

Knockdown of heme oxygenase-1 promotes apoptosis and autophagy and enhances the cytotoxicity of doxorubicin in breast cancer cells

XIAO-FENG ZHU^{1*}, WEN LI^{2,3*}, JIE-YI MA², NAN SHAO¹, YUN-JIAN ZHANG¹, RUI-MING LIU^{2,3},
WEI-BIN WU¹, YING LIN¹ and SHEN-MING WANG^{1,3}

¹Department of Breast and Thyroid Surgery, ²Laboratory of General Surgery,
³Guangdong Engineering Laboratory for Diagnosis and Treatment of Vascular Disease,
The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510080, P.R. China

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Abstract. Heme oxygenase-1 (HMOX-1) is a microsomal enzyme that exerts anti-apoptotic and cytoprotective effects. In the present study, HMOX-1 was demonstrated to be over-expressed and able to be induced by doxorubicin in breast cancer cell lines. Knockdown of *HMOX-1* using short interfering (si)RNA enhanced the cytotoxicity of doxorubicin in MDA-MB-231 and BT549 cells. Knockdown of *HMOX-1* downregulated B cell lymphoma (Bcl)-2 and Bcl-extra large expression, and significantly enhanced doxorubicin-induced apoptosis in MDA-MB-231 and BT549 cells. Additionally, knockdown of *HMOX-1* upregulated light chain 3B expression and markedly increased the accumulation of autophagic vacuoles in MDA-MB-231 and BT549 cells treated with doxorubicin. These results indicated that HMOX-1 may be involved in conferring the chemoresistance of breast cancer cells, by preventing apoptosis and autophagy. Therefore, HMOX-1 may represent a potential therapeutic target for enhancing the cytotoxicity and efficacy of doxorubicin during the treatment of breast cancer.

Introduction

Breast cancer is the most frequently diagnosed cancer amongst females and the second most common cause of

cancer-related mortality among women (1). One third of novel cancer diagnoses in females are breast cancer and a total of one in eight women will be diagnosed with breast cancer, with a lifetime risk of mortality due to breast cancer of 3.4% (1). Chemotherapy is one of the major systemic therapies available for the treatment of breast cancer. A recent meta-analysis of the outcome of 100,000 patients with breast cancer, confirmed the benefit of cyclophosphamide-, methotrexate- and fluorouracil- therapies and anthracycline-based therapies with absolute reduction of mortalities of 6.2 and 6.5%, respectively, at 10 years (2). Despite progress in the improvement of chemotherapeutic strategies, the ability to treat advanced breast cancer remains poor, mainly due to the chemoresistance of cancer cells to standard chemotherapy. Numerous anti-apoptotic and cytoprotective pathways have been associated with the chemoresistance of cancer cells (3,4).

Heme oxygenase (HMOX) is a microsomal enzyme, which catalyzes the initial, rate-limiting step in the degradation of heme, and has a crucial role in the recycling of iron (5). The enzymatic activity of HMOX also produces CO, ferrous iron and biliverdin. Thus, HMOX is able to reduce oxidative stress, attenuate inflammatory responses and lower the rate of apoptosis (6). HMOX-1, an isoform of HMOX, may be induced in response to cellular stress and diverse oxidative stimuli (7). HMOX-1 is frequently overexpressed in a range of cancers, including hepatoma, prostate cancer, melanoma and brain tumors (8-11). HMOX-1 promotes proliferation in human melanoma and pancreatic cancer cell lines (10,12). As HMOX-1 is a potent inducer of vascular endothelial growth factor (VEGF), a crucial factor involved in tumor angiogenesis, it has also been recognized to stimulate angiogenesis and thus support tumor progression (13). Additionally, overexpression of HMOX-1 promoted metastasis in a murine model of melanoma (14).

Expression of HMOX-1 may be induced by radiation and chemotherapy in cancer cells (15), and the expression of HMOX-1 was predictive of poorer survival in patients with breast carcinoma (16). HMOX-1 has also been found to protect cancer cells against apoptosis by exerting

Correspondence to: Professor Shen-Ming Wang or Professor Ying Lin, Department of Breast and Thyroid Surgery, The First Affiliated Hospital of Sun Yat-Sen University, 58 Zhongshan 2nd Road, Guangzhou, Guangdong 510080, P.R. China
E-mail: shenmingwang@hotmail.com
E-mail: frostlin@hotmail.com

*Contributed equally

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anti-apoptotic activities (17,18). Studies have indicated that upregulation of HMOX-1 is correlated with the inhibition of autophagy (19,20). Autophagy, which describes the process of cellular self-digestion, is considered to be a cytoprotective response, which may occur following the withdrawal of growth factors or under stressful conditions (21). However, under certain conditions, autophagy may function as a mechanism of cell death (22). HMOX-1 and autophagy are induced in response to cell stress (15,21). Therefore, HMOX-1 may function as a specific regulator of autophagy in breast cancer and may have a role in the survival of breast cancer cells during chemotherapy.

