Original Article
Silencing of HMOX1 enhances apoptosis caused by arsenic trioxide in HCC

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Abstract: Arsenic trioxide (ATO) is an effective commercial chemotherapeutic drug used to treat different cancers. ATO can induce apoptosis in cancer cells by causing the accumulation of intracellular reactive oxygen species (ROS). However, the therapeutic effect of ATO on hepatocellular carcinoma (HCC) is unsatisfactory, largely due to chemoresistance. In this study, the antioxidant enzyme heme oxygenase 1 (HMOX1), whose function is to remove ROS, was found to be upregulated in SMMC-7721 cells after ATO intervention. The effect of knocking down HMOX1 on the effects of ATO treatment was also assessed. Cell viability, ROS concentrations, and apoptosis were examined in SMMC-7721 cells treated with or without ATO and transfected with or without HMOX1-siRNA. The results showed that HMOX1-siRNA downregulated HMOX1 expression in the presence of ATO. In addition, silencing of HMOX1 promoted ATO treatment-induced cell death by causing the accumulation of ROS and augmenting apoptosis. This study indicates that inhibition of HMOX1 could strengthen the therapeutic effect of ATO in hepatocellular carcinoma (HCC).

Keywords: Arsenic trioxide, heme oxygenase 1, hepatocellular carcinoma, reactive oxygen species

Introduction

Hepatocellular carcinoma (HCC) is a worldwide disease that causes nearly 745,000 deaths every year [1]. Due to the strong compensatory system in the liver, metastases are found in most cases during the first diagnosis. These cases are not suitable for surgery. Sorafenib was approved by the Food and Drug Administration (FDA) in 2007 for HCC treatment and has been a first-line choice for more than 10 years. Although sorafenib is a representative drug in HCC treatment, the price of clinical application is still unmanageable for patients in developing countries. Arsenic trioxide (ATO) is an effective and economical choice.

ATO has successfully demonstrated efficacy in leukemia [2, 3] as well as breast cancer [4], lung cancer [5], and prostate cancer [6]. In HCC, ATO has also found to be effective in different ways. Cui L reported that ATO can prevent HCC progression and tumorigenesis by downregulating B7-H4 [7]. Liu ZM suggested that inhibition of the PI3K/Akt pathway leads to a decrease in TGIF, which can strengthen the therapeutic effect of ATO in HCC [8]. On the other hand, several groups have reported that ATO can induce ROS in different cancers. Mesbahi Y suggested that ATO enhances ROS levels in AML cells [9]. Dugo EB demonstrated that the cytotoxicity of ATO in HCC relies on oxidative stress and the mitochondrial apoptotic pathway [10].

However, some obstacles have restricted the application of ATO. For example, chemoresistance reduces the induction of apoptosis ATO [8]. In addition, the application of ATO may damage the kidney [11] and liver [12] in a dose-dependent manner. Most HCC patients have other fundamental diseases, such as hepatitis infection and liver cirrhosis; therefore, their liver functions are not normal, and it is difficult for them to endure chemotherapy. Therefore, pursuing new therapy with more efficacy and less hepatotoxicity is an urgent mission for chemotherapy.
Heme oxygenase 1 (HMOX1) is an antioxidant enzyme, and its function is to remove intracellular reactive oxygen species (ROS) [13]. Overexpression of ROS can lead to cell death. Owing to the important function of HMOX1 in scavenging ROS, HMOX1 probably acts as a barrier to cell death in ATO treatment. However, whether HMOX1 is involved in the therapeutic efficacy of ATO remains unknown. Based on a GEO database (GEO ID: GSE6907) containing data on the effects of heavy metals on HCC cell lines, we analyzed microarray results between control cells and arsenic-treated cells. HMOX1 was found to be upregulated over 15.4-fold (logFC=3.94). Therefore, it is possible that the upregulation of HMOX1 restricts the induction of ROS by ATO. Consistent with this hypothesis, silencing of HMOX1 promotes ATO treatment-induced cell death by enabling the accumulation of ROS and augmenting apoptosis. This study indicates that inhibition of HMOX1 could strengthen the therapeutic effect of ATO on HCC.

