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Breast cancer metastasis suppressor 1 (BRMS1) suppresses prostate cancer progression by inducing apoptosis and regulating invasion

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Abstract. – OBJECTIVE: Breast cancer metastasis suppressor 1 (BRMS1) was originally identified as a metastasis suppressor gene in human breast cancer.

MATERIALS AND METHODS: A recent study has established an association between BRMS1 with the clinical stage and different pathology grades of prostate cancer. However, whether BRMS1 plays a role in prostate cancer has not been elucidated.

RESULTS: In this study, we found that overexpression of BRMS1 in PC-3 cells induced apoptosis and inhibited invasion; moreover, we found that overexpression BRMS1 was associated with the suppressed expression of EMMPRIN.

CONCLUSIONS: Taken together, our results show that BRMS1 may suppress progression and metastasis of prostate cancer through modulating EMMPRIN expression.

Key Words:
BRMS1, Prostate cancer, Apoptosis, EMMPRIN.

Introduction

Prostate cancer, the second leading cause of cancer death accounting for 28% of all male cancer cases and 10% of all male cancer deaths, is estimated to cause 630,000 deaths in 2035[1]. The diagnosis rate of prostate cancer in Chinese male has been increasing since the beginning of this century[2]. Despite employment of a variety of therapeutic regimens including surgery, radiotherapy, chemotherapy and hormonal therapy, a large proportion of patients with prostate cancer develop complications, leading to the treatment failure and poor prognosis[3-5]. Up to date, the etiology of prostate cancer is not fully understood; however, accumulating evidence have indicated that changes in expression pattern of specific genes contribute to the development and progression of prostate cancer[6-8]. Therefore, there is an urgent demand to identify new, more effective molecular target, which can guide clinical treatment.

The metastasis suppressors comprise a growing class of genes whose down-regulation triggers metastatic progression. Up to date, a number of genes have been identified as metastasis suppressors, which have been found to be valuable prognostic markers and therapeutic targets in cancer treatment[9]. The breast cancer metastasis suppressor 1 (BRMS1), firstly identified in breast cancer[10,11], has been found to function in a variety of human malignancies, including melanoma, ovarian carcinoma, non-small lung cancer, colon cancer and mammary carcinoma[12-17]. In these human malignancies, BRMS1 expression level is negatively associated with tumor progression and metastasis[15,18-21], suggesting the role of BRMS1 as a prognosis marker. A recent study has established an association between BRMS1 with the clinical stage and different pathology grades of prostate cancer[22]; nevertheless, little is known about the role of BRMS1 in prostate cancer.

Extracellular matrix metalloproteinase inducer (EMMPRIN), a cell-surface glycoprotein belonging to the immunoglobulin superfamily, is implicated in many biological functions, including embryo implantation, spermatogenesis[23], retinal development[24], inflammation[25], wound healing[26] and tumor progression[27]. Mounting evidence has established the correlation among the elevated EMMPRIN expression, the clinically aggressive behaviors, and the poor prognoses in human malignancies[28-30], which might be attributable to the inducing effect on the synthesis of matrix metalloproteinase (MMP) in the surrounding stromal cells[31]. In a case of prostate cancer, it has been reported that high level of EMMPRIN indicates

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unfavorable prognosis[32]. *In vitro* studies[33,34] also showed that EMMPRIN regulates cytoskeleton reorganization and cell adhesion as well as mediates metastasis and chemoresistance in prostate cancer. The current study is designed to explore the role of BRMS1 in prostate cancer and identify the downstream signaling elicited by BRMS1. Human prostate cancer cell line PC-3 was used as the model cell line to examine the effect of BRMS1 on cellular behaviors, including proliferation, migration, and invasion. Data from the present study suggested that BRMS1 suppress prostate cancer progression and metastasis by inhibiting expression of EMMPRIN.

Materials and Methods

Cell Culture

The human prostate cancer cell lines PC-3 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells are cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum, 100 U penicillin, and 100-mg/ml streptomycin in 5% CO_2 atmosphere at 37°C.

