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Downregulation of ROS1 enhances the therapeutic efficacy of arsenic trioxide in acute myeloid leukemia cell lines

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Abstract. The present study investigated the function of ROS proto-oncogene 1 receptor tyrosine kinase (ROS1) in regulating the migration and proliferation of acute myeloid leukemia (AML) cells through Wnt/β-catenin signaling, and in arsenic trioxide (ATO) treatment. The migration and proliferation of multiple ROS1-silenced leukemic cell lines was assessed, and the expression levels of proteins associated with Wnt/β-catenin signaling were determined using western blot analysis. Compared with the AML control cells, ROS1-knockdown cells exhibited increased migration and proliferation, and the significant downregulation of β-catenin expression. Additionally, ROS1 knockdown sensitized AML cells to the effects of chemotherapeutic ATO. The results of the present study demonstrated that, in leukemic cell lines, ROS1 counteracted the effects of ATO on migration and proliferation, suggesting that ROS1 may be a potential therapeutic target in patients with AML undergoing ATO treatment. The results of the present study provided novel insight into the function of ATO and ROS1 in regulating AML progression.

Introduction

Acute myeloid leukemia (AML) is a malignancy characterized by abnormal excessive proliferation of hematopoietic cells in the bone marrow (1). Cytotoxic chemotherapy and bone marrow hematopoietic stem cell transplantation are among the primary therapeutic treatments for AML (2). However, the majority of patients do not have access to treatment options other than chemotherapy, due to the limited medical access and economic conditions in China.

Arsenic trioxide (ATO) was the first successful treatment for acute promyelocytic leukemia (APL), and has improved the clinical outcomes and prolonged the survival of patients with APL (3-8). Physicians have begun to use ATO to treat types of hematological malignancies other than APL, with AML being the most common (9). However, the suitability of treating hematological malignancies other than APL with ATO, and the molecular mechanism underlying ATO therapy, remains unknown. An improved understanding of the mechanisms associated with ATO may reveal its potential therapeutic value for treating AML.

A number of malignant tumor types exhibit abnormal expression of ROS proto-oncogene 1 receptor tyrosine kinase (ROS1), a gene associated with the Wnt/β-catenin signaling pathway (10-14). ROS1 is considered a potential target for cancer treatment and targeted anticancer therapies are under development (15). If ROS1 inhibitors may be used in combination with ATO to treat patients with AML, the therapeutic efficacy may be improved and adverse effects may be decreased. In addition, novel applications of approved drugs may decrease the costs associated with researching and developing novel agents. Thus, in the present study, the function of ROS1 in the ATO-treatment of AML cells has been evaluated.

Materials and methods

Cell lines. The AML cell lines used in this study include THP-1 (organism, *Homo sapien*/human; cell type, monocyte; tissue, peripheral blood; disease, acute monocytic leukemia; product format, frozen), HL60 (organism, *Homo sapien*/human; cell type, promyeloblast; tissue, peripheral blood; disease, acute promyelocytic leukemia; product format, frozen) and Kasumi-6 (organism, *Homo sapien*/human; cell type: myeloblast; tissue, peripheral blood; disease, acute myeloid leukemia subtype M2; product format, frozen). All cell lines were purchased from Shenyang Yike Biological Technology Co., Ltd. (Shenyang, China).

Transfection. AML cells were transfected with human ROS1-specific or control small interfering (si)RNAs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Functionally-validated siRNA (1 nmol) against ROS1 gene with target sequence of 5'-AAGGTAATTGCTCTAACT TTA-3'. The ROS1-silenced (ROS1-si) and siRNA-transfected control (ROS1-c) cells were harvested 48 h following transfection, and the efficacy of ROS1 silencing was determined using western blot analysis.

