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Original Article

Down-regulation of BRMS1 by DNA hypermethylation and its association with metastatic progression in triple-negative breast cancer

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Abstract: Breast cancer metastasis suppressor 1 (BRMS1) is a metastasis suppressor gene in several solid tumors. However, the expression and function of BRMS1 in triple-negative breast cancer (TNBC) have not been reported. In this study, we found that BRMS1 was down-regulation in breast cancer cell lines and primary TNBC, while decreased expression of BRMS1 mRNA was significantly associated with lymph node metastasis. And this down-regulation was found to be in accordance with aberrant methylation of the gene. Hypermethylation of the gene was observed in 53.4% (62/116) of the TNBC primary breast carcinomas, while it was found in only 24.1% (28/116) of the corresponding nonmalignant tissues. In addition, BRMS1 expression was restored in MDA-MB-231 after treatment with the demethylating agent, 5-aza-2-deoxycytidine (5-Aza-dC), and demethylation of the highly metastatic cells MDA-MB-231 induced invasion suppression of the cells. Furthermore, the suppression of BRMS1 by siRNA transfection enhanced cancer cells invasion. Collectively, our results suggest that the aberrant methylation of BRMS1 frequently occurs in the down-regulation of BRMS1 in TNBC and that it may play a role in the metastasis of breast cancer.

Keywords: Triple-negative breast cancer, breast cancer metastasis suppressor 1, methylation, metastasis

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, accounting for 23% of the total new cancer cases and 14% of the total cancer deaths in 2008 [1]. Breast cancers are routinely classified by stage, pathology, grade, and expression of estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor (Her2/neu). Current successful breast cancer therapies include hormone-based agents that directly target ER, PR, or Her2/neu [2]. Triple-negative breast cancer (TNBC) is a heterogeneous subset of neoplasms that is defined by the absence of these targets [3]. Studies have shown that TNBC tumors are in poor overall prognoses [4, 5]. To improve outcomes of TNBC, we need to unravel its biological pathways and modes of progression and use that knowledge to develop novel targets and therapies.

Breast cancer metastasis suppressor 1 (BRMS1) was identified by differential display comparing metastasis-suppressed chromosome 11 hybrids with metastatic, parental MDA-MB-435 human breast carcinoma cells [6, 7], and BRMS1 has subsequently been shown to suppress metastasis, but not tumorigenicity, of human melanoma and ovarian cancer cells in nude mice [8, 9]. Recent studies have indicated that BRMS1 expression was reduced in breast cancer, melanoma, ovarian cancer, nasopharyngeal carcinoma and non-small cell lung cancer (NSCLC), and seemed to be associated with cancer cell invasion, metastasis and patients' prognosis [10, 11]. There are several proposed mechanisms of action for BRMS1 and its role in the regulation of tumor metastasis; these include restoration of gap junctions, reduction of phosphoinositide signaling, interaction with the histone deacetylase complex and regulation of the nuclear factor-kB (NF-kB) pathway [12, 13]. Specially, several metastasis-related

genes were found to be downregulated by BRMS1 through modulating the activity of NF-kB, including osteopontin (OPN), urokinase-type plasminogen activator (uPA), microRNA-146, interleukin-6 (IL-6) and chemokine receptor 4 (CXCR4) [14, 15]. Until recently, limited data existed on the role of BRMS1 in TNBC.

In this study, we aimed to verify whether decreased BRMS1 expression in TNBC is related to DNA methylation. Three breast cancer cell lines and 42 paired normal and TNBC tissue samples were used to examine BRMS1 mRNA expression. In addition, TNBC specimens and corresponding nonmalignant breast tissues were recruited to observe the DNA methylation of BRMS1 and its clinical significance. In addition, the effect of 5-Aza-dC on the invasion ability of the highly metastatic basal-like breast cancer cells MDA-MB-231 was demonstrated in vitro using the Transwell assay.