Materials and methods

Materials

Arsenic trioxide was purchased from Beijing SL Pharmaceutical Co., Ltd. (Beijing, China). High-glucose Dulbecco's modified Eagle's medium (H-DMEM) was purchased from HyClone (GE Healthcare Life Science, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Biological Industries, USA). PCR primers were purchased from Shanghai Sunya Biotechnology Co., Ltd. (Shanghai, China). An EZ-press RNA Purification Kit and an RT reaction kit were purchased from EZBioscience (Roseville, USA). Lipofectamine™ 2000 was purchased from Invitrogen Life Technologies (Carlsbad, USA). HMOX1-siRNA was purchased from Ribobio (Guangzhou, China). A Cell Counting Kit-8 (CCK-8) was purchased from Yeasen (Shanghai, China). Phosphate-buffered saline (PBS) was purchased from HyClone (GE Healthcare Life Science, USA). A rabbit anti-human HO-1 polyclonal antibody, a mouse anti-human β-actin monoclonal antibody, a horseradish peroxidase (HRP)-labeled anti-rabbit IgG, and an anti-mouse IgG were purchased from Proteintech Group (Rosemont, USA). A heme oxygenase 1 (HO-1) rabbit polyclonal antibody was purchased from HuaAn Biotechnology Co., Ltd. (Hang Zhou, China). An alpha tubulin rabbit polyclonal antibody, HRP-conjugated Affinipure goat anti-mouse IgG (H+L) and rabbit anti-goat IgG polyclonal antibody were purchased from Proteintech Group (Chicago, USA). Trypsin (0.25%, phenol red) and trypsin-EDTA (0.25%, phenol red) were purchased from Yeasen (Shanghai, China).

Cell culture and treatments

Two human HCC cell lines were studied in this research. SMMC-7721 and Huh-7 were kindly provided by Dr. Zhang Shizhe at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China. The two cell lines were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) mixed with 10% fetal bovine serum (FBS). Double antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin) were also added to the H-DMEM at 37°C in a humidified incubator at a concentration of 5% carbon dioxide. For ROS and apoptosis assays, cells were cultured in 60 mm diameter dishes (5×10^5 cells/dish) for 24 hours to incubate. Then, we refreshed the medium with medium containing ATO and/or siRNA for the following experiments.

Transfection

On the basis of the protocol from the manufacturer (Ribobio, Guangzhou, China), siRNA (HMOX1-siRNA or negative control siRNA) was transfected into cells in 6-well plates (5×10^5 cells per well) or 96-well plates (1×10^4 cells per well) with Lipofectamine™ 2000 (Invitrogen GmbH, Karlsruhe, Germany). The sequence of HMOX1-siRNA is GCGCATTTTCTCATACCATA.
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The medium contained no FBS or antibiotics but did contain ATO, if applicable. Six hours after transfection, the medium was replaced with fresh medium containing 10% FBS and double antibiotics with/without ATO for subsequent culturing. Thirty-six hours after the exchange of medium, cells were collected for the next steps. Negative control siRNA was used to establish the control cells.

Western blot analysis

Cells were collected and lysed with RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) and a protease inhibitor cocktail (APE-xBIO, Shanghai, China) and then centrifuged at 12,000×g at 4°C for 20 minutes to discard the sediment and extract the supernatant. SDS loading buffer (Yeasen, Shanghai, China) was mixed with the supernatant completely. Then, the mixture was heated at 95°C for 15 minutes to make the protein sample. A BCA protein assay kit was used to measure the concentration of each protein sample. The supernatant was stored in a heat block at 70°C until use. The protein samples were loaded in different lanes in 12% SDS-PA gels for electrophoresis. The voltage of electrophoresis was 60 V for 115 minutes. Then, a voltage of 120 V was applied until the migration distance of the target marker was satisfactory. After that, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA). After electrotransfer, 5% milk (milk powder dissolved in PBST) was used to block the PVDF membranes. After 8 hours of blocking, the PVDF membranes were washed with PBST 3 times for 10 minutes per wash. Primary rabbit anti-human HO-1 polyclonal (1:10000) and mouse anti-human β-actin monoclonal (1:5000) antibodies were used to incubate the PVDF membranes for 1 hour. The same washing procedures used after incubation with the HO-1 antibody were again performed. After washing the PVDF membranes, immunoreactive bands were visualized with ECL reagents (Tanon, Shanghai, China) in an automatic gel imaging analysis system (Tanon, Shanghai, China). The expression of protein in Western blot is judged by densitometry using Image J (1.51T) software normalized to control.

qPCR

An EZ-press RNA Purification Kit and an RT reaction kit were purchased from EZBioscience (Roseville, USA). Total RNA purification was performed as described according to the protocol of the EZ-press RNA Purification Kit (EZBioscience, Roseville, USA). The RT reaction procedures were also performed following the instructions provided with the RT reaction kit (EZBioscience, Roseville, USA). Real-time quantitative PCR was performed in a 7500 Real-Time PCR system (Applied Biosystems, Mannheim, Germany). PowerUp™ SYBR Green Master Mix was purchased from Applied Biosystems Life Technologies (Thermo Fisher Scientific, USA). The primer sets used in this research are listed in Table 1.