In the present study, HMOX-1 expression and the effects of doxorubicin in breast cancer cell lines were evaluated. In addition, the effects of HMOX-1 knockdown in breast cancer cells were investigated.

Materials and methods

Cell culture. The human breast cancer cell lines MDA-MB-231, BT549, HS578T, MDA-MB-468, MCF-7 and ZR751, as well as the non-malignant MCF-10A breast cell line were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Gibco, Karlsruhe, Germany) in a 5% CO₂ atmosphere at 37°C. Doxorubicin was obtained from Keygen Biotech (Nanjing, China), dissolved in water and stored at 4°C.

Transfection of short interfering (si)RNAs. HMOX-1 siRNA and control siRNA was synthesized by Guangzhou RiboBio Co., Ltd (Guangzhou, China). The sequence of HMOX-1 siRNA was as follows: Sense, 5'-CCAGCAACAAAGUGC AAGAdTdT-3' and antisense, 3'-dTdTGGUCGUUGUUU-CACGUUCU-5'. Breast cancer cell lines were transfected with 50 nM siRNA using RNAiMAX transfection reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Reverse transcription quantitative-polymerase chain reaction (RT-qPCR) for mRNA quantification. Total RNA was extracted from the cells using TRIzol (Invitrogen Life Technologies) and complementary (c)DNA was synthesized from 1,000 ng total RNA using the PrimeScript RT reagent kit (Takara Biotechnology, Co., Ltd, Dalian, China), according to the manufacturer's instructions. PCR was performed using the PrimeScript RT Master Mix (Takara Biotechnology, Co., Ltd) on a Bio-Rad CFX96 real-time PCR machine (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequences were as follows: HMOX-1 forward, 5'-TACCACATC TATGTGGCCCTG-3' and reverse, 5'-TGGCTGGTGTAGGGGAT-3'; GAPDH forward, 5'-GCACCGTCA AGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGC CAGTGA-3'. Data analysis was performed using the comparative C_T method (23) with Bio-Rad Manager 2.1 software (Bio-Rad Laboratories, Inc.).

Western blotting. Cells were lysed in radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology, Inc.

Danvers, MA, USA) supplemented with protease inhibitor (Roche Diagnostics, Basel, Switzerland). The concentration of total protein was determined using a bicinchoninic acid assay kit (Keygen Biotech). Equal amounts of protein (35 µg) were separated by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA) and blotted with the following monoclonal IgG rabbit anti-human antibodies at a dilution of 1:1,000: Anti-HMOX-1 (cat. no. ab52947; Abcam, Cambridge, UK), anti-B cell lymphoma-2 (Bcl-2; cat. no. 2870), anti-Bcl-extra large (xL; cat. no. 2764), anti-Bcl-2-like protein 4 (Bax; cat. no. 5023), anti-Becclin-1 (cat. no. 3495), anti-LC3B (cat. no. 3868) or anti-β-tubulin (cat. no. 2128) (Cell Signaling Technology, Inc.), followed by incubation overnight at 4°C. The bands were visualized using Luminol reagent (Pierce Biotechnology, Inc.; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and imaged using GE ImageQuant Las 4000 mini (GE Healthcare Life Sciences, Chalfont, UK).

Drug sensitivity assay. MDA-MB-231 and BT549 cells were seeded in 96-well culture plates at a density of 5,000 and 10,000 cells/well, respectively, and transfected with siRNA, 12 h prior to treatment with 0.02, 0.05, 0.10, 0.15 or 0.20 µg/ml doxorubicin for 48 h. Cell viability was assessed using the Cell Counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance values were measured at 450 nm using a Sunrise Absorbance Reader (Tecan US, Inc., Morrisville, NC, USA).

