Gene Silencing of the Tyrosine Phosphatase SHP1 Gene by Aberrant Methylation in Leukemias/Lymphomas

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Abstract

High-frequent silencing of hematopoietic cell-specific protein-tyrosine phosphatase SHP1 gene by promoter methylation was detected in various kinds of leukemias and lymphomas, as well as in many hematopoietic cell lines, which is supported by our previous observation of strong decrease of SHP1 mRNA and protein. The promoter methylation of the SHP1 gene was clearly correlated with the clinical stage. Loss of heterozygosity with microsatellite markers near the SHP1 gene was shown in 79% of informative acute lymphoblastic leukemia cases. These results suggest that functional loss of SHP1 is associated with the pathogenesis of leukemias/lymphomas.

Introduction

The SHP1 gene, which is also called the PTPN6, HCP, SH-PTP1, or PTP1C gene, is located on human chromosome 12p13 and codes a M, 68,000 protein of a nonreceptor type protein-tyrosine phosphatase containing two tandem Src homology (SH2) domains, a catalytic domain, and a COOH-terminal tail of ~100 amino acid residues (1). SHP1 is expressed primarily in hematopoietic cells with hematopoietic cell-specific manner from a promoter for exon 1b (2) and usually functions as a negative regulator in signal transduction (3-5). SHP1 is known to be associated with multiple signaling molecules, including ZAP70 (5), CD3e (6, 7), CD5 (7), and IL-2R (8) in T cells; IL-3 receptor β chain (9) and erythropoietin receptor (10) in hematopoietic cells; CD22 (11), B-cell receptor (12), SLP76 (13, 14), and CD72 (15, 16) in B cells; and the killer cell inhibitory receptor (17) in NK cells (18). These interactions appear to exert primarily inhibitory effects on their signaling cascades. SHP1 is thought to be capable of terminating an activating signal by dephosphorylating some molecules involved early in signal transduction; however, little is known about the functional role of SHP1 in the malignant transformation process in the hematopoietic cells. To investigate the lymphomagenesis and leukemogenesis, we comprehensively and systematically analyzed the mRNA and protein expression pattern in the human lymphoma cell lines by cDNA array and tissue microarray, respectively (18). We detected some genes that showed significant changes in the gene expression of these cell lines. Among these genes, we found a strong decrease of SHP1 mRNA by cDNA-array and RT-PCR (18). Additional analysis with standard immunohistochemistry and tissue microarray, which used 207 paraffin-embedded specimens of various kinds of malignant lymphomas, showed that SHP1 protein was decreased in most of the malignant lymphomas, but that SHP1 protein was strongly expressed in the mantle zone and interfollicular zone lymphocytes in reactive lymphoid hyperplasia specimens (18). In addition, various kinds of hematopoietic cell lines, particularly the highly aggressive lymphoma and leukemia lines, lacked SHP1 expression (18), suggesting that loss of the SHP1 expression may be related to not only malignant transformation but also to tumor cell aggressiveness. Here, we analyzed the promoter region of the SHP1 gene in the lymphoma and leukemia cells in which the SHP1 expression was decreased, and demonstrated that one of the mechanisms for SHP1 gene silencing is an aberrant methylation of the promoter region.

Materials and Methods

Cell Culture. Human NK cell lines, NK-YS and NK-TY2, were maintained in Iscove's modified Dulbecco's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS, 100 units/ml of recombinant human IL-2, 100 units/ml of kanamycin, and 100 μg/ml of streptomycin. Other cell lines were maintained in RPMI 1640 supplemented with 10% FCS, 100 units/ml of kanamycin, and 100 μg/ml of streptomycin. Human B-ALL cell line KCA was obtained from Dr. Eugene C. Butcher (Department of Pathology, Stanford University, Stanford, CA). Human CML cell line K562, human AML cell line KG1, human B-ALL line BALL1, Burkitt lymphoma line Ramos and Daudi, human T-lymphoma line Jurkat, ATL line EDS, and T-cell line immortalized with HTLV-I, ATL16T, and TomJim were from Dr. Yoshinobu Matsuo (Fujisaki Cell Center, Hayashibara Biomedical Laboratory, Okayama, Japan). Human ATL line ILT10M cells were from Dr. Kazuo Sugamura (Tohoku University Medical School) and Riken Cell Bank (Wako, Japan).

