

Role and mechanism of decitabine combined with tyrosine kinase inhibitors in advanced chronic myeloid leukemia cells

LI-CAI JIANG and JIAN-MIN LUO

Department of Hematology, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei 050000, P.R. China

Received September 6, 2015; Accepted January 13, 2017

DOI: 10.3892/ol.2017.6318

Abstract. Patients with advanced chronic myeloid leukemia (CML) have a poor prognosis, with the use of tyrosine kinase inhibitors (TKIs) to treat CML demonstrating poor results. The results of the present study revealed that, following Cell Counting Kit-8 analysis, treatment of K562 cells with decitabine (DAC) combined with TKIs exhibits synergic effects. Co-immunoprecipitation indicated that tyrosine-protein phosphatase non-receptor type 6 (SHP-1) and BCR-ABL fusion protein (BCR-ABL) (p210) form a complex in the K562 cell line, and in the primary cells derived from patients with CML. These results suggested that SHP-1 serves a role in regulating the tyrosine kinase activity of BCR-ABL (p210). In addition, SHP-1 expression increased, while BCR-ABL expression decreased in the group treated with DAC and TKIs combined group compared with the TKI monotherapy group. Treatment with imatinib (IM) demonstrated no effect on SHP-1 methylation in the K562 cell line; however, the methylation of SHP-1 was not determined in the combined IM and DAC therapy group. Treatment with DAC demonstrated the ability to activate the expression of silenced SHP-1 through demethylation, thus decreasing BCR-ABL tyrosine kinase activity, resulting in an improved therapeutic effect on CML.

Introduction

Chronic myeloid leukemia (CML) is a clonal bone marrow stem cell disorder in which the proliferation of mature granulocytes and their precursor myeloid cells occurs. CML accounts for 15% of all leukemia cases in adults (1). The global annual incidence rate of CML is between 1.6 and 2 cases/100,000 people (2), while epidemiological surveys demonstrate an

annual incidence of 0.39-0.55 cases/100,000 people in several areas of China (2). Patients with CML in China are younger compared with those in western countries (3).

CML is associated with the presence of the Philadelphia (Ph) chromosome. This chromosomal translocation is termed t(9, 22)(q34, q11). This translocation generates an oncogenic BCR-ABL fusion gene, encoding the BCR-ABL fusion protein (BCR-ABL) of 210 kDa (4). As the ABL gene encodes a domain that can add phosphate groups to tyrosine residues, BCR-ABL possesses tyrosine kinase activity (5,6). Other molecules typically control the activity of tyrosine kinases, but the mutant tyrosine kinase of the BCR-ABL transcript encodes for a continuously activated protein, resulting in unregulated cell division and consequent tumor development. Therefore, tyrosine kinase inhibitors such as imatinib (IM), became the first-line treatment against a variety of cancers, including CML. Although IM demonstrated a successful effect in the majority of patients, 10-15% of patients developed drug resistance or exhibited disease progression into the CML accelerated phase due to a late and thus less effective treatment. Once the tumor progresses into the blast phase, the median progression-free survival time of patients is 4 months with a median overall survival time of 7 months (7).

At present, allogeneic stem cell transplantation, bone marrow transplantation and tyrosine kinase inhibitors (TKIs) represent the treatments available for patients with advanced CML. In addition to these treatments, other targeted drugs have been considered as potential candidates. The demethylating drug decitabine (DAC) has been successfully used in the treatment of myelodysplastic syndrome and acute myeloid leukemia in the elderly (8). Several clinical studies (9-13) demonstrated that DAC monotherapy exhibits a substantial effect during all the phases of CML. DAC is a DNA methylation inhibitor, enabling the expression of silenced tumor suppressor genes (8). Src homology region 2 (SH2) domain-containing tyrosine phosphatase-1 (SHP-1) is a gene primarily expressed in hematopoietic cells and is considered as a hematopoietic cell malignancy tumor suppressor gene (14,15). It serves an important role in the negative regulation of growth factor receptor-mediated signal transduction processes. (14,15). The results of a previous study (16) demonstrated that there is a significant decrease in SHP-1 expression in patients with advanced CML compared with chronic phase CML, suggesting that SHP-1 serves a role in the onset of the blast crisis of CML. Previous studies have suggested that the decreased SHP-1 expression in a variety of

Correspondence to: Professor Jian-Min Luo, Department of Hematology, The Second Hospital of Hebei Medical University, 215 Heping Xi Road, Shijiazhuang, Hebei 050000, P.R. China
E-mail: luojm2011@163.com

Key words: tyrosine-protein phosphatase non-receptor type 6, demethylation, tyrosine kinase inhibitors, chronic myeloid leukemia, immunoprecipitation

hematologic malignancies is associated with the methylation of the SHP-1 gene promoter (17-21). A previous study demonstrated that SHP-1 forms a complex with BCR-ABL through immunoprecipitation in immortalized myeloblast-like 32D and fibroblast 3T3 cell lines, suggesting that SHP-1 regulates BCR-ABL (22). Imbalanced levels of tyrosine phosphatase and tyrosine kinase serve an important role in cell proliferation and apoptosis. As BCR-ABL is continuously activated, patients with CML-blastic phase (CML-BP) exhibit enhanced tyrosine kinase activity (23). As DAC enables the expression of silenced tumor suppressor genes due to its DNA methylation inhibitor function, it may possess a role in SHP-1 expression and function. Therefore, the present study aimed to investigate the mechanism of action of DAC on restoring the expression of SHP-1 in CML, compared with the effect of tyrosine kinase inhibitors, to provide a novel strategy to prevent the progression of CML and treat patients with CML.