Cell apoptosis assay. Apoptosis was assessed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (Dojindo) according to the manufacturer's instructions. Briefly, the cells were transfected with siRNA for 12 h prior to treatment with 0.2 µg/ml doxorubicin for 48 h. The cells were harvested, resuspended in 500 µl binding solution, and incubated with 5 µl Annexin V and 5 µl propidium iodide (PI) for 10 min in the dark. Apoptosis was analyzed by flow cytometry using a Beckman Coulter EPICS XL-MCL and the results were analyzed using Kaluza software version 1.2 (both from Beckman Coulter Inc., Brea, CA, USA).

Cell autophagy assay. MDA-MB-231 and BT549 cells were seeded in 24-well plates at a density of 20,000 and 40,000 cells/well, respectively, and transfected with siRNA for 12 h prior to treatment with 0.1 µg/ml doxorubicin for 48 h. The cells were then washed in phosphate-buffered saline (PBS; Gibco Life Technologies, Shanghai, China), fixed in 4% formaldehyde (Xilong Chemical Co., Ltd., Shantou, China) for 15 min, permeabilized with 0.1% Triton X-100 (Beyotime Institute of Biotechnology, Shanghai, China) for 10 min, blocked with 0.1% bovine serum albumin (Beyotime Institute of Biotechnology) for 1 h and incubated with anti-light chain 3B (LC3B) antibody (Cell Signaling Technology, Inc.) overnight at 4°C. Subsequently, cells were incubated with a green fluorescent protein (GFP)-tagged secondary antibody (Life Technologies, Grand Island, NY, USA) for 1 h at room temperature, then incubated with DAPI (Roche Diagnostics) for 10 min and images were acquired using a fluorescence microscope (Carl Zeiss Axio Observer Z1; Carl Zeiss, Jena, Germany).