Materials and methods

Cell line and culture

Three breast cancer cell lines with a basal-like transcriptional profile (MDA-MB-435, MDA-MB-231, and HCC-1937) and a normal mammary epithelial cell line (MCF-10A) were obtained from the Cancer Research Institute of Beijing, China. These cells were cultivated in T75 tissue culture flasks in DMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 20 mM hydroxyethyl piperazine ethanesulfonic acid, and incubated in humidified incubator containing 5% CO_2 at 37°C. The cells were incubated in culture media with 5-aza-2-deoxycytidine (5-Aza-dC) for 3 days using daily media changes. Cells were harvested and RNA and DNA were extracted on day 3. MDA-MB-231 cells were plated at 2×10^3 cells in 24-well culture plates, and cultured as described above. After overnight incubation, Cells were transfected with siRNA- BRMS1 or siRNA-negative control using RNAiMAX transfection reagent according to manufacturer's recommendations. BRMS1-specific small interfering RNA (siRNA) and negative control siRNA were purchased from Gene- Pharma Co., Shanghai, China.

Clinical tissue samples

TNBC Primary breast carcinoma samples and normal breast tissues were collected in the Affiliated Hospital of Qingdao University from 2011 to 2014. None of the patients had undergone preoperative chemotherapy or radiation. Written informed consent was obtained from all participants, and research protocols for the use of human tissue were approved by and conducted in accordance with the policies of the Institutional Review Boards at Qingdao University. The histological subtype was determined according to the World Health Organization classification. The TNM stage was determined postoperatively according to the American Joint Committee on Cancer, and the histological grade was determined according to the Scarff-Bloom-Richardson grading system.

RNA extraction and real-time RT-PCR

The cells were grown to subconfluence and then starved for 15 h in serum-free medium to attain quiescence. Total RNA was isolated from these cells using the TRIzol reagent according to the manufacturer's instructions. One microgram of the total cellular RNA was then reverse-transcribed into cDNA for PCR amplification using a kit from Sigma. Amplification consisted of an initial 5 min incubation at 95°C and then 30 cycles of amplification using 30 s of denaturation at 95°C, 30 s at 56°C, and 60 s at 72°C. The final extension was set for 10 min at 72°C. All data were expressed as the relative differences between control and treated cells after normalization to GAPDH expression.

Protein extraction and Western blotting

Total cellular protein was extracted using a lysis buffer and quantified using protein quantification reagents from Bio-Rad. Next, 50 μg of the protein were suspended in 5 × reducing sample buffer, boiled for 5 min, electrophoresed on 10% SDS-PAGE gels, and then transferred to polyvinylidene difluoride membrane by electroblotting. The membrane was blocked in 1% BSA/0.05% Tween/PBS solution overnight at 4°C, followed by incubation with the primary antibody for 24 h. A horseradish peroxidase-labelled goat anti-mouse IgG was used as the secondary antibody. The blots were then developed by incubation in a chemiluminescence substrate and exposed to X-ray films.

Immunohistochemistry

For immunohistochemical detection of BRMS1, 4 μm histological sections were deparaffinized with xylene and rehydrated through a graded series of alcohol. The sections were then boiled