Western Blot

Upon treatment, the cells were harvested from flasks and lysed with ice-cold lysis buffer (Invitrogen, Carlsbad, CA, USA) for 30 minutes on ice. The cell lysate was then collected after centrifugation at 4°C, 12,000 rpm for 5 minutes. Equal amounts (50 μg) of lysate proteins were separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Proteins were probed with specific antibodies following standard protocol. After another washing with Tris Buffered Saline with Tween (TBST), the secondary antibody was added and incubated for 2 hours. The blots were washed with TBST before signals were detected by using a chemiluminescent substrate (KPL, Guildford, UK). BandScan software (Glyko, Novato, CA, USA) was used to quantify the blot density.

Plasmid Construction and Transfection

To generate BRMS1 overexpressing vectors, CDS region of BRMS1-coding sequences were obtained by RT-PCR and cloned into pCMV vector (Beyotime Institute of Biotechnology, Shanghai, China). The resulting plasmid was named pCMV-BRMS1. PC-3 cells were transfected with the pCMV-BRMS1 vector to induce enforced BRMS1 expression or pCMV vector to generate stable clones expressing BRMS1 constitutively as a control. The resulting cell lines were named PC-3/pCMV-BRMS1 and PC-3/pCMV, respectively. Two days after transfection, G418 solution was added to cells for selection of stable clones (PC-3/pCMV-BRMS1 and PC-3/pCMV cells). Stable clones were selected and maintained in a medium containing G418. Overexpression of BRMS1 was verified by quantitative RT-PCR and Western blot analysis.

Quantitative RT-PCR Analysis (qRT-PCR)

Gene expression was determined by quantitative real-time PCR (qRT-PCR) using gene-specific primers as described previously. In brief, the total RNA was extracted by using a commercial kit (RNeasy Mini kit, Qiagen, Dusseldorf, Germany). mRNA expression was detected by using a master mix that included a SYBR GREEN master mix (Solarbio Co., Beijing, China), a forward primer, a reverse primer, and template cDNA (10 ng), on a BioRad iCycler (Hercules, CA, USA). Primers for BRMS1 were: forward seq., 5'-GTGTCCCCTCAGAAGAGAAAATCG-3'., reverse seq., 5'-CTCCTCGCCCTTGCTCA CC-3'.

Colony Formation Assay

PC-3 cells (3.0 ×104) suspended in 1.6 mL Roswell Memorial Park Institute (RPMI) agarose medium (10% FBS and 0.33% agarose) containing the shogaol analogs or dimethylsulfoxide (DMSO) were plated in each well of a 6-well plate over a bottom layer of solidified RPMI agarose medium (10% FBS and 0.5% agarose). Cultures were maintained for 2 weeks without fresh medium feeding at 37°C in a humidified atmosphere of 95% air and 5.0% CO_2, after which cell colonies >0.1 mm were enumerated and photographed using the Nikon Eclipse TE2000-U microscope fitted with Nikon Digital Camera DXM1200 at a 109 objective (Nikon, Tokyo, Japan).

Cell Apoptosis Analysis

The cell apoptosis was evaluated by flow cytometry by using a fluorescein isothiocyanate (FITC) Annexin V apoptosis kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to manufacturer's instructions. In brief, the cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM sodium chloride, and

2.5 mM calcium chloride) at a concentration of 1.0×106 cells/mL. Cells were stained with annexin V-FITC and propidium iodide (PI) for 15 min in the dark before being analyzed by flow cytometry. Annexin V-FITC positive cells were considered to be undergoing apoptosis and those negative for FITC were considered to be alive.

Wound Scratch Assay

Each well of 24-well tissue culture plate was seeded with cells to a final density of 1×10^5 cells per well and these cells were maintained at 37°C and 5% CO_2 for 24 hours to permit cell adhesion and the formation of a confluent monolayer. These confluent monolayers were then scored with a sterile pipette tip to leave a scratch of approximately 0.4-0.5 mm in width. The wound closure was monitored by collecting digitized images at 0 and 24 hours after the scratch was performed. The digitized images were captured with an inverted microscope (Motic China Group Co., Xiamen, China) and digital camera (Nikon, Tokyo, Japan). The digitized images were then analyzed by Image-J software.