CCK-8 assay

We digested SMMC-7721 cells with trypsin-EDTA and collected the cells to determine the cell densities. Then, 10,000 cells per well were seeded into 96-well plates. After attachment, ATO was added into the wells to achieve the indicated final concentrations. For wells receiving siRNA interference, ATO at the same concentration was added when exchanging the medium. The cells were incubated until analysis with a Cell Counting Kit-8 (Yeasen, Shanghai, China) following the protocol provided by the manufacturer. The experiment was repeated three times.

Apoptosis

An Annexin V-FITC/PI Apoptosis Detection Kit was purchased from Yeasen (Shanghai, China). Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen, Shanghai, China) was used for the apoptosis experiments. Trypsin without EDTA was used for digestion, because Ca2+ is needed.

Table 1. The prime of β-actin and HMOX1 for qPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTIN-F</td>
<td>CATGTACGGTGCTATCCAGGC</td>
</tr>
<tr>
<td>ACTIN-R</td>
<td>CTCCCTAATGTCACGCACTG</td>
</tr>
<tr>
<td>HMOX1-F</td>
<td>AAGACTCGGCCTCTGTCAAC</td>
</tr>
<tr>
<td>HMOX1-R</td>
<td>AAAGCCCTACAGCAACTGTCG</td>
</tr>
</tbody>
</table>
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A

B

[Graph depicting gene expression changes with annotations]

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Figure 1. HMOX1 is positively induced by ATO treatment in HCC. A. Heat map illustrating the top 49 differentially expressed genes (threshold log fold change of 3) as determined by microarray analysis between 3 control samples and 3 arsenic-treated samples (GSE6907). B. Volcano plot showing the 49 genes; the top 15 genes are labeled. C. Dot plot displaying the counts and expression changes of the top 10 genes.
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According to the manufacturer's instructions. After arsenic treatment, the cells were trypsinized, washed with PBS, and then incubated with DCFH-DA at a final concentration of 10 μM in H-DMEM for 30 minutes at 37°C. Fluorescence was measured by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. For each analysis, 10,000 events were recorded. Intracellular ROS levels were expressed as the average DCF fluorescence intensity of the cells.

Statistical analysis

All data calculations and statistical analyses in this study were performed in GraphPad Prism 7.0 software (San Diego, CA, USA). Student's t-test was used to analyze the significance of any differences between two groups. Student's t-tests were used to determine the significance of the respective groups for the different study conditions. P<0.05 (*), P<0.01 (**), P<0.001 (***). ** or P<0.001 (****) indicated a significant difference.

Results

To gain further insight into the cell death caused by ATO with regard to oxidative stress, we investigated data from NCBI (GEO ID: GSE6907; website: https://www.ncbi.nlm.nih.gov/geo/). The datasets contained gene expression changes induced by heavy metals and other chemicals, including arsenic. From the series, 6 datasets (GSM159303, GSM159305, GSM159306, GSM159312, GSM159314 and GSM159315) were chosen for further analysis. Gene expression levels and selected those with a log fold change ≥3. Ultimately, a total of 49 differentially expressed genes (29 downregulated and 20 upregulated). Figure 1A shows a heat map of

when Annexin V-FITC combines with cells. Trypsin-EDTA chelates Ca^{2+} while digesting, which influences the accuracy of apoptosis detection. The emitted green fluorescence of annexin (FL1) and red fluorescence of PI (FL3) were detected at excitation wavelengths of 488 and 535 nm and at emission wavelengths of 525 and 615 nm, respectively. The levels of early apoptosis, late apoptosis, and necrosis were determined based on the percentages of annexin V/PI^−, annexin V/PI^+, and annexin V/PI^+ cells, respectively.

ROS detection

A Reactive Oxygen Species Assay Kit was purchased from Yeasen (Shanghai, China). Intracellular ROS levels were measured by flow cytometry using the cell-based ROS assay kit

![Figure 2](image-url)
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the analysis of the selected genes, and Figure 1B shows a volcano plot. Both figures feature an important enzyme related to oxidative stress, HMOX1. Figure 1C displays the 10 genes with the greatest absolute fold changes from the analyzed data. HMOX1 not only changed up to 3.949-fold (log fold change) but also had greater counts than the other genes. This might imply a concrete change in biological function. These results indicate that HMOX1 might play an important role in arsenic treatment in HCC.