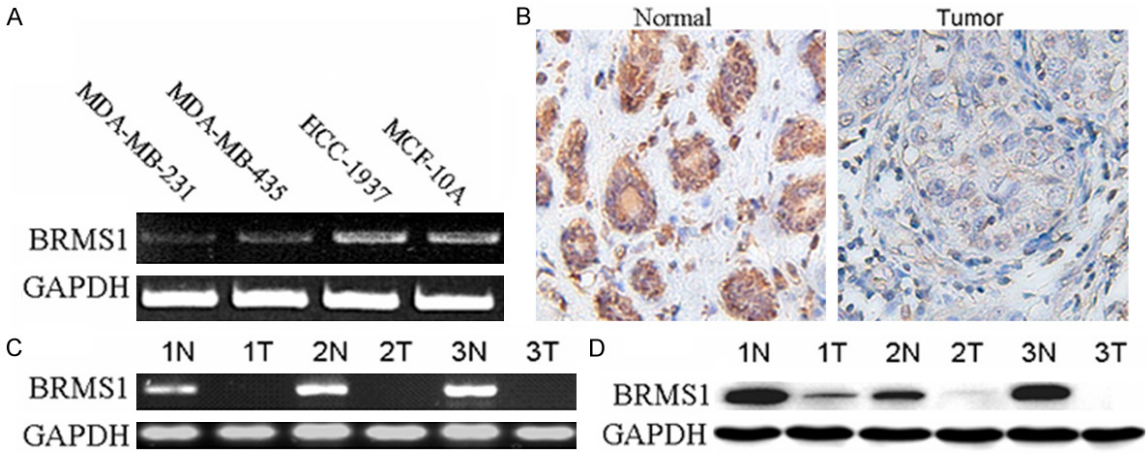

Figure 1. BRMS1 expression in human breast cancer cells and tissues, taking GAPDH as control. (A) Quantitative reverse-transcription polymerase chain reaction (RT-PCR) analyses on the expression of BRMS1 in MCF-10A and three breast cancer cell lines (HCC-1937, MDA-MB-435, and MDA-MB-231). The expression of BRMS1 in MCF-10A was higher than the three different types of breast cancer cell lines, and was lowest in MDA-MB-231. (B) Immunohistochemical analysis of BRMS1 protein in primary breast carcinomas and corresponding nonmalignant tissues. BRMS1 mRNA (C) and protein (D) expression in three cases of breast cancer tissues and corresponding non-tumorous tissues. N nonmalignant breast tissues, T primary breast tissues.

Table 1. Correlation of BRMS1 expression and clinicopathological parameters of TNBC samples

Clinicopathological variables	Cases (n = 67)	Expression of BRMS1	*P* value
Age (years)			0.822
≤ 45	29	1.301 ± 0.862	
> 45	38	1.344 ± 0.669	
Tumor size (cm)			0.715
≤ 2	22	1.270 ± 0.749	
> 2	45	1.344 ± 0.800	
Histological grade			0.302
Well/moderate	15	1.504 ± 0.877	
Poor	52	1.267 ± 0.749	
Lymph node status			0.018*
No	27	1.590 ± 0.816	
Yes	40	1.137 ± 0.705	
TNM stage			0.119
I + II	42	1.434 ± 0.812	
III	25	1.127 ± 0.693	

(*$P < 0.05$) (Data are shown as $\log_{10}$ of relative ratio change of breast cancer tissues relative to normal tissues).

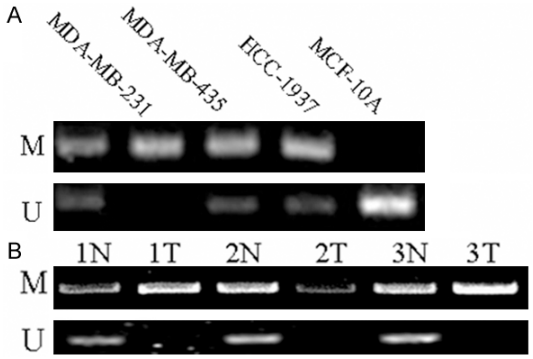

Figure 2. BRMS1 methylation analyses in TNBC cell lines and tumors and their corresponding nonmalignant breast tissues by methylationspecific PCR (MSP). A. DNA methylation of BRMS1 in MCF-10A and breast cancer cell lines. BRMS1 was hypermethylated in MDA-MB-231, and partially methylated in HCC-1937 and MDA-MB-435, but not methylated in MCF-10A. B. PCR showing DNA methylation of BRMS1 in TNBC specimens and corresponding nonmalignant breast tissues. M methylation, U unmethylation, N nonmalignant breast tissue, T tumor specimens.

for 10 min in 0.01 M citrate buffer and endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 in methanol for 30 min. Nonspecific binding was blocked by incubating slides with normal goat serum for 30 min at room temperature. The sections were incubated overnight at 4°C with a 1:150 dilution of rabbit-BRMS1 antibody. The sections were exposed to biotin-labeled secondary antibody for 1 h, to a streptavidin-peroxidase reaction system, and then developed with DAB-H_2O_2.