Materials and methods

Drugs and reagents. IM and nilotinib were provided by Novartis International AG (Basel, Switzerland). DAC was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The Cell Counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

Cell culture. The human immortalized myelogenous leukemia K562 cell line (Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China) was preserved in the Province Key Laboratory of Experimental Hematology (The Second Affiliated Hospital of Hebei Medical University, Shijiazhuang, China). The K562 cells were suspended in RPMI-1640 medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated at 37°C with 5% CO₂. Subsequently, cells were seeded into 96-well plates at a density of 1x10⁵ cells/well and incubated for 2-3 days prior to treatment with 0.1 μM IM, 5 nM nilotinib, 5 μM DAC, 0.1 μM IM + 5 μM DAC, or 5 nM nilotinib + 5 μM DAC at 37°C for 48 h. Subsequently, the cells were incubated with 10 μl CCK-8 reagent for 2 h at 37°C and the optical density (UV-visible NanoDrop 8000 spectrophotometer; Thermo Fisher Scientific, Inc.) was measured at 450 nm. The % of cell proliferation was calculated as follows: (Untreated negative control group-experimental group)/(untreated negative control group-blank group)x100%.

Primary cell culture of mononuclear cells from patients with CML. A total of 10 patients (4 male, 6 female; median age, 40.5 years; age range, 21-60 years) with CML-chronic phase (CML-CP) and 6 patients (5 male, 1 female; median age, 42.5 years; range, 35-50 years) with CML-BP were selected between October 2013 and October 2014 at The Second Affiliated Hospital of Hebei Medical University (Shijiazhuang, China). This study was approved by the ethics committee of The Second Hospital of Hebei Medical University. The definitions of chronic and blast phase CML were obtained from European Leukemia Net 2013 (24). A total of 4 ml fresh heparin marrow was taken from patients with chronic, accelerated or blast phase CML using an aseptic technique. An

equal volume of RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) was added to each sample, then mixed and dried. Subsequently, the bone marrow was diluted with 4 ml of lymphocyte separation medium (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and centrifuged at 670.8 x g for 20 min at 4°C. The middle layer containing the mononuclear cells was collected. Following joining the high-pressure liquid PBS 167.7 (x g) rinsing 5 min at 4°C. Subsequently, mononuclear cells were seeded into the flasks at a density of 1x10⁶ in RPMI-1640 medium containing 20% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.).

Measuring SHP-1 expression through reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Following harvesting of each group, total RNA was extracted using the TRIzol method. Reverse transcription was performed using the All-in-One™ First-Strand cDNA Synthesis kit (GeneCopoeia Inc., Rockville, MD, USA), according to the manufacturer's protocol. The protocol was as follows: RNA degeneration at 65°C for 10 min and reverse transcription at 37°C for 60 min. Complementary (c) DNA was synthesized in a total volume of 25 μl of working solution (5 μl 5X RT Reaction Buffer, 1 μl 250 μM random primers, 1 μl 25 mM dNTP, 1 μl 25 U/μl RNase inhibitor, 1 μl 200 U/μl Moloney murine leukemia virus reverse transcriptase, 1 μg total RNA and deionized H₂O to make up to 25 μl). The qPCR reaction was performed in a total volume of 20 μl of working solution [9 μl SYBR Green Real-Time MasterMix (Invitrogen; Thermo Fisher Scientific, Inc.) solution, 0.5 μl SHP-1 forward primer (AACAGCCGTGTCATC GTCAT), 0.5 μl SHP-1 reverse primer (ATCAGGTCTCCA TTGTCCAGC), 2 μl cDNA, 8 μl deionized H₂O] at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 68°C for 45 sec. The reaction was performed in a Rotor-Gene Q 6000/3000 Quantitative PCR instrument (Qiagen GmbH, Hilden, Germany). Relative SHP-1 expression in the treated and control groups was calculated using the 2^{-ΔΔC_q} method (25) using β-actin (forward primer, GAGCTA CGAGCTGCCTGAC; reverse primer, GGTAGTTTCGTG GATGCCACAG) as a reference gene.

RT-qPCR determination of absolute BCR-ABL gene expression. Following harvesting each group of cells, RNA was extracted using the TRIzol method and the RNA concentration was measured using the NanoDrop 8000 spectrophotometer. The RT-qPCR reaction to amplify the BCR-ABL gene was performed in a total volume of 25 μl working solution using a BCR-ABL210 Fusion Gene Detection kit (Shanghai YuanQi Biomedical Technology Co., Ltd., Shanghai, China), according to the manufacturer's protocol. The reaction was performed in a Rotor-Gene Q 6000/3000 Quantitative PCR instrument. BCR-ABL expression was calculated as follows: BCR-ABL (p210) mRNA levels (%)=(BCR-ABL copy number/ABL copy number)x100.

Immunoprecipitation and western blot analysis. A total of 200 μl RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP40, 0.5% sodium deoxycholate) and 2 μl phenylmethylsulfonyl fluoride were added to the CML and K562 cells, and incubated on ice for 30 min to obtain the cell lysate. Next,

the cells were centrifuged at $10,732.8 \times g$ for 10 min at 4°C , and the supernatant containing the proteins was collected. The protein was quantified using a Coomassie (Bradford) Protein Assay kit Nanjing Jiancheng Bioengineering Institute, China). Subsequently, $10 \mu\text{l}$ c-ABL rabbit polyclonal IgG antibody (cat. no. sc-131; dilution, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was added to $100 \mu\text{g}$ of each protein sample. Intracellular pH (IPH) buffer [10 ml 1 M Tris-HCl (pH 8.0), 15 ml 2 M NaCl, 2 ml 0.5 M EDTA, 1 ml NP-40, 172 ml triple-distilled water] was added to reach a total volume of $300 \mu\text{l}$ in 1.5 ml eppendorf (EP) tubes. The EP tubes were subsequently shaken for 1 h at 4°C . Then, $25 \mu\text{l}$ protein A/G PLUS-Agarose (cat. no. sc-2003; Santa Cruz Biotechnology, Inc.) was added and the EP tubes were shaken overnight at 4°C . EP tubes were subsequently washed with IPH buffer 3-4 times.

