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DNA methyltransferase 1 mediated aberrant methylation and silencing of SHP-1 gene in chronic myelogenous leukemia cells

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ABSTRACT

Introduction: Extensive studies on SHP-1 protein and SHP-1 mRNA revealed that the diminishment or abolishment of the expression of SHP-1 in leukemias/lymphomas was due to aberrant promoter methylation. Thus far, the mechanism of epigenetic silencing of the SHP-1 tyrosine phosphatase gene that occurs in chronic myelogenous leukemia cells remains poorly understood.
Methods: The expressions of the target molecules were determined by quantitative real time PCR and western blot, respectively. Bisulfite sequencing PCR was used to detect methylation status of DNA CpG. The lentiviral vectors were applied to modify gene expression.
Results: In the present study, we found that the promoter 2 of SHP-1 gene is located between positions from −577 bp to +300 bp, and 22 CpG sites contained in positions −353 bp ~ +182 bp are aberrantly methylated in K562 cells. *In vitro*, we demonstrated that DNMT1 silencing induced demethylation of the 22 CpG sites located in the SHP-1 promoter and re-expression of SHP-1 gene in K562 cells. Moreover, we proved that the expression levels of DNMT1 and SHP-1 mRNA and protein were negatively correlated in K562 cells and BM aspirates mononuclear cells from CML patients.
Conclusion: Collectively, these results indicate that DNMT1 mediates aberrant methylation and silencing of SHP-1 gene in chronic myelogenous leukemia cells, and provide a novel therapeutic target for CML.

1. Introduction

The SHP-1 gene is located on human chromosome 12p13 and codes a *M*r 68,000 protein of a non-receptor type protein-tyrosine phosphatase negatively regulating growth-promoting signaling molecules [1,2]. Two different and mutually exclusive tissue-specific promoters regulate the expression of the two forms of SHP-1 protein, and promoter 2 is active exclusively in cells of hematopoietic tissue [3,4]. We found the decrease of SHP-1 expression due to aberrant promoter hypermethylation in K562 cells and samples from advanced stages CML patients [5]. More studies by others demonstrated that SHP-1 promoters were methylated in a large spectrum of hematopoietic malignancies [6–8]. However the mechanism of the SHP-1 gene silencing in the malignant cells is currently unknown.

Epigenetic gene silencing plays a key role in inhibiting the expression of tumor suppressor genes in cancer cells, and methylation of DNA enriched in the CpG sequences is an important mechanism of affecting gene promoter region [9–11]. Whereas the CpG methylation is mediated by DNA methyltransferases (DNMTs) family [9,12], and it has been reported that DNMTs were overexpressed in AML and in acute phase CML [13,14]. The latest research shows that DNMT1 and EZH2 mediated methylation silences the microRNA-200b/a/429 gene and promotes tumor progression [15], and increasingly studies show that epigenetic aberrations in leukemia are early key events during leukemogenesis and disease progression [16].

In this study, we demonstrate that (1) DNMT1 silencing induced demethylation of the 22 CpG sites located in the SHP-1 promoter contained in positions −353 bp ~ +182 bp; (2) The expression levels of DNMT1 and SHP-1 mRNA and protein was negatively correlated in K562 cells and BM cells from CML patients; (3) Up-regulated DNMT1 may contribute to the disease progression in CML by inducing aberrant hypermethylation of SHP-1 promoter.

Abbreviations: CML, chronic myelogenous leukemia; DNMT, DNA methyltransferase; PCR, polymerase chain reaction; BSP, bisulfite sequencing PCR; MSP, methylation-specific PCR.

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2. Materials and methods

2.1. Cell culture

The human CML blast crisis cell line (erythroleukemia cell line) K562 (maintained in our laboratory) was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37 °C. The cells were passed or the medium was renewed every 2–3 days, and the cells were prepared for experimental procedures when they reached log-phase growth and 80% confluence.

2.2. Bisulfite sequencing PCR (BSP)

The genomic DNA was first extracted using DNA Extraction Kit (TIANGEN Biotechn Co., Ltd. Beijing, China) and then first modified by sodium bisulfite (EpiTect Fast DNA Bisulfite Kit, QIAGEN, Hilden, Germany) to convert all Cytosine (C) to uracil (U) or thymidine (T) nucleotides (U) except methylated cytosine (mC). The bisulfite-modified DNA was amplified by PCR. BSP primers were designed with MethPrimer (http://www.urogene.org/methprimer/index1.html) software based on the sequence of human SHP-1 promoter 2 from NCBI (NC_000012.12) and were synthesized by Invitrogen Corporation (Shanghai, China). PCR reaction system (20 μl): AmpliTaq Gold DNA Polymerase (ABI, USA) (5U/μl) 0.25 μl, 10 × PCR Buffer II 2 μl, bisulfite-treated DNA 1 μl (approximately 50 ng), $MgCl_2$ (25Mm) 2 μl, Forward primer, (10 μmol/L) 0.5 μl, Reverse primer, (10 μmol/L) 0.5 μl, ddH_2O 13.75 μl. Initial denaturation at 95 °C for 8 min was followed by 30 cycles of denaturation at 95 °C for 30 s, an annealing step at 65 °C (each cycle reduced 0.5 °C) for 20 s, an extension step at 72 °C for 1 min, and 20 cycles of denaturation at 95 °C for 20 s, an annealing step at 50 °C for 20 s, an extension step at 72 °C for 1 min, followed by a final extension step of 72 °C for 5 min. The PCR products were recovered by Gel DNA Extraction Kit (Dongsheng BIOTEH, Guangzhou, China) after separation by electrophoresis in 1% agarose gel. Furthemore, the recycled DNA was cloned using pMD18-T Vector Cloning Kit (TaKaRa Clontech, Dalian, China), transformed into DH5, and 10 positive clones per sample were selected for verification of the correct-sized insert via PCR using T connection-specific primers. All sequence reactions were analyzed using an automated DNA sequencer (ABI 377, Applied Biosystems). The methylation status of DNA CpG was analyzed using the bisulfite sequencing web-based tool QUMA software (http://quma.cdb.riken.jp/).