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Western blot analysis. ROS1-si and ROS1-c AML cells were collected 48 h after transfection. GAPDH and β-actin were used for control. The protein concentration in the lysates was quantified using an enhanced bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.) with bovine serum albumin (BSA; Invitrogen; Thermo Fisher Scientific, Inc.) as a standard; 30 μg protein was added per lane. The protein extracts were subjected to 7.5% SDS-PAGE at 200 V for 40 min and the resolved proteins were electro-transferred for 12 min at 18 V to nitrocellulose membranes (Thermo Fisher Scientific, Inc.) using a western blotting semi-dry transfer unit (Hoefer, San Francisco, CA, USA). The membranes were blocked for 1 h at room temperature with TBST (10X buffer; 200 mM Tris base, 1.5 M NaCl in MiliQ water; 0.1% Tween-20 was added and pH was adjusted to 7.5 for 1X buffer) containing 5% BSA The membranes were then incubated with one of the primary antibodies (1:200) [rabbit anti-ROS 1 (cat no. MA5-26180; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-Axin (cat no. 34-5900; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-GSK-3β (cat no. A170132; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-APC (cat no. 17-9956-42; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-β-catenin (cat no. 71-2700; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-c-Myc (cat no. MA1-980; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-Cyclin-D1 (cat no. PA5-16777; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-PPAR-α (cat no. PA1-820; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-MMP-7 (cat no. PA1-9069; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-GAPDH (cat no. PA1-988; Invitrogen; Thermo Fisher Scientific, Inc.) and rabbit anti-β-actin (cat no. PA1-183; Invitrogen; Thermo Fisher Scientific, Inc.)] in TBST with 5% BSA overnight at 4˚C with gentle shaking. Membranes were washed twice with TBST for 10 min each and additionally incubated with secondary antibody (goat anti-rabbit; cat no. A11008; 1:5,000; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Then the membrane was washed 2 times for 10 min each with TBST to remove any non-bound secondary antibody. The membrane was incubated with Pierce ECL western-blotting Detection Reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at room temperature for 5 min. Excess detection reagent was removed and the membrane was exposed to X-ray film placed on top of the membrane for 6 min in an X-ray film cassette (Amersham; GE Healthcare, Chicago, IL, USA). Membranes were also assessed for equal loading. The degree of target protein downregulation in ROS1-si cells relative to the control cells was determined by gray level analysis, which was calculated by area and pixel values, using Image J software (National Institutes of Health, Bethesda, MD, USA).

Measurement of cell growth. THP-1, HL60 and Kasumi-6 cells ($3x10^3$ cells/well) were transferred to 96-well plates and treated with 2.5 μM ATO (Harbin Pharmaceutical Group Co., Ltd., Harbin, China) for 72 h at 37˚C in an atmosphere containing 5% CO_2. The effect of ROS1 knockdown on cell proliferation in THP-1, HL60 and Kasumi-6 cells was determined using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), as described previously (16,17).

For cell migration assays, transfected THP-1, HL60 and Kasumi-6 cells ($5x10^5$ cells/well) were seeded in 250 μl RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), without fetal bovine serum (FBS), into the upper chamber of a Transwell plate (with 8 μm pores; Corning Incorporated, Corning, NY, USA). RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) was added into the lower chamber. These cells were incubated at 37˚C in an atmosphere containing 5% CO_2 for between 48 and 72 h. Cells were stained at room temperature for ~10 min and observed using light microscopy at magnification, x100. Each experiment was repeated in triplicate.

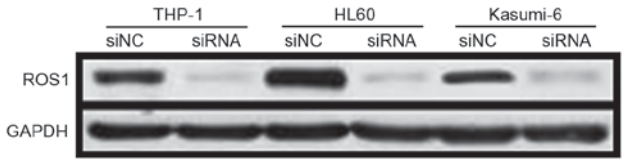

Figure 1. Efficacy of ROS1 knockdown in THP-1, HL60 and Kasumi-6 cell lines by siRNA. ROS1 was successfully silenced in all three cell lines. GADPH was used as the loading control. siRNA, short interfering RNA; NC, negative control.

Statistical analysis. All data were analyzed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Data from the three different samples are presented as the mean ± standard deviation, and the differences among them were compared by analysis of variance, followed by. a Student Newman Keuls post-hoc test, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

ROS1 is silenced successfully in THP-1, HL60 and Kasumi-6 cells. In order to determine the efficacy of gene silencing, the expression levels of ROS1 in THP-1, HL60 and Kasumi-6 cell lines were determined using western blot analysis. ROS1 expression was markedly decreased in cells following transfection with human ROS1-specific siRNA compared with control cells (Fig. 1).