The supernatant was discarded and $35 \mu\text{l}$ 2X SDS buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added, centrifuged at $24148.8 \times g$ for 1 min at 4°C , boiled at 100°C for 7-8 min, and separated on an 8% gel using SDS-PAGE (300 mA) for 2 h, prior to being transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was removed and blocked in 5% bovine serum albumin (Roche Diagnostics, Basel, Switzerland) for 1 h at 4°C . The PVDF membrane was transferred to a hermetic bag, rabbit polyclonal IgG primary antibody directed against SHP-1 (cat. no. sc-287; dilution, 1:200; Santa Cruz Biotechnology, Inc.) was added and the membrane was incubated in the dark for 1 h at 4°C . The membrane was washed with 1X TBS-Tween 20 (TBS-T) 3 times and incubated with goat anti-rabbit IgG HRP-conjugated secondary antibody (cat. no. sc-2004; dilution, 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at 4°C . Finally, the membrane was washed with 1X TBS-T 3 times, and the fluorescence was developed using Potent ECL kit [Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China]. The software used to analyze the protein band density was Image-ProPlus (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Methylation-specific PCR (MSP). The following SHP-1 primers (26) were used: SHP-1 methylated MSP forward, 5'-GAACGTTATTATAGTATAGCGTTC-3' and SHP-1 methylated MSP reverse, 5'-TCACGCATACGAACCCAAACG-3'; SHP-1 demethylation MSP forward, 5'-GTGAATGTTATTATAGTATAGTGTGTTGG-3' and SHP-1 demethylation MSP reverse, 5'-TTCACACATACAAACCCAAACAAT-3'. MSP was performed according to the EZ-DNA Methylation kit (Zymo Research Corp, Irvine, CA, USA) protocol. The total PCR reaction volume was $25 \mu\text{l}$ and consisted of the following: $5 \mu\text{l}$ bisulfite-converted DNA; $2 \mu\text{l}$ forward primers; $2 \mu\text{l}$ reverse primers; $12.5 \mu\text{l}$ Green PCR system (Thermo Fisher Scientific, Inc.); and $3.5 \mu\text{l}$ ddH₂O. PCR thermocycling conditions were as follows: Initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 45 sec; annealing at 54°C for 45 sec; extension at 72°C for 45 sec; and a final extension at 72°C for 10 min. A total of $10 \mu\text{l}$ of the PCR amplification product was collected from each group and the products were detected using GoldView™ nucleic acid dyes in a 2% agarose gel electrophoresis running at 90 V for 60 min, then visualized using a gel imaging system (Dinco Ltd., Tirat Carmel, Israel).

Calculation of combination index (CI). The CI of two drugs was calculated to evaluate their potential synergistic effect, using the calculation previously described by Soriano *et al* (27): DA/ICX, A + DB/ICX, B. A and B represent two drugs separately; ICX, A and ICX, B are the concentrations of two drugs used alone to achieve the drug growth inhibition rate X; and DA and DB are the combinations of two drugs that achieve growth inhibition rate X. According to Soriano *et al* (27) the synergistic effect is classified as follows: $0.9 \leq \text{CI} \leq 1.1$, additive effect; $0.8 \leq \text{CI} < 0.9$, slight synergism; $0.6 \leq \text{CI} < 0.8$, moderate synergism; $0.4 \leq \text{CI} < 0.6$, synergism; $0.2 \leq \text{CI} < 0.4$, strong synergism.

Statistical analysis. Each experiment was repeated ≥ 3 times. All data are presented as the mean \pm standard deviation, and the statistical analysis was performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). The one-way analysis of variance with Tukey's post hoc test was used for multiple comparisons. The Student's t-test was used to compare two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

K562 cell proliferation inhibition rate is significantly increased in the DAC+TKI groups (IM+DAC, and nilotinib+DAC) compared with the TKI monotherapy group. OD values of K562 cells following 48 h drug treatment were measured using a CCK-8, and the EC₅₀ values were calculated (DAC, 6,457 nM, Fig. 1A; nilotinib EC₅₀, 10.55 nM, Fig. 1B; IM EC₅₀, 227.5 nM, Fig. 1C). K562 cells treated with $5 \mu\text{M}$ DAC combined with $0.1 \mu\text{M}$ IM ($P < 0.0001$; vs. IM monotherapy) or 5 nM nilotinib ($P = 0.0015$; vs. nilotinib monotherapy) demonstrated a significant inhibition in proliferation compared with the corresponding IM or nilotinib monotherapy treatment groups (Fig. 1D). According to the Chou and Talalay calculation method, the results of the present study revealed that the drug combinations used exhibited a synergistic effect as follows: IM+DAC $\text{CI} = (0.1/0.625) + (5/20) = 0.41$, synergism; nilotinib+DAC $\text{CI} = (0.01/0.1) + (5/22) = 0.33$, strong synergism.

Determination of SHP-1 protein expression in K562 cells, and CML-CP and CML-BP mononuclear cells through immunoprecipitation. The immunoprecipitation analysis revealed that SHP-1 protein was expressed in K562 cells, and CML-CP and CML-BP mononuclear cells (Fig. 2A). The expression of SHP-1 was significantly lower in K562 cells compared with CML-CP mononuclear cells ($P < 0.001$), and significantly lower in CML-BP compared with CML-CP cells ($P < 0.01$; Fig. 2B). These results indicate that decreased SHP-1 expression is associated with CML disease progression.