Invasion Assay

The 24-well Transwells coated with Matrigel (8-μm pore size; BD Biosciences, San Jose, CA, USA) were used for cell invasion assays following the standard protocol. Equal numbers (1×10^5) of cells were plated onto separate well. The cells were starved overnight in a serum-free medium, trypsinized and washed three times in Dulbecco's Modified Eagle Medium (DMEM) containing 1% fetal bovine serum (FBS). A total of 1×10^5 cells were then resuspended in 500 μl DMEM containing 1% FBS and added to the upper chamber, while minimum essential medium (MEM) with 10% FBS was added to the lower chamber as a chemoattractant. For the control, the medium containing 1% FBS was added to the lower chamber. The cells on the lower surface of the membrane were fixed in formaldehyde and stained with hematoxylin staining solution. The cells in at least five random microscopic fields (magnification, ×200) were counted and photographed.

Statistical Analysis

All statistical analysis was performed by SPSS Statistical software (SPSS Inc., Chicago, IL, USA). Values were presented as the mean ± SD. Statistical comparisons were performed by one-way ANOVA. Tukey's post hoc test was used for multiple group comparisons, and Student's *t*-test was used for single comparisons. $p<0.05$ was considered to be statistically significant.

Results

Confirmation of Over-expressing BRMS1 in PC-3 Cells

The over-expression of BRMS1 in PC-3 cells was examined by using RT-PCR and Western blot. As shown in Figure 1A, the mRNA level of BRMS1 was remarkably increased in PC-3/BRMS1 cells relative to control cells. Also, a significant increase in BRMS1 protein expression

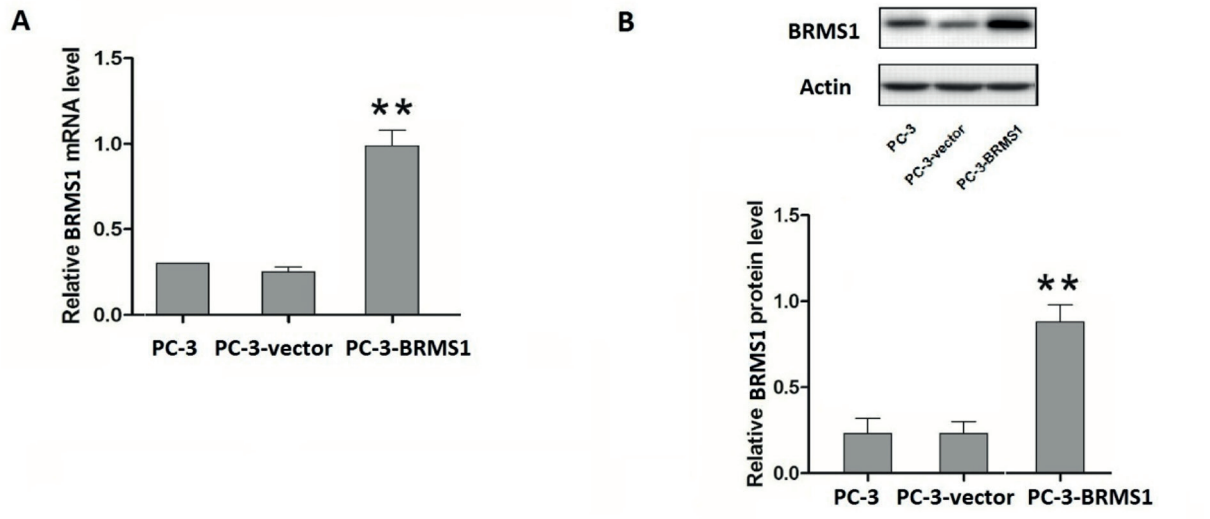

Figure 1. Verification of PC-3 cells stably overexpressing BRMS1. $^{**}p<0.01$ *vs*. controls.