To explore whether ATO could induce the upregulation of HMOX1 in HCC, two common HCC cell lines were selected, SMMC-7721 and Huh-7. As shown in Figure 2, HMOX1 increased dozens-fold in a dose-dependent manner and in a time-dependent manner. Figure 2A shows that HMOX1 mRNA expression increased approximately 20-fold in cells treated with 5 µmol ATO and approximately 50-fold in cells treated with 8 µmol ATO after 24 hours. Figure 2B illustrates the time-dependent effect of 5 µmol ATO. After 12 hours, HMOX1 had increased by approximately 10-fold; after 24 hours, HMOX1 had increased by approximately 20-fold; and after 36 hours, HMOX1 had increased by 40-fold. As shown in Figure 2C and 2D, HMOX1 was upregulated in both SMMC-7721 and Huh-7 cells more than 10-fold. These results proved that ATO treatment does upregulate HMOX1 in HCC.

HMOX1 is an antioxidant enzyme, and its function is to remove ROS. We then determined the effect of knocking down HMOX1 on the effects of ATO treatment. siRNA was used to investigate changes in HMOX1 in SMMC-7721 cells. The consequences of HMOX1 silencing are shown in Figure 3. As shown in Figure 3A, some SMMC-7721 cells were transfected with the negative control siRNA (scramble), and the mRNA expression of HMOX1 was approximately 30-fold higher in the ATO treatment group than in the non-ATO treatment group (p<0.05). However, HMOX1 expression in the SMMC-7721 cells transfected with HMOX1-siRNA (HMOX1-siRNA) was upregulated no more than 1.5-fold compared with that in the cells without ATO treatment (p=not significant). As shown in Figure 3B and 3C, the Western blot results showed the same tendency at the protein level as at the mRNA level. The two columns, the scramble group and the HMOX1-siRNA group, both of which received ATO, showed significant differences (p<0.05). These results indicate that HMOX1-siRNA blocked ATO-mediated HMOX1 upregulation in SMMC-7721 cell lines at both the mRNA and protein levels.
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Cell Counting Kit-8 was used to explore cell viability changes caused by ATO with HMOX1-siRNA at different concentrations. As shown in Figure 4A, the pink curve (scramble) represents SMMC-7721 cells treated with ATO and negative control siRNA, while the blue curve (HMOX1-siRNA) represents SMMC-7721 cells treated with ATO and HMOX1-siRNA. The scramble curve had higher OD values between 0 µmol and 50 µmol than the HMOX1-siRNA curve. A concentration of 12.355 µmol ATO in the scramble group and a concentration of 8.093 µmol ATO in the HMOX1-siRNA group led to 50% cell death. Quantification of the IC\textsubscript{50} values is shown in Figure 4B and the IC\textsubscript{50} for the HMOX1-siRNA group was significantly lower than that for the scramble group (**p<0.01). As shown in Figure 4C, the ATO-induced variation between the scramble group and the HMOX1-siRNA group was time-dependent. The HMOX1-siRNA curve is significantly lower than the scramble curve (**p<0.01), indicating that HMOX1-siRNA helps to enhance cell death caused by ATO. These results demonstrate that silencing HMOX1 might overcome chemoresistance to ATO in SMMC-7721 HCC cell lines.

Several studies [14, 15] have reported that the accumulation of ROS can induce apoptosis in different cancers. HMOX1 has been recognized as an indispensable enzyme that prevents oxidative stress by scavenging ROS [16]. ROS levels were detected to determine the differences between the scramble and HMOX1-siRNA groups at different concentrations of ATO (Figure 5). ROS accumulated in a dose-dependent manner at 5 µmol and 10 µmol but was not increased by much at 20 µmol and 40 µmol. At lower concentrations (5 µmol and 10 µmol), the ROS levels in the HMOX1-siRNA group were higher than those in the scramble group, while at higher concentrations (20 µmol and 40 µmol), the ROS levels in the HMOX1-siRNA gr-
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Figure 6A demonstrates 12 flow cytometry figures under four different conditions. Figure 6B reveals the total apoptotic rates under these four conditions. Under ATO treatment, HMOX1-siRNA (the pink column) greatly increased the proportion of apoptotic cells in the population compared with scramble siRNA (the blue column). These results illustrate that decreased HO-1 expression enhances apoptosis under ATO treatment.

