



Role of heme oxygenase-1 in demethylating effects on SKM-1 cells induced by decitabine

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ABSTRACT. We evaluated the influence of heme oxygenase-1 (HO-1) gene inhibition in myelodysplastic syndrome (MDS) cell line SKM-1 on enhancement of the demethylating effects of decitabine on p15, and explored the possible mechanism. *DNMT1* gene expression in SKM-1 cells was silenced by being transfected by a constructed siRNA with liposomes. The proliferation inhibition rates after drug treatment were detected by cell counting kit-8 assay. The apoptotic rates were detected by Annexin V/PI assay with flow cytometry. The expressions of p16, p15, TP73, CDH1, ESR1, and PDLIM4 mRNAs were detected by real-time PCR, and those of HO-1, DNMT1, DNMT3A, DNMT3B, HDAC, and p15 proteins were measured by western blot. The degree of methylation of the *p15* gene was analyzed by

using methylation-specific PCR (MSP). CCK-8 assay showed that after *HO-1* gene expression was inhibited; the proliferation rate of SKM-1 cells treated by decitabine ($70.91 \pm 0.05\%$) was significantly higher than that of the control group ($53.67 \pm 0.05\%$). Flow cytometry showed that the apoptotic rate of SKM-1 cells treated by decitabine in combination with *HO-1* expression inhibition ($44.25 \pm 0.05\%$) exceeded that of the cells treated by this drug alone ($37.70 \pm 0.05\%$). MSP showed that inhibiting *HO-1* expression significantly increased the degree of methylation of the *p15* gene. As suggested by western blot, the degree of methylation of the p15 protein was changed after decitabine treatment when the expression of the *HO-1* protein was changed, being associated with the affected DNMT1 expression. Inhibited *HO-1* expression attenuated the hypermethylation of *CDKN2B* by suppressing DNMT1, which was conducive to treatment by cooperating with decitabine. In conclusion, the findings of this study provide valuable experimental evidence for targeted MDS therapy, and a theoretical basis for further studies.

Key words: Myelodysplastic syndrome; Heme oxygenase-1; Methyltransferase; p15

INTRODUCTION

As a heterogeneous group of hematopoietic stem cell-related clonal diseases, myelodysplastic syndrome (MDS) is typified by myeloid dysplasia, ineffective hematopoiesis, increasing number of primitive cells, and high risk of transformation to acute myelocytic leukemia (AML). MDS is clinically manifested as an increase in hematopoietic cell number, accompanied by monolineage or multilineage dysplasia involving one or more abnormal myeloid cell types, and peripheral blood cytopenias. The pathogenesis of MDS remains unclear, and has been attributed to exogenous and endogenous factors (Dalamaga et al., 2002; Nimer, 2008; Vardiman et al., 2009; Bejar et al., 2011). Most patients have karyotype abnormalities (Ebert et al., 2008); some of these are affected with mutations in the oncogenes and anti-oncogenes, as well as aberrant expressions of apoptosis-related genes (Kao et al., 2011; Fuchs, 2012). Epigenetic changes involving DNA methylation are also correlated with the onset of hematological tumors (Olk-Batz et al., 2011).

At the molecular level, MDS is a result of malignant cell clones arising because of silenced expressions of anti-oncogenes after the demethylation of anti-oncogene promoters and mutations in the relevant regulatory genes (Wang et al., 2011). For instance, anti-oncogenes such as p15 are deactivated because of over-methylation, leading to cell cycle abnormalities, which, together with increased cell proliferation and reduced apoptosis and differentiation, finally induce the generation of tumor clones (Fandy et al., 2009). *p15*, which belongs to the cyclin-dependent kinase inhibitor (CDKI) family, codes for the p15 protein that directly binds to CDK4, thereby blocking the binding of CDK to cyclin D, and subsequently arresting the cells at the G1/S transition phase (Rual et al., 2005). Kim et al. (2013) reported that *CDKN2B* was ubiquitously hypermethylated in children and adults suffering from MDS. Zhang et al. (2013) also discovered that the MDS patients treated with low doses of decitabine in addition to daunorubicin and cytarabine showed a significantly better prognosis, compared to those treated individually

with decitabine. In addition, demethylation was observed to play an important role in the clinical treatment of MDS. However, the detailed mechanism remains to be clarified. Since this disease is particularly sensitive to DNA methyltransferase inhibitors, DNA methylation is believed to be vital for its onset (Zhang et al., 2013).