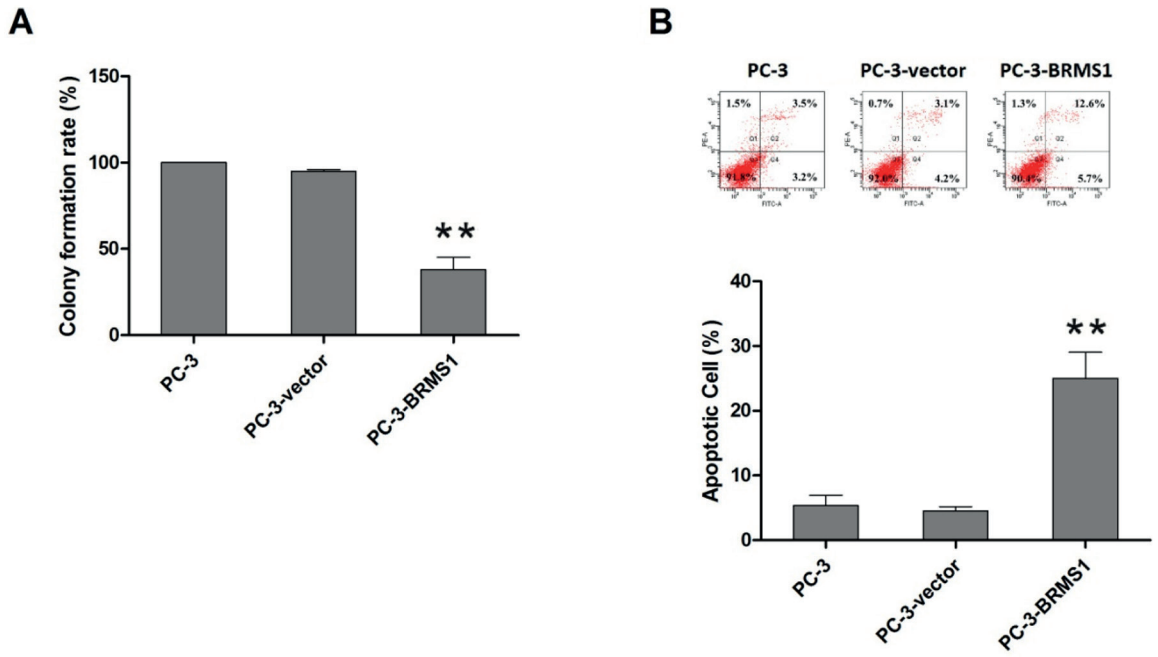

Figure 2. Effect of BRMS1 overexpression on cell growth and apoptosis. $^{**}p<0.01$ *vs*. controls.

was also found in PC-3/BRMS1 cells, as shown in Figure 1B.

Over-expression of BRMS1 Suppresses Cell Growth and Induces Apoptosis

The effect of overexpression of BRMS1 on cell proliferation was determined by the colony formation assay. As shown in Figure 2A, BRMS1 overexpression in PC-3 cells was associated with significantly fewer colonies relative to parent PC-3 cell and PC-3 cells transfected with vector only, indicating that BRSM1 functioned as a tumor suppressor and inhibited cell proliferation in PC-3 cells. Next, the effect of overexpressing BRSM1 on apoptosis was assessed by flow cytometry analysis. As shown in Figure 2B, PC-3 cells over-expressing BRMS1 showed a significantly lower rate of apoptosis, suggesting that BRMS1 suppressed proliferation at least partly, due to inducing apoptosis.

BRMS1 Suppresses Cell Migration and Invasion in PC-3 Cells

The effect of BRMS1 on migration and invasion of PC-3 cells were determined by wound healing and Transwell assay, respectively. As shown in Figure 3A, PC-3 cells stably overexpressing BRMS1 migrated significantly slower than the control cells at 24 hours. Transwell experiments reflected cell trans-membrane abilities, including migration and invasion. Also, BRMS1 overexpression significantly suppressed the invasive abilities of PC-3 cells compared with the Control cells, as shown in Figure 3B.

EMMRPRIN Expression Is Down-Regulated in BRMS1-Overexpressing Cells

To investigate the effect of BRMS1 on EMMPRIN expression, the EMMPRIN level was assessed by Western blot. As shown in Figure 4, the expression of EMMPRIN was significantly suppressed in the expression of EMMPRIN. Given the crucial role of EMMPRIN in the cellular functions of tumor cells, our results suggested that BRMS1 might regulate cellular activities including proliferation, apoptosis, migration and invasion via modulating EMMPRIN.

