Silencing of Bmi-1 gene by RNA interference enhances sensitivity to doxorubicin in breast cancer cells

Xiangmei Wu1,2, Xing Liu3, Joyeeta Sengupta2, Youquan Bu2, Faping Yi2, Changdong Wang2, Yanyan Shi2, Yong Zhu5, Qingfang Jiao3 & Fangzhou Song2*

1Department of Physiology, Chongqing Medical University, Chongqing 400016, PR China
2Molecular Medicine and Cancer Research Center, Chongqing Medical University, Chongqing 400016, PR China
3Department of Pediatric Urology, Chongqing Children’s Hospital, Chongqing Medical University, Chongqing 400014, PR China

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Breast cancer is one of the neoplasms with worst prognosis worldwide. Owing to factors like aggressive invasion, early metastasis, resistance to existing chemotherapeutic agents and radiation therapy, the mortality rate associated to it is very high. Bmi-1 (B-cell-specific moloney murine leukemia virus insertion site 1) was originally identified as an oncogenic partner of c-Myc in murine lymphomagenesis1,2. Bmi-1 was involved in regulation of stem-cell-associated genes to control cell self-renewing and differentiation3,4. Bmi-1 is overexpressed in several malignancies such as non-small cell lung cancer5, colorectal cancer6, nasopharyngeal carcinoma7 and oral cancer8. On the other hand, loss of Bmi-1 with RNA interference (RNAi) was effective in suppressing cancer cells (such as SH-SY5Y neuroblastoma and ovary adenocarcinoma) growth and tumorigenicity9. This is consistent with the present study, where downregulation of Bmi-1 impaired the ability of neuroblastoma cells to grow in soft agar as well as tumorigenicity in immunodeficient mice10. Therefore, silencing of Bmi-1 may be of good importance in the designing and development of anticancer therapy. It is hypothesized that the abrogation of Bmi-1 expression may be an effective strategy for sensitizing human cancer cells, including breast cancer cells, to cancer chemotherapy. Effective enhancing of chemosensitivity of 5-fluorouracil by downregulation of Bmi-1 has been confirmed in nasopharyngeal carcinoma cells11.

Doxorubicin has emerged as one of the most widely used chemotherapeutic agent for breast cancer. Although there is initial response to this chemotherapeutic agent, resistance is seen to develop over a period of time. This leads to a more phenotypically aggressive cell variant which enters metastasis very fast. Therefore, increase in chemosensitivity is an important factor for breast cancer therapy.

Increased resistance to apoptosis is a hallmark of many tumor cells. Inhibition of apoptosis is...
considered as a survival advantage on cells harboring genetic alternations and may promote acquisition of further mutations that induce neoplasm progression and also promote resistance to chemotherapy\textsuperscript{12}. Therefore, inhibition of specific antiapoptotic factors may provide a rational basis for the development of new therapeutic strategies in cancer\textsuperscript{13}.

In the present study, retroviruses vector pSuper-retro-puro expressing short hairpin RNA (shRNA) has been introduced to silence Bmi-1 gene with an aim to study the combined effect of RNAi and doxorubicin treatment on breast cancer in vivo and in vitro. The results obtained suggest that suppression of Bmi-1 gene expression followed by treatment with doxorubicin may be used as a potential and specific therapeutic tool for the treatment of breast cancer.

Materials and Methods

Cell line and animals—MCF-7 cell line was maintained as a monolayer culture in RPMI-1640 medium with L-glutamine (2 mM) and 10% fetal calf serum in a humidified atmosphere supplemented with 5% CO\textsubscript{2} at 37°C. PT67 was maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal calf serum, and cultured at 37°C in a 5% CO\textsubscript{2} humidified incubator. BALB/c nude mice, 4–6-weeks-old, weighing 18–22 g were used as subject.