ATO inhibits the migratory and proliferative abilities of THP-1, HL60 and Kasumi-6 cells. The effects of ATO on the migratory and proliferative capabilities of THP-1, HL60 and Kasumi-6 cells was determined *in vitro*. The results demonstrated that ATO treatment significantly inhibited cell migration and proliferation in these three cell lines ($P= 0.003$, 0.007, and 0.014, respectively) (Fig. 2).

ROS1 knockdown increases the sensitivity of AML cells to ATO treatment. Following ROS1 knockdown by siRNA, THP-1, HL60 and Kasumi-6 cells were treated with 2.5 μM ATO. The results revealed that cell migration and proliferation was significantly inhibited by the combination of ROS1 knockdown and ATO treatment, compared with ATO treatment alone (all $P<0.001$) (Fig. 3). This result suggests that ROS1 knockdown sensitized AML cells to the effects of ATO, and that ROS1 is involved in the migration and proliferation of leukemia cells.

ROS1 silencing inhibits Wnt/β-catenin signaling in AML cells. Proliferation and migration of AML cells are regulated by a number of signaling pathways, including the Wnt/β-catenin

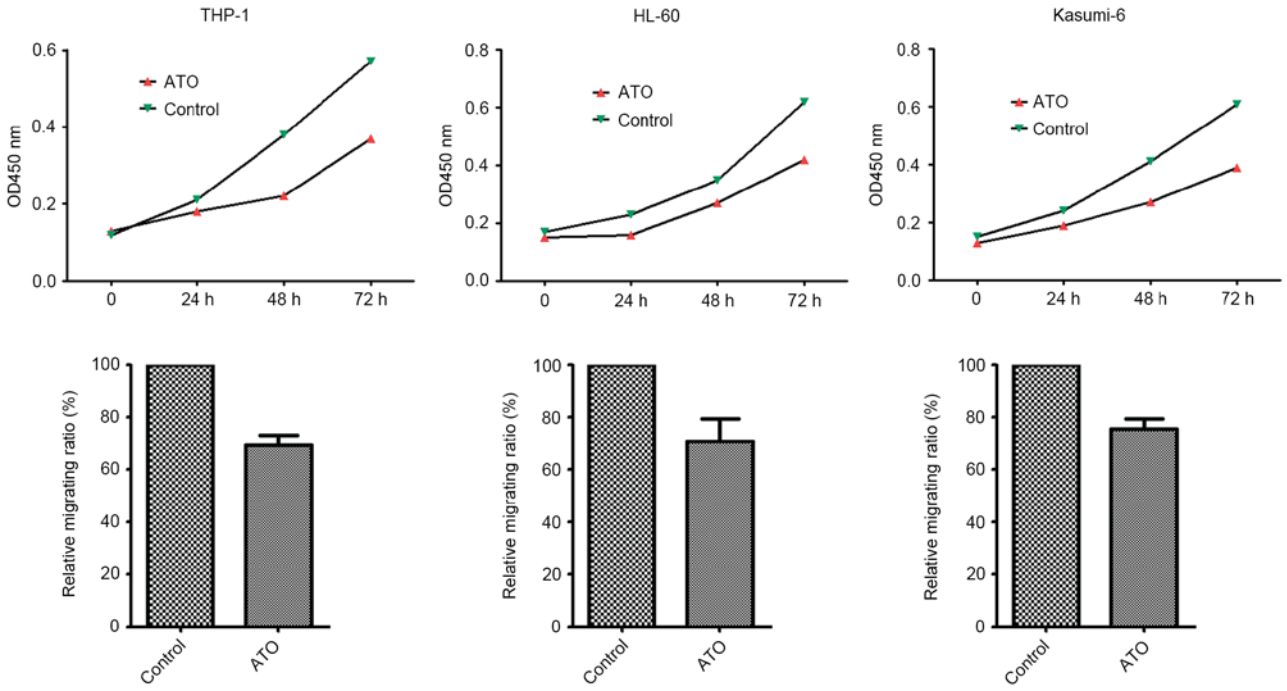

Figure 2. ATO treatment inhibited the proliferation and migration of THP-1, HL60 and Kasumi-6 cells. The bars represent mean ± standard deviation. ATO, arsenic trioxide; OD, optical density.