Discussion

BRMS1, located in the chromosome region 11q13-q14, was firstly identified in metastatic breast carcinomas patients[10]. Later experimental evidence proved that BRMS1 functions as a regulating gene in a variety of human malignancies such as melanoma, ovarian carcinoma, and non-small lung cancer, among others[35] besides breast carcinomas. Mechanistically, as a component of the mSin3-HDAC complex, BRMS1 regulates chromatin status

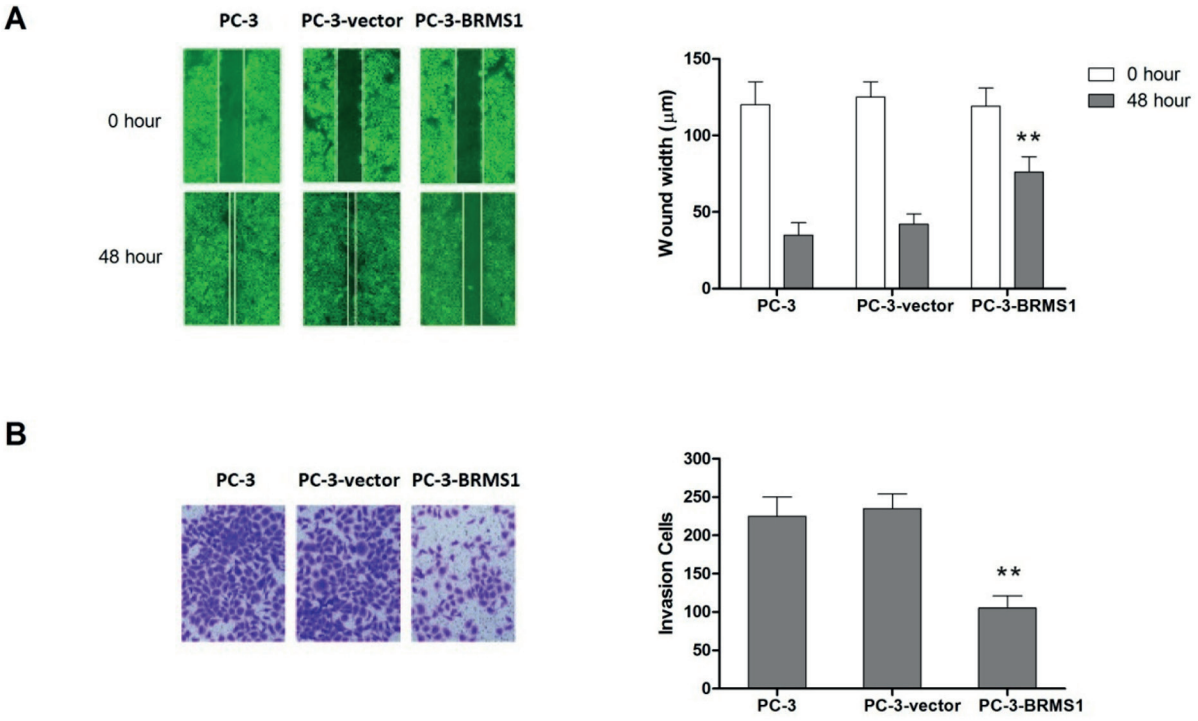

Figure 3. Effect of BRMS1 overexpression on cell migration and invasion. $^{**}p<0.01$ *vs*. controls.