Heme oxygenase-1 (HO-1), an isozyme of heme oxygenase, is the rate-limiting enzyme of the heme catabolic process, and can be expressed upon being induced by multiple external factors. Under disease or stress conditions, HO-1 protects the cells from apoptosis, promotes cell proliferation, and mitigates the inflammatory response (Ryter et al., 2006; Alam and Cook, 2007). The *HO-1* gene has been associated with the progression of AML (Miyazaki et al., 2010; Ma et al., 2014). In addition, it has been known to shield the AML cells from tumor necrosis factor-induced apoptosis through the regulation of the transcription factors Nrf2, NF- κ B, and AP-1, and the reduction in reactive oxygen species accumulation in these cells (Fang et al., 2003; Heasman et al., 2011). It has also been reported that the Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein contributed to the resistance of AML cells to apoptosis, via the regulation of HO-1 expression (Rushworth et al., 2010).

We have previously discovered high HO-1 expression in MDS patients with CDKN2B methylation (and vice versa). The treatment of SKM-1 cells with decitabine led to the downregulation of HO-1 expression, in addition to CDKN2B demethylation. Therefore, we postulated a correlation between the *HO-1* gene and MDS progression and over-methylation of tumor suppressor gene promoters, aiming to prove that the *HO-1* gene can function as a potential target for MDS therapy.

MATERIAL AND METHODS

Cell line and culture conditions

The SKM-1 cell line was purchased from the Japanese Collection of Research Bioresources, and cultured in Dulbecco's modified Eagle's medium and F12, supplemented with 15% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. All reagents and antibiotics were obtained from Thermo Scientific Fisher (Waltham, MA, USA). All cells were maintained in a 37°C incubator with 95% humidity and 5% CO₂.

Construction of DNMT1 siRNA

siRNA was constructed to transfect the cells for the knockdown of *DNMT1* expression. The siRNA sequences specific for DNMT1 were GGAUGAGAAGAGACGUAGAtt, UCUACGUCUCUUCUCAUCctg, GCACCUCAUUUGCCGAAUAtt, and UAUUCGGCAAUGAGG UGCtg.

Cell proliferation inhibition assay

The cells were inoculated onto 6-well plates at the density of 10⁸ cells/mL, and incubated with different doses of ZnPP IX and Hemin, in combination with decitabine, for 24 and 48 h. The inhibitory effects were determined using the cell counting kit-8 (CCK-8) (Beyotime Institute of Biotechnology, Shanghai, China) as per the manufacturer protocols.

Western blot

The cells were collected, lysed with western and IP lysates as well as 10X phenylmethanesulfonyl fluoride solution, incubated on ice for 20 min, and centrifuged at 4°C and 12,000 rpm for 10 min. The protein concentrations were measured using a microplate reader, and the sodium dodecyl sulfate (SDS) loading buffer was added 10 min prior to the boiling water treatment. The protein bands obtained after separation by SDS polyacrylamide gel electrophoresis (10% SDS-PAGE) were transferred to a nitrocellulose membrane, incubated with the HO-1, p15, DNMT1, DNMT3A, DNMT3B, HDAC antibodies, and detected with electrochemiluminescent substrates (for western blotting). All reagents were purchased from Beyotime Institute of Biotechnology.

Real-time fluorescent quantitative polymerase chain reaction (PCR)

The cells were collected and treated with (shaken) a mixture of TRIzol reagent (Beyotime Institute of Biotechnology) and chloroform. This mixture was then allowed to stand still, and subsequently centrifuged at 4°C and 12,000 rpm for 15 min. The supernatant was mixed with an equal volume of isopropanol, allowed to stand still for 20 min, and centrifuged again at 4°C and 12,000 rpm for 10 min. Afterwards, the resulting total RNA was washed with 75% ethanol and reverse-transcribed to cDNA with Revert Aid First-Strand cDNA Synthesis Kit (Thermo Scientific Fisher). The obtained cDNA was used for real-time quantitative PCR, using primers provided by Airui Technology Corporation (Guiyang, China) and iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA).