2.3. Cell line transfection

The lentiviral vectors, HSH004407-HIVmU6 (psiHIV-mU6-shDNMT1) and CSHCTR001-HIVmU6 (psiHIV-mU6-empty control) were designed, constructed and packaged by GeneCopoeia Inc., Guangzhou, China. One day before transfection, ogarithmic growth K562 cells were washed twice with serum and antibiotic-free RPMI-1640, then seeded in 6-well plates to a final concentration of 50×10^4 cells/ml. Viral infections were carried out at a MOI (multiplicity of infection) of 200. Equal volume of RPMI-1640 containing 20% fetal bovine serum was added into the transfection system after incubating for 2 h with being shaked every 30 min. All lentiviral knock-downs were selected by culturing cells in the presence of puromycin (1 μg/mL) 72 h after transfection. Finally, the transfection efficiency was evaluated using inverted fluorescence microscope to observe the cherry fluorescent protein.

2.4. SYBR Green-based qRT-PCR

Mononuclear cells were isolated from BM aspirates by centrifugation on a Ficoll-Hypaque gradient. Total RNA extraction, first strand cDNA synthesin, and SYBR Green-based quantitative PCR referred to the protocol as previously described (TRIzol reagent, Invitrogen Life Technologies, USA. All-in-One First-Strand cDNA synthesis kit and All-in-One™ qPCR Mix kit, GeneCopoeia Inc., Guangzhou, China). The primer sequences are listed in Table 1. β-actin was used as housekeeping control gene. SHP-1 and DNMT1 mRNA was standardized to β-actin levels to obtain the relative expression level. All reactions were amplified in triplicate on an ABI Prism 7900 Sequence Detector (Applied Biosystems, Foster City, CA).

Table 1
MSP and SYBR Green-based qRT-PCR: primer sequences and products.

Gene	Sense primer: (5′-3′)	Antisense primer: (5′-3′)	Products (bp)
β-actin	gagctacgagctgcctgac	ggtagtttcgtggatgccacag	121
DNMT1	gatcgagaccacggttcctc	ccggcctcgtcataactctc	114
SHP-1	aacagccgtgtcatcgtcat	atcaggtctccattgtccagc	191
SHP-1M-MSP	gaacgttattatagtatagcgttc	tcacgcatacgaacccaaacg	158
SHP-1U-MSP	tcacgcatacgaacccaaacg	ttcacacatacaaacccaaacaat	158

2.5. Methylation-specific polymerase chain reaction (MSP)

Genomic DNA was isolated, sodium bisulfite-modified and purified with the EZ DNA Methylation-Direct™ Kit (ZYMO RESEARCH, USA) according to the manufacturer's protocol. Primers (Table 1) design and PCR were referred to the previously described protocol [5].

2.6. Western blot analysis

Whole-cell protein extracts were analyzed by Western blotting with adequate mouse anti-human antibodies (SHP-1, DNMT1 and GAPDH antibodies, Santa Cruz, CA, USA), as previously described [5], using horseradish peroxidase (HRP)–labeled immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech Inc., USA), and X-ray film (Kodak, USA). Intensities of bands were quantified using Sicon ImageJ software. All experiments were performed 3 times.

2.7. Patients

Between December 2012 and June 2015, bone marrow (BM) aspirate cells were collected from 36 consecutive patients (median age of 46 years, ranging from 20 to 76; male: 20; female: 16; 19 in chronic phase (CML-CP); 7 in accelerated phase (CML-AP) and 10 in blastic phase (CML-BP)) with CML treated at Harrison International Peace Hospital under an approved institutional protocol. The diagnosis was based on morphology, karyotype analysis and subsequently confirmed by the presence of BCR-ABL1P^{210} fusion gene. This study was approved by the Medical Ethics Committee of Harrison International Peace Hospital.

2.8. Statistical analysis

Values represent the mean ± SD of at least 3 independent experiments. Data were analyzed statistically using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA, 5695502). Data were tested for normal distribution. One-way ANOVA analysis (LSD), Independent-Samples T test and Linear regression analysis were applied, p-values < 0.05 were considered statistically significant.