Patients. Blood samples were obtained at Second Department of Internal Medicine of Okayama University Hospital after an acquisition of informed consent from each patient. PB, BM, or lymph node tissue was obtained from patients with ATL, NK/T-cell lymphoma, AML, ALL, or CML at diagnosis. Mononuclear cell fraction was isolated from these PB or BM samples by Ficoll-Hypaque density centrifugation as manufacturer's protocols. Fresh normal NK cell fraction was additionally purified by the magnetic beads method with anti-CD56 monoclonal antibody. The purity of this NK cell-enriched fraction was about 60–70%. In the case of AML and ALL, the diagnostic BM samples contained blast cells in the ratio of at least 70%. BM or PB samples were also obtained after hematological complete remission achieved by chemotherapy.

Analysis of mRNA Expression by RT-PCR. A 2-μg aliquot of DNase-I treated total cellular RNA was reverse-transcribed with SuperScript II reverse transcriptase (Life Technologies, Inc., Rockville, MD). Portions (0.5 μl) of single-stranded cDNA were amplified by PCR using SHP1-specific primer pairs (BD Biosciences Clontech, Palo Alto, CA) with Platinum PCR Super

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3 The abbreviations used are: NK, natural killer; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATL, adult T-cell leukemia; CML, chronic myelogenous leukemia; MSP, methylation-specific PCR; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription-PCR; LOH, loss of heterozygosity; IL, interleukin; BM, bone marrow; PB, peripheral blood; 5azaCdr, 5-aza-2′-deoxycytidine; FISH, fluorescence in situ hybridization.
Mix (Life Technologies, Inc.). The amplification condition consisted of 2 min pretreatment at 94°C, and 30 cycles of denaturation (94°C, 30 s), annealing (59°C, 30 s), and extension (72°C, 1 min 30 s) followed by final extension step (72°C, 10 min).

**Western Blot Analysis.** Western blot analysis was performed as described in the previous paper (18). Briefly, after 12.5% PAGE of cellular protein lysate from 7.5 x 10⁶ cells of each culture, banded proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Immobilon; Millipore, Ltd., Bedford, MA), and then reacted with mouse monoclonal antibody against the COOH terminal of SHPI (D11; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and monoclonal anti-β-actin (Sigma, St. Louis, MO). The immuno-reactive bands were incubated with peroxidase-labeled goat antimouse immuno-globulin (Amersham-Pharmacia Co., Ltd., Piscataway, NJ), followed by reactions with the substrate of the enhanced chemiluminescence-SuperSignal Western blotting system (Pierce, Rockford, IL) and exposed to X-ray film.

**Methylation Analysis of Promoter Region with Restriction Enzyme-mediated PCR.** A pair of primers, sense 5'-GAAACGACTGCTTCTGG-GAACC and antisense 5'-ATCCTGAGAATCCCTAGGAGT, was designed to enclose MspI/HpaII restriction sites in the promoter region of the SHPI gene. DNA (50 ng) was incubated with 20 units of MspI, HpaII, or dH2O in 1 x buffer at 37°C overnight. The enzymes were inactivated by heating at 70°C for 20 min. PCR was carried out in 25 μl of reaction mixture with 10 pmol of each primer, 5 ng of genomic DNA, 1 x μM of each deoxynucleotide triphosphate, and 0.5 units of Taq DNA polymerase (Takara, Tokyo, Japan). Initial denaturation at 94°C for 3 min was followed by 35 cycles of a denaturation step at 94°C for 30 s, an annealing step at 60°C for 1 min, and an extension step at 72°C for 1 min, and a final extension step of 72°C for 7 min was added. The products were separated by electrophoresis on 2% agarose gel.

