Knockdown of HOXA10 reverses the multidrug resistance of human chronic myelogenous leukemia K562/ADM cells by downregulating P-gp and MRP-1

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Abbreviations: GFP, green fluorescent protein; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; HOXA10 shRNA, homeobox A10 short hairpin RNA; control shRNA, control short hairpin RNA

Key words: multidrug resistance, homeobox A10, short hairpin RNA, reverse, P-glycoprotein, multidrug resistance protein-1

Abstract. Multidrug resistance (MDR) of leukemia cells is a major obstacle in chemotherapeutic treatment. The high expression and constitutive activation of P-glycoprotein (P-gp) and multidrug resistance protein-1 (MRP-1) have been reported to play a vital role in enhancing cell resistance to anticancer drugs in many tumors. The present study aimed to investigate the reversal of MDR by silencing homeobox A10 (HOXA10) in adriamycin (ADR)-resistant human chronic myelogenous leukemia (CML) K562/ADM cells by modulating the expression of P-gp and MRP-1. K562/ADM cells were stably transfected with HOXA10-targeted short hairpin RNA (shRNA). The results of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis showed that the mRNA and protein expression of HOXA10 was markedly suppressed following transfection with a shRNA-containing vector. The sensitivity of the K562/ADM cells to ADR was enhanced by the silencing of HOXA10, due to the increased intracellular accumulation of ADR. The accumulation of ADR induced by the silencing of HOXA10 may be due to the down-regulation of P-gp and MRP-1. Western blot analysis revealed that downregulating HOXA10 inhibited the protein expression of P-gp and MRP-1. Taken together, these results suggest that knockdown of HOXA10 combats resistance and that HOXA10 is a potential target for resistant human CML.

Introduction

Chronic myelogenous leukemia (CML) is one of the most common types of cancer in humans, and multidrug resistance (MDR) is a major factor that limits the efficacy of chemotherapeutic agents used to treat CML. MDR is a phenomenon characterized by the development of resistance in cancer cells to a variety of unrelated drugs following exposure to a single chemotherapy drug (1). MDR has many causes; however, the most widely studied mechanism involves the increased efflux of cytotoxic drugs, which is mediated by ABC transporters (2). P-glycoprotein (P-gp) and multidrug resistance protein (MRP)-1 are members of the ABC transporter family, which are mainly expressed in the plasma membrane and pump the cytotoxic drugs out of the cells through active transportation. The increased expression of P-gp and MRP-1 has been demonstrated to cause reductions in the concentration of chemotherapeutic drugs inside tumor cells, thus reducing the clinical effectiveness of chemotherapy drugs (4-10). P-gp, the first member of the family to be identified, is a 170-kDa energy-dependent drug efflux pump which mediates resistance to a variety of pharmacologically unrelated anticancer drugs, such as doxorubicin, epirubicin and paclitaxel (1-3). MRP-1, a drug-transporting MRP, is a 190-kDa protein and is encoded by the MRP-1 gene, which is located on chromosome 16p13. MRP-1 overexpression has emerged as an important contributor to chemoresistance. In tumor cells, MRP-1 was found to cause resistance not only to doxorubicin, but also to many other chemotherapeutic drugs, including methotrexate, etoposide and vincristine (11).

Homeobox (HOX) proteins are homeodomain transcription factors that are highly conserved in many species, from Drosophila to humans. The human and murine HOX genes are found in four groups on four different chromosomes with 9-11 genes in each group (referred to as HOXA-D) (12-15). The HOX genes are important regulators encoding transcription factors that influence embryologic development. HOXA10...
belongs to the HOX gene family. HOXA10 is maximally expressed in the primitive hematopoietic cell compartment and decreases when the cells differentiate. In addition, it has been demonstrated that the overexpression of HOXA10 is involved in myelopoiesis and blocks differentiation, which may ultimately lead to myeloid leukemia (13,16). Previous studies have confirmed that HOXA10 expression was associated with temozolomide resistance in glioblastoma (GBM) (17,18). However, the role of HOXA10 in multidrug-resistant human CML K562/ADM cells was previously unknown. Thus, in the present study, we explored the effects of HOXA10 knockdown on multidrug resistance and the underlying molecular mechanism.