Retroviral constructs and infection—Human Bmi-1 gene shRNA-expressing plasmid pSuper-retro/Bmi-1 si was used for downregulation of Bmi-1. The Bmi-1 siRNA sequence was 5′-AATGGACATACTAATTCT-3′, position 546 to 564 bp relative to the start codon. pSuper-retro/GFP si expressing Green Fluorescent Protein (GFP) shRNA was used as a negative control\textsuperscript{10}. The GFP target sequence was 5′-GCAAGCTTGACCTGAAGTTC-3′. Retroviruses were produced using PT67 packaging cell line. Briefly, PT67 cells were plated into 6-well plates (4×10\textsuperscript{3}/well), incubated for 24 h. When the density of cells was 80%, transfections were carried out with Lipofectamine 2000 reagent (Invitrogen, USA) using 2 µg of a retroviral plasmid as described by the manufacturer. After 48 h of transfection, the retrovirus-containing medium was filtered through a 0.45 µm filter (Millipore, USA) and supplemented with 4 µg/ml Polybrene (Sigma, USA). For retroviral infections, MCF-7 cells (2×10\textsuperscript{5}) were seeded in a 10 cm dish and incubated overnight. The cultured medium was then replaced by the retrovirus containing medium. After 48 h, the viral supernatant was removed, and fresh culture medium containing 0.6 µg/ml puromycin was added to select puromycin-resistant clones. The selection medium was changed every 3 days, and puromycin-resistant cells were pooled. The stable Bmi-1-knockdown cell line and stable cell line transfected with pSuper-retro/GFP si were named as MCF-7/Bmi-1si and MCF-7/GFPsi respectively. The MCF-7 cells devoid of transfection were used as blank control.

RNA preparation and semiquantitative reverse transcription polymerase chain reaction (RT-PCR)—The total RNA was extracted from MCF-7, MCF-7/Bmi-1si and MCF-7/GFPsi cells using Trizol method (Invitrogen, USA) according to the manufacturer’s specification, and quantified by spectrophotometry at 260 nm. cDNA was generated from 3–5 µg of total RNA using SuperScript II reverse transcriptase and random primers as per the manufacturer’s protocol (Invitrogen, USA). PCR-based amplification was carried out under standard conditions with rTaq DNA polymerase (Takara, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for the integrity and uniformity of the sample preparation. The PCR conditions maintained were as follows: predenaturing at 94°C for 5 min then followed by 30 cycles of reaction at 94°C for 30 s, 51°C for 30 s, 72°C for 30 s and final extension at 72°C for 5 min. PCR products were analysed on a 1.5% agarose gel stained with ethidium bromide and the resultant target bands were analyzed densitometrically by using Vistra Fluor Imager SI (Molecular Dynamics Inc., USA), the ratio of target gene to GAPDH OD value was used to represent relative intensity of the target product. Primers were designed with Primer Premier 5.0 software according to the human sequences obtained from Medline. The primers were designed as follows: Bmi-1, 5′-GACCACTACTAATATAAGTGA-3′ (sense), and 5′-CATTTGTAGTCCATCCTCTC-3′ (anti-sense). GAPDH, 5′-CATGAGAATTGTGACAACAGCCT-3′ (sense), and 5′-CGTCTCTCTCCAGATACCAGT-3′ (anti-sense).

Western blot analysis—Total protein of cells was isolated and quantified respectively. Cells were harvested, washed with ice-cold PBS and lysed in lysis buffer containing 25 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100 and protease inhibitor mixture (Sigma, USA) at 4°C for 30 min, followed by brief sonification. After centrifugating at 14,000 rpm at 4°C for 20 min, supernatants were collected, and the protein concentration was measured.
by the BCA assay reagent (Biotek, China) according to the manufacturer’s protocol. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred onto PVDF membrane (Immobilon, USA). After saturation, the membranes were incubated at room temperature for 2 h in TBS with 0.1% Tween-20 (TBS-T) containing 5% nonfat dry milk, and subsequently incubated with primary antibodies against Bmi-1 (1:600 dilution, Upstate, USA), phosphorylated Akt (pAkt), total Akt (tAkt), Bcl-2 and Bax (1:200 dilution, Santa Cruz, USA) were incubated overnight at 4°C. Second peroxidase conjugated IgG (MultiSciences Biotech, China) were used as secondary antibodies. The protein was detected using the ECL detection kit (Keygen, China) following the manufacturer’s protocol. β-actin immunoblotting was used as a protein loading control.