Methylation-specific PCR

The genomic DNA was prepared from cell lines and tissues by the phenol/chloroform protocol and was modified by bisulfite treatment as

Table 2. Clinicopathologicalparameters of TNBC samples and BMRS1 methylation (*$P < 0.05$)

Clinicopathological variables	Cases (n = 116)	BMRS1 methylation			*P* value
		U (%)	P (%)	M (%)	
Age (years)					0.342
≤ 45	45	16 (35.6)	6 (13.3)	23 (51.1)	
> 45	71	15 (21.1)	17 (23.9)	39 (54.9)	
Tumor size (cm)					0.000*
≤ 2	43	23 (53.5)	14 (32.6)	6 (14.0)	
> 2	73	8 (11.0)	9 (12.3)	56 (76.7)	
Histological grade					0.497
Well/moderate	36	7 (19.4)	9 (25.0)	20 (55.6)	
Poor	80	24 (30.0)	14 (17.5)	42 (52.5)	
Lymph node status					0.410
0	9	0 (0.0)	3 (33.3)	6 (66.7)	
1-3	59	17 (28.8)	12 (20.3)	30 (50.8)	
>3	48	14 (29.2)	8 (16.7)	26 (54.2)	
TNM stage					0.003*
I	15	7 (46.7)	7 (46.7)	1 (6.7)	
II	82	20 (24.4)	13 (15.9)	49 (59.8)	
III	19	4 (21.1)	3 (15.8)	12 (63.2)	

*$P < 0.05$.

described previously [16]. Then, DNA (2 μg) was purified using a Wizard DNA Clean-Up System, precipitated with ethanol, and resuspended in 30 μl of Tris-EDTA buffer. PCR amplification was performed using 2.0 μl bisulfite-modified DNA in a volume of 50 μl containing 10 × DreamTaq buffer, 2 mM dNTP Mix, 0.4 μM of each primer, and 1.25 U of DreamTaq. The PCR conditions were as follows: 95°C for 10 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing for 30 s at 60°C, extension for 30 s at 72°C, and then a final extension for 10 min at 72°C. CpG genome Universal Methylated and Unmethylated DNA was used as a positive control for the methylated and unmethylated genes, respectively. The amplification products were separated on 2.5% agarose gels.

Invasion assay

Invasion assays were performed using the Chemicon Cell Invasion Assay Kit according to the manufacturer's protocol. Briefly, cells (1×10^4) were plated onto a Matrigel-coated transwell invasion chamber and incubated at 37°C for 24 h. Non-invading cells were removed by wiping the upper side of the transwell. Invading cells were fixed with methanol and stained with hematoxylin. Three independent invasion assays were performed in triplicate. Six random fields on average were counted using a light microscope.

Statistics

For statistical analysis, we used the X^2 test and Fisher's exact test for categorical variables, and the Student's t test or one-way ANOVA test for continuous variables. Relative mRNA expression levels (BRMS1/GAPDH) were calculated from quantified data. Data are expressed as mean ± SD. For statistical analysis SPSS version 18.0 was used throughout, and *P* values < 0.05 were considered significant.

Results

BRMS1 expression is downregulated in breast cancer cell lines and tissues

The mRNA expression of BRMS1 was downregulated in HCC-1937, MDA-MB-435, and MDA-MB-231 cells compared to the normal mammary epithelial cell line MCF-10A. The lowest BRMS1 expression was found in MDA-MB-231. We then examined 67 paired breast cancer specimens and corresponding nonmalignant breast tissues by RT-PCR. We examined the relationship between BRMS1 mRNA expression and clinicopathological factor. BRMS1 mRNA expression was just related to lymph node metastasis. BRMS1 mRNA expression level in patients with lymph node metastasis were significantly lower than those in no lymph node metastasis tumors (*P* = 0.018) (**Figure 1**; **Table 1**).

Low expression of BRMS1 is related to DNA methylation

To identify whether low BRMS1 expression was due to DNA methylation, we first examined the DNA methylation of BRMS1 in breast cancer cell lines. Data from our MSP analysis showed hypermethylation of BRMS1 in MDA-MB-231 cells, and partial methylation in HCC-1937 and MDA-MB-435 cells. No methylation was obser-

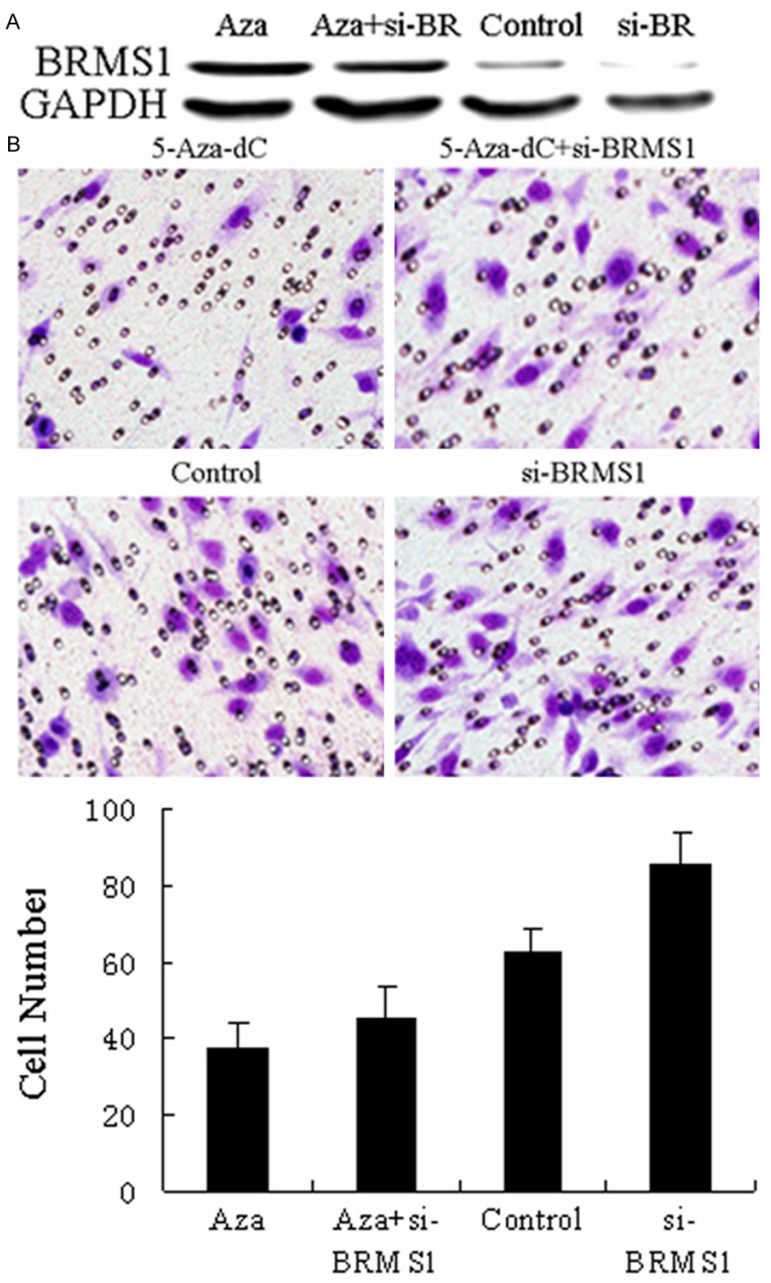

Figure 3. Effect of 5-Aza-dC on MDA-MB-231 cells invasion. A. Comparison of BRMS1 protein expression in human breast cancer cell line MDA-MB-231 after treatment with 5-Aza-cytidine (10 μM) and siRNA-BRMS1. B. Demethylation effect of BRMS1 on the invasion ability of the highly metastatic cell line MDA-MB-231 was observed by the invasion assay after treatment with 5-Aza-dC and siRNA- BRMS1. The columns indicate the number of cells invaded at the 24 h time point. The number of invading MDA-MB-231 cells was significantly reduced after treatment with 5-Aza-Dc compared with the control group. The values represent the mean values ± SD.