**Discussion**

HCC is not very sensitive to chemotherapeutic drugs compared with other cancer types. Arsenic trioxide (ATO) is a commercial chemotherapeutic drug that has been applied in various kinds of tumors [19]. In hepatocellular carcinoma, ATO has proven to be beneficial in inducing cell death in different ways [10].
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However, the use of ATO is restricted for several reasons. For example, some severe side effects related to dose may drastically disrupt homeostasis, especially in HCC patients. The occurrence of acute liver dysfunction in patients with ATO treatment ranges from 33% to 75% [20-22] and can only be relieved by reducing the application of ATO [23]. In this study, the dose of ATO in combination with HMOX1-siRNA giving rise to 50% cell death was only 65.50% of the dose required for 50% cell death with negative control siRNA. Therefore, the study is clinically significant and provides new evidence supporting reduced doses of ATO with certain targets.

Figure 6. Silencing of HMOX1 strengthens apoptosis induced by ATO. (A) Four different treatment conditions were applied to SMMC-7721 cells as indicated. Apoptosis was examined by flow cytometry with Annexin V-FITC/PI dual staining for 36 hours after 6 hours of transfection. Representative plots of flow cytometry analysis. Q4, normal cells; Q3, cells in early apoptosis; Q2, cells in late apoptosis; Q1, cells in the nonspecific staining region. (B) Bar graph of 3 repeated experiments displaying the percentages of apoptotic cells, including the cells in early apoptosis (Q3 in A) and late apoptosis (Q2 in A). The data are expressed as the mean ± SD, N=2, ***P<0.001 between cells treated with ATO but not siRNA and cells treated with ATO and HMOX1-siRNA.
C Zou demonstrated that HMOX1 prevents the progression of the HCC cell line HepG2 both in vivo and in vitro. Overexpression of HMOX1 could inhibit IL-6 and p38MAPK, thereby inhibiting the migration of HepG2 cells [24]. Others have shown that HMOX1 slows HCC progression by silencing miR-30d/miR-107. The PI3K/Akt pathway may play the final effective role [25]. Some scientists suggest that inducible HMOX1 could be regarded as a cytoprotective protein against oxidative stress [26, 27]. Induction of HMOX1 can degrade the prooxidant heme to clean biliverdin and bilirubin [28]. However, the mechanisms by which specific pathways mediate HCC biological processes after ATO treatment remain to be fully explored.

In this study, chemoresistance to ATO in HCC was partially caused by upregulation of HMOX1, and silencing of HMOX1 during ATO treatment increased cell death in a time-dependent manner. Furthermore, increased accumulation of ROS after HMOX1 silencing indicates that oxidative stress is an important pathway for ATO treatment in HCC.

Ghavamzadeh proposed that the clinical dose of ATO should be 0.15 mg/kg in HCC, which is equivalent to nearly 10.8 µmol in human plasma [29]. Our results demonstrate that a lower concentration (under 20 µmol) of ATO with inhibition of HMOX1 can enhance the accumulation of ROS; at higher (20–40 µmol) concentrations of ATO, although the tendency was not dose-dependent, the proportion of negative cells was increased. This might indicate that cells had died after sufficient accumulation of ROS. Herein, evidence of sensitization to ATO treatment and the elimination of chemoresistance in HCC is provided, which makes HMOX1 a potential therapeutic target. Furthermore, considering the clinical dosing of ATO, these results suggest the possibility of a reduced dose of ATO in combination with an HMOX1 inhibitor, which might ameliorate the side effects of ATO to some extent.

The study has some limitations. First, the types of cell lines were limited and were insufficient to elucidate whether downregulation of HMOX1 can universally synergize ATO treatment in HCC. Second, an in vivo study is needed to determine whether the side effects of this combination can be endured. Finally, the oxidative pathway is a significantly important pathway that contains various important enzymes, including HMOX1. It is imperative to study the upstream and downstream mechanisms of HMOX1 to understand the intrinsic mechanisms involved in the effects of ATO treatment in HCC. Therefore, further exploration of the underlying mechanisms is needed in the future.

In conclusion, silencing of HMOX1 promotes ATO treatment-induced cell death through generating ROS and enhancing apoptosis. The study provides new proof that inhibition of HMOX1 could strengthen the therapeutic effect of ATO on HCC.

Disclosure of conflict of interest

None.

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