polyacrylamide gels accompanied with the several bands derived from one DNA species. Moreover, we visualized PCR-amplified bands by ethidium bromide staining, instead of using isotopes or fluorescence, to obtain genes with increased level of mRNA compared with other genes, avoiding too many meaningless bands which hindered further analysis. Many genes were differentially expressed between TF-1 and ID-1 cells even with our method. Among them a gene which was apparently overexpressed in ID-1 cells was designated as HOIMS-1 and analyzed in this study. Cloning and sequencing showed that HOIMS-1 had a high homology with several genes reported in GenBank.

Ikeda et al. reported a gene, PRAME, encoding a melanoma tumor antigen [9]. PRAME-encoding antigen was recognized by cytotoxic T lymphocytes (CTL) in a HLA-24-restricted manner. The gene was expressed ubiquitously in normal human tissues at a low level and highly expressed in testis. PRAME was also expressed in many neoplastic tumors including melanoma, lung cancer, renal cancer, and acute leukemia [9]. In the present study, we showed that HOIMS-1 was a PRAME gene and a candidate BCR/ABL-inducible gene.

The character of ID-1 was reported to be close to that of leukemic blasts in CML-BC [3]. The results of RT-PCR for PRAME mRNA expression using primary leukemic cells supported this hypothesis. All the cases of CML-BC and Ph-positive ALL tested were positive for PRAME mRNA although other cases including CML-CP and Ph-negative ALL were only weakly positive or negative. Generally leukemic blast cells of CML-BC and Ph-positive ALL are positive for CD34, rising the possibility that expression of PRAME is increased in CD34-positive populations in the leukemic cells. Therefore we compared PRAME mRNA expression levels in CD34-positive cells with different clinical stages of CML. As shown in Fig. 4B, a high level of PRAME mRNA expression was not ubiquitous in CD34-positive cells, but seemed to be marked in leukemic blast cells of CML-BC in myeloid crisis. In fact, CD34-positive cells from a male patient with CML-AP with a cytogenetic clonal evolution, implying that his clinical feature was close to CML-BC, were positive for PRAME, but the cells from another patient with CML-AP without any cytogenetic change were negative for PRAME. These results did not show that the mRNA was absent in the cells with CML-AP or CP since the level of PRAME mRNA expression could be low and merely under the sensitivity of PCR in those cells. However, the results suggested that the level of PRAME expression was increased in the leukemic blasts with CML-BC, reflected the acceleration of CML, and was useful to diagnose the clinical stage of CML.

Anti-leukemia immunotherapy has been extensively investigated and clinically practiced. However, few tumor antigens, which could be specifically recognized by CTL, have been identified in leukemia. PRAME was reported to be expressed in various types of acute leukemia, especially in acute myeloid leukemia with a specific karyotype of t (8;21), which generates a fusion gene of AML-1/ETO [14]. It is interesting to note that the report and our results suggested that AML1/ETO might share its signal transduction pathway with BCR/ABL. AML with t (8;21) is a representative of leukemia with a good prognosis while CML-BC and Ph-positive ALL are well known as leukemias with very poor prognosis. In the AML patients with t (8;21), PRAME might be recognized as tumor specific antigen by CTL. Whether CTL activity against PRAME-positive leukemic cells is higher in AML patients with AML-1/ETO than CML patients with BCR/ABL requires further investigation. As mentioned above, PRAME mRNA expression was not restricted to leukemia, but distributed to various types of malignant tumors [9]. Hence development of CTL which recognizes PRAME could be useful and applicable for the treatment of various neoplasms as well as leukemia.

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