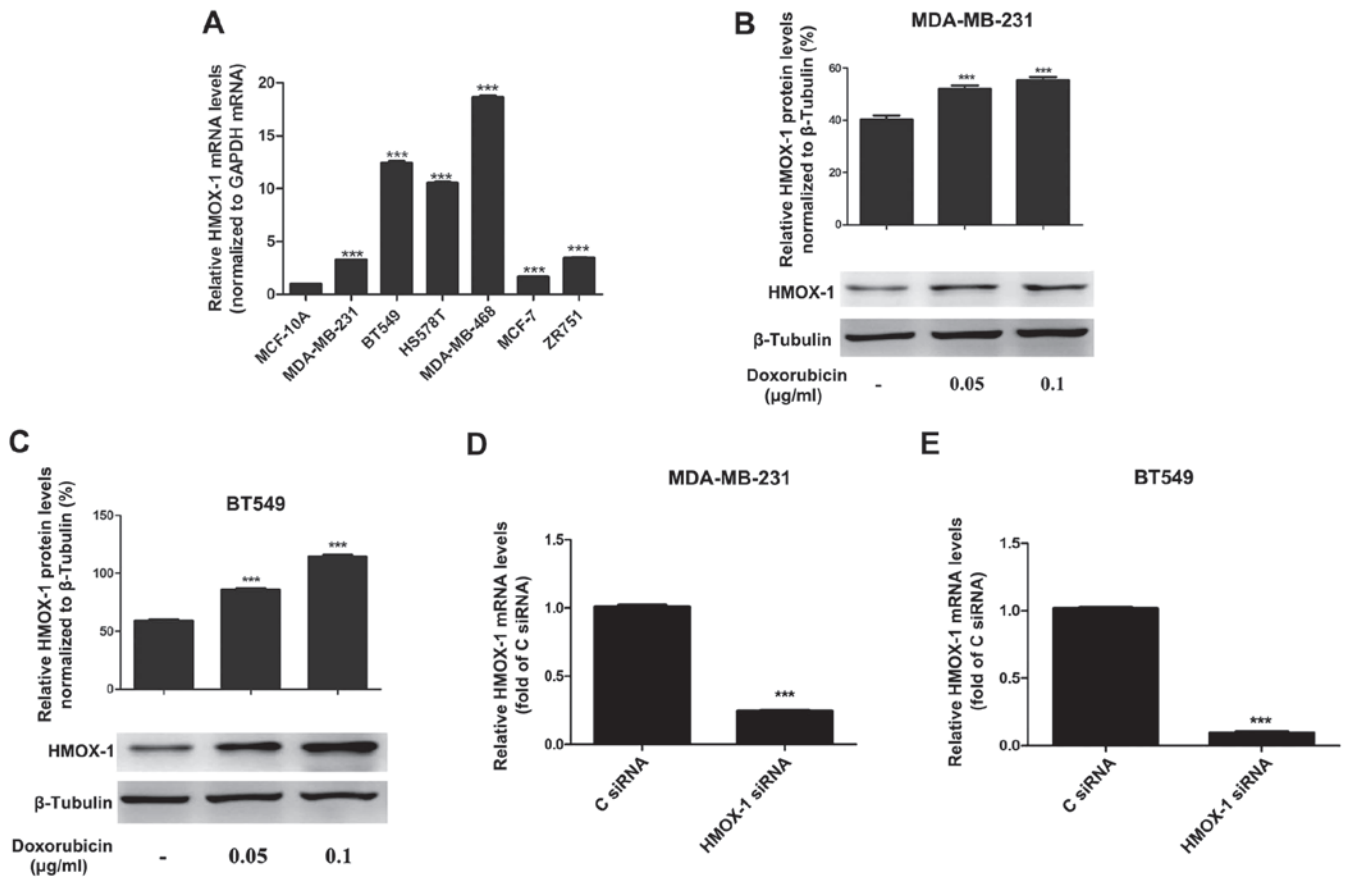


Figure 1. HMOX-1 is overexpressed and is induced by doxorubicin in breast cancer cell lines. (A) RT-qPCR analysis of *HMOX-1* expression in breast cancer cell lines and MCF-10A non-malignant breast cells. (B and C) Western blot analysis of HMOX-1 protein expression in (B) MDA-MB-231 and (C) BT549 cells treated with various concentrations of doxorubicin for 48 h. (D and E) RT-qPCR analysis of *HMOX-1* expression in (D) MDA-MB-231 and (E) BT549 cells 48 h following transfection with 50 nM *HMOX-1* siRNA or control siRNA. All experiments were repeated at least three times. Data are presented as the mean \pm standard deviation. *** P <0.001 vs. control cells. HMOX-1, heme oxygenase-1; RT-PCR, reverse transcription-quantitative polymerase chain reaction; mRNA, messenger RNA; siRNA, short interfering RNA; C, control.

Statistical analysis. Data are expressed as the mean \pm standard deviation. Differences between the treatment groups and control group were assessed with Student's t-test using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). P <0.05, P <0.01 and P <0.001 were considered to indicate statistically significant differences.

Results

HMOX-1 is overexpressed and able to be induced by doxorubicin in breast cancer cell lines. RT-qPCR analysis indicated that *HMOX-1* was overexpressed in the breast cancer cell lines evaluated. Compared with that of the non-malignant breast cell line MCF-10A, the expression of *HMOX-1* was 3.3-fold higher in MDA-MB-231 cells, 12.4-fold higher in BT549 cells, 10.5-fold higher in HS578T cells, 18.6-fold higher in MDA-MB-468 cells, 1.6-fold higher in MCF-7 cells and 3.4-fold higher in ZR751 cells (Fig. 1A). In addition, doxorubicin treatment significantly upregulated HMOX-1 protein expression in MDA-MB-231 and BT549 cells (Fig. 1B and C).

Silencing HMOX-1 increases the sensitivity of breast cancer cell lines to doxorubicin. A cytotoxicity assay was performed to investigate the effects of HMOX-1 on the sensitivity of breast cancer cell lines to doxorubicin. Breast cancer cells

were transfected with 50 nM *HMOX-1* siRNA or the negative control siRNA. At 48 h post-transfection, *HMOX-1* messenger (m)RNA expression was significantly knocked down in the MDA-MB-231 and BT549 cell lines (Fig. 1D and E). The transfected cells were subsequently exposed to 0.02, 0.05, 0.1, 0.15 or 0.2 μ g/ml doxorubicin for 48 h. The CCK-8 assay demonstrated that knockdown of *HMOX-1* significantly enhanced the cytotoxicity of doxorubicin in MDA-MB-231 and BT549 cells (Fig. 2).

HMOX-1 knockdown enhances doxorubicin-induced apoptosis in breast cancer cell lines. MDA-MB-231 and BT549 cells were transfected with *HMOX-1* siRNA or control siRNA, treated with 0.2 μ g/ml doxorubicin for 48 h, then stained using Annexin V and PI and subjected to flow cytometric analysis to quantify apoptotic cells. Silencing of *HMOX-1* significantly increased the apoptotic rate in doxorubicin-treated MDA-MB-231 and BT549 cells (Fig. 3A and B). Furthermore, western blot analysis indicated that knockdown of *HMOX-1* reduced the expression of Bcl-xL and Bcl-2, but did not alter the expression of Bax (Fig. 3C).