SHP-1 protein expression is significantly increased in the TKI+DAC group compared with the TKI monotherapy group in K562 cells. SHP-1 protein expression was significantly increased in the IM+DAC group compared with the IM monotherapy group ($P < 0.01$; Fig. 2C and D). SHP-1 protein expression was significantly increased in the nilotinib+DAC group compared with the nilotinib monotherapy group ($P < 0.05$). No significant difference was identified in SHP-1

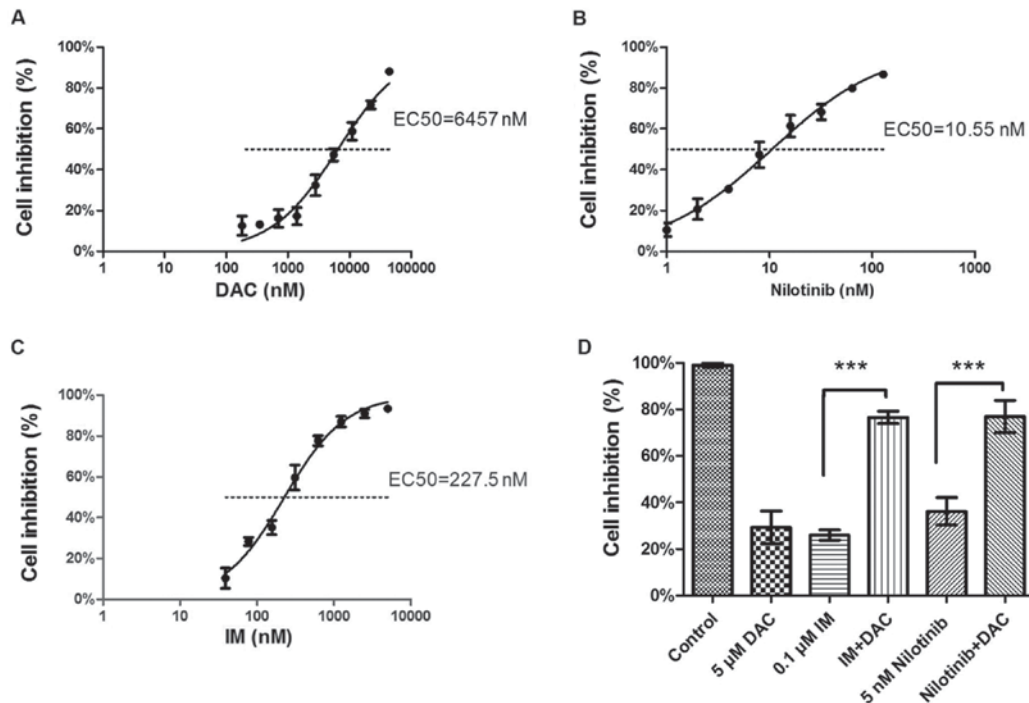


Figure 1. Optical density values of K562 cells following 48 h drug-treatment with (A) DAC (EC₅₀, 6457 nM), (B) nilotinib (EC₅₀, 10.55 nM) and (C) IM (EC₅₀, 227.5 nM) were measured using a Cell Counting Kit-8. (D) K562 cells treated with 5 μM DAC combined with 0.1 μM IM or 5 nM nilotinib exhibited proliferation inhibition that was statistically significant compared with the IM or nilotinib monotherapy treatment groups. ***P<0.001. DAC, decitabine; IM, imatinib; EC₅₀, half maximal effective concentration.

protein expression between the control and IM monotherapy group (Fig. 2C and D).

SHP-1 and BCR-ABL mRNA expression in K562 cells. RT-qPCR results revealed no significant difference in SHP-1 gene expression between IM and nilotinib monotherapy treatment groups and the control group (Fig. 3A). The DAC group demonstrated a marked increase in SHP-1 expression compared with the control group. IM+DAC group demonstrated a significant increase in SHP-1 expression compared with IM monotherapy group, while BCR/ABL mRNA expression decreased in a marked manner in the combined group compared with the IM monotherapy group. The nilotinib+DAC group revealed a significant increase in the expression of SHP-1 compared with in the nilotinib monotherapy group, while BCR-ABL and ABL copy number ratio levels decreased in a significant manner in the combined group respect nilotinib monotherapy group. No significant differences were identified in the nilotinib monotherapy BCR-ABL and ABL copy number ratio compared with in the DAC+IM group (Fig. 3A).

SHP-1 and BCR-ABL gene and protein expression in patients with CML 48 h following drug treatment. SHP-1 gene expression was measured using RT-qPCR analysis in patients with CML (control), IM, DAC and IM+DAC groups. The results revealed that SHP-1 gene expression was significantly increased in the IM+DAC group compared with the IM monotherapy group (P<0.001; Fig. 3B), while BCR-ABL gene expression was significantly decreased in IM+DAC group compared with the IM monotherapy group (P<0.05; Fig. 3C). SHP-1 protein expression was measured through immunoprecipitation in

patients with CML (control group), IM, DAC and IM+DAC groups. SHP-1 protein expression was markedly increased and BCR-ABL (p210) protein expression was markedly decreased in the IM+DAC group compared with the IM monotherapy group (Fig. 4).

SHP-1 gene methylation levels in K562 cells 48 h following drug treatment. SHP-1 gene methylation levels of K562 cells were measured using MSP analysis in the control, IM, DAC and IM+DAC groups. The results demonstrated the presence of SHP-1 gene methylation in the control and IM monotherapy group, while no methylation was demonstrated in the IM+DAC and DAC monotherapy group (Fig. 5).

Discussion

K562 cells are derived from Philadelphia chromosome-positive chronic myelogenous leukemia blast erythro-megakaryoblastic leukemia cell lines (28). A CCK-8 assay was used to investigate the proliferative ability of the K562 control and the IM, nilotinib, DAC, IM+DAC, and nilotinib+DAC treatment groups. DAC demonstrated a proliferation inhibition, which was dosage-dependent. A significant difference in cell proliferation was identified between 0.1 μM IM and IM + 5 μM DAC, in addition to between 10 nM nilotinib and nilotinib + 5 μM DAC. Therefore, the combinations of these drugs possess high synergy. The concentration of DAC used was according to the results obtained through CCK-8 analysis, and confirmed by a previous study by Momparler *et al* (29). The concentration of IM and nilotinib used was according to the result obtained through CCK-8 analysis, and confirmed by a previous study by