**MSP and Unmethylation-specific PCR Assay.** Reaction with sodium bisulfite was followed by CpGenome DNA Modification kit (Intergen Co., Purchase, NY). Briefly, 1 μg of genomic DNA was denatured by NaOH and modified by sodium bisulfite, which converts all unmethylated cytosines to uracils, whereas methylated cytosines remain unchanged. The modified DNA was treated with NaOH to disulfonate and purified. A pair of primers, MF2 5'-GGACCTTATATTGATAGGTC and MR2 5'-TCAGGTATAC-GAACCCCAACCG, was designed for the methylated sequence of promoter region for exon 1b of the SHPI gene (nucleotide numbers 6857–7015 in GenBank accession no. X828818). A pair of primers, UF22 5'-ATCCTGAGAATCCCTAGGAGT and R22 5'-TTC ACA CAT ACA AAC ATT ATA GTA TAG TGT TTG G, was designed for the unmethylated sequence of the same region also. Initial denaturation at 94°C for 3 min was followed by 40 cycles of a denaturation step at 94°C for 30 s, an annealing step at 60°C for 1 min, and an extension step at 72°C for 2 min, and a final extension step of 72°C for 7 min was added. The products were separated by electrophoresis on 2% agarose gel.

**DNA Sequencing.** The PCR products were purified by using GeneClean III kit (Bio101; Carlsbad, CA), phosphorylated by T4 DNA kinase, and cloned with pBluescript KS. Independent plasmid clones were purified from several bacterial colonies, and were subjected to sequencing reaction with BigDye Terminator Cycle Sequencing Ready Reaction kit V2.0 (Applied Biosystems, Foster City, CA) and applied on ABI Prism 3100 DNA sequencer (Applied Biosystems).

**5AzaCdr Treatments.** Ten hematopoietic cell lines were incubated with or without 5AzaCdr (Sigma, St. Louis, MO) at 1 μM for 5 days. Cells were harvested at the end of day 5 and tested for protein expression with enhanced chemiluminescence Western blotting.

**Southern Blot Analysis.** Cultured hematopoietic cell lines were examined with Southern blot analysis for the confirmation of the presence of the complete SHPI1 gene. According to the standard method, 5 μg of genomic DNA was digested with EcoR1 and subjected to 0.8% agarose gel electrophoresis, transferred to nylon membrane, and UV cross-linked. Transferred DNA was hybridized with full-length SHPI cDNA probe labeled with fluorescein-11-dUTP, washed three times 5 x SSC, and was detected using a chemiluminesence detection kit (Gene Images random prime labeling and detection system; Amersham Life Science, Buckinghamshire, United Kingdom) following the manufacturer’s protocol.

**FISH.** The 10 kb of genomic SHPI1 DNA was amplified with primer 5'-GAC TAG CTG CAC CTC CTC ATT C-3' for exon1b and primer 5'-CAT CGC GAA ATG CTT CCA CAG G-3' for exon17 by LA-Taq DNA polymerase (Takara, Kyoto, Japan), and nick-translated with Nick Translation kit (Vysis, Inc., Downers Grove, IL). Cells were smeared on the slides and fixed in acetone for 5 min. Aging was performed in 2 x SSC/0.1% NP40 (Vysis, Inc.) at 37°C for 30 min. Denature was performed in 70% formamide/2 x SSC at 73°C for 5 min. The cells were hybridized with 10 μl of the chromosome enumeration of DNA FISH probe 12 probe (D12Z3; Vysis) and the SHPI1 genomic probe, and incubated in a humid chamber at 42°C for 16 h. The spectrum green-labeled probe specific for a-satellite of the chromosome 12 was used. Nuclei were counterstained with 4',6-diamidino-2-phenylindole II (Vysis), and the slides were covered with a glass coverslip and sealed. The FISH slides were examined by using an Olympus BH2 fluorescence microscope. Cells (200–250) were examined for each cell line, and the signals were counted for each cell.

**Microsatellite Analysis.** LOH examination was performed by using two microsatellite markers by which the SHP1 gene on chromosome 12p13 was surrounded. Primers for microsatellite markers, D12S336 and D12S356, are available through an internet genome database. After the sense primers were labeled with 5-iodoacetamidofluorescein, PCR was carried out in 20 μl of reaction mixture with 10 pmol of each primer, 40 ng of genomic DNA, 1 x...
PCR buffer, 200 μM of each deoxynucleotide triphosphate, and 0.5 units of Taq DNA polymerase (Takara). The PCR products were applied on ABI Prism 3100 DNA sequencer (Applied Biosystems) and analyzed by Genescan analysis software version 3.7 (Applied Biosystems). Expected 227-bp bands were shown.

