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Research on epigenetic mechanism of SFRP2 in advanced chronic myeloid leukemia

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ABSTRACT

Secreted frizzled-related protein 2 (SFRP2) has been reported to act as a tumor suppressors. This study aims to detect the biological role of SFRP2 in advanced chronic myeloid leukemia (CML). In this study we examined bone marrow samples from 45 CML patients and 10 healthy donors. K562 and KCL22 cells were cultured and treated with demethylation drug and histone deacetylase inhibitor (HDACi). KCL22 and K562 cells were transfected with lentiviral vector (LV)-SFRP2, LV-control. The cells were then subjected to proliferation and apoptosis assays, real time polymerase chain reaction (PCR), Methylation-specific PCR (MSP), Western blotting, co-immunoprecipitation (CoIP) and Chromatin immunoprecipitation (ChIP), We found that SFRP2 was down-regulated in the accelerated and blast phase of CML, whereas, the levels of WNT1, WNT3 and WNT5A were up-regulated in the accelerated and blast phase of CML. Overexpression SFRP2 inhibited proliferation, promoted apoptosis and activated the WNT pathway. CoIP-MS results showed that SFRP2 interacted with WNT1 and WNT5A. ChIP-seq result indicated that the promoter of H3K4me3 and H3K27me3 were able to interact with SFRP2. In conclusion, our findings demonstrated the SFRP2 act as a potential therapeutic target for advanced CML. Furthermore, our results support the use of demethylation drugs and HDACi as a potential CML treatment strategy.

1. Introduction

Chronic myeloid leukemia (CML) is a clonal disease characterized by the Philadelphia chromosome, which contains the BCR/ABL1 fusion gene that encodes the BCR/ABL1 P210 protein [1]. Typically, patients with CML pass through three phases: a chronic phase (CP), an accelerated phase (AP), and a blast phase (BP), accelerated phase and blast phase means the progression of the disease [2]. Patients who progress to blast phase even receive standard a regiment similar to acute leukemia; the effects were still poor and always had a poor prognosis [3]. The development of CML was very complex and involved gene mutations, chromosomal variations, and epigenetic regulations of genes that were reported to contribute to the progression of CML [2,4].

Epigenetics involves heritable changes in gene expression, such as DNA methylation and chromatin conformation, without any corresponding changes in the DNA sequence [5]. Abnormal DNA promoters were reported to be involved in tumorigenesis [6]. SFRPs were reported to inhibit invasion but had no effect on proliferation, which function as tumor suppressor. In colorectal cancer, the methylation of SFRP2 promoter was detected in tumor tissue [7,8]. In non−small cell lung cancer, low level of SFRP2 was found in the tumor tissue of non−small cell lung cancer [9,10]. Although, the biological role of SFRP2 in many kinds of cancers had been elucidated, the role of SFRP2 had not been detected. Therefore, we aimed to explore the epigenetic regulation of SFRP2 in advanced chronic myeloid leukemia.

2. Materials and methods

2.1. Specimen collection

Bone marrow samples were collected from 40 CML patients, who were admitted to the Department of Hematology of the Second Hospital of Hebei Medical University between May 2015 and June 2017 (Table 1). Bone marrow samples from 10 healthy donors were selected to serve as controls. Bone marrow mononuclear cells were isolated via lymphocyte separation. This study was approved by the Ethics Committee of the Department of Hematology of the

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Table 1
Characteristics of the patients included in the study.

Item	CML-CP (n = 20)	CML-AP (n = 10)	CML-BP (n = 10)
Age (years), median (range)	41.4 (9–65)	49.1 (13–69)	51.9 (20–69)
Male/female, (n/n)	15/5	6/4	6/4
WBCs $\times 10^9$/median (range)	221.4 (30.2–517)	263.5 (47.4–396)	69.5 (27.4–224)
Hemoglobin level (g/L)	94 (76–120)	75 (61–105)	62.4 (52–79)
Platelet count,10^9/median (range)	518 (99–809)	305 (52–725)	35.5 (19–71)

AP, accelerated phase; BP, blast phase; CML, chronic myeloid leukemia; CP, chronic phase; WBC, white blood cells.

Second Hospital of Hebei Medical University, and each patient signed informed consent. The inclusion criteria were (i) diagnosis of CML via bone marrow morphology, immunology, molecular biology and cytogenetic; (ii) clear pathological staging; and (iii) availability of intact clinical data. The exclusion criteria were (iv) significant organ dysfunction; (v) pregnancy (in females); and (vi) failure to provide informed consent. No chemotherapy was administered before the specimens were collected.

2.2. Cell culture

KCL22 and K562 cells were maintained in our laboratory. KCL22 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco, Beijing, China), which contained 10% fetal bovine serum (FBS) (Clark Bio, Claymont, DE, USA), 100units/ml penicillin and 100 μg/ml streptomycin. K562 cells were cultured in RPMI 1640 medium (Gibco, Beijing, China) supplemented with 10% FBS and the two antibiotics listed above.

