SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 42: 2777-2787, 2019

RSK4 inhibits breast cancer cell proliferation and invasion *in vitro*, and is correlated with estrogen receptor upregulation in breast cancer

HONG HUO[1*], XINQING YE[2*], HONGHE YANG[1], QIUYUN LI[1] and YI JIANG[1]

Departments of [1]Breast Surgery and [2]Pathology, Guangxi Medical University Cancer Hospital, Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China

Received April 12, 2019; Accepted August 20, 2019

DOI: 10.3892/or.2019.7328

Abstract. Estrogen (E2) receptor (ER) upregulation has been associated with tumor progression and is the most commonly used clinical biomarker in breast cancer. X-linked ribosomal S6 kinase 4 (RSK4) is downregulated in breast cancer and may act as a tumor suppressor gene. In order to understand the association between the ER and RSK4, the present study studied the effects of RSK4 on ER-positive (ER^+) breast cancer cell function, and the effects of E2 on RSK4 function and RSK4 methylation. Furthermore, the disease-free survival of patients with breast cancer with RSK4 hypermethylation/hypomethylation was investigated to establish the link between RSK4 methylation on patient prognosis. The expression levels of RSK4 were increased and RSK4 promoter methylation was decreased in ER^+ breast cancer tissues and cell lines compared with ER-negative breast cancer tissues and cell lines, respectively. ER expression was negatively correlated with RSK4 expression and positively associated with RSK4 methylation. *In vitro* overexpression of RSK4 decreased the proliferation, clone formation, migration and angiogenesis and increased apoptosis of breast cancer cells. Patients with RSK4 hypomethylation exhibited a longer disease-free survival compared with patients with RSK4 hypermethylation. E2 stimulation of breast cancer cells increased ER expression and RSK4 methylation, which was associated with decreased RSK4 expression. Furthermore, ER upregulation was proposed to be related to the decreased expression of RSK4 in ER^+ breast cancer. E2 signaling may therefore act upstream of RSK4 to promote cancer progression. The results obtained in the current study suggested that RSK4 inhibited breast cancer cell invasiveness and that RSK4 promoter hypomethylation may serve as a novel prognostic marker for patients with breast cancer.

Correspondence to: Dr Qiuyun Li or Dr Yi Jiang, Department of Breast Surgery, Guangxi Medical University Cancer Hospital, 71 Hedi Road, Nanning, Guangxi Zhuang Autonomous Region 530021, P.R. China
E-mail: qyli1020@sina.com
E-mail: cat7921@sina.com

[*]Contributed equally

Key words: breast cancer, estrogen receptor, X-linked ribosomal S6 kinase 4 methylation

Introduction

Breast cancer is one of the most common types of cancer in females, with 2.1 million newly diagnosed cases in 2018 alone, accounting for ~25% of all female cancer diagnoses (1). Approximately 15% of all female cancer-associated mortalities in the United States of America were attributed to breast cancer (2). Current treatment therapies include surgical treatment, chemotherapy, radiotherapy and endocrine therapy (3,4). However, treatment may be ineffective, particularly due to the development of chemotherapy and endocrine therapy resistance. There is therefore a requirement for the identification of novel therapeutic strategies for the treatment of breast cancer.

X-linked ribosomal S6 kinase 4 (RSK4) is a serine-threonine protein kinase (5) that participates in the regulation of cellular senescence (6) and in tumor protein P53-dependent cell growth arrest signaling, acting as an inhibitor during embryogenesis (7). The distribution RSK4 in normal and tumor tissues has not been systematically investigated. Aberrant RSK4 expression has been reported in several types of cancer, including breast cancer (8), colorectal cancer (9), acute myeloid leukemia (10), non-small cell lung carcinoma (11), endometrial cancer (12) and ovarian cancer (13,14). Several studies have demonstrated that RSK4 is downregulated in breast cancer and may therefore act as a tumor suppressor gene (8,15-17). Additionally, downregulation of RSK4 in breast cancer is associated with hypermethylation of the RSK4 promoter (8,15).

