Reduced BMP6 expression by DNA methylation contributes to EMT and drug resistance in breast cancer cells

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Abstract. Bone morphogenetic protein 6 (BMP6) is an important regulator of cell growth, differentiation and apoptosis in various types of tumor. In breast cancer, it was considered as a tumor suppressor. Our previous study also confirmed that BMP6 was a critical regulator of breast cancer drug resistance. However, little is known about how its expression is regulated and its mechanisms in breast cancer drug resistance. In the present study, we assessed the DNA methylation regulation of BMP6 based on the presence of a large CpG island in the BMP6 gene promoter. Quantitative DNA methylation analyses showed a significantly increased DNA methylation level in the drug-resistant cell line MCF-7/ADR compared to their parental cells MCF-7. Moreover, the drug-resistant cell line MCF-7/ADR showed an EMT phenotype confirmed by morphology and the expression of EMT marker gene. MCF-7 cells transfected with BMP6-specific shRNA vector also showed an EMT phenotype. The MCF-7/ADR cells treated with the recombinant BMP6 proteins reversed their EMT phenotype. These data indicated that hypermethylation modifications contributed to the regulation of BMP6 and induced an EMT phenotype of breast cancer during the acquisition of drug resistance.

Introduction

The epithelial-mesenchymal transition (EMT) is a complex biological process by which the epithelial cells go through multiple biochemical changes mainly including the loss of epithelial characteristics and acquisition of a mesenchymal motile phenotype, which enhance migratory capacity, invasiveness, elevate resistance to apoptosis and increase production of the extracellular matrix components (1-5). It has been confirmed that EMT is closely related to cancer development and progression. Increasing evidence suggests that the cancer cell acquisition of EMT phenotype is critically involved in tumor invasion and metastasis (6,7). Recently, the EMT characteristics were also found in cancer cells which acquired chemoresistance after chemotherapy (8). For example, EMT is reportedly associated with the resistance to gefitinib and erlotinib in NSCLC (9-11). In breast and ovarian cancer, chemoresistance can also induce EMT via upregulation of Snail, which is a key regulator of EMT (12,13). These data indicated that EMT is strongly involved in cancer cell drug resistance, but the underlying mechanisms remain unknown.

Breast cancer is the main cause of cancer-related mortality among women and drug resistance is the major clinical obstacle to the successful treatment. There are a series of molecular mechanisms which are involved in breast cancer cell drug resistance including gene mutation, gene amplification, epigenetic change and microRNAs (14,15). In our previous study, we found that BMP6 played a critical role in breast cancer progression and drug resistance (16). However, during the acquisition of chemoresistance of breast cancer, the regulation of BMP6 expression and the function of BMP6 remained unknown. DNA methylation is an efficacious mechanism to regulate gene expression during development, differentiation and carcinogenesis of various tumors. DNA methylation has previously been described to act during the EMT program in the suppression of epithelial markers and the conversion of epithelial cells into aggressive, invasive tumor cells (17,18).

Herein, we performed analyses to identify BMP6 which was regulated by DNA methylation and which regulated the EMT of drug-resistant cells. We found that the promoter of BMP6 is hypermethylated significantly in drug-resistant breast cancer cells that have undergone EMT. These results suggest that aberrant DNA methylation of BMP6 is critical in the drug resistance and EMT phenotype in breast cancer.
Materials and methods

Patients and tissue specimens. A total of 16 fresh breast cancer tissues and patient-matched adjacent normal breast cancer tissues from West China Hospital of Sichuan University were used in this study. None of the patients received chemotherapy or radiotherapy prior to surgery. The study was approved by the local ethical standards of the institutional review board.

RNA isolation and quantitative RT-PCR. The expression of BMP6 was analyzed using SYBR-Green PCR master mix (Tiangen). Real-time PCR was performed on Mastercycler ep realsplex (Eppendorf). Levels of mRNA expression were quantified after normalization with endogenous control GAPDH using the 2-ΔΔCT method (19). The primer sequences used for PCR were: GAPDH: 5'-ctttggtatctggaagacct-3', 5'-gtggagatggtggtggttct-3' (product size, 132 bp); bmp6: 5'-gtttacagagcatagacag-3', 5'-ggagcacaacccacagttg-3' (product size, 128 bp); ZEB1: 5'-ctgatcccaaccgttgctca-3', 5'-ggggactgtgatgagttttct-3' (product size, 168 bp); Twist1: 5'-agtgtcttcgcgccatctc-3', 5'-gtggactgtggtgccaaagtgatc-3' (product size, 161 bp); E-cadherin: 5'-ctgtgactgtgagttgctgctc-3', 5'-ctgggtggtgaggtgacact-3' (product size, 175 bp); Vimentin: 5'-cgccatactcagacaagcctc-3', 5'-ggcttttgctggtgctgtagct-3' (product size, 166 bp). Experiments were performed independently for each sample and at least 3 technical replicates were run for each treated sample and controls.

Cell culture. MCF7 cell line was purchased from American Type Culture Collection (ATCC) and doxorubicin-resistant subline (MCF7/Adr) was generated by continuously culturing the drug sensitive parental cell line (MCF7) in medium containing incrementally increasing concentrations of doxorubicin (Sigma-Aldrich). The cells were cultured in RPMI-1640 (Gibco) supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C and 5% CO2. The drug-resistant cell line was cultured in drug-free medium for ≥2 weeks before subsequent experiments to avoid the influence of the drug. MCF-7/ADR were treated with 5 µM 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich) or 200 ng/ml rhBMP-6 (R&D Systems) for 72 h.

BMP6 small hairpin RNA (shRNA) transfection and isolation of clones stably expressing BMP-6 shRNA. MCF-7 cells grown on 24-well plates were transfected either with pGPU6/GFP/NEO-BMP6 or negative control plasmid (GenePharma) using Lipofectamine 2000. After another 12-h incubation, the cells were re-plated at 1/10 dilution onto the 6-well plates. Selection with G418 (500 µg/ml) started on the next day and the process lasted for 2 weeks. MCF-7 cell clones with stably decreased expression of BMP6 as well as the control clones were obtained for further study. BMP6 knockdown was evaluated by real-time PCR and western blotting.

Western blot analysis. Tissue samples or cell pellets were lysed in RIPA buffer containing complete protease inhibitor cocktail (Millipore). Proteins were quantified with BCA Protein Assay (Bio-Rad) and separated by SDS-PAGE. Blots were probed using anti-BMP6 (ab155963, Abcam), anti-E-cadherin (ab53033, Abcam) and anti-Vimentin (sc-6260, Santa Cruz). Proteins were detected using horseradish peroxidase-conjugated secondary antibodies.

Quantitative DNA methylation analyses. Genomic DNA of 16 cancers and adjacent normal colorectal tissues or cells were extracted by Tissue DNA kit (Qiagen) according to the manufacturer's instructions. A total of 200 ng genomic DNA from each sample was bisulfite-treated with the Methylamp DNA Modification kit (Epigentek). The quality of the bisulfite conversion was controlled by using PCR products. The Sequenom MassARRAY platform was used to perform the quantitative methylation analysis of BMP6. We analyzed BMP6 promoter region and selected three fragments in CpG islands, the following primers were designed. The first (to amplify fragment 1 base pairs between -1346 to -870) was 5'-aggaagagag GAGGTTGTTTTTTATATTTAGTA-3' and 3'-cattatacaactcactagagggaaagctACTCAAATACCTCCTCCA AACCACATATAAATTTCCCCC-5'. The second (to amplify fragment 2 base pairs between -892 to -498) was 5'-aggaagagagGGTTGTAGTTAGTTAGTTAGT TTGAGT-3' and 3'-cattatacaactcactagagggaaagctC AACCACATATAAATTTCCCCC-5'. The third (to amplify fragment 3 base pairs between -521 to -177) was 5'-aggaagagag GGGTAAATTTATGTTGTTTTTT-3' and 3'-cattatacag actcactagagggaaagctACTCAAATACCTCCTCCA AACCACATATAAATTTCCCCC-5'. The spectra methylation ratios were generated by Epityper software version 1.0 (Sequenom).

Transwell migration assay. Cell migration was assessed using 24-well inserts (Corning Inc., Corning, NY, USA) with 8-µm pores according to the manufacturer's protocol. Brieﬂy, cells (1x10⁵ cells) were seeded into the upper chamber of the system. After 72 h of incubation, the cells in the upper chamber were removed, and the cells which invaded through the Matrigel matrix membrane were stained with Giemsa assay. After slightly washing, the migrating cells were examined by microscopy at a magnification of x40 with a Nikon microscope (tsl100-F, Japan).

Statistical analyses. Data are expressed as the mean ± SD. The paired t-test was applied for statistical analysis using the SPSS software (version 13.0). Results were considered statistically significant if 2-tailed P-values were <0.05.

Results

DNA methylation suppresses BMP6 expression in primary breast tumors. To determine the epigenetic silencing of the BMP6 gene in primary breast cancer, firstly, we analyzed BMP6 expression in 16 paired breast cancer samples and matched adjacent normal tissues. Real-time PCR showed that 12/16 (75%) breast cancer tissues decreased expression of BMP6 compared to paired adjacent normal tissues; there was a significant difference between tumor tissues and the paired adjacent normal tissues (Fig. 1A). Secondly, we detected BMP6 methylation status in primary breast cancer tissues using Sequenom MassARRAY platform. The mean methylation level of BMP6 of breast cancer tissues was significantly higher than paired adjacent normal tissues (Fig. 1B). Fig. 2 shows the methylation level at different CpG sites of tumor tissues and adjacent normal tissues and 29/51 (56.86%) CpG sites showed
a higher methylation level in tumor tissues compared to the paired adjacent normal tissues.

DNA hypermethylation of BMP6 is related to breast cancer cell drug resistance. In our previous study (16), we found that BMP6 expression was downregulated in the multidrug-resistant breast cancer cell line MCF-7/ADR compared to its parental MCF-7 cells and knockdown of the expression of BMP6 in MCF-7 cells enhanced the chemoresistance to doxorubicin. Here, we found that downregulation of BMP6 in MCF-7/ADR was reversed by treatment of DNA methyltransferase inhibitor 5-aza-dC (Fig. 3A and B). This indicated that downregulation of BMP6 in the drug-resistant cell line MCF-7/ADR might be involved in epigenetic mechanisms. Subsequently, we examined BMP6 methylation status in MCF-7 and MCF-7/ADR cells. Higher level methylation status was detected in MCF-7/ADR cells compared to the MCF-7 cells (Fig. 3C). In addition, we found that 20/51 (39.21%) CpG sites showed a higher methylation level in MCF-7/ADR compared to the MCF-7 cells (Fig. 4). It is of note that the methylation level of CpG

Figure 1. Real-time PCR and Sequenom MassARRAY analysis of the mRNA expression and promoter DNA methylation level of BMP6 in breast cancer tissues and adjacent normal tissues. (A) Real-time PCR analysis showed that BMP6 mRNA expression was significantly decreased in tumor tissues compared to the paired adjacent tissues. (B) Quantitative methylation analysis showed that DNA methylation level of BMP6 promoter was significantly increased in tumor tissues compared to the paired adjacent tissues. (C) Schematic diagram of the BMP6 promoter and the fragment for the methylation analysis. (D-F) Details of the BMP6 promoter region sequence: (D) base pairs between -1346 to -870; (E) base pairs between -892 to -498; (F) base pairs between -521 to -177; **P<0.01.
Figure 2. Methylation level of CpG sites in BMP6 gene promoter in tumor tissues and adjacent tissues. (A) Fragment between -1346 to -870; (B) fragment between -892 to -498; (C) fragment between -521 to -177; *P<0.01.

Figure 3. Real-time PCR, western blot and Sequenom MassARRAY analysis of the expression and promoter DNA methylation level of BMP6 in breast cancer cell line MCF-7 and drug-resistant cell line MCF-7/ADR. Real-time PCR (A) and western blotting (B) showed that BMP6 was downregulated in MCF-7/ADR cells and 5-aza-dc treatment reversed the decrease of BMP6 expression. (C) Quantitative methylation analysis showed that DNA methylation level of BMP6 promoter was significantly increased in MCF-7/ADR cells compared to MCF-7 cells; **P<0.01.
site 1, 2, 3, 25, 26, 27 and 28 in fragment 1 of MCF-7 cells was higher than MCF-7/ADR cells (Fig. 4A).

**Downregulation of BMP6 is involved in EMT in MCF-7/ADR cells.** In the drug-resistant breast cancer cells MCF-7/ADR, the BMP6 expression was signficantly lower than in the MCF-7 cell lines (Fig. 5A and B). In addition, our previous results indicated that downregulation of BMP6 contributed to the breast cancer cell drug resistance. In the present study, low expression BMP6 cells MCF-7/ADR or MCF-7 cells transfected BMP6-specific shRNA showed EMT phenotype. We found that higher BMP6 expression cells (MCF-7 or MCF-7/shNC cells) displayed a cobblestone-like appearance and tight cell–cell junctions (Fig. 5D). In contrast, the lower BMP6 expression cells MCF-7/ADR cells or MCF-7 cells transfected with BMP6-specific shRNA vector) appeared to have spindle-cell morphology; MCF-7/ADR cells treated with BMP6 protein reversed the morphologic changes (Fig. 5D).