Methylation-specific PCR (MSP)

The cells were collected; the total DNA extracted using the TIANamp Blood DNA Kit (Tiagen Biotech Co., Ltd., Beijing, China), and the samples modified with the BisulFlash™ DNA Modification Kit (Epigentek Group Inc.). Primers specific for methylation and demethylation were designed and amplified, and the products were subjected to agarose gel electrophoresis. The designed primers were as follows: methylation, forward 5'-TTACGAGGGGCGGTCGTACGCGGG-3' and reverse 5'-AAAACGACCGACGCGAACGCCTCC-3'; and demethylation, forward 5'-TTATGAGGGGTGGTGTATGTGGG-3' and reverse 5'-AAAACAACCAACACAAACACCTCC-3'.

Detection of cell apoptosis using the Annexin V/PI staining assay

The cells from the control-, Hemin-, and ZnPP IX-treated groups were collected, washed twice with PBS, and counted. Subsequently, 10⁷ cells of each group were suspended with 500 µL 1X binding buffer, into which was added 5 µL Annexin V-fluorescein isothiocyanate and 5 µL PI. These treated cells were placed in the dark for 15 min, and subsequently analyzed by flow cytometry after 30 min.

RESULTS

Upregulation of *HO-1* protects the SKM-1 cell line against decitabine-induced apoptosis via the inhibition of p15

The endogenous expression of the *HO-1* gene in SKM-1 cells was successfully up-

regulated by the Hemin enhancer, and downregulated by the ZnPP IX inhibitor. The control-, Hemin-, and ZnPP IX-treated groups were co-cultured with different doses of decitabine. The increase in decitabine dosage led to sensitivity of SKM-1 cell inhibition. The inhibitory effects of decitabine were less obvious on the *HO-1* upregulation group, compared to those on the control group. However, these effects were more evident in the downregulation group. Meanwhile, of the detected anti-oncogenes, only p15 was differentially expressed in the control and two of the experimental groups (treated with decitabine). The increase in the HO-1 protein led to a corresponding decrease in p15 expression, and vice versa (Figure 1).

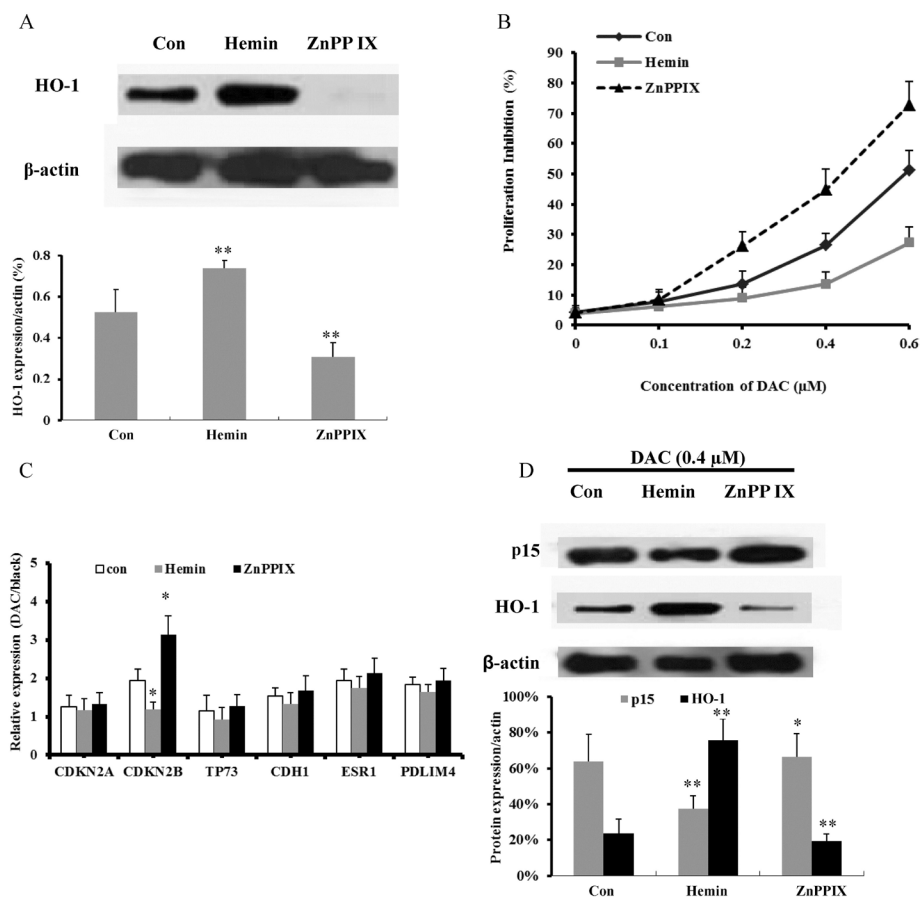


Figure 1. Regulation of HO-1 expression affecting the inhibitory effects of decitabine on SKM-1 cells and p15 expression. **A.** Endogenous HO-1 expression upregulated and downregulated by Hemin and ZnPP IX, respectively. **B.** Experimental groups co-cultured with 0, 0.1, 0.2, 0.4, and 0.6 μ M decitabine. The SKM-1 cells were inhibited with greater efficiency upon increasing the dosage of decitabine. Decitabine-induced inhibition was less obvious in HO-1 upregulated cells compared to the cells of the control group. On the other hand, decitabine exerted a greater inhibitory effect on the HO-1 downregulated cells compared to the cells of the control group. **C.** Anti-oncogenes *CDKN2A*, *CDKN2B*, *TP73*, *CDH1*, *ESR1*, and *PDLIM4* detected by real-time PCR; however, only *CDKN2B*, coding for the anti-oncogene p15, was differentially expressed. **D.** Cells treated with 0.4 μ M decitabine. The results of the western blot analysis revealed that p15 expression was upregulated and downregulated when HO-1 expression was inhibited or promoted, respectively. Con = control.

HO-1 upregulation resulted in a reduced level of decitabine-induced p15 demethylation, by increasing the DNMT1 expression

The cell groups were treated with placebo and decitabine. The levels of methylation of *p15* gene were elevated upon upregulation of the *HO-1* gene, as detected by MSP. The HO-1 upregulation group resisted the demethylating effects of decitabine, while the control and HO-1 downregulation group did not. In addition, DNMT1 was differentially expressed in all groups. The expression of DNMT1 was observed to increase and decrease with the upregulation and downregulation of HO-1, respectively. In contrast, the cell cycle initiation factor- and anti-oncogene methylation-related gene expression (i.e., DNMT3A, DNMT3B and HDAC) was similar in all groups (Figure 2).