The sex hormone estrogen (E2) is involved in the growth, development and physiology of the mammary glands. E2 binds to the E2 receptor (ER) α or β and regulates cellular proliferation and differentiation. ERα upregulation occurs in ~70% of breast cancer cases (18,19) and is associated with tumor progression (20,21). ER upregulation is the most commonly used clinical biomarker for breast cancer and ER-positive (ER^+) breast cancer is amenable to endocrine therapy (22). E2 stimulation promotes the expression of RSK4 in normal

mammary epithelial cells and the expression of RSK4 in breast cancer cells is closely associated with RSK4 methylation (15). ER signaling may therefore affect the expression of RSK4 or the methylation of the RSK4 promoter.

The present study explored the association between ER status, RSK4 expression and promoter methylation in breast cancer tissues. Additionally, the role of RSK4 and the effect of ER signaling on RSK4 promoter methylation in ER^+ breast cancer cells were investigated.

Materials and methods

Tumor tissue specimens. A total of 60 female patients who underwent breast surgery at the Guangxi Medical University Cancer Hospital between January 2013 to December 2014 were included in the current study. The clinical characteristics of the patients are presented in Table I. The patients had a mean age of 45.3 years (range, 25-71 years). The inclusion criteria were as follows: i) Pathological diagnosis of luminal A or luminal type B invasive breast cancer; and ii) no family history of breast cancer. Patients who had received tumor-associated treatment (including radiotherapy and chemotherapy) prior to admission were excluded from the current study.

Surgical resection specimens were obtained in the Department of Breast Surgery at the Guangxi Medical University Cancer Hospital. Breast cancer tissue and non-cancerous glandular tissue samples (at a distance of ≥2 cm from the tumor edge) were obtained from all patients. All samples were immediately placed in liquid nitrogen, stored at 80˚C and subsequently embedded in paraffin. The present study was approved by the Research Ethics Committee of the Guangxi Medical University Cancer Hospital and patients provided written informed consent.

Cell culture. The ER^+ human breast cancer cell line MCF-7 and the ER^- human breast cancer cell lines MDA-MB-231 and MDA-MB-453 were purchased from the Chinese Academy of Sciences. The breast cancer cell lines were cultured in Dulbecco's Modified Eagles medium (DMEM; HyClone; GE Life Sciences) containing 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA). 293T and human umbilical vein endothelial cells (HUVECs) were purchased from the Chinese Academy of Sciences. All cells were maintained at 37˚C and 5% CO_2.

Lentiviral packaging and cell transfection. The RSK4 lentiviral expression vector carrying the RSK4 coding sequence (pLenti-RSK4; 650 ng/μl; Thermo Fisher Scientific, Inc.) was co-transfected into 293T cells together with the packaging plasmids (Invitrogen; Thermo Fisher Scientific, Inc.). Medium containing lentiviral particles was subsequently harvested 48 h following transfection. Lentivirus stock was stored at -80˚C and used to infect MCF-7 cells in subsequent experiments.

MCF-7 cells were seeded in 6-well plates at a density of $3x10^5$ cells/well. Upon reaching 70% confluence, the cells were transfected with the lenti-RSK4-eGFP virus (RSK4-OE) or a lenti-eGFP virus as a negative control (NC) at a multiplicity of infection of 30. Nontransfected MCF-7 cells served as additional controls. Transfections were performed in triplicate. Cells were observed 24, 48 and 72 h following transfection using bright field/fluorescence microscopes (magnification, x100; three fields), and the transfection efficiency was further assessed by western blotting.

Immunohistochemistry. Paraffin blocks containing fixed tissues were sliced into 4-μm sections, placed on a glass slide, dewaxed with xylene and dehydrated using an alcohol gradient (100, 95, 70, 50%). Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide at 37˚C for 10 min. Samples were microwaved at high power for 4 min and low power for 20 min and allowed to reach room temperature to allow antigen recovery. Sections were immersed in normal goat serum (Abcam) for 10 min and subsequently incubated overnight at 4˚C with primary antibodies against ER (1:500; Abcam, Ab75635) and RSK4 (1:1,000; Abcam, Ab76117). Following primary antibody incubation, samples were incubated with rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories Inc., cat. no. 117360), stained with DAB (Beijing Solarbio Science & Technology Co., Ltd.) for 2 min and counterstained with hematoxylin for 5 min at room temperature. Samples were visualized using a DM750 microscope (Leica, Germany).