Moreover, Transwell assay showed MCF-7/shRNA or MCF-7/ADR cells displayed markedly increased migration ability in comparison with their parental MCF-7 cells and BMP6 recombinant protein treatment significantly inhibited the migration ability of MCF-7/ADR cells (Fig. 5C). Real-time PCR showed that EMT transcription factors such as ZEB1, Snail1 and Twist1 expression were significantly upregulated in MCF-7/ADR or MCF-7 cells transfected with BMP6 specific-shRNA vector (Fig. 5E). The epithelial cell marker E-cadherin expression was significantly down regulated but the mesenchymal marker vimentin was significantly upregulated (Fig. 5E and F). MCF-7/ADR cells treated with BMP6 protein effectively reversed the expression of marker of EMT (Fig. 5E and F).
Discussion

In the present study, we found that DNA methylation can regulate the expression of BMP6 in breast cancer. These findings showed a decreased expression of BMP6 and an increase of methylation level of its promoter in breast cancer tissue compared to the paired adjacent normal tissues. In the breast cancer cell line, doxorubicin-resistant MCF-7/ADR cells showed a higher methylation level of BMP6 promoter compared with their parental MCF-7 cells accompanied by the lower expression of BMP6. Moreover, the cells with lower expression of BMP6, MCF-7/ADR cells or MCF-7 cells transfected with BMP6-specific shRNA vector, showed a typical EMT phenotype. Collectively, our results indicated that hypermethylation of promoter downregulated the expression of BMP6 and then induced the cells to undergo EMT during the acquisition of drug resistance of breast cancer cells.
BMP6 is a member of the signaling molecules of the TGF-β superfamily that are important regulators of cell proliferation and apoptosis in various types of cells including breast cancer, myeloma and lymphoma (16,20,21). BMP6 transfer signals through ligation of type I and type II serine-threonine kinase receptor, which then propagate the signal downstream by phosphorylating receptor-activated Smad protein (22). Previous studies by us and others showed that BMP6 might act as a suppressor of cancer to inhibit the cancer cell proliferation and metastasis (23). Furthermore, we also confirmed that BMP6 played a critical role in breast cancer cell aberrant proliferation and chemoresistance. With the development of breast cancer cell drug resistance, the expression of BMP6 was markedly decreased. However, the regulation of BMP6 downregulation remains unknown in breast cancer since the promoter sequence of BMP6 was identified as a target for aberrant DNA methylation (24).

Herein, we analyzed the methylation status of BMP6 promoter region in primary breast cancer tissues and breast cancer cell line MCF-7 and the drug-resistant cell line MCF-7/ADR. We found the level of methylation of BMP6 in cancer tissues was significantly higher than the adjacent normal tissues. In cell lines, the MCF-7 cells showed a lower level of methylation of BMP6 than the MCF-7/ADR cells. These indicated that hypermethylation of BMP6 promoter might be involved in breast tumorgenesis and drug resistance. In fact, previous studies also confirmed that aberrant methylation of BMP6 is involved in various cancers including malignant lymphoma, malignant pleural mesothelioma, T-cell leukemia and multiple myeloma (20,21,25-27). In the present study, we noted that aberrant methylation of BMP6 promoter was involved in breast cancer cell drug resistance. The findings suggest that DNA methylation may be important molecular events in the acquisition of drug resistance in breast cancer and the use of hypomethylation agents may be a novel therapeutic strategy for reversal of drug resistance.

Epithelial-mesenchymal transition (EMT) is an essential process for embryonic development and is implicated in tumor progression, invasion and metastasis (28). Previous studies suggested that EMT phenotype was closely related to chemoresistance in cancer cells. An EMT phenotype has been detected in gemcitabine-resistant pancreatic cancer cells (29,30), gefitinib-resistant non-small cell lung cancer (11), oxaliplatin-resistant colorectal cancer cells (31), paclitaxel-resistant ovarian cancer cells (32) and 5-fluorouracil or tamoxifen-resistant breast cancer cells (12,33). In our study, we found BMP6 expression inhibition caused an EMT phenotype of MCF-7 cells and resistance to doxorubicin. MCF-7/ADR cells or MCF-7 cells transfected with BMP6-specific shRNA vector displayed elongated, irregular fibroblastoid morphology. In contrast, MCF-7 cells or MCF-7 cells transfected with control shRNA vector had a rounded shape, typical of epithelial cells. These changes in phenotype suggest that MCF-7/ADR cells underwent EMT. Results from real-time PCR and western blotting also confirmed the phenotype of EMT in MCF-7/ADR cells. When the MCF-7/ADR cells were treated with the BMP6 recombinant protein, the morphologic change and EMT-related gene expression could be reversed. These indicated that BMP6 was an important regulator of EMT during the breast cancer cell acquisition of drug resistance.

In conclusion, our data highlight the importance of DNA methylation modifications in determining the expression of BMP6. In addition, downregulation of BMP6 caused by hypermethylation induced an EMT phenotype subsequently during the breast cancer cell acquisition drug resistance. To the best of our knowledge, this is the first study on BMP6 suppression by DNA methylation that modulates the breast cancer chemosensitivity via EMT. The findings suggest that DNA methylation and EMT may be important molecular events in the acquisition of drug resistance in breast cancer and the use of hypomethylation agents may be a novel therapeutic strategy for the reversal of drug resistance.

References