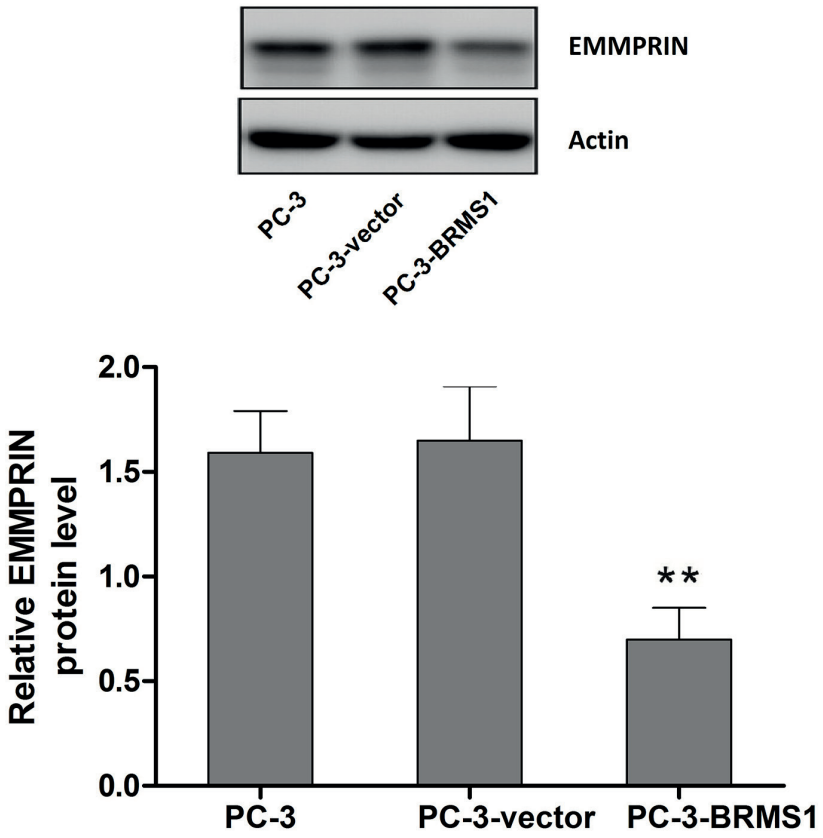

Figure 4. Effect of BRMS1 overexpression on EMMPRIN expression. $^{**}p<0.01$ *vs*. controls.

and therefore modulates the expression of genes functioning in cell apoptosis, cell-cell communication and cell migration[36-39]. In addition to its role in metastasis, it has also been reported that BRMS1 also plays a role in apoptosis. Wu et al[40] reported that BRMS1 induced apoptosis in hepatocellular carcinoma cells through suppressing osteopontin expression. Another study by using non-small cell lung cancer cell model showed that BRMS1 regulate apoptosis in NSCLC cells by modulating Stat3 activation[41]. A study carried out by Shi et al[22] showed that the expression of BRMS1 was negatively correlated with the Gleason score, T stage and M stage of prostate cancer, which triggered the present study to investigate the role of BRMS1 in the cellular behavior of prostate cancer using PC-3 cell line as *in vitro* model. To elucidate the functional role of BRMS1 in prostate cancer, we investigated the effect of BRMS1 on cellular behaviors in PC-3 cells line.

Metastasis is a biological event that involves many critical steps. The tumor metastasis suppressor genes, including BRMS1, can exert their effects through blocking any one (or more) of these steps; for example, they can sensitize cells to an apoptotic stimulus. In PC-3 cells, overexpressing BRMS1 resulted in a slight decrease in cell growth, which is not statistically significant; however, the overexpression of BRMS1 in PC-3 cells significantly induced apoptosis. Given the fact that the ability of cancer cells to escape apoptosis is required for metastasis[42], our results suggested that BRMS1 may exert activity in regulating NSCLC cell metastasis by modulating apoptosis. Moreover, we examined the effect of overexpression BRMS1 on migration and invasion. We found BRMS1 over-expression significantly impaired the capability of PC-3 cells to migrate and invade through the ECM to the surrounding tissue. Taken together, our results showed that BRMS1 over-expression inhibited progression and metastasis by inducing apoptosis and suppressing migration and invasion.

EMMPRIN plays a central role in tumor progression and early metastasis, with a positive correlation between EMMPRIN expression and the invasive and metastatic potential of malignant tumors[43,44]. Wang et al[45] reported that siRNA targeting to EMMPRIN mRNA could interfere with the transcription and translation of EMMPRIN gene, thus decrease the productions of MMPs to inhibit the invading of prostate cancer cells through reconstituted basement membrane *in vitro*. Prostate cancer cells could reduce the potential invasion of cells. In this study, we found that over-expression BRMS1 was associated with the suppressed expression of EMMPRIN. Given the role of EMMPRIN in cell proliferation, apoptosis, and invasion, these results suggested that BRMS1 may exert its effect by modulating EMMPRIN levels. It has been found that STAT3 and NF-κB signaling were regulated by BRMS1[41, 46]; therefore, we postulated that these signaling might be involved in the regulatory effect of BRMS1 on EMMPRIN expression, although further studies were needed.

Conclusions

We found that that over-expression of BRMS1 in PC-3 cells induced apoptosis and inhibited invasion; moreover, we found that over-expression BRMS1 was associated with the suppressed expression of EMMPRIN. Taken together, our results showed that BRMS1 might suppress progression and metastasis of prostate cancer through modulating EMMPRIN expression.

Ethical Statement

The authors declare no conflicts of interest.

Conflict of Interest

The authors declare no conflicts of interest.

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