Results and Discussion

We found a strong reduction of hematopoietic cell-specific SHP1 mRNA and protein in 93% of diagnostic samples of malignant lymphomas and leukemias, including NK/T-cell lymphoma, diffuse large cell lymphoma, follicular lymphoma, Hodgkin’s disease, mantle cell lymphoma, peripheral T-cell lymphoma, adult T-cell lymphoma and leukemia, and plasmacytoma in the previous investigation (18). In Fig. 1A, no expression of the SHP1 gene was shown in NK/T lymphoma (NK-YS and NK-TY2) and CML (K562) cell lines, and reduced expression of the SHP1 gene was detected in ATL (EDS and ATL1K) and B-cell ALL (BALL1) cell lines by RT-PCR, whereas transcript of the SHP1 gene was detected in normal fresh PBMCs. SHP1 protein also showed an underdetection level in NK-YS, NK-TY2, K562, EDS, and ATL1K cells (Fig. 1A). To investigate a mechanism for the reduced expression of the SHP1 gene, we analyzed the promoter region for the SHP1 exon 1b, which is known to express with hematopoietic-specific manner. By MSP and unmethylation-specific PCR, CpG island of the SHP1 promoter was revealed to be highly methylated in NK-YS, NK-TY2, and K562 cell lines in addition to patient samples of ATL and NK/T-cell lymphoma (Fig. 1, A and C, left), which was clearly in contrast to no SHP1 promoter methylation in normal PBMCs (Fig. 1, B and C, left). Some cell lines including EDS and ATL1K showed both methylation-specific and unmethylation-specific signals, suggesting that these cell lines are mix population in terms of SHP1 DNA methylation. The discrepancy between protein and mRNA expression of the SHP1 gene in these cells might be caused by the heterogeneity as well as differences of post-translational modification. It has been reported that there is a poor correlation between mRNAs and their respective proteins, generally <0.5 (19), because degradation rates of individual mRNAs and proteins differ. Extensive modification can be introduced during and after translation: e.g., phosphorylation, glycosylation, lipid attachment, peptide cleavage, complex formation, and translocation within

### Table 1
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>SHP1 gene methylation*</th>
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<tbody>
<tr>
<td></td>
<td>++ (+)</td>
</tr>
<tr>
<td>ATL</td>
<td>13/20 (65%)</td>
</tr>
<tr>
<td>NK/T lymphoma</td>
<td>9/11 (82%)</td>
</tr>
<tr>
<td>AML</td>
<td>2/8 (25%)</td>
</tr>
<tr>
<td>CML</td>
<td>5/10 (50%)</td>
</tr>
<tr>
<td>Healthy volunteer</td>
<td>8/11 (73%)</td>
</tr>
<tr>
<td></td>
<td>0/0 (0%)</td>
</tr>
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* ++, strong methylation; +, weak methylation; −, no methylation.
the cells may have an effect on the stability of these products. The PCR products were cloned with plasmid, and the methylation status of the independent clones was analyzed by sequencing and shown in Fig. 1C (right). Fig. 1C (bottom) demonstrates a representative of sequencing data. To clarify a frequency of the promoter methylation in diagnostic samples of ATL and NK/T-cell lymphoma, methylation analysis of the promoter region was performed by restriction enzyme-mediated PCR (Fig. 2, A and B). NK-YS, NK-TY2, K562, L58 (ATL patient), and L1688 (NK/T-lymphoma patient), which were shown the promoter methylation by MSP method in Fig. 1, A and C, still demonstrated the promoter methylation by PCR with HpaII-digested DNA in Fig. 2, A and B, indicating that both methods are useful to detect DNA methylation. One ATL cell line, EDS, showed partial methylation, which is not contradict with the low level of the SHP1 mRNA and protein in EDS cells as shown in Fig. 1A. About 90.0% (18 of 20) of ATL, 90.9% (10 of 11) of NK/T-cell lymphoma, 62.5% (5 of 8) of ALL, 90.0% (9 of 10) of AML, and 100.0% (11 of 11) of CML patient specimens showed methylation at the CpG island of the SHP1 gene promoter (Fig. 2, A and B; Table 1). This quite high frequency of the SHP1 promoter methylation is well correlated with the high frequency of reduced expression of SHP1 protein in these leukemias and lymphomas (18), indicating that silencing of the SHP1 gene expression by DNA methylation might be one of the critical events in the leukemogenesis and lymphomagenesis. Furthermore, methylation of the SHP1 CpG island was clearly correlated with the clinical stage of ALL, AML, and CML patients; i.e., the SHP1 gene was strongly methylated in the diagnostic PBMCs of ALL patients and BM of AML or CML patients, on the other hand no methylation of the SHP1 promoter was detected in the hematological complete remission (Fig 2C). These results indicate that methylation of the SHP1 gene might be used for the monitoring or diagnosis of these leukemias as the clinical marker of expansion of malignant cells.