2.3. Cell treatment

5-Azacytidine and chidamide were purchased from ApexBio, (Houston, TX, USA). KCL22 and K562 cells were seeded in 6-well plates at a density of 1×10^6 cells per well. MTT assays were performed to detect the EC50 concentrations of 5-azacytidine. K562 and KCL22 were treated with drugs according to the respective EC50 values. KCL22 cells were treated with 60, 80 and 100 μmol/l 5-azacytidine; K562 cells were treated with 120, 160 and 200 μmol/l 5-azacytidine. Both KCL22 and K562 were treated with 5, 10 and 15 μmol/l chidamide. The KCL22 and K562 cells were treated with 5-azacytidine and chidamide for 48 h.

2.4. Cell transfection

The full-length cDNA sequence of SFRP2 was synthesized by Invitrogen and then subsequently cloned into the pLVX-hSFRP2-Puro lentiviral overexpression vector, which was constructed as described previously. KCL22 and K562 cells were seeded onto 6-well plates (5×10^5/well) and then subsequently transfected with the LV-SFRP2 or LV-control.

2.5. MTT assays

We suspended KCL22 and K562 cells and seeded them into 96-well plates (1×10^5 cells/well). We cultured them for 0, 24, 48, 72, 96 h using IMDM medium and 1640 with 10% FBS at 37 °C. The proliferation of KCL22 and K562 cells was determined by using an MTT assay. Briefly, following cell culture, 10 μl of 3-[4,5-dimethylthiazole-2-yl]-2,5- diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) was added to each well and the 96-well plates were incubated at 37 °C in a humidified 5% CO2 atmosphere for 4 h. Absorbance was then read at 490 nm, in a microplate reader (Thermo Fisher USA).

2.6. Apoptosis assays

KCL22 and K562 cells were seeded into 6-well plates after transfection and were treated according to the manufacturer's instructions. The cells were stained with 5 μl AnnexinV-APC AnnexinV-FITC and 5 μl PI (BD Bioscience Pharmingen USA) and then analyzed with a BD FACSCanto II system (BD USA). The apoptosis were performed using BDFAC-Diva (BD, USA).

2.7. Real-time quantitative RT-PCR

Total RNA was isolated with Trizol (Life Technologies, Invitrogen, USA), and RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher, USA) was used to synthesize cDNA according to the manufacturer's instructions. The reaction was performed at 42 °C for 60 min, followed by 25 °C for 5 min and then 70 °C for 5 min. Polymerase chain reaction (PCR) amplification was performed in 20 μl final volumes containing 1 μl of template cDNA, 1 μl of sense primer, 1 μl of antisense primer, 10 μl of SYBR Green Mix and 8 μl of DEPC water. Quantitative real-time RT–PCR was carried out using ABI 7500 real-time rotary analysis (ABI Life Science). Real-time PCR primers are shown in Table 2. For each run, each reaction was repeated independently at least three times. RT-PCR was performed under the following conditions: denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 35 s, and 72 °C for 20 s. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression.

2.8. Methylation-specific PCR

Genomic DNA was extracted and the specific steps were performed according to the instructions of the manufacturer (Shanghai Generay Biotech Co. Ltd, Shanghai, China). DNA concentration was detected at −80 °C. The sulfite conversion of DNA was performed according to the instructions of the EZ DNA methylation-Gold kit (Zymo USA Inc, Fleming Island, FL, USA). For all reactions, the reaction mix contained bisulfite-modified DNA (2 μl), Zymo Taq PerMix (12.5 μl), water (8.5 μl), upstream primer (1 μl), and downstream primer (1 μl). The PCR conditions were as follows: 95 °C for 10 min; followed by 35 cycles of 30 s at 95 °C, 45 s at 54 °C for annealing, and 45 s at 72 °C; and a final extension

Table 2
Primer sequences for RT-PCR.

Gene	Primer	Product
SFRP2	Forward: 5′-CTCCCCTTCTAGCGCTCACG-3′ Reverse: 5′-CTAGCCGCCGTCTATACTACCGGCT-3′	154 bp
WNT1	Forward: 5′-AGACACTCGCTCAGCTTCTTG-3′ Reverse: 5′-CAATTGCTGCTGGGATTCATC-3′	116 bp
WNT3	Forward: 5′-CTGCTTTGTATTCCCTTTTGCA-3′ Reverse: 5′-TTGATTTCTCCTGGCTGTCTC-3′	141 bp
WNT5A	Forward: 5-GTGCCTAGTAGAGGGTTTG-3′ Reverse: 5- TTATACCCCACAACGCGTAG-3′	138bp
ACTB	Forward: 5′-GAGCTACGAGCTGCCTGAC-3′ Reverse: 5′-GGTAGTTTCGTGGATGCCACAG-3′	121 bp

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