Materials and methods

Chemicals and reagents. Adriamycin (ADR; Melone Pharmaceutical Co., Ltd., Dalian, China) was dissolved at a concentration of 2 g/l with ddH₂O and divided into 25 aliquots. Rabbit polyclonal antibodies against HOXA10 (cat. no. bs-2502R), P-gp (cat. no. bs-0563R), MRP-1 (cat. no. bs-0657R) were obtained from Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Rabbit polyclonal antibodies against GAPDH were obtained from Hangzhou Goodhere Biotechnology Co., Ltd. (Hangzhou, China).

Cell lines and cell culture. The human CML cell line, K562, was obtained from the Key Laboratory of Tumour Molecular Biology of Binzhou Medical University (Binzhou, China) and its MDR subline, K562/ADM, was obtained from the Department of Pharmacology at the Institute of Hematology of the Chinese Academy of Medical Sciences (Tianjin, China).

Determination of multidrug resistance in K562/ADM cells. A Cell Counting kit 8 (CCK8; Dojindo Molecular Technologies, Inc., Shanghai, China) was used to determine the survival rate of cells incubated with ADR at various concentrations (0.2-1.6 mg/l for the K562 cells and 16-128 mg/l for the K562/ADM cells), as previously described (7). After dilution in RPMI-1640 medium for 24 h, 10 µl CCK8 solution was added to each well and incubated for 1-4 h. The absorbance was then measured at 570 nm with a fluorescence spectrofluorometer (F-7000; Hitachi High-Technologies Corp., Tokyo, Japan). A blank well containing cells and medium. In addition, the cells treated with

| Gene Primer sequence Product length (bp) |
|------------------------------------------|------------------|
| **HOXA10** F: 5’-CTCACGGCAAAAGATGTC-3’ R: 5’-AGTTTTCATCCTGGCGTTCTG-3’ | 182 |
| **GAPDH** F: 5’-TGACCTTCAAAGCGACACCCA-3’ R: 5’-CACCCCTGTTCGTAGCCAAA-3’ | 121 |

HOXA10, homeobox A10; F, forward; R, reverse.

Reverse transcription was performed with a PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan). PCR amplification was performed on an Eppendorf Mastercycler personal (Eppendorf China Co., Ltd., Shanghai, China) using Premix Taq™ (Takara Bio, Inc.). The reaction system contained diethyl pyrocarbonate, forward primer, reverse primer, Premix Taq and template cDNA. The amplification was as follows: 95°C for 2 min, then 35 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, followed by a full extension cycle of 72°C for 5 min. The PCR products were electrophoresed on 1.5% agarose gels (Takara Biotechnology Co., Ltd., Dalian, China), and stained with ethidium bromide for 15 min. The images were captured with a Tanon gel imaging system. The results are expressed for each sample as band intensity relative to that of GAPDH.

qPCR was performed on an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using a SYBR-Green reaction kit (Takara Bio, Inc.). The PCR reaction system consisted of SYBR-Green reagent, forward primer, reverse primer, template cDNA and nuclease-free distilled water. The PCR conditions were as follows: 95°C for 30 sec, followed by 45 cycles of 95°C for 5 sec and 60°C for 30 sec. GAPDH was used as an internal control. qPCR for each gene of each cDNA sample was assayed in triplicate. The results were calculated using the 2−ΔΔCt method. The following equations were used: ΔCt = Ct(target gene) - Ct(GAPDH); ΔΔCt = Ct[short hairpin RNA (shRNA) cells] - Ct(ununtreated control).