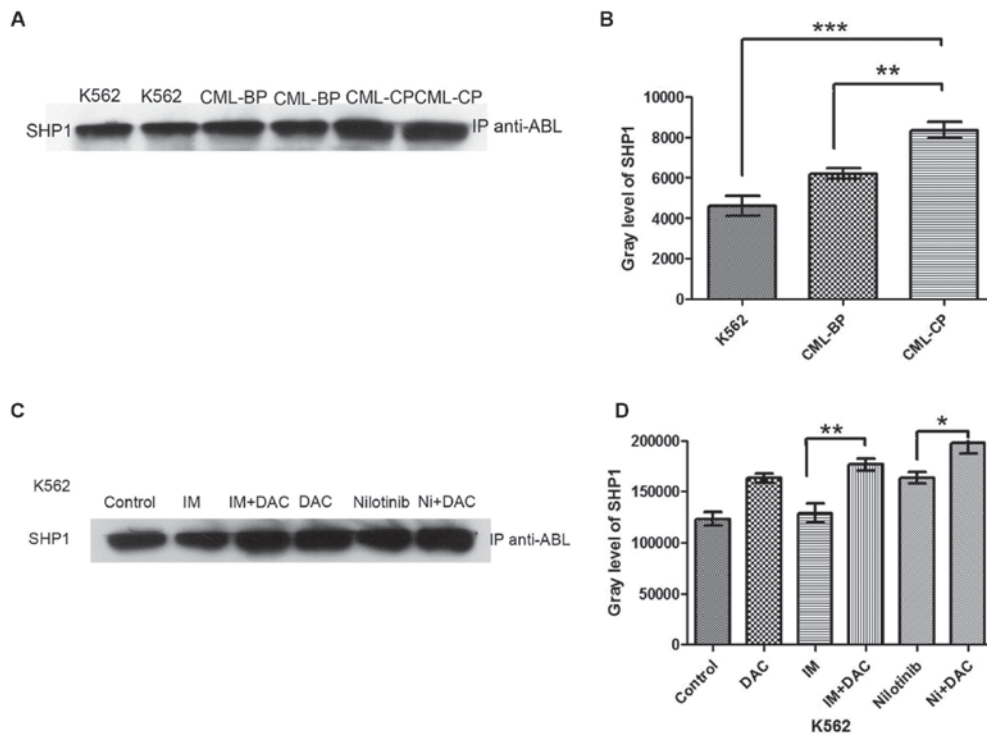


Figure 2. Western blotting analysis of SHP-1 expression. (A) Representative image and (B) quantitative analysis of SHP-1 expression in K562 cells, and CML-CP and CML-BP mononuclear cells through immunoprecipitation. (C) Representative image and (D) quantitative analysis of SHP-1 expression in K562 cells through immunoprecipitation following 48 h drug-treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. DAC, decitabine; IM, imatinib; CML, chronic myeloid leukemia; CP, chronic phase; BP, blastic phase; SHP-1, tyrosine-protein phosphatase non-receptor type 6.

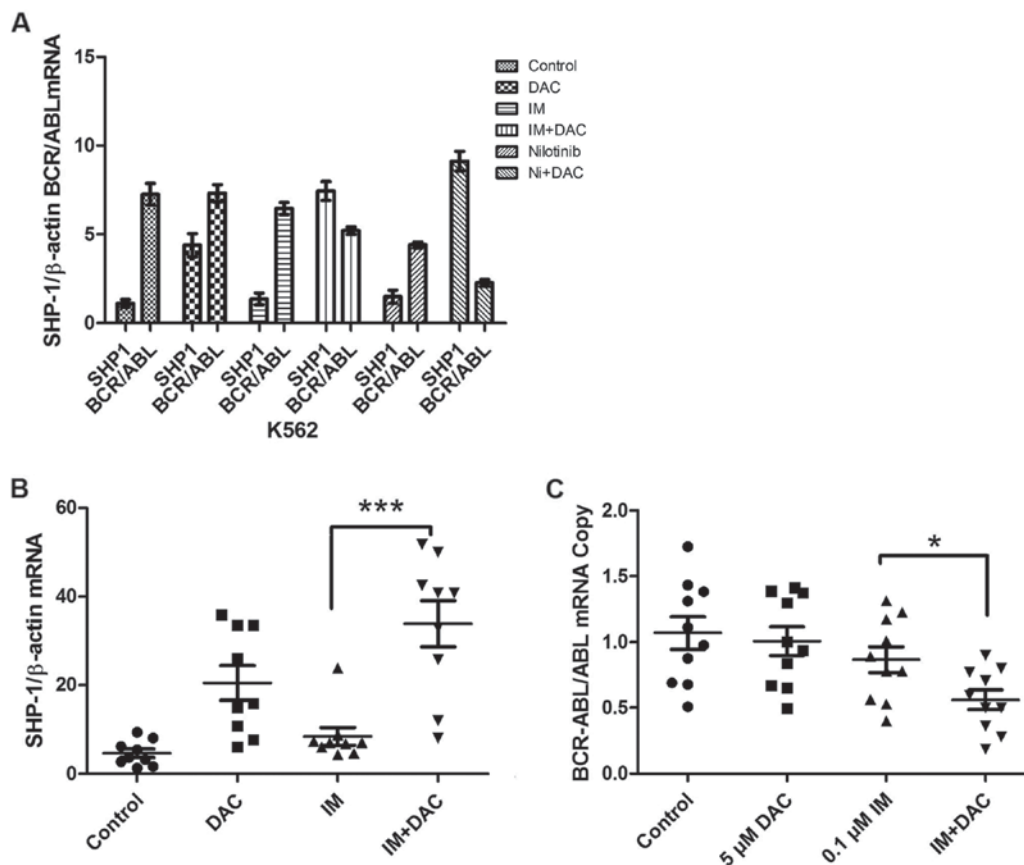


Figure 3. RT-qPCR analysis of SHP-1 and BCR/ABL mRNA expression. (A) SHP-1 and BCR/ABL mRNA expression in K562 cells following 48 h drug-treated through RT-qPCR analysis. Expression of (B) SHP-1 and (C) BCR/ABL mRNA expression in chronic myeloid leukemia-chronic phase mononuclear cells following 48 h drug treatment through RT-qPCR analysis. * $P < 0.05$, *** $P < 0.001$. SHP-1, tyrosine-protein phosphatase non-receptor type 6; RT-qPCR, real-time quantitative polymerase chain reaction; mRNA, microRNA; DAC, decitabine; IM, imatinib; BCR/ABL, BCR-ABL fusion protein.