ved in MCF-10A cells (**Figure 2A**). Meanwhile, the results of MSP showed that both un-methylated and methylated bands existed in two breast cancer lines, HCC-1937 and MDA-MB-435 cells, but un-methylated bands did not appear in the MSP was also used to examine BRMS1 methylation in breast specimens, including 116 tumor and corresponding nonmalignant breast tissues. DNA methylation occurred in 53.4% (62/116) of primary breast cancer tissues and 24.1% (28/116) of nonmalignant breast tissues. We also found partial methylation of BRMS1 in 23 (19.8%) primary breast tissues and 16 (13.8%) nonmalignant breast tissues. No methylation was observed in 31 (26.7%) primary breast cancer tissues and 72 (62.1%) nonmalignant breast tissues. The difference in methylation between primary breast cancer and nonmalignant breast tissue specimens was significant ($P < 0.001$). Furthermore, DNA methylation of BRMS1 was related to the size of tumor and TNM stage. Compared with T1, the larger size of tumor were more frequently methylated (76.7 vs. 14.0 %, $P = 0.000$). For TNM stage, only 6.7 % (1/15) of tumor showed hypermethylation of BRMS1 in stages I, compared with stages II (59.8%) and III (63.2%) (**Table 2**).

Reactivation of BRMS1 expression after treatment with 5-Aza-dC

To confirm that aberrant methylation was responsible for silencing BRMS1 expression, we treated the MDA-MB-231 breast cancer cell lines with the demethylating agent 5-Aza-dC. The methylation status of BRMS1 of breast cancer cells was modified from methylated to unmethylated by the 5-Aza-dC treatment. As shown in **Figure 3A**, BRMS1 expression significantly increased in

MDA-MB-231 cells after treatment with 5-Aza-dC, with the highest expression occurring at a concentration of 10 μM (**Figure 3**).

Reactivation of BRMS1 expression with 5-Aza-dC inhibits the invasion ability of highly metastatic MDA-MB-231 cells

To further examine whether the reactivation of BRMS1 expression can regulate breast cancer invasion, we analyzed the invasion capability of the highly metastatic MDA-MB-231 cells using the methods described above. The number of MDA-MB-231 cells in the untreated group that migrated through the membrane was 62.30 ± 5.92/HP. The number of invading cells was significantly decreased when MDA-MB-231 cells were treated with 5-Aza-dC (37.30 ± 6.75/HP). A significant reduction in the number of invasive cells was observed for 24 h when the cells were treated with 72 h of 5-Aza-dC exposure compared to the control ($P = 0.001$). We further used the BRMS1-specific small inhibitory RNA to knock down BRMS1 expression in the MDA-MB-231 cell line, Western blot-conformed BRMS1 was effectively down-regulated after 48 h transfection. Invasion assay showed the number of invading cells was significantly increased after transfection with siRNA-BRMS1 (85.79 ± 7.83/HP) compared with the control group (**Figure 3**).

Discussion

It is well known that carcinoma results from the combined forces of both genetic and epigenetic events. DNA methylation is the most widely studied epigenetic event. DNA methylation, especially in CpG islands, leads to transcriptional gene silencing. There is increasing evidence that the inactivation of most tumor suppressor genes (TSGs) is related to DNA methylation [17-19], and the silencing of these genes possibly contributes to the development and progression of tumors. It has also been shown that aberrant methylation of TSGs is a common event in the development and progression of cancer [20]. Thus, DNA methylation has been a promising biomarker in detecting the disease-associated changes in cells [21, 22].

Metastasis suppressors are a growing family of molecules that are functionally defined by their ability to suppress metastasis without blocking orthotropic, primary tumor growth when repressed [23]. BRMS1 gene was originally identified as a true metastasis suppressor gene in breast cancer cell lines as stable overexpression of BRMS1 suppressed pulmonary metastasis. Subsequent studies have indicated that BRMS1 remarkable suppresses the metastatic phenotype in vitro in cells from several other types of cancer, including melanoma, ovarian cancer, bladder cancer, lung cancer [8-11]. BRMS1 was also shown to inhibit metastasis in xenograft models of these tumors, And BRMS1 expression in ovarian serous adenocarcinoma was significantly lower than in both normal ovarian tissue and benign ovarian tumor tissue [24]. Another study found that BRMS1 expression was diminished in NSCLC compared to the adjacent non-cancerous lung [25]. The relevance of BRMS1 in cancer was reinforced by the identification that BRMS1 downregulation was correlated with poor patient survival in breast cancer, ovarian cancer, melanoma, NSCLC, and nasopharyngeal carcinoma [10, 11]. However, the role of BRMS1 in TNBC has not been clearly studied.