**In vitro transfection with shRNA.** The HOXA10-specific shRNA and the control shRNA were synthesized with recombinant plasmids containing the green fluorescent protein (GFP) vector, pGPH1, purchased from Shanghai Genepharma Co., Ltd., (Shanghai, China). The target sequence of HOXA10 shRNA was as follows: 5’-GGCCAAAUUACCCACAACA-3’. Prior to transfection, the cells were cultured in RPMI-1640 medium free of serum and antibiotics. shRNA transfection (at a final concentration of 1 µg in all experiments) was performed using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, shRNAs and lipofectamine (2.5 µl) were diluted in RPMI-1640 separately and incubated for 5 min at room temperature. The diluted solutions were then mixed and incubated for 15 min at room temperature. The mixtures were then added to each well containing cells and medium. In addition, the cells treated with
only Lipofectamine were considered as the blank control. The cell culture plates were subsequently incubated for 6 h at 37°C in an incubator. Subsequently, RPMI-1640 medium containing 20% FBS was added and the cells were then incubated under the abovementioned conditions. Transfection efficiency was examined under a fluorescence microscope (Olympus DP71; Olympus, Tokyo, Japan). RT-qPCR and western blot analysis were performed to determine the inhibitory efficacy. G418 (500 ng/ml; Life Technologies, Carlsbad, CA, USA) was then added to the medium after 48 h transfection, and the cells were cultured for 1 month to permit selection. The cells successfully transfected with HOXA10 shRNA and control shRNA were named HOXA10 shRNA and control shRNA cells.

**Assay of the reversal efficacy of HOXA10 knockdown.** The K562/ADM cells as well as the cells transfected with HOXA10 shRNA and the control shRNA were seeded into 96-well plates in the presence of various concentrations of ADR (0-128 µg/ml) for 24 h at 37°C in 5% CO₂, and the quantity and percentages of viable cells were determined using the CCK8 assay. Each group consisted of five parallel wells. ADR IC₅₀ was calculated using the untreated cells as a 100% viable control. The reversal fold (RF) values, as a measure of the potency of reversal, were obtained using the following formula: RF = IC₅₀ of K562/ADM/IC₅₀ of HOXA10 shRNA.

**Enhancement of intracellular ADR accumulation.** The intracellular accumulation of ADR was monitored using a standard procedure. The K562/ADM cells, and cells transfected with HOXA10 shRNA and control shRNA were incubated for 1 h at 37°C with ADR (3 mg/l). The cells were then harvested by centrifugation (1,500 rpm for 5 min at 4°C) and washed twice with ice-cold phosphate-buffered saline (PBS). The cell-associated mean fluorescence intensity (MFI) of ADR was detected by a flow cytometer (FACS FC500; Beckman Coulter, Brea, CA, USA) with excitation/emission wavelengths of 485/585 nm.

**Western blot analysis.** The cells were harvested, a total of 100 µl lysis buffer was added and the protein concentration of the lysate was determined using a bicinechonic acid protein assay kit (Beyotime Biotechnology, Shanghai, China). The lysed samples (50 µg) were separated by 6-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime Biotechnology) with a constant voltage of 80 V for 0.5 h which was then switched to 100 V for another 1.5 h. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA) and blocked with 5% skimmed milk for 2 h. Subsequently, the membranes were incubated overnight at 4°C with specific antibodies. The primary antibodies used were rabbit polyclonal antibodies against HOXA10 (1:500), P-gp (1:500), MRP-1 (1:500) and GAPDH (1:1,000). The following day, the membranes were incubated in horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:5,000; cat. no. ZB-5301; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 2 h at room temperature. Finally, the images were captured using a FluorChem FC2 gel imaging system (Alpha Innotech, San Leandro, CA, USA). The intensity of each band was normalized to GAPDH for their respective lanes.

**Data analysis.** Statistical analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as the means ± SD. Statistical comparisons were evaluated by one-way ANOVA. A P-value <0.05 was considered to indicate a statistically significant difference.

**Results**

**Comparison of multidrug resistance in K562/ADM cells and K562 cells.** Compared with the non-resistant K562 cells, the K562/ADM cells exhibited significant resistance to ADR. As shown in Table II, a 31.2747-fold increase in resistance was observed in the K562/ADM cells in comparison with that in the non-resistant K562 cells (P<0.05) (Table II).

**Expression of HOXA10 in K562 cells and K562/ADM cells.** We determined the expression of HOXA10 in the non-resistant K562 cells and the resistant K562/ADM cells. We demonstrated that the K562 cells and K562/ADM cells exhibited high expression levels of HOXA10, while the K562/ADM cells expressed higher levels of HOXA10 than the K562 cells (Fig. 1) (P<0.05).

### Table II. Determination of multidrug resistance according to the sensitivity of K562/ADM and K562 cells to ADR (means ± SD of triplicate experiments).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>K562/ADM IC₅₀ (µg/ml)</th>
<th>K562 IC₅₀ (µg/ml)</th>
<th>Fold resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR</td>
<td>43.6783±0.33094</td>
<td>1.3966±0.01526</td>
<td>31.2747</td>
</tr>
</tbody>
</table>

*P<0.05 vs. K562 cells. ADR, adriamycin.
Suppression efficacy of HOXA10 shRNA. After transfection, more than 80% of cells were GFP-positive, indicating high transfection efficiency (Fig. 2). RT-qPCR analysis revealed that mRNA levels of HOXA10 in the K562/ADM cells transfected with HOXA10 shRNA decreased by 46.15±1.245%, while the control shRNA had almost no influence on the HOXA10 mRNA levels in the K562/ADM cells (P<0.05) (Fig. 3A). Western blot analysis revealed that transfection with HOXA10 shRNA resulted in a reduction to 26.1±0.489% compared with that in the parental K562/ADM cells and the K562/ADM cells transfected with control shRNA (P<0.05) (Fig. 3B and C).

Evaluation of ADR-induced cytotoxicity by knockdown of HOXA10. To determine whether the downregulation of HOXA10 affected multidrug resistance in vitro, the effect of HOXA10 shRNA on ADR-induced cytotoxicity was assessed by a CCK8 assay. The results are shown in Table III and Fig. 4. They indicated that after 24 h, the K562/ADM cells transfected with HOXA10 shRNA had a slower rate of cell proliferation compared with the cells transfected with control shRNA and the parental K562/ADM cells (P<0.05) (Fig. 4), suggesting that the transfection of HOXA10 shRNA increased ADR-induced cytotoxicity in the K562/ADM cells. Knockdown of HOXA10 caused a 2.587-fold reversal in the sensitivity of K562/ADM cells to ADR according to the results of a CCK8 assay (P<0.05) (Table III).

Knockdown of HOXA10 increases intracellular accumulation of ADR. It was previously noted that the intracellular accumulation of ADR decreased significantly in K562/ADM cells compared to the parental K562 cells (7). In the present study, we determined that knockdown of HOXA10 increased the intracellular accumulation of ADR in K562/ADM cells in comparison with K562/ADM cells transfected with control shRNA and parental K562/ADM cells. These results indicated that knockdown of HOXA10 increased the sensitivity of the K562/ADM cells to ADR through increasing the intracellular accumulation of ADR (P<0.05) (Fig. 5).

Knockdown of HOXA10 decreases the protein expression of P-gp and MRP-1 in K562/ADM cells. P-gp and MRP-1 are ABC transporters, which are overexpressed in many drug-resistant cells (9,11). In the present study, the K562/ADM cells expressed P-gp and MRP-1 at high levels. Western blot analysis revealed that after knockdown of HOXA10, P-gp and MRP-1 expression decreased markedly to 20.85±8.258% and 20.55±1.144%, respectively (Fig. 6A-C). These results indicate that knockdown of HOXA10 decreases the protein expression of P-gp and MRP-1 by increasing the intracellular accumulation of ADR.

Discussion

We noted that silencing of HOXA10 significantly increased the cytotoxicity of ADR in K562/ADM cells. The effect of silencing HOXA10 is associated with the increased intracellular accumulation of ADR, as well as the inhibition of the expression of P-gp and MRP-1.
CML is a stem cell disorder characterized by chronic and blast-crisis phases (20). Chemotherapy plays a vital role in CML treatment; however, it is always accompanied by MDR which results in treatment failure. MDR in tumors is characterized by the ability of the tumor cells to exhibit simultaneous resistance to a number of structurally and functionally unrelated chemotherapeutic agents (21). There are multiple mechanisms of MDR in cancer, including the high expression of members of the ABC transporter family, and abnormalities in enzymatic systems and apoptosis. ADR is an effective chemotherapy drug that has been used extensively in the treatment of CML despite the emergence of MDR, which considerably limits the therapeutic efficacy of ADR (3). As shown in Table II, the K562/ADM cells demonstrated significant resistance to ADR-induced cytotoxicity. Overexpression of P-gp and MRP-1 is one of the best known causes of MDR. Significant efforts have been made to identify novel MDR-inhibiting genes, modulating the expression of P-gp and MRP-1. It has been
previously suggested that HOX genes are important regulators in normal and leukemic stem cells. HOXA10, a member of the HOX gene family, which has been reported to be an important regulator in normal and leukemic stem cells, is frequently overexpressed in myeloid leukemia (22-24). It has been reported that overexpression of HOXA10 may exert both pro-differentiation and anti-differentiation effects in a dose-dependent spatiotemporal manner (25). High levels of HOXA10 expression were predictive of tumor resistance to treatment. Studies have demonstrated that HOXA10 is associated with resistance in GBM (17,18). The inhibition of HOXA10 reinforced temozolomide sensitivity independent of MGMT status in GBM cell lines (17). High expression levels of the HOXA9/HOXA10 genes were noted in pediatric GBM patient samples as well as in a TMZ-resistant pediatric GBM cell line (18). Temozolomide resistance in the high HOXA9/HOXA10-expressing GBM cell line was independent of MGMT status, and the PI3K pathway was considered to be an upstream regulator of HOX genes that is targeted to overcome resistance (18). In the present study, we determined that the expression of HOXA10 was higher in the K562/ADM cell line than in the K562 cell line (Fig. 1).

The present study was designed to investigate the cellular functions of HOXA10 in order to elucidate the mechanism by which it contributes to reversing MDR of human CML-derived K562/ADM cell lines. The results revealed that the expression of HOXA10 was higher in the K562/ADM cells than in the K562 cell lines, which suggested that HOXA10 was involved in MDR of the K562/ADM cell lines. This study used shRNA to reduce the expression of HOXA10 in the human CML K562/ADM cell line. As shown in Fig. 3, the shRNA-containing vector efficiently suppressed HOXA10 expression at both the mRNA and protein levels. The effect of HOXA10 knockdown on the cellular functions of K562/ADM cells was then explored. The results of the CCK8 assay indicated that ADR-induced cytotoxicity in the K562/ADM cells transfected with HOXA10 shRNA was significantly increased compared with that in the control cells, which confirmed that knockdown of HOXA10 was capable of reversing MDR in the K562/ADM cells (Fig. 4 and Table III). The flow cytometric analysis indicated that the silencing of HOXA10 increased the intracellular accumulation of ADR. These results indicated that knockdown of HOXA10 reversed MDR by increasing the intracellular accumulation of ADR. To further examine the mechanism by which knockdown of HOXA10 increased the intracellular accumulation of ADR within the K562/ADM cells, the expression of P-gp and MRP-1 were measured. Overexpression of P-gp and MRP-1, members of the ABC transporter family, lower intracellular drug accumulation and decrease the cellular toxicity of chemotherapeutic agents, such as adriamycin, daunorubicin, epirubicin, mitoxantrone, bisantrene, vincristine, vinblastine, etoposide and paclitaxel (11,25). P-gp is one of the most well-known MDR-associated proteins, which functions as an ATP-dependent transmembrane drug transporter that reduces intracellular drug accumulation by pumping drugs out of cells (3). MRP-1, another energy-dependent drug pump, plays an important role in MDR by decreasing the intracellular accumulation of chemotherapeutic agents (5). In the present study, western blot analysis revealed that transfection with HOXA10 shRNA significantly decreased the expression of P-gp and MRP-1 compared with the expression levels in the cells transfected with control shRNA and the parental K562/ADM cells (Fig. 6). The decreased expression of P-gp and MRP-1 increased the intracellular accumulation of ADR in the K562/ADM cells. Silencing of HOXA10 may increase the intracellular accumulation of ADR as a result of the downregulation of P-gp and MRP-1 proteins. Hence, the results confirmed that knockdown of HOXA10 reversed MDR through modulating the expression of P-gp and MRP-1.

In conclusion, HOXA10 was expressed at a high level in the K562/ADM cells, and knockdown of HOXA10 enhances the sensitivity of the K562/ADM cells to cytotoxic killing by the therapeutic drug, ADR, as a result of the increased intracellular accumulation of ADR. The accumulation of
ADR induced by HOXA10 silencing was associated with the downregulation of P-gp and MRP-1 proteins. To the best of our knowledge, we are the first to suggest that knockdown of HOXA10 is a novel and potent therapeutic target that may be used for reversing MDR in human CML-derived myelogenous leukemia K562/ADM cells.

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