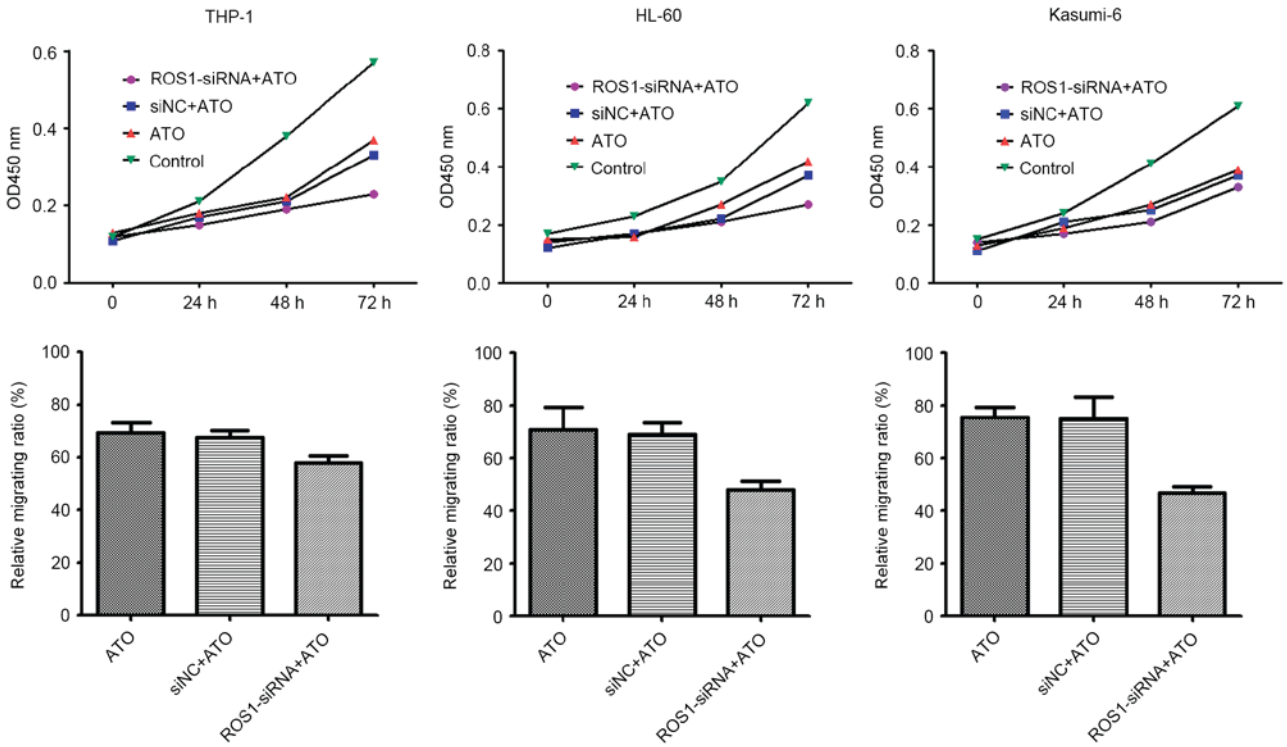

Figure 3. ROS1 knockdown sensitized THP-1, HL60 and Kasumi-6 cells to ATO treatment. The bars represent mean ± standard deviation. ROS1, ROS proto-oncogene 1 receptor tyrosine kinase; ATO, arsenic trioxide; OD, optical density; siRNA, short interfering RNA; NC, negative control.

pathway. Therefore, in the present study, the effect of ROS1 silencing on Wnt/β-catenin activation in AML cells was investigated using western blot analysis (Fig. 4). The relative levels of Axin, glycogen synthase kinase-3β, Adenomatous polyposis coli and peroxisome proliferator-activator receptor α were markedly increased in ROS1-si cells, compared with in ROS1-c cells. The relative levels of β-catenin, c-Myc, cyclin D1, and matrix metalloproteinase-7 were markedly decreased in ROS1-si cells, compared with in ROS1-c cells. These data indicate that ROS1 silencing inhibits Wnt/β-catenin activation, which is crucial for the proliferation and migration of AML cells.