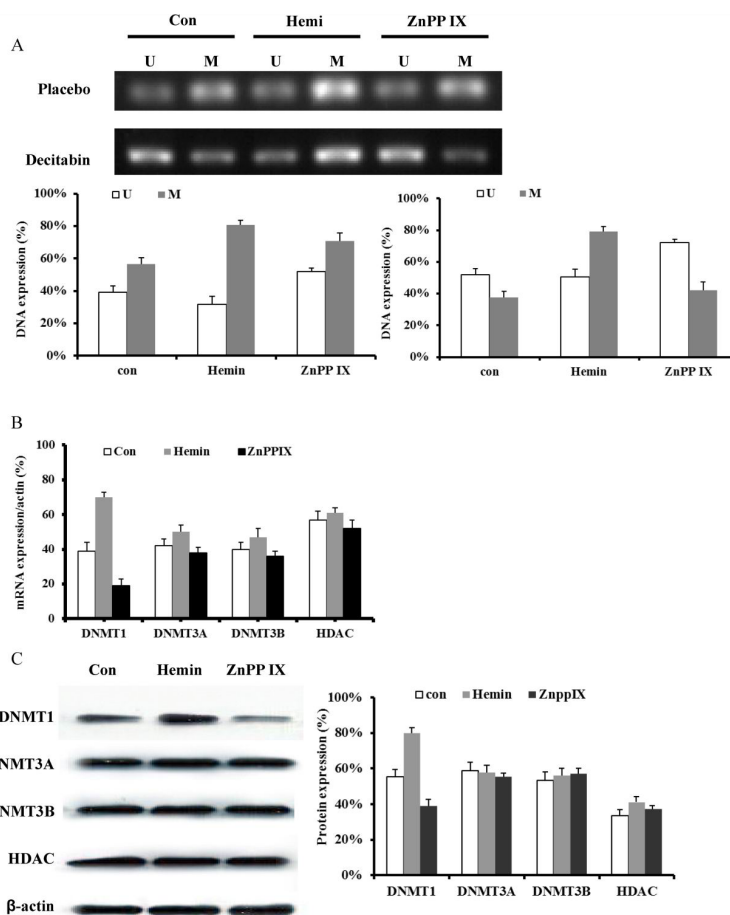


Figure 2. HO-1 affecting p15 methylation by altering the DNMT1 expression. **A.** MSP revealed an increase in p15 demethylation upon upregulation of HO-1 expression. The treatment of cells with decitabine resulted in a reduction in the demethylation in the HO-1 upregulated group; however, the control and HO-1 downregulated groups did not show any such decrease. **B.** Real-time PCR of DNA methylation-related genes showing significant differences in DNMT1 expression following HO-1 regulation. **C.** Western blot analysis revealing a decrease (or increase) in DNMT1 expression corresponding to the increase (or decrease) in HO-1 expression. Con = control.

Inhibition of DNMT1 attenuated the effects of HO-1 on p15 expression

Following inhibition (using siRNA in combination with HO-1 regulation), DNMT1 protein expression was observed to be lower than in the group wherein only the HO-1 protein was regulated. This resulted in an increase in p15 protein expression, suggesting that changes in HO-1 expression affected p15 expression by altering DNMT1 expression. Similarly, MSP revealed that the demethylating effect of decitabine on the group with inhibited DNMT1 expression was greater than that on the group without inhibited DNMT1 expression (Figure 3).