In our study, we found that BRMS1 expression was significantly reduced in Triple-negative breast cancer cell lines compared to the normal breast mucosa cell line, MCF-10A. BRMS1 expression was lost in the undifferentiated breast cancer cell line, MDA-MB-231, and the less-differentiated cell line, MDA-MB-435, was weakly expressed in the HCC-1937 cancer cell lines, but was strongly expressed in MCF-10A. The differential expression between the three breast cancer cell lines may be related to cell type. We further found that the expression of BRMS1 was also lower in breast cancer tissues compared with corresponding nonmalignant breast tissues, and the expression level was statistically significantly inverse correlated with lymph node metastasis. This provides further evidence that BRMS1 may function as a tumor suppressor in breast cancer, and indicates that BRMS1 inactivation may contribute to the development of breast cancer.

Upon examining DNA methylation status in breast cancer cell lines, breast cancer tissues, and corresponding nonmalignant breast tissues by MSP, we found that BRMS1 was hypermethylated in MDA-MB-231, and partially methylated in HCC-1937 and MDA-MB-435, but not methylated in MCF-10A. In our study,

BRMS1 methylation was observed in 53.4% of the primary breast cancer specimens and 24.1% of corresponding nonmalignant breast tissues, and the difference in methylation between primary breast cancer and nonmalignant breast tissue specimens was significant. Meanwhile, we found partial methylation of BRMS1 in 19.8% of primary breast tissues and 13.8% nonmalignant breast tissues. Furthermore, our results also showed that DNA methylation of BRMS1 was related to the size of tumor and TNM stage. Compared with T1, the larger size of tumor was more frequently methylated. For TNM stage, only 6.7% of tumor showed hypermethylation of BRMS1 in stages I, compared with stages II (59.8%) and III (63.2%). It suggests that the malignancy degree of breast cancer may be higher when the methylation frequency of BRMS1 is high.

To further explore the DNA methylation, we treated cancer cells with 5-Aza-dC, a DNA methyltransferase inhibitor, which was sufficient to cause demethylation of the promoter region and reactivate the expression of the hypermethylated silenced gene. After 5-Aza-dC treatment, we observed a complete reversal of BRMS1 protein expression in MDA-MB-231 cells. As shown in this study, the action of 5-Aza-dC on the breast cancer cell lines MDA-MB-231 resulted in the demethylation of the BRMS1 gene, accompanied by the up-regulation of mRNA and protein. This confirmed that 5-Aza-dC regulated the transcription of the BRMS1 gene. In addition, the effect of 5-Aza-dC on the invasion ability of the highly metastatic breast cancer cells MDA-MB-231 was demonstrated in vitro using the Transwell assay. Our data suggest that the invasion ability suppression effect of 5-Aza-dC may result from the demethylation and reactivation of MDA-MB-231. The down-regulation of MDA-MB-231 was correlated with its promoter methylation in both breast cancer cell lines and breast tumor tissues. To further investigate the functions of BRMS1, cancer cells were knocked down by the BRMS1-specific siRNA. These results showed that cell invasion effect was remarkably enhanced when BRMS1 was silencing. It suggests that BRMS1 may be involved in the metastasis progress of breast cancer, and BRMS1 expression may inhibit the invasion of breast cancer.

In summary, we confirmed that BRMS1 was down-regulated in TNBC, which inactivation mainly caused by aberrant DNA methylation, may be involved in the development and progression of breast cancer. DNA demethylation and reactivation of BRMS1 after treatment with 5-Aza-dC inhibited the invasion ability of cells, while silencing of BRMS1 after transfection with siRNA promoted its progression. These findings provide a new method for detecting and treating TNBC. Further studies are needed to determine the precise mechanism underlying the role of BRMS1 in the progression of tumors, and particularly, triple-negative breast cancer.

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Disclosure of conflict of interest

None.

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