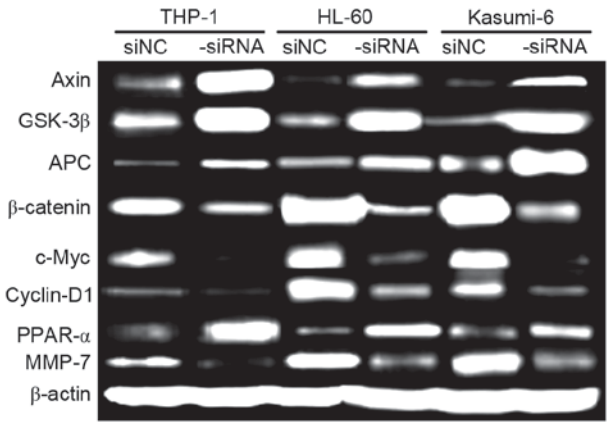

Figure 4. Relative levels of target proteins in ROS1-si and ROS1-c AML cells, cultured for 72 h, as determined using western blot analysis. β-catenin was the loading control. ROS1, ROS proto-oncogene 1 receptor tyrosine kinase; -si, ROS1-silenced; -c, siRNA-transfected control; AML, acute myeloid leukemia; GSK-3β, glycogen synthase kinase-3β; APC, adenomatous polyposis coli; PPAR-α, peroxisome proliferator-activator receptor α; MMP-7, matrix metalloproteinase-7.

Discussion

In the present study, it was identified that ATO treatment inhibited the migration and proliferation of THP-1, HL60 and Kasumi-6 leukemia cell lines, which was mediated by ROS1. Knockdown of ROS1 expression sensitized the three leukemia cell lines to ATO treatment and inhibited the Wnt/β-catenin signaling pathway. Knockdown of ROS1 expression alone decreased the migration and proliferation of the three cell lines, but not significantly (data not presented).

ATO promotes apoptosis and other biological responses in tumor cells, which has prompted its use in the treatment of APL and other types of hematological malignancies, including multiple myeloma (16-20), and cancer of the breast, brain, liver, stomach, prostate, kidney and bladder (21,22). However, toxicity to the liver, heart and level 3/4 peripheral nerves rises with increasing ATO dosage (19,23), which effects therapeutic efficacy and patient compliance. Therefore, increasing the therapeutic window of ATO is important. In this respect, ROS1 downregulation represents a potential therapeutic strategy that requires additional investigation.

A number of signal transduction pathways regulate the biology of leukemia. Among them, the Wnt signaling pathway is one of the best characterized. Wnt signaling serves functions in the development of leukemia and is important for the survival and self-renewal of leukemia cells (24-26). Notably, the Wnt signaling pathway is associated with the maintenance of leukemia stem cells, which is directly associated with disease progression (27,28). β-catenin is maintained in an activated state in these leukemia cells and is expressed in a number of types of tumor (29-32). Previous studies have suggested that ROS1 may be involved in the regulation of Wnt signaling (33). The results of the present study provided insights into the regulation of Wnt signaling, although additional studies are required. For example, in the present study, it was observed that alterations in ROS1 expression resulted in alterations to the expression of proteins associated with Wnt signaling. However, the association between ROS1 and Wnt signaling remains unknown and, in particular, the functions ROS1 serves in the regulatory network of Wnt signaling. In addition, the regulation of Wnt signaling by ROS1 and sensitization of AML cells to ATO treatment by ROS1 downregulation requires validation in animal studies.

The present study demonstrated that knockdown of ROS1 expression enhanced the migration and proliferation of leukemia cell lines, and suppressed Wnt/β-catenin activation. The results of the present study suggested that ROS1 may be a therapeutic target and a sensitizer for ATO treatment in patients with AML. Therefore, the present study provided novel insight into the function of ROS1 in regulating AML progression and ATO treatment.

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL designed the study, conducted the statistical analysis and drafted of the article, and read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author declares that he has no competing interests.

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