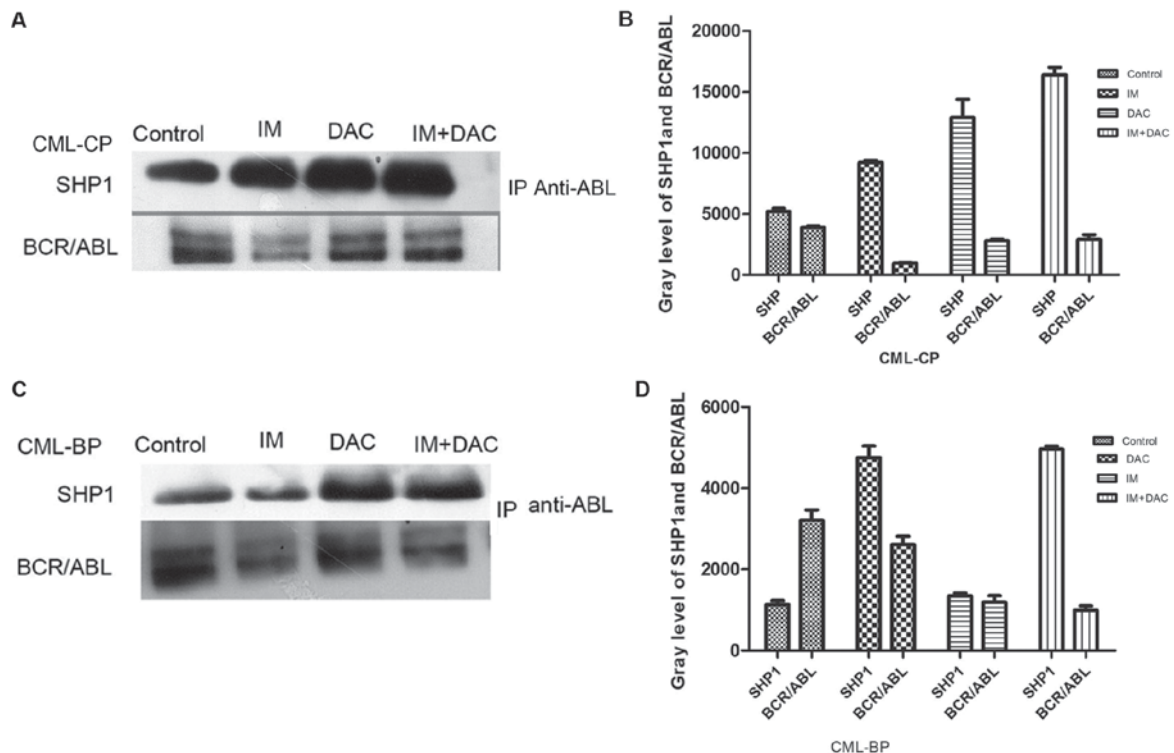


Figure 4. Western blotting analysis of SHP-1 and BCR/ABL expression. (A) Representative image and (B) quantitative analysis of SHP-1 expression in CML-CP mononuclear cells following 48 h drug treatment through immunoprecipitation. (C) Representative image and (D) quantitative analysis of SHP-1 expression in CML-BP mononuclear cells following 48 h drug treatment through immunoprecipitation. SHP-1 protein expression increased in the combined groups compared with the monotherapy groups, while BCR-ABL expression decreased. CML, chronic myeloid leukemia; CP, chronic phase; BP, blastic phase; SHP-1, tyrosine-protein phosphatase non-receptor type 6; DAC, decitabine; IM, imatinib; BCR/ABL, BCR-ABL fusion protein.

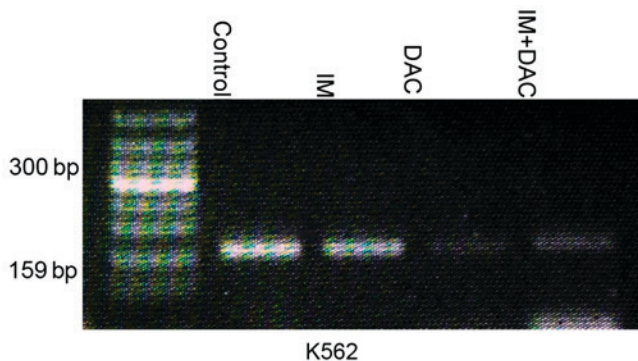


Figure 5. SHP-1 gene methylation levels of K562 cells were measured through methylation-specific PCR in the control, IM, DAC, and IM combined with DAC group. The results demonstrated the presence of SHP-1 gene methylation in the control and IM monotherapy group, while no methylation was demonstrated in the IM+DAC and DAC monotherapy group. DAC, decitabine; IM, imatinib; SHP-1, tyrosine-protein phosphatase non-receptor type 6.

Savage and Antman (30). The effects of TKIs in combination with low dose DAC were more effective than monotherapy in the K562 cell line.

DAC is a hypomethylating cytosine analogue used in the treatment of myeloid malignancies due to its ability to activate silent tumor suppressor genes through demethylation. SHP-1 is a non-transmembrane protein tyrosine phosphatase containing two N-terminal SH2 domains that is primarily expressed in hematopoietic cells (31). It has been

demonstrated to be associated with the negative regulation of signaling pathways mediated by growth factor, cytokine and antigen receptors (31). Several studies have demonstrated that the expression of SHP-1 is decreased in lymphoma (17), leukemia (18), multiple myeloma (19), cervical cancer (20) and colorectal cancer (21) due to the methylation of the promoter region of the SHP-1 gene. In a previous study, SHP-1 was identified in a complex with BCR-ABL in 3T3 cells and 32D/Bcr-Abl cells through immunoprecipitation analysis, and an association between SHP-1 and Bcr-Abl expression was demonstrated (22). The results of the present study obtained through immunoprecipitation demonstrated that SHP-1 forms a complex with BCR-ABL in K562 cells and primary cells derived from patients with CML. SHP-1 may be a type of tyrosine phosphatase, which control the activity of BCR/ABL (p210) tyrosine kinase since in the present study, the expression of SHP-1 was demonstrated to be significantly decreased in K562 cells compared with in patients with CML-CP. A previous study revealed that there was no expression of SHP-1 in K562 cells (32). The expression of SHP-1 was restored by drug induced differentiation, therefore inactivation of SHP-1 may serve a role in the progression to blast crisis in CML. The results of the present study revealed that the expression of SHP-1 is low as opposed to non-existent. The conflict between the two results may be due to the sensitivity of the test methods used. In addition, the expression of SHP-1 was identified to be decreased in CML-BP compared with CML-CP primary cells, thus it may be associated with CML disease progression and is consistent with the studies

of Amin *et al* (16). The results of the present study revealed that SHP-1 mRNA expression increased while BCR-ABL expression decreased in the IM+DAC treated group compared with the IM group. This was confirmed through analysis of SHP-1 and BCR-ABL protein expression. SHP-1 mRNA and protein expression increased, while BCR-ABL expression decreased in the nilotinib+DAC compared with the nilotinib group. No significant difference was identified in SHP-1 and BCR-ABL expression between the nilotinib and IM+DAC treatment groups. These results demonstrated that treatment with DAC combined with TKIs possesses an improved ability to enhance SHP-1 mRNA and protein expression, and decreasing BCR-ABL expression. This suggests that DAC restores SHP-1 expression by demethylation, which enhances TKIs anti-tumor effect. A previous study revealed that SHP-1 expression is significantly lower in TKI-resistant compared with TKI-sensitive cell lines (33). In KCL22-R cells, SHP-1 ectopic expression restores responsiveness to imatinib (IM) (33). Data from the present study demonstrated that the increase in SHP-1 and decrease in BCR-ABL expression in response to IM+DAC, as compared with IM monotherapy, in K562 cells was consistent with that in primary cells from patients with CML *in vitro*.

In conclusion: i) DAC was demonstrated to inhibit the proliferation of K562 cells in a dose-dependent manner. ii) IM+DAC and nilotinib+DAC exhibited high synergy. iii) Immunoprecipitation demonstrated that SHP-1 and BCR-ABL (p210) proteins form a complex in K562 cells and in the primary cells derived from patients with CML, suggesting there is an interaction between SHP-1 and BCR-ABL (p210) protein. Therefore, SHP-1 may be considered a tyrosine phosphatase that controls the activity of BCR-ABL (p210) tyrosine kinase. SHP-1 expression was identified to be decreased in K562 cells and CML-BP primary cells compared with the expression in CML-CP primary cells, suggesting that it is also associated with CML disease progression. iv) SHP-1 mRNA and protein expression increased in the combined groups (IM+DAC/nilotinib+DAC) compared with the monotherapy groups, while BCR-ABL expression decreased. Methylation of SHP-1 was observed in K562 cells, although it was not identified in the IM+DAC therapy group. Hence, it is suggested that DAC recovers SHP-1 expression through demethylation, thereby enhancing the therapeutic effect of TKIs. The results of the present study suggest that DAC combined with TKIs provide a novel strategy for managing TKI tolerance and the progression of CML in patients.

Acknowledgements

The authors of the present study would like to thank Dr Luo Jian Min (Department of Hematology, The Second Affiliated Hospital of Hebei Medical University, Shijiazhuang, China) for reading the manuscript.