3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay—The MTT assay was performed to detect the effect of Bmi-1 on chemosensitivity to doxorubicin. Untransfected or stably transfected cells (1×10⁶) were plated in 96-well plates and allowed to attach for 24 h, cells were then placed in media containing different concentrations of doxorubicin (0.03–100 µg/ml) and incubated for another 72 h. Every time, 10 µl MTT stock solution (5 mg/ml in PBS) was added to each microtiter well and incubated for 4 h at 37°C. After aspiration of the medium, 150 µl dimethyl sulfoxide was added, and absorbances were measured at 570 nm. The percent growth inhibition was calculated as per the following formula: \[ \frac{A_{570}(\text{control}) - A_{570}((\text{drug})/A_{570}(\text{drug}))}{100\%} \], where \( A_{570} \) is the absorbance of the cells exposed to doxorubicin and \( A_{570}(\text{control}) \) is the absorbance of the cells without doxorubicin treatment.

Analysis of apoptosis—Apoptosis of MCF-7 cells was performed using Annexin V-FITC Apoptosis Kit (Keygen, China) according to the manufacturer’s instructions. Briefly, the cells were harvested after treating with 1 µg/ml doxorubicin for 48 h and washed twice with PBS, followed by resuspension in Annexin-V binding buffer, then FITC-conjugated Annexin V and PI was added. After incubating for 10 min at room temperature in the dark, another binding buffer was added, and the samples were immediately analyzed using FCM. Terminal transferase dUTP nick end labeling (TUNEL) analysis for the detection of apoptotic cells in solid tumor tissue was performed using in situ apoptosis detection kit (Roche, Germany) on fixed paraffin-embedded tissues, after which deparaffinized and rehydrated slides were placed in citrate buffer (0.1M, pH6.0) and subjected to microwave irradiation for 1 min. Double distilled water was used for cooling the slides and was immersed in 0.1M Tris-HCl, pH7.5, 3% BSA, 20% normal bovine serum for 30 min. The slides were rinsed twice with PBS, and TUNEL reaction mixture was added on the sections and incubated for 60 min at 37°C in the dark. Converter-POD was added on sections and incubated for 30 min at 37°C, after which DAB colour was developed followed by counterstaining with methyl green. Each experiment was repeated at least twice. Number of apoptotic cells was calculated under five high power fields, considering at least more than 1000 cells per field chosen randomly. Percentage of positive cells to total cells was used apoptosis index (AI).

In vivo treatments—The sensitivity of the tumor tissues against doxorubicin was evaluated by subcutaneous injection of MCF-7, MCF-7/Bmi-1si and MCF-7/GFPsi cells in athymic nude mice. Each aliquot of approximately 2.0×10⁶ cells suspended in 200 µl PBS were injected subcutaneously into the right flank of the male BALB/c nude mice aged 4-6 weeks, with 12 animals in each group. At 1 week postinoculation, the mice of each group were divided into two groups, with 6 animals in each group. The diameter of the tumor was 0.5 cm approximately. The mice in group 1 were used as control which received normal saline intraperitoneally, where as mice in group 2 received doxorubicin intraperitoneally at a dose of 1.5 mg/kg once a week for 3 weeks. Tumor growth was monitored in both the groups. Two orthogonal diameters of the tumors were measured with vernier calipers at day 4, 7, 11, 14, 18 and 21. Each tumor volume (mm³) was calculated by the following formula: \[ V = 0.5 \times D \times d^2 \], where \( V \) is volume, \( D \) is longitudinal diameter, and \( d \) is latitudinal diameter. On the 21st day after the first intraperitoneal injection, all mice were sacrificed and the tumor tissues were removed, weighed, and embedded in paraffin. Tumor sections were then subjected to haematoxylin and eosin (H&E) staining for histological examination and immunohistochemical investigation to study the Bmi-1 expression in tumor tissues. The relative tumor volume (RTV) was calculated as the tumor volume at the time of measurement divided by that of treatment. The mean RTV value was plotted as function of time for various treatment groups. Tumor growth inhibition was determined as the ratio of treated: control (T:C) tumor
RNAi technique was an effective way of modulating Bmi-1. siRNAs markedly decreased, suggesting that Bmi-1 expression in MCF-7/Bmi-1si was significantly less and showed 72% reduction as compared to the blank control. There were significant differences between MCF-7/Bmi-1si and controls (P<0.05). The results shown are representative of three independent experiments.

Bmi-1 silencing made the cells more sensitive to doxorubicin—To examine the effect of doxorubicin on the survival of Bmi-1 knockdown cells, an MTT assay was performed after the MCF-7/Bmi-1si, MCF-7/GFPsi and MCF-7 cells were treated with various concentrations of doxorubicin for 72 h. MCF-7/Bmi-1si cells showed higher percent growth inhibition than that in MCF-7/GFPsi and MCF-7 cells (P<0.05). The IC_{50} value of doxorubicin in MCF-7/Bmi-1si cells and MCF-7/GFPsi cells were 0.15±0.02 µg/ml and 0.87±0.02 µg/ml respectively, compared to 0.81±0.06 µg/ml in blank control MCF-7 cells (Fig. 2). There were significant differences between the IC_{50} of MCF-7/Bmi-1si cells and that of controls (P<0.05).

Bmi-1 silencing enhanced doxorubicin-induced apoptosis—Percent apoptosis was examined in the MCF-7/Bmi-1si and MCF-7 cells after treating them with 1 µg/ml doxorubicin for 48 h. The FCM data showed that the percent apoptosis in MCF-7/Bmi-1si treated with doxorubicin was 41%, as compared to 27% in MCF-7 treated with doxorubicin and 25% in MCF-7/GFPsi treated with doxorubicin. The levels of apoptosis in MCF-7, MCF-7/GFPsi and MCF-7/Bmi-1si were 5.1, 5.4 and 6.2%, respectively.
There were significant differences between MCF-7/Bmi-1si treated with doxorubicin and MCF-7 treated with doxorubicin ($P<0.05$).

**Bmi-1 silencing down-regulated Akt Phosphorylation and regulated the expression levels of Bcl-2 and Bax**—To further explore the mechanism underlying the enhancement of doxorubicin-induced apoptosis by the silencing of Bmi-1, the expression levels of pAkt, tAkt, Bcl-2 and Bax were examined after cells were treated with 1 µg/ml doxorubicin for 48 h. Bmi-1 silencing decreased Akt phosphorylation without affecting tAkt expression (Fig. 4). There were decreased expression of Bcl-2 and increaseation of Bax in the Bmi-1 knockdown cells exposed to doxorubicin, and Bcl-2/Bax ratio was down-regulated.

Fig. 3—Effect of Bmi-1 silencing on doxorubicin-induced apoptosis. (a) MCF-7 cells, (b) MCF-7 cells treated with 1 µg/ml doxorubicin for 48 h, (c) MCF-7/GFPsi, (d) MCF-7/GFPsi treated with 1 µg/ml doxorubicin for 48 h, (e) MCF-7/Bmi-1si, (f) MCF-7/Bmi-1si treated with 1 µg/ml doxorubicin for 48 h. The FCM data showed that the percent apoptosis in MCF-7/Bmi-1si treated with doxorubicin was 41%, as compared to 27% in MCF-7 treated with doxorubicin and 25% in MCF-7/GFPsi treated with doxorubicin. The levels of apoptosis in MCF-7, MCF-7/GFPsi and MCF-7/Bmi-1si were 5.1, 4.6 and 6.2%, respectively. There were significant differences between MCF-7/Bmi-1si treated with doxorubicin and MCF-7 treated with doxorubicin ($P<0.05$).

Fig. 2—Effect of Bmi-1 silencing on chemosensitivity of cells to doxorubicin. The X-bar indicates concentration of doxorubicin, the Y-bar indicates percent growth inhibition. The 50% inhibitory concentration (IC$_{50}$) value of doxorubicin in MCF-7/Bmi-1si was 0.15±0.02 µg/ml, compared to 0.87±0.02 and 0.81±0.06 µg/ml in MCF-7 and MCF-7/GFPsi, respectively. There were significant differences between the IC$_{50}$ of MCF-7/Bmi-1si and that of controls ($P<0.05$).
Bmi-1 siliencing increased antitumor effect of doxorubicin in nude mice model—The expression of Bmi-1 in parent MCF-7 and MCF-7/GFPsi negative control solid tumors was strong, whereas it was weak in MCF-7/Bmi-1si solid tumors (Fig. 5A). This clearly suggest that RNAi technique targeting Bmi-1 is effective in nude mice model. The growth of MCF-7/Bmi-1si tumor after doxorubicin treatment was notably inhibited compared to other two control groups (P<0.05) (Fig. 5B). After doxorubicin was administered, the growth in MCF-7/Bmi-1si tumor was arrested from 18th day onwards, with a T:C value of 39% on 18th day and 36% on the 21st day (Fig. 5C). The T:C value of MCF-7/Bmi-1si tumor was significantly lower than that of other two control groups (P<0.05). These results suggest that Bmi-1 siliencing can efficiently mediate the sensitivity of doxorubicin to MCF-7 cell line in vivo.

Bmi-1 siliencing increased doxorubicin-induced apoptosis in nude mice model—AI in solid tumor tissue was calculated. AI of MCF-7/Bmi-1si solid tumor treated with doxorubicin was significant higher than control groups which indicated that MCF-7/Bmi-1si tumor treated with doxorubicin exhibited significantly increased apoptosis than MCF-7 tumor treated with doxorubicin and MCF-7/GFPsi tumor treated with doxorubicin. (Fig. 6) (P<0.05).

Discussion

Polycomb group (PcG) proteins are chromatin-modifying proteins which play an important role in the development of cancer. Bmi-1 is the first functional mammalian PcG proto-oncogene to be recognized, it is overexpressed in a number of human malignancies including breast cancer. Amplification of the Bmi-1 gene is detected in approximately 85% of human invasive ductal breast cancer and this genomic alteration is predictive of poor clinical outcome. Bmi-1 may enhance tumor cell proliferation or survival by repressing transcription of p16\(^{INK4a}\) and p19\(^{ARF}\), which sequesters the p53 inhibitor MDM2 and thereby prevents the degradation of p53, resulting in p53-mediated apoptosis. So, loss of Bmi-1 may promote cell apoptosis.

In this study, specific inhibition of Bmi-1 expression by RNAi was applied to investigate the role of Bmi-1 for the sensitivity of breast cancer cell line MCF-7 towards chemotherapy doxorubicin in vitro and in vivo. The expression of MCF-7 and MDA-MB-231 in

Fig. 5—Effect of Bmi-1 silencing on antitumor effect of doxorubicin in nude mice model[(a) Expression of Bmi-1 in tumor tissues (Immunohistochemistry method, DAB staining, the magnification of a is ×400). The expression of Bmi-1 in MCF-7 and MCF-7/GFPsi tumors was strong, whereas it was weak in MCF-7/Bmi-1si solid tumors, (b) Efficacy of doxorubicin on growth of tumors transplanted in nude mice. The efficacy was evaluated by relative tumor volume (RTV). The growth of MCF-7/Bmi-1si tumor after doxorubicin treatment was notably inhibited compared to other two control groups (P<0.05), (c) The treated: control (T: C) ratio of tumor volume for tumors transplanted in nude mice. After doxorubicin was administered, the growth in MCF-7/Bmi-1si tumor was arrested from 18th day onwards, with a T: C value of 39% on 18th day and 36% on the 21st day]
The Bcl-2/Bax ratio was also found to be downregulated. Simultaneously pAkt was also found to be downregulated, and this result is consistent with the previous finding observed in nasopharyngeal carcinoma cells. Simstein et al. reported that Akt activation is a key determinant in defining sensitivity to chemotherapeutic drugs, and phosphatidylinositol-3-kinase (PI3K)/Akt pathway played an important role in drug sensitivity of MCF-7 cells. Result of this present study suggest that PI3K/Akt pathway may be involved in the sensitization of Bmi-1 to doxorubicin treatment, and this assumption should be confirmed in further study.

The in vivo model showed that the growth of MCF-7/Bmi-1si tumor after doxorubicin treatment was markedly slower and apoptosis of the same tumor was increased compared to that of the controls. This indicate that solid tumor of MCF-7/Bmi-1si with weak positive Bmi-1 expression was more sensitive to doxorubicin treatment than that of parent MCF-7 and negative control with positive Bmi-1 expression. These results suggest that Bmi-1 repression may play as a viable approach to sensitize MCF-7 cells to doxorubicin. Bmi-1 knockdown in combination with doxorubicin may lead to an increased potency and efficiency of this agent.

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