HMOX-1 silencing enhances doxorubicin-induced autophagy in breast cancer cell lines. Doxorubicin is able to promote cell death in cancer cells via autophagy (21). Silencing

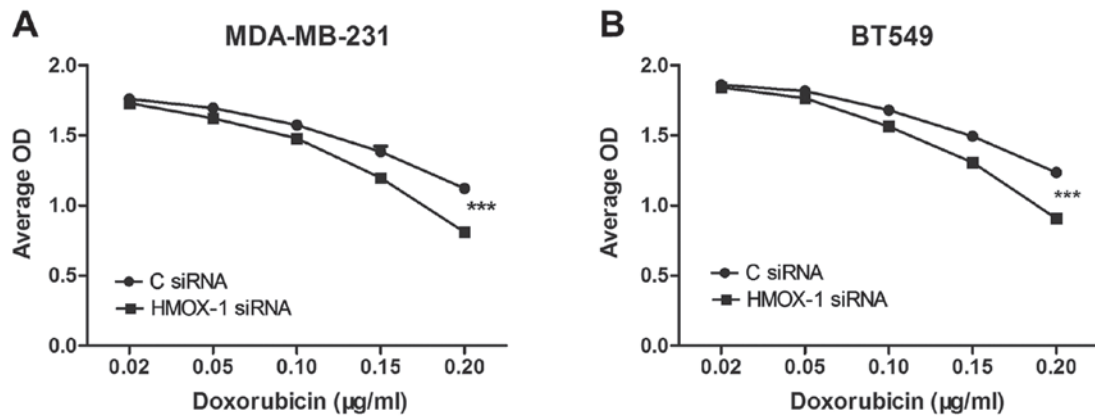


Figure 2. Knockdown of *HMOX-1* increases the cytotoxicity of doxorubicin in breast cancer cell lines. (A) MDA-MB-231 and (B) BT549 cells were transfected with C siRNA or *HMOX-1* siRNA and then exposed to various concentrations of doxorubicin for 48 h. Cell viability was analyzed using the cell counting kit-8 assay. All experiments were repeated at least three times. Data are presented as the mean ± standard deviation. ***P<0.001 vs. control cells. HMOX-1, heme oxygenase-1; C, control; siRNA, short interfering RNA; OD, optical density.