To investigate whether the reduced expression of the SHP1 gene can be rescued with the demethylation procedure, hematopoietic cell lines were treated with 5AzaCdR (Fig. 3A). Several cell lines (NK-TY2, IWA3, TomJim, and MT1) were induced to express SHP1...
protein with this treatment, and a weak induction was detected in some cell lines (ILTMoT and EDS), suggesting that the SHP1 expression is repressed by aberrant methylation. However, as no induction was observed in NK-YS, some other mechanism for gene silencing including chromosomal loss and rearrangement, chromatin modification, and translational regulation may have an effect on the expression of the SHP1 gene. Therefore, we performed Southern blot analysis to make clear whether both allele of the SHP1 gene are lost in some cells like NK-YS (Fig. 3B). We detected the retention of at least one SHP1 gene allele, whereas it was impossible to quantify the allele number of the SHP1 gene technically with this method. Furthermore, FISH analysis of NK-YS and ILTMoT detected two signals in each cell with the SHP1 gene itself and chromosome 12 probe, indicating each cell line has no numerical aberration of the SHP1 gene on chromosome 12 (Fig. 4A).

We still investigated LOH with microsatellite markers located on the both sides of the SHP1 gene in only the limited cases with hematological complete remission sample. The telomeric D12S356 marker showed LOH of 15 cases in 19 informative cases (79%), and the centromeric D12S336 marker showed 6 cases of 16 informative cases (38%) by using diagnostic BM samples and the PB samples obtained after hematological complete remission from ALL patients. One of the representative data is shown in Fig. 4B. Telomeric D12S356 is at a distance of ~4.4 cM, and centromeric D12S336 is at a distance of ~2.4 cM from the SHP1 gene on chromosome 12p13. It may be possible that transcriptional repression of the SHP1 gene by DNA methylation might be one of the critical events of lymphomagenesis or leukemogenesis after the loss of one allele of the SHP1 gene. We have preliminary results of transfection analysis with the SHP1 cDNA-expression vector, which showed the tendency of growth inhibitory effects (data not shown). Bruecher-Encke et al. (20) also reported the similar observation that expression of the exogenous SHP1 gene inhibits K562 cell proliferation. In addition to the growth inhibitory effect of the transfected SHP1 gene on hematopoietic cells, high frequency of LOH, that of DNA methylation, and that of no or decreased SHP1 gene expression in lymphomas and leukemias strongly suggest that the SHP1 gene is one of the tumor suppressor genes. DNA sequencing analysis of the coding region of the SHP1 gene revealed no mutation in some specimens of lymphoma patients and cell lines thus far, which is still ongoing for the analysis of various specimens.

DNA methylation has been thought to be one of the important mechanisms of gene silencing and development of cancer. Present investigation revealed that reduced expression of the SHP1 gene in various types of leukemias and lymphomas mainly occurred by promoter methylation. This finding is consistent with the methylation of the SHP1 gene promoter in malignant T-cell lymphoma cells (21). A greater understanding of the relationship between silencing of the SHP1 gene through the promoter methylation and the clinicopathological data may provide the basis for the additional advancements in the surgical and pharmacological treatment of malignant lymphomas and leukemias.

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References

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