References

- National Comprehensive Cancer Network: NCCN Clinical Practice Guidelines in Oncology: Chronic Myelogenous Leukemia, V.2.[DB/OL].(2007-10-25), 2008).
- Druker BJ and Lee SJ: Chronic myelogenous leukemia. In: Cancer: Principles and Practice of Oncology, 2007.
- Incidence survey of leukemia in China. Chinese Epidemiologic Study Group of Leukemia and Aplastic Anemia. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 14: 12-19, 1992 (In Chinese).
- Goldman JM and Melo JV: Chronic myeloid leukemia-advances in biology and new approaches to treatment. N Engl J Med 349: 1451-1464, 2003.
- McWhirter JR and Wang JY: An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias. EMBO J 12: 1533-1546, 1993.
- Van Etten RA, Jackson PK, Baltimore D, Sanders MC, Matsudaira PT and Janney PA: The COOH terminus of the c-Abl tyrosine kinase contains distinct F- and G-actin binding domains with bundling activity. J Cell Biol 124: 325-340, 1994.
- Foroni L, Gerrard G, Nna E, Khorashad JS, Stevens D, Swale B, Milojkovic D, Reid A, Goldman J and Marin D: Technical aspects and clinical applications of measuring BCR-ABL1 transcripts number in chronic myeloid leukemia. Am J Hematol 84: 517-522, 2009.
- Hackanson B and Daskalakis M: Decitabine. Recent Results Cancer Res 201: 269-297, 2014.
- Kantarjian HM, O'Brien SM, Keating M, Beran M, Estey E, Giral S, Kornblau S, Rios MB, de Vos D and Talpaz M: Results of decitabine therapy in the accelerated and blastic phases of chronic myelogenous leukemia. Leukemia 11: 1617-1620, 1997.
- Sacchi S, Kantarjian HM, O'Brien S, Cortes J, Rios MB, Giles FJ, Beran M, Koller CA and Keating MJ: Chronic myelogenous leukemia in nonlymphoblastic phase: Analysis of the results of first salvage therapy with three different treatment approaches for 162 patients. Cancer 86: 2632-2641, 1999.
- Kantarjian HM, O'Brien S, Cortes J, Giles FJ, Faderl S, Issa JP, Garcia-Manero G, Rios MB, Shan J, Andreeff M, *et al*: Results of decitabine (5-aza-2-deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. Cancer 98: 522-528, 2003.
- Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J and Kantarjian HM: Phase I study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 103: 1635-1640, 2004.
- Issa JP, Gharibyan V, Cortes J, Jelinek J, Morris G, Verstovsek S, Talpaz M, Garcia-Manero G and Kantarjian HM: Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate. J Clin Oncol 23: 3948-3956, 2005.
- Wu C, Sun M, Liu L and Zhou GW: The function of the protein tyrosine phosphatase SHP-1 in cancer. Gene 306: 1-12, 2003.
- Oka T, Yoshino T, Hayashi K, Ohara N, Nakanishi T, Yamaa Y, Hiraki A, Sogawa CA, Kondo E, Teramoto N, *et al*: Reduction of hematopoietic cell specific tyrosine phosphatase SHP-1 gene expression in natural killer cell lymphoma and various types of lymphomas/leukemia. Combination analysis with cDNA expression array and tissue microarray. Am J Pathol 159: 1495-1505, 2001.
- Amin HM, Hoshino K, Yang H, Lin Q, Lai R and Garcia-Manero G: Decreased expression level of SH2 domain-containing protein tyrosine phosphatase-1 (Shp1) is associated with progression of chronic myeloid leukaemia. J Pathol 212: 402-410, 2007.
- Zhang Q, Wang HY, Marzec M, Raghunath PN, Nagasawa T and Wasik MA: STAT3- and DNA methyltransferase 1-mediated epigenetic silencing of SHP-1 tyrosine phosphatase tumor suppressor gene in malignant T lymphocyte. Proc Natl Acad Sci USA 102: 6948-6953, 2005.
- Uhm KO, Lee ES, Lee YM, Park JS, Kim SJ, Kim BS, Kim HS and Park SH: Differential methylation pattern of ID4, SFRP1, and SHP1 between acute myeloid leukemia and chronic myeloid leukemia. J Korean Med Sci 24: 493-497, 2009.
- Chim CS, Fung TK, Cheung WC, Liang R and Kwong YL: SOCS1 and SHP1 hypermethylation in multiple myeloma: Implications for epigenetic activation of the Jak/STAT pathway. Blood 103: 4630-4635, 2004.
- Kim JH, Choi YD, Lee JS, Lee JH, Nam JH and Choi C: Assessment of DNA methylation for the detection of cervical neoplasia in liquid-based cytology specimens. Gynecol Oncol 116: 99-104, 2010.
- Xu SB, Liu XH, Li BH, Zhang Y, Yuan J, Yuan Q, Li PD, Yang XZ, Li F and Zhang WJ: DNA methylation regulates constitutive expression of Stat6 regulatory genes SOCS-1 and SHP-1 in colon cancer cells. J Cancer Res Clin Oncol 135: 1791-1798, 2009.

22. Liedtke M, Pandey P, Kumar S, Kharbanda S and Kufe D: Regulation of Bcr-Abl-induced SAP kinase activity and transformation by the SHPTP1 protein tyrosine phosphatase. *Oncogene* 17: 1889-1892, 1998.
23. Cramer K, Nieborowska-Skorska M, Koptyra M, Slupianek A, Penserga ET, Eaves CJ, Aulitzky W and Skorski T: BCR/ABL and other kinases from chronic myeloproliferative disorders stimulate single-strand annealing, an unfaithful DNA double-strand break repair. *Cancer Res* 68: 6884-6888, 2008.
24. Baccarani M, Deininger MW, Rosti G, Hochhaus A, Soverini S, Apperley JF, Cervantes F, Clark RE, Cortes JE, Guilhot F, *et al*: European LeukemiaNet recommendations for the management of chronic myeloid leukemia: 2013. *Blood* 122: 872-884, 2013.
25. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
26. Oka T, Ouchida M, Koyama M, Ogama Y, Takada S, Nakatani Y, Tanaka T, Yoshino T, Hayashi K, Ohara N, *et al*: Gene silencing of the tyrosine phosphatase SHP1 gene by aberrant methylation in leukemias/lymphomas. *Cancer Res* 62: 6390-6394, 2002.
27. Soriano AF, Helfrich B, Chan DC, Heasley LE, Bunn PA Jr and Chou TC: Synergistic effects of new chemopreventive agents and conventional cytotoxic agents against human lung cancer cell lines. *Cancer Res* 59: 6178-6184, 1999.
28. Lozzio CB and Lozzio BB: Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* 45: 321-334, 1975.
29. Momparler RL, Côté S and Eliopoulos N: Pharmacological approach for optimization of the dose schedule of 5-Aza-2-deoxycytidine (Decitabine) for the therapy of leukemia. *Leukemia* 11 (Suppl 1): S1-S6, 1997.
30. Savage DG and Antman KH: Imatinib mesylate-a new oral targeted therapy. *N Eng J Med* 346: 683-693, 2002.
31. Tonks NK and Neel BG: From form to function: Signaling by protein tyrosine phosphatases. *Cell* 87: 365-368, 1996.
32. Bruecher-Encke B, Griffin JD, Neel BG and Lorenz U: Role of the tyrosine phosphatase SHP-1 in K562 cell differentiation. *Leukemia* 15: 1424-1432, 2001.
33. Esposito N, Colavita I, Quintarelli C, Sica AR, Peluso AL, Luciano L, Picardi M, Del Vecchio L, Buonomo T, Hughes TP, *et al*: SHP-1 expression accounts for resistance to imatinib treatment in Philadelphia chromosome-positive cells derived from patients with chronic myeloid leukemia. *Blood* 118: 3634-3644, 2011.