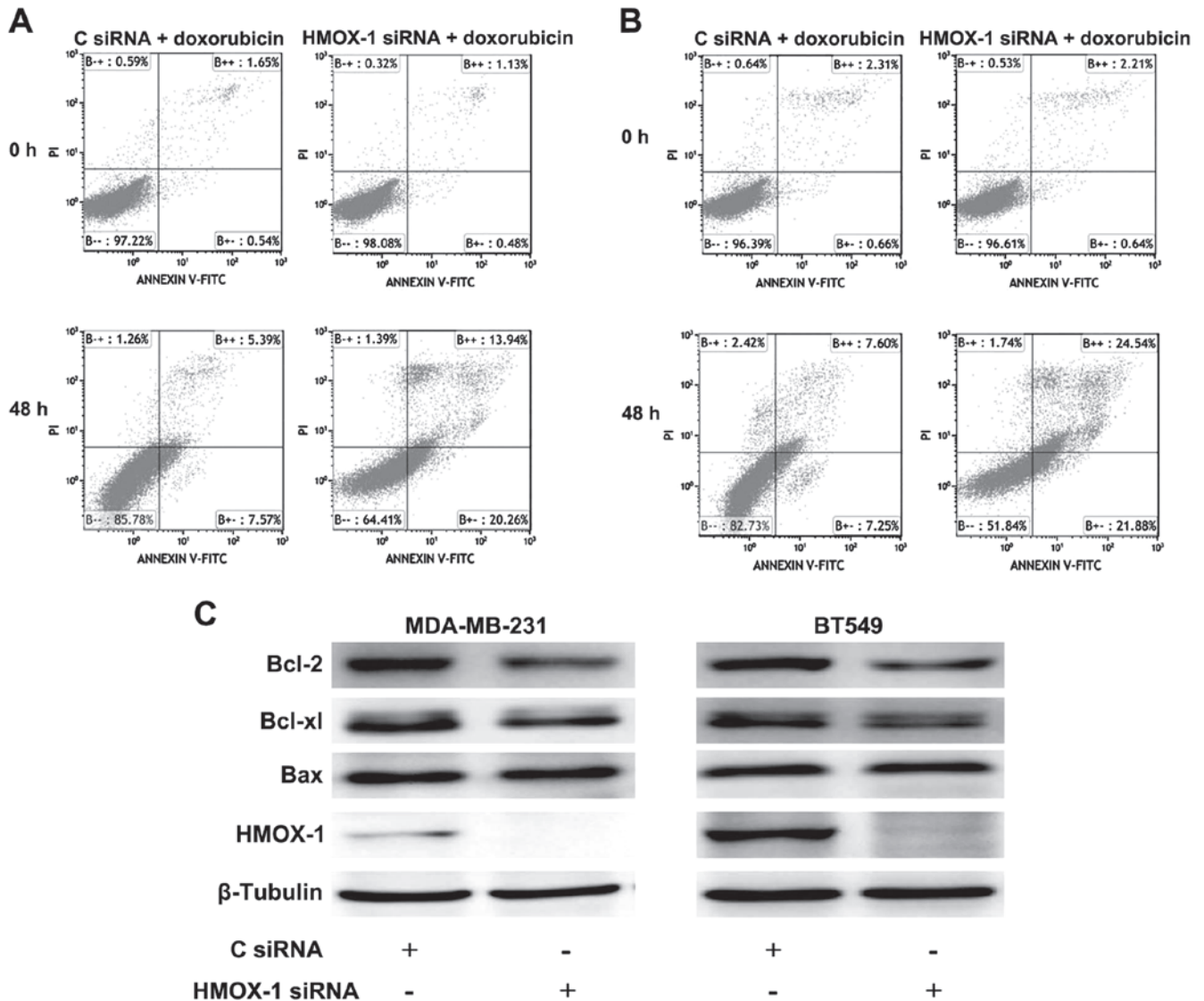


Figure 3. Knockdown of *HMOX-1* enhances doxorubicin-induced apoptosis in breast cancer cell lines. (A and B) Rate of apoptosis in (A) MDA-MB-231 cells and (B) BT549 cells transfected with C siRNA or *HMOX-1* siRNA and then treated with 0.20 μg/ml doxorubicin for 48 h. The rates of apoptosis were determined using Annexin V and PI staining and flow cytometry. (C) Western blotting revealed that siRNA knockdown of *HMOX-1* downregulated HMOX-1, Bcl-2 and Bcl-xl protein expression, but not Bax protein expression, in MDA-MB-231 and BT549 cells at 72 h post-transfection. Results are representative of three independent experiments. HMOX-1, heme oxygenase-1; C, control; PI, propidium iodide; Bcl-2, B cell lymphoma-2; Bcl-xl, B cell lymphoma-extra large; Bax, Bcl-2-like protein 4; FITC, fluorescein isothiocyanate.