ER and RSK4 staining was independently assessed by two senior pathologists. Three randomly selected fields were scored under 100x magnification using a light microscope. A total of 200 cells were scored per field, with ≥600 cells scored per section. ER and RSK4 staining was scored as follows: i) <10% Positively stained cells, 0 points; ii) 10-30% positively stained cells, 1 point; iii) 31-60% positively stained cells, 2 points; and iv) >61% positively stained cells, 3 points. ER and RSK4 staining was additionally scored for color intensity as follows: i) Colorless, 0 points; ii) light yellow, 1 point; iii) brown, 2 points; and iv) tan, 3 points. The overall level of ER and RSK4 expression was determined by multiplying the two scores as follows: i) 0-1 Points, negative; ii) 1.1-2.0 points, weakly positive; iii) 2.1-3.0, moderately positive; and iv) 3.1-5.0 staining, strongly positive.

RNA extraction and reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from patient tissue samples and transduced cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA was reverse-transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. qPCR was subsequently performed using SYBR® Green mix (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol at Mx3000P Real-time fluorescence quantification PCR instrument (Agilent Technologies, Inc.). The thermocycling conditions were as follows: 95˚C for 2 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 20 sec and 72˚C for 20 sec and a final extension step 60.5˚C for 30 sec. mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (23) and normalized to β-actin. Primer sequences were as follows: RSK4 forward, 5'-AGATTCTCCCGGTTTGCC-3' and reverse, 5'-AAGGGTCTCGCTTACTTTTGT-3'; β-actin forward, 5'-GGCACTCTTCCAGCCTTCC-3' and reverse, 5'-GAGCCG CCGATCCACAC-3'.

Western blotting. Cells were lysed with cell lysis buffer (Beyotime Institute of Biotechnology) and the protein

Table I. Characteristics of 60 patients with breast cancer, stratified by molecular subtype.

Characteristic	Total	Luminal A	Luminal B1	Luminal B2	P-value
n (%)	60 (100)	21 (35)	19 (32)	20 (33)	
Age (years), n					0.858[a]
<45	24	9	8	10	
≥45	36	12	11	10	
Median (range)		45 (30-65)	45 (28-71)	46 (25-69)	
Menopausal status, n					0.859[a]
Premenopausal	28	11	10	12	
Postmenopausal	32	10	9	8	
Pathology type, n					
Invasive ductal carcinoma	60	21	19	20	
T stage, n					0.744[b]
T1	8	2	4	2	
T2	23	8	8	7	
T3	29	11	7	11	
N stage, n					0.922[b]
N0	6	2	3	1	
N1	28	10	8	10	
N2	26	9	8	9	
TNM stage, n					0.783[b]
IA	3	1	2	0	
IIA	9	3	4	2	
IIB	25	8	7	10	
IIIA	23	9	6	8	
Hormone receptor status, n					
Positive	60	21	19	20	
Negative	-	-	-	-	
Tumor nuclear grade, n					0.387[b]
I	18	5	6	7	
II	20	6	9	5	
III	22	10	4	8	

[a]P-value were determined using Pearson's χ^2 test. [b]P-values were determined using the Mann-Whitney U test.

concentration was determined using a Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Protein aliquots (30 μg/lane) were separated via SDS-PAGE on a 10% gel. The separated proteins were subsequently transferred onto a polyvinylidene difluoride membrane and blocked with 5% skim milk powder in Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature. The membranes were incubated with primary antibodies against RSK4 (1:1,000; Abcam), ER (1:400; Abcam) and GAPDH (1:1,000; Sungene), β-actin (1:2,000; Boster Biological Technology) or tubulin (1:2,000; Sungene) overnight at 4˚C. Following three washes with TBST, the membranes were incubated with rabbit HRP secondary antibodies against RSK4 and ER (1:3,000; Jackson ImmunoResearch Laboratories Inc.), and GAPDH, β-actin and tubulin) (1:3,000; Jackson ImmunoResearch Laboratories Inc.) for 1 h at room temperature. The membranes were washed three times with TBST and protein bands were visualized using the Tanon-4200 automatic chemiluminescence image analysis system (Tanon-4200 Fully Automatic Chemiluminescence Image Analyzer, Tianneng).

DNA extraction and bisulfite sequencing. Genomic DNA was extracted from patient tissues and cells and treated with bisulfite using the EZ DNA Methylation-Gold™ kit (Zymo Research Corp.) according to the manufacturer's protocols. The bisulfite-treated DNA was subsequently amplified by PCR using the following primer pair: