

Aberrant promoter DNA methylation inhibits bone morphogenetic protein 2 expression and contributes to drug resistance in breast cancer

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Abstract. Bone morphogenetic protein 2 (BMP2) is a growth factor that is involved in the development and progression of various types of cancer. However, the epigenetic regulation of the expression of BMP2 and the association between BMP2 expression and drug resistance in breast cancer remains to be elucidated. The present study reported that the expression of BMP2 was significantly decreased in primary breast cancer samples and the MCF-7/ADR breast cancer multidrug resistance cell line, which was closely associated with its promoter DNA methylation status. The expression of BMP2 in MCF-7/ADR cells markedly increased when treated with 5-Aza-2'-deoxycytidine. Knockdown of BMP2 by specific small interfering RNA enhanced the chemoresistance of the MCF-7 breast cancer cell line. These findings indicated that epigenetic silencing of BMP2 in breast cancer may be involved in breast cancer progression and drug resistance, and provided a novel prognostic marker and therapeutic strategy for breast cancer.

Introduction

Breast cancer is one of the most common types of cancer among females (1). Although progress has been made in diagnosis and adjuvant therapies, the pathogenesis of breast cancer remains unknown. Furthermore, drug resistance also remains a major clinical obstacle for the successful treatment of breast cancer. Bone morphogenetic proteins (BMPs), belonging to the TGF- β superfamily, had been confirmed in several studies to be important in cell proliferation, differentiation and apoptosis during tumor progression (2-4). In breast cancer, it was

revealed that BMP2, BMP4, BMP6, BMP7, BMP9 and BMP10 were involved in breast cancer development, progression and drug resistance (5-10). However, few studies focusing on the epigenetic regulation of the expression of BMPs have been conducted. Recently, it was recognized that DNA methylation was a common event in human cancer, which induced the loss of tumor suppressor gene expression (11-12). Previous studies have also demonstrated that the downregulation of BMP2 was silenced by aberrant DNA methylation in bone formation and gastric carcinomas (13,14). To date, the epigenetic regulation of BMP2 in breast cancer and its association with tumor progression and drug resistance remains to be elucidated.

The present study detected the mRNA expression and the promoter methylation status of BMP2 in breast cancer tissues and breast cancer cell lines. In addition, BMP2 expression and cancer cell chemoresistance was also evaluated in an *in vitro* cell model. Our data indicated that the downregulation of BMP2 caused by promoter hypermethylation may contribute to breast cancer progression and cancer cell drug resistance.

Materials and methods

Patients and tissue specimens. A total of 32 breast cancer tissues and patient-matched adjacent normal breast tissues were obtained from patients who underwent surgery at the Cancer Hospital of Sichuan Province (Chengdu, China). All tumor tissues were reviewed by an experienced pathologist using World Health Organization recommendations on histopathological typing. For total RNA isolation, the specimens were frozen in liquid nitrogen and stored at -80°C. There were no patients who received chemotherapy or radiotherapy prior to surgery. The study protocol conformed to the local ethical standards of the institutional review board of the The PLA General Hospital of Chengdu Military Region (Chengdu, Sichuan, China).

Cell culture. The MCF-7 human breast cancer cell line was purchased from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and MCF-7/ADR, a doxorubicin resistant subline of MCF-7, was generated by continuously culturing the drug-sensitive parental cell line MCF-7 in medium containing incrementally

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increasing concentrations of doxorubicin (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured in RPMI-1640 (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C and 5% CO₂. To avoid the effects of the drug, the MCF-7/ADR drug resistant cell line was cultured in drug-free medium for >2 weeks prior to subsequent experiments.

Treatment of cells with 5-aza-2'-deoxycytidine (5-aza-dc). The cells were seeded at a density of 1x10⁵ cells in 6-well plates. Following 24 h, the cells were treated with 5 µM 5-aza-dc (Sigma-Aldrich). Total cellular RNA and protein were isolated from the cells 0 and 3 days after the addition of 5-aza-dc as described above.

RNA isolation and quantitative polymerase chain reaction (qPCR). The total RNA of 32 cancer and adjacent normal breast tissues were extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was retrotranscribed using the RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. qPCR was performed with the SYBR-Green PCR master mix (Takara Bio, Inc., Shiga, Japan) using a Mastercycler ep realplex (Eppendorf, Hamburg, Germany). The levels of mRNA expression were quantified following normalization with endogenous control GAPDH using the 2^{-ΔΔCT} method (15). The primer sequences used for PCR were as follows: Forward: 5'-CTTTGGTATCGTGGAAGGACTC-3' and reverse: 5'-GTAGAGGCAGGGATGATGTTCT-3' for GAPDH (product size: 132 bp); and forward: 5'-CCCAGCGTGAAAAGAGAGAC-3' and reverse: 5'-GAGACCGCAGTCCGTCTAAG-3' for bmp2 (product size: 168 bp). The experiments were performed independently for each sample and at least three technical replicates were run for each treated sample and controls.

Western blot analysis. Whole cell extracts were lysed using lysis buffer pulsed protease inhibitor (Millipore, Billerica, MA, USA). Equivalent quantities of protein were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (GE Healthcare Biosciences, Piscataway, NJ, USA), inhibited with phosphate-buffered saline (PBS) containing 0.2% Tween-20 and 5% non-fat dry milk and incubated with primary antibodies against BMP2 (1:1,000; rabbit polyclonal to BMP2) and actin (1:2,000, mouse monoclonal to actin) obtained from Abcam (Cambridge, MA, USA). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Invitrogen Life Technologies) and an Enhanced Chemiluminescence Plus immunoblotting detection system (GE Healthcare Biosciences). The signals were quantified using NIH Image J 1.63 software (National Institutes of Health, Bethesda, MA, USA).

Methylation-specific PCR (MSP) and bisulfite genomic sequencing. Genomic DNA of MCF-7 or MCF-7/ADR was extracted using a TIANamp Genomic DNA kit (Tiangen

Biotech (Beijing) Co., Ltd., Beijing, China). Bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. MSP was performed on bisulfite-modified DNA. The primer sequences of BMP-2 for the unmethylated reaction were: Forward: 5'-GGGTTAGTGTGAGTGGATTATT-3' and reverse: 5'-CACAACTAAAAATCAACTCC-3' and for the methylated reaction forward: 5'-TTAGCGTCGAGTGGATTATC-3' and reverse: 5'-GACGCTAAAAATCGACTCC-3'. PCR products were analyzed by electrophoresis in a 2.0% agarose gel containing golden view (SBS Genetech Co., Ltd., Beijing, China). For bisulfite sequencing of DNA samples, sodium bisulfite-treated genomic DNA was amplified using the following primers: Forward: 5'-GTGGTTTTTGTGTTGTYGG-3' and Reverse: 5'-TCTACCTTACTCCAATACACCC-3'. The PCR reaction products were gel purified and cloned into the pGEM-T Vector system according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). The colonies were sequenced on a genetic analyzer (Applied Biosystems, Foster City, CA, USA) to analyze the methylated cytosine level.

Small interfering RNA (siRNA) transfection. siRNAs (Guangzhou RiboBio Co., Ltd., Guangzhou, Guangdong, China) were transfected using Lipofectamine™ 2000 and OptiMEM (Invitrogen Life Technologies) according to the manufacturer's instructions. Following 72 h of siRNA transfection, the cell lysate was prepared and western blotting was performed as described above.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. MCF-7 cells were seeded in 96-well plates at a density of 1x10⁴ cells per well and incubated overnight in 10% fetal bovine serum medium. The cells were transfected with BMP2 specific siRNA or negative control siRNA. Then, the cells were treated with different concentrations of adriamycin (0-200 mg/l). Following incubation for 48 h at 37°C, 20 µl MTT (Sigma-Aldrich) solution (5 mg/ml in PBS) was added to each well and incubation continued for a further 4 h at 37°C. The medium was then removed and the MTT crystals were solubilized using 100 µl dimethylsulfoxide. Absorbance was measured at 560 nm using a microplate reader (Thermo Fisher Scientific). Absorbance readings were subtracted from the value of blank wells. The reduction in cell growth was calculated as a percentage of control absorbance in the absence of any drug. Data are presented as the mean ± standard deviation (SD) of at least three independent experiments.

Statistical analysis. Data are expressed as the mean ± SD. The paired t-test and independent sample t-test were applied for statistical analysis using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

DNA methylation suppresses BMP2 expression in breast cancer tissues. The present study performed quantitative

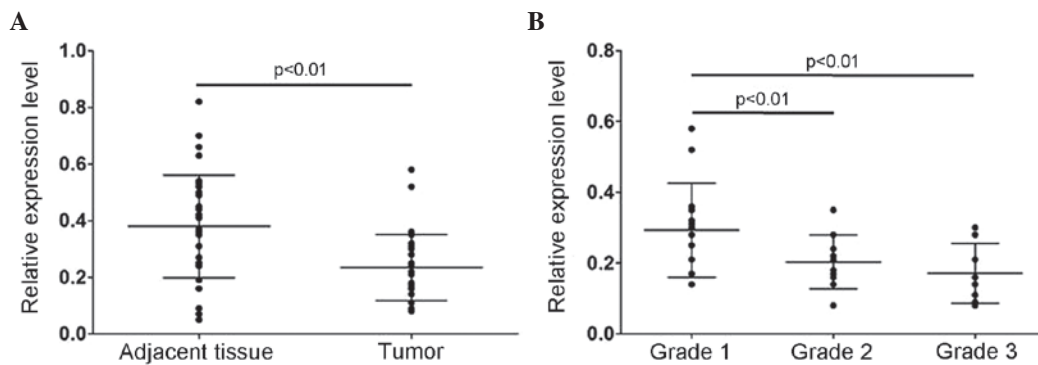


Figure 1. (A) Quantitative polymerase chain reaction demonstrated that the mRNA expression of BMP2 was significantly decreased in breast cancer tissues compared with the adjacent normal tissues. (B) In tumor tissues, the BMP2 expression level was significantly higher in grade 1 tumors than in grade 2 and grade 3 tumors. BMP2, bone morphogenetic protein 2.

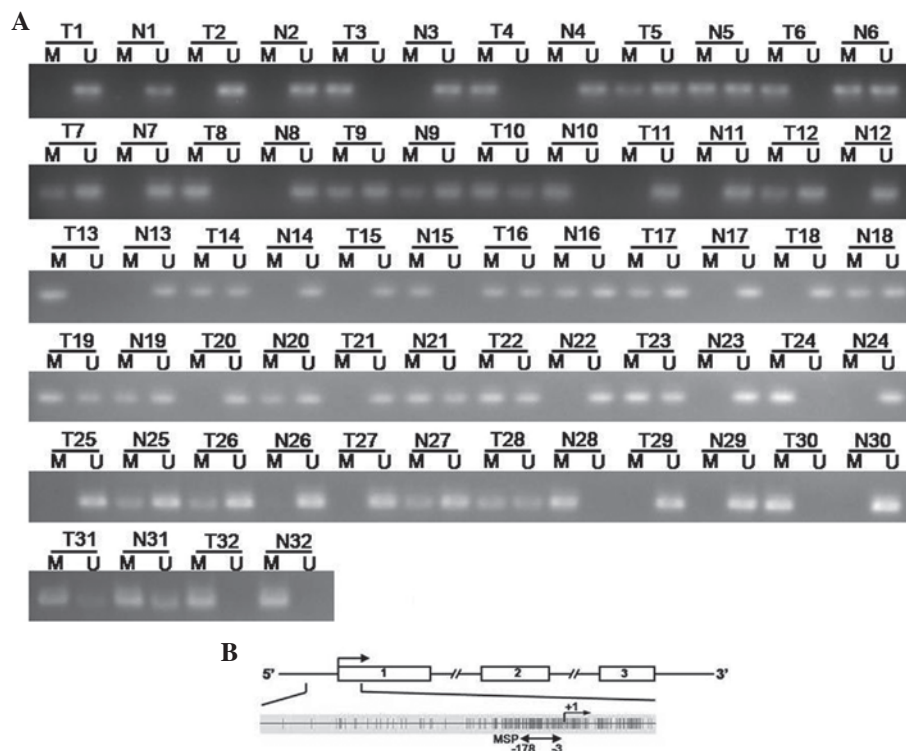


Figure 2. (A) MSP analyzed the BMP2 promoter methylation status in 32 breast cancer tissues and matched adjacent normal tissues. (B) Schematic diagram of the BMP2 promoter and the fragment for MSP analysis. BMP2, bone morphogenetic protein 2; MSP, methylation-specific polymerase chain reaction; M, methylated, U, unmethylated.

analysis of BMP2 mRNA in 32 breast cancer tissues and matched adjacent normal tissues by qPCR. Among the 32 pairs of samples, nine samples of breast cancer tissue demonstrated a higher expression of BMP2 compared with the adjacent normal tissue, and 23 samples of breast cancer tissue demonstrated a lower expression of BMP2 compared with the adjacent normal tissue. Compared with the normal tissues, BMP2 mRNA expression was significantly lower in cancer tissues ($P<0.01$; Fig. 1A). Furthermore, data demonstrated that the mRNA expression of BMP2 decreased between tumor stage 1 and stage 3. There was a significantly higher level of BMP2 mRNA in stage 1 than in stage 2 and 3 tumors (Fig. 1B). Subsequently, the BMP2 methylation status was detected by MSP. Aberrant methylation was detected in 22 out of 32 (68.75%) tumors, which was more

frequent than that in the paired adjacent normal tissues [15 out of 32 (46.87%); Fig. 2A].

DNA methylation suppresses the expression of BMP2 in drug resistant breast cancer cells. qPCR and western blot analysis demonstrated that BMP2 was expressed in the breast cancer cell line MCF-7 and was downregulated in doxorubicin resistant MCF-7/ADR cells (Fig. 3A and B). Following treatment with a DNA methyltransferase inhibitor 5-aza-dc, the downregulation of BMP2 was reversed in MCF-7/ADR cells (Fig. 3A and B). This indicated that the expression of BMP2 may be involved in DNA methylation regulation. Thus, MSP and bisulfite-sequencing PCR (BSP) were employed to investigate the promoter methylation status of BMP2. MSP analyses revealed that CpG islands

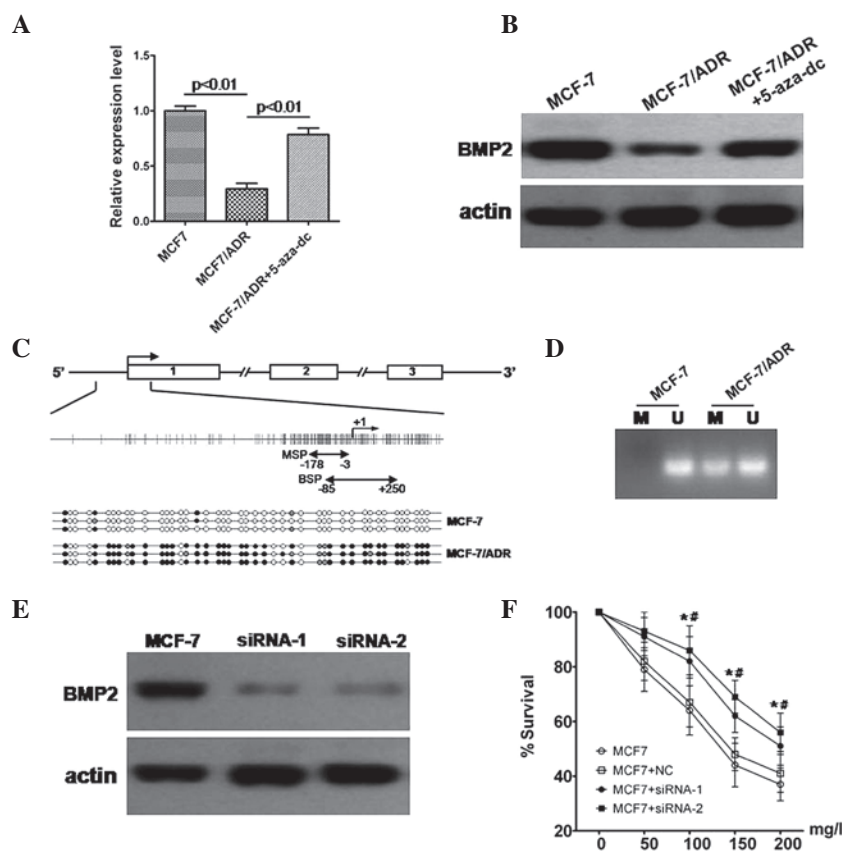


Figure 3. (A) Quantitative PCR and (B) western blot analysis demonstrated that BMP2 was downregulated in MCF-7/ADR drug resistant breast cancer cells and this downregulation may be reversed by treatment with 5-aza-dc. (C) Bisulfite-sequencing PCR and (D) methylation-specific PCR demonstrated an aberrant hypermethylation status of the BMP2 promoter. (E) Specific siRNA inhibited the expression of BMP2 and (F) enhanced the chemoresistance of MCF-7 breast cancer cells. * $P < 0.01$ MCF-7 + siRNA-1 vs. MCF-7; # $P < 0.01$ MCF-7 + siRNA-2 vs. MCF-7. BMP2, bone morphogenetic protein 2; 5-aza-dc, 5-aza-2'-deoxycytidine; siRNA, small interfering RNA; PCR, polymerase chain reaction; M, unmethylated; U, methylated.

of the BMP promoter were unmethylated in MCF-7 cells. However, in MCF-7/ADR cells, they were partially methylated (Fig. 3D). Furthermore the MSP results were confirmed by BSP. The results demonstrated that MCF-7/ADR cells showed dense methylation of the promoter CpG islands (Fig. 3C).

Knockdown of BMP2 by siRNA increases the chemoresistance of MCF-7 cells. In order to evaluate the effect of BMP2 downregulation in MCF-7 cells, the negative control siRNA and BMP2-specific siRNA were transfected into the MCF-7 cells and qPCR and western blot analysis detected the efficacy of the downregulation of expression of the BMP2 gene. The results indicated that siRNA was able to significantly downregulate the expression of BMP2 (Fig. 3E). Then, the MTT assay was employed to detect the effects of BMP2 expression downregulation by siRNA transfection on doxorubicin resistance. The survival ratio of MCF-7 cells, MCF-7 cells transfected with negative control siRNA and BMP2-specific siRNA-1 and siRNA-2 were analyzed following treatment with a range of increasing concentrations of doxorubicin for 48 h. The survival ratio of cells transfected with BMP2-specific siRNA-1 or siRNA-2 was significantly higher than cells transfected with negative control siRNA or MCF-7 cells (Fig. 3F). No significant difference between the MCF-7 cells and cells transfected with negative control siRNA was identified.

Discussion

The present study found that BMP2 expression was decreased in breast cancer tissues and was closely associated with DNA hypermethylation. Furthermore, the downregulation of BMP2 induced by DNA hypermethylation was able to enhance the chemoresistance of the MCF-7 breast cancer cell line. All these results indicated that aberrant methylation regulated BMP2 expression and was important in breast cancer development and drug resistance.

BMPs are a group of growth factors that belong to the TGF- β superfamily. Previous studies confirmed that BMPs are important in cell proliferation, differentiation and apoptosis during development of the embryo and tumor progression (2-4). In breast cancer, the role of BMPs has not been well characterized, although several studies have focused on this subject (16,17). BMP2 was reported to inhibit breast cancer cell proliferation through p21 and PTEN (18,19). In addition, BMP2 may be involved in tumor angiogenesis, invasion and hormone-independent growth of breast cancer (20,21). The data of the present study also confirmed that BMP2 was involved in breast cancer development and progression. However, there are few studies investigating the association between BMP2 and cancer cell drug resistance, and the mechanisms underlying the regulation of BMP2 expression. In the present study, the *in vitro* cell model revealed that the downregulation

of BMP2 was able to enhance the chemoresistance of MCF-7 breast cancer cells. All these data indicated that BMP2 may be important in breast cancer development and drug resistance.

DNA methylation was recognized as one of the most common forms of gene expression regulation (22). In various types of human cancer, DNA methylation patterns are associated with cancer development and progression (23). In breast cancer, numerous studies have confirmed that DNA methylation is involved in tumorigenesis by inhibition of tumor suppressor gene expression, including estrogen receptor, *hox5a*, *twist* and *E-cadherin* (24-28). The present study revealed that DNA hypermethylation of BMP2 was closely associated with the downregulation of BMP2 in clinical breast cancer specimens. Furthermore, in an *in vitro* cell model, DNA hypermethylation led to the downregulation of BMP2 and resulted in enhanced chemoresistance of MCF-7 cells. These data indicated that DNA hypermethylation may cause breast cancer cell drug resistance through silencing BMP2 expression.

In conclusion, the results of the present study indicated that promoter hypermethylation suppression of BMP2 is a frequent event in human breast cancer. This aberrant epigenetic event may be important in the development and drug resistance of breast cancer, and our data may provide a novel prognostic marker and therapeutic strategy for breast cancer.

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