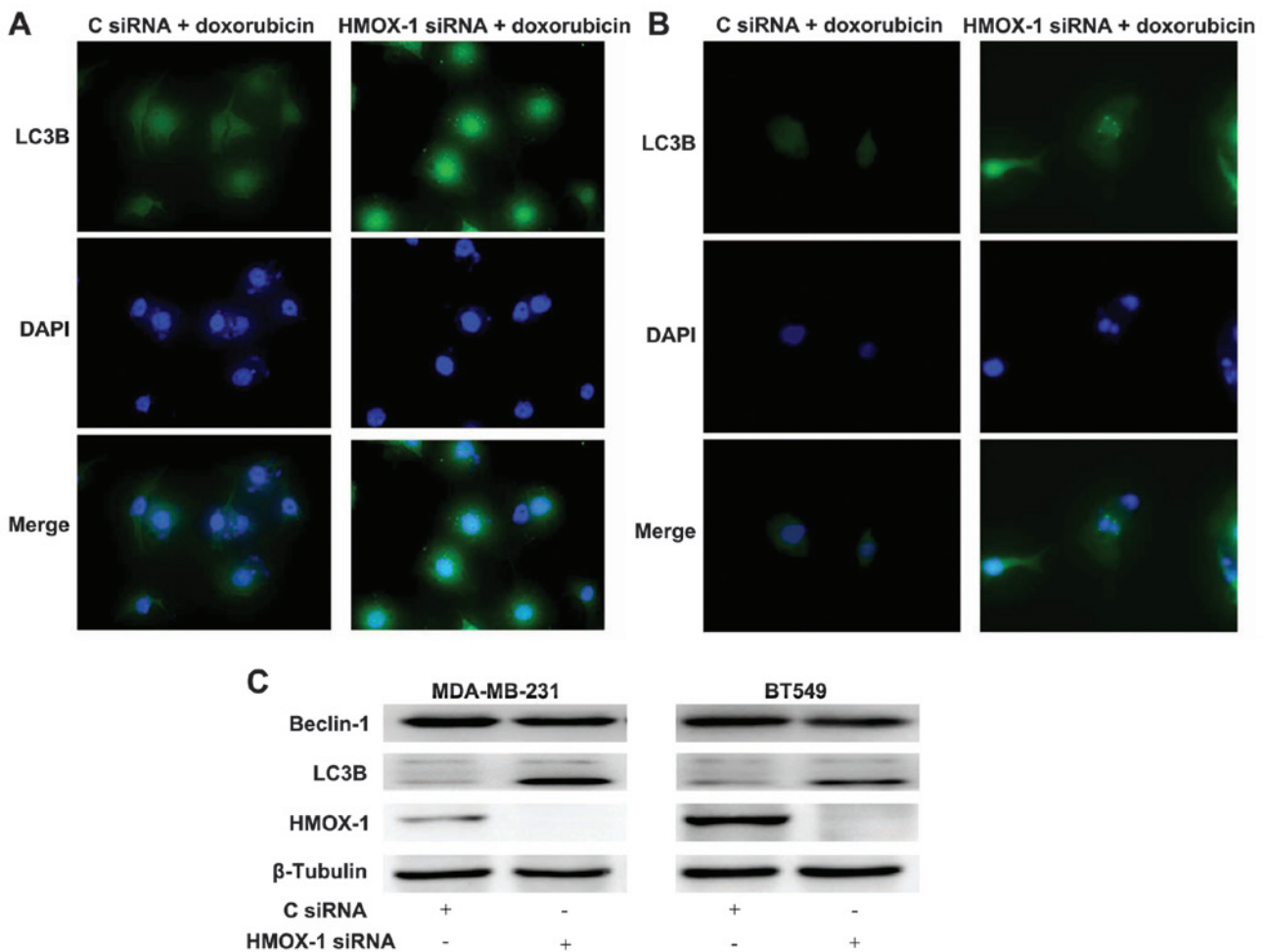


Figure 4. Knockdown of *HMOX-1* enhances doxorubicin-induced autophagy in breast cancer cells. (A) MDA-MB-231 or (B) BT549 cells were transfected with C siRNA or *HMOX-1* siRNA and then treated with 0.1 μ g/ml doxorubicin for 48 h. Immunofluorescent staining using a green fluorescent protein-tagged anti-LC3B antibody and fluorescence microscopy (magnification, x400) revealed that knockdown of *HMOX-1* markedly increased the accumulation of autophagic vacuoles in the cancer cells. (C) Western blotting indicated that siRNA knockdown of *HMOX-1* downregulated *HMOX-1* and enhanced LC3B protein expression, but not Beclin-1 protein expression, in MDA-MB-231 and BT549 cells at 72 h post-transfection. Results are representative of three independent experiments. *HMOX-1*, heme oxygenase-1; LC3B, light chain 3B; C, control; siRNA, short interfering RNA.

HMOX-1 markedly increased the accumulation of autophagic vacuoles in doxorubicin-treated MDA-MB-231 and BT549 cells (Fig. 4A and B). Furthermore, western blotting confirmed that the expression of LC3B was upregulated in *HMOX-1*-silenced MDA-MB-231 and BT549 cells compared with that of the respective control cells, while Beclin-1 expression was unaffected (Fig. 4C).

Discussion

HMOX-1 is involved in the maintenance of cellular homeostasis and has a significant cytoprotective role (5). *HMOX-1* is frequently upregulated in tumor tissues and promotes tumor growth and metastasis (8-14). Induction of *HMOX-1* is often associated with chemoresistance in cancer cells, and the inhibition of *HMOX-1* in combination with chemotherapy may enhance the efficacy of cancer treatment (24-26).