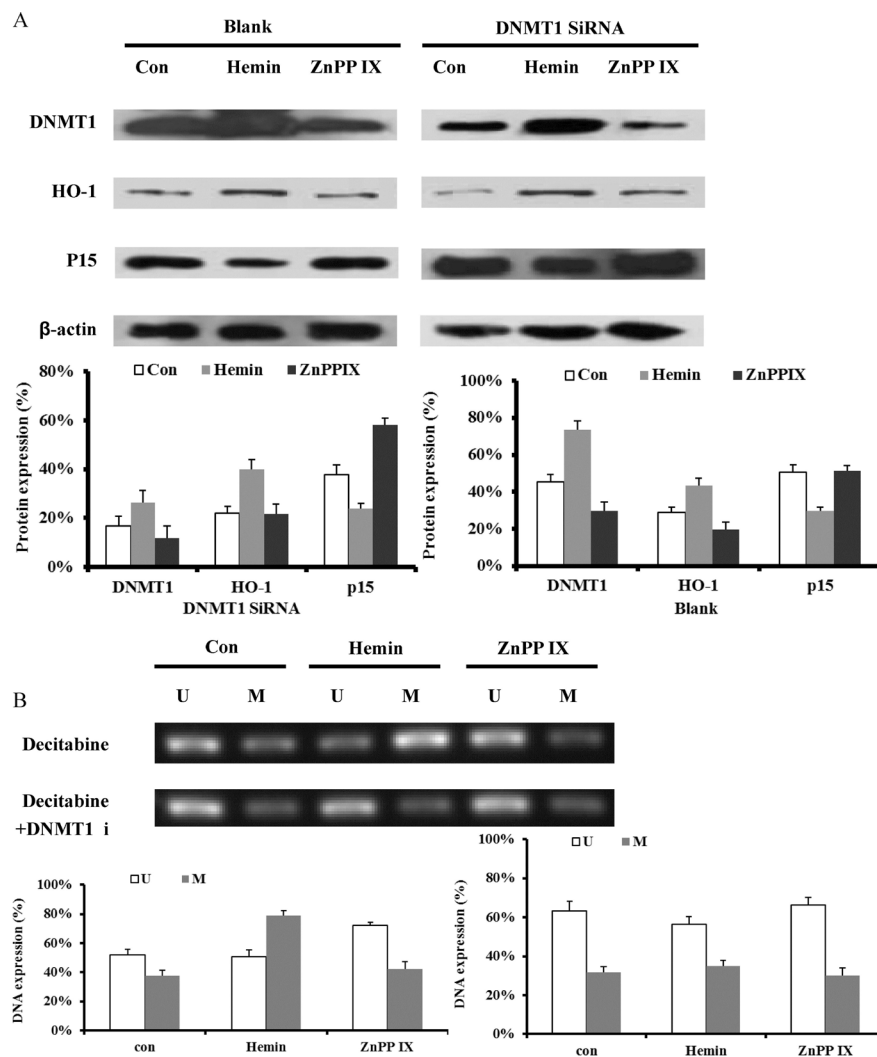


Figure 3. Inhibition of DNMT1 conducive to p15 expression. **A.** Western blot analysis performed following the inhibition of DNMT1 expression by treatment with small interfering RNA (siRNA) revealing a moderate change in the HO-1 protein expression and an increase in the p15 protein expression. **B.** MSP showing that the inhibition of DNMT1 expression boosted the demethylating effects of decitabine. Con = control.

Inhibition of HO-1 induced SKM-1 cell apoptosis via the reduction of DNMT1 expression

The regulation of HO-1 expression led to an increase in the apoptotic rate of the cells treated with decitabine compared to the untreated group. In addition, the combination of DNMT1 inhibition and decitabine treatment augmented the apoptotic rate, compared to that caused by decitabine alone. Therefore, DNMT1 inhibition weakened the over-methylation of tumor cells and enhanced the demethylating effects of decitabine, which ultimately facilitated cell apoptosis (Figure 4).