In the present study, it was demonstrated that *HMOX-1* was overexpressed in breast cancer cell lines, and was also induced by doxorubicin. Therefore, the effect of *HMOX-1* on the chemoresistance of breast cancer cells was investigated. Knockdown of *HMOX-1* significantly enhanced the

cytotoxicity of doxorubicin in MDA-MB-231 and BT549 cells. Therefore, the mechanism underlying the effects of knockdown of *HMOX-1* on the cytotoxicity of doxorubicin was investigated. The Bcl-2 family includes pro-apoptotic (Bax, Bcl-2-antagonist/killer 1 and Bcl-2-associated death promoter) and anti-apoptotic (Bcl-2 and Bcl-xL) members and is a key regulator of apoptosis in cancer cells. Additionally, Bcl-2 and Bcl-xL have been implicated in the resistance of cancer cells to chemotherapeutic drugs (27,28). The results of the present study indicated that knockdown of *HMOX-1* significantly enhanced doxorubicin-induced apoptosis and downregulated the expression of Bcl-2 and Bcl-xL in breast cancer cells. These findings indicated that *HMOX-1* may modulate the survival of breast cancer cells via regulation of the expression of Bcl-2 and Bcl-xL.

Furthermore, knockdown of *HMOX-1* enhanced doxorubicin-induced autophagy, without altering the expression of autophagic regulator Beclin-1 in MDA-MB-231 and BT549 cells. Autophagy is the process of lysosomal degradation, by which cells self-destruct, and has been associated with cell survival in various types of cancer (21). Autophagy is also able to protect cells from apoptosis and induce drug-resistance.

Blocking autophagy restored anti-estrogen sensitivity to an MCF-7-derived anti-estrogen resistant cell line (29). Autophagy has also been implicated in the development of trastuzumab resistance in human epidermal growth factor receptor 2-positive breast cancer (30). A number of chemotherapeutic agents (anthracyclines and taxanes) are able to induce autophagy, and inhibition of autophagy increases drug toxicity via the induction of apoptosis in breast cancer cells (31,32).

However, studies have indicated that excessive autophagy may result in autophagic cell death (also known as type II programmed cell death), which occurs in association with increased autophagosome formation in a caspase-independent manner (33). Scarlatti *et al* (34) reported that resveratrol, which is found in grapes and peanuts, induced Beclin-1-independent cytotoxic autophagy in MCF-7 cells. Di *et al* (35) demonstrated that breast cancer cells treated with doxorubicin and z-VAD-FMK, a general inhibitor of caspases, underwent cell death via a non-apoptotic pathway, characterized by dramatic accumulation of autophagic vacuoles.

Doxorubicin is one of the most commonly used chemotherapeutic drugs, and primarily functions by inhibiting topoisomerases and intercalating into the DNA double helix to interfere with DNA uncoiling, which induces cell death (36). Cell lines resistant to doxorubicin often exhibit defective apoptosis due to overexpression of Bcl-2 family proteins; therefore, chemotherapeutic agents that evoke autophagic cell death may be more cytotoxic in apoptosis-defective and drug-resistant tumor cells (37). In the present study, knockdown of *HMOX-1* induced accumulation of autophagic vacuoles and markedly increased the expression of LC3B in breast cancer cells, without altering the expression of Beclin-1. Therefore, knockdown of *HMOX-1* induced Beclin-1-independent autophagy in breast cancer cells. Under certain conditions, autophagy and apoptosis contribute to the rate of cell death as parallel pathways, and silencing Bcl-2 may induce autophagic cell death (31). Knockdown of *HMOX-1* also reduced the expression of Bcl-2 and promoted autophagy in breast cancer cells, suggesting that depletion of *HMOX-1* may induce autophagic cell death by regulating the levels of Bcl-2. These results demonstrate that knockdown of *HMOX-1* enhanced the cytotoxic effects of doxorubicin and induced apoptosis and autophagy in breast cancer cells. These results indicate that HMOX-1 may have a role in tumor cell survival and chemoresistance in breast cancer. Additionally, HMOX-1 represents a potential therapeutic target, as targeting HMOX-1 may enhance the therapeutic efficacy of chemotherapy in breast cancer.

In conclusion, the present study demonstrated that HMOX-1 is overexpressed and able to be induced by doxorubicin in breast cancer cell lines. Knockdown of *HMOX-1* enhanced the cytotoxicity of doxorubicin by downregulating anti-apoptotic genes and promoting apoptosis and autophagy in breast cancer cells. Therefore, HMOX-1 may represent a potential therapeutic target for breast cancer.

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