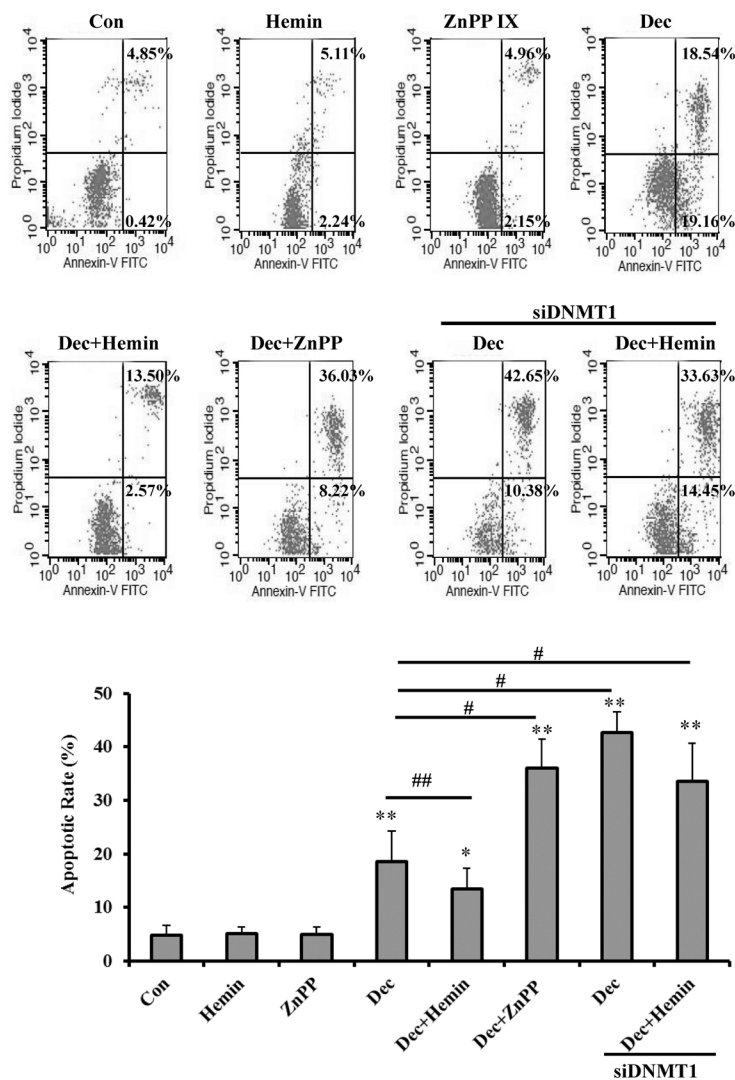


Figure 4. Reduction in HO-1 over-expression facilitated tumor cell apoptosis. Apoptosis rate of un-transfected/transfected cells treated with Hemin/ZnPP IX and decitabine detected using flow cytometry. All experiments were repeated thrice. ** $P < 0.01$; * $P < 0.05$; ## $P < 0.01$; and # $P < 0.05$. Con = control; Dec = decitabine.

DISCUSSION

So far, the onset and progression of MDS has been mainly ascribed to over-methylation (Griffiths and Gore, 2013). In this study, we have attempted to clarify the *in vitro* relationship between HO-1 and methylation, and analyze the possible mechanism by regulating the endogenous expression of the HO-1 gene using an inhibitor, in order to verify the applicability of HO-1 as a potential target for MDS therapy, as well as to discover novel treatment protocols.

The studies conducted so far have correlated HO-1 expression with AML or CML, but never with MDS. Specifically, the mechanism governing the relationship between HO-1 and methylation has not been unraveled so far. MDS is currently being treated by chemotherapy and hematopoietic stem cell transplantation; however, these methods have serious drawbacks, highlighting the need for targeted therapy. The aim of this study was to confirm the participation of HO-1 in the regulation of anti-oncogene promoter methylation. We also attempted to provide experimental proof that the downregulation of HO-1 sensitized MDS toward demethylation, and a theoretical basis for the use of HO-1 as a potential target for the treatment of MDS.

The inhibition of HO-1 expression significantly affected the therapeutic effects of decitabine on MDS. Decitabine (2'-deoxy-5-azacytidine) is known to exert a demethylating effect via the suppression of both DNA methyltransferase activity and over-methylation of anti-oncogenes, thereby promoting cell differentiation and apoptosis (Li et al., 2014; Martinez-Galan et al., 2014). Decitabine reactivates the anti-oncogenes for MDS, by reducing their methylation levels. However, the relationship between decitabine and the recovery of p15 gene expression (Daskalakis et al., 2002; Raj et al., 2007) remains a controversial topic. In general, decitabine cannot effectively treat human MDS under conditions of high HO-1 expression. In this study, the DNA methyltransferase expression was augmented in addition to high *HO-1* gene expression, which resisted the demethylating effects of decitabine. This resulted in over-methylation and deactivation of the anti-oncogene p15. Subsequently, the tumor cells became prone to proliferation. On the contrary, inhibition of the endogenous HO-1 expression by ZnPP IX led to a reduction in DNA methyltransferase expression, subsequently resulting in an increase in *p15* gene methylation. The application of decitabine under these conditions resulted in easier tumor cell differentiation and apoptosis. The simultaneous inhibition of *HO-1* and DNMT1 expressions was observed to further enhance the demethylating effects of decitabine, and raise the apoptotic rates of tumor cells.

In addition to shielding the cells from a variety of stimuli and inflammations, HO-1 is known to protect normal tissues as well as cancer cells (Choi et al., 2014; Tertilt et al., 2014). In summary, we theorize that MDS patients with barely alleviated symptoms may have high levels of HO-1 expression. Further studies are still ongoing in our group to validate this presumption, as well as to clarify the pathway with which HO-1 affects DNA methyltransferase, and to provide an experimental basis and possible therapeutic target for epigenetic modification of MDS.

Conflicts of interest

The authors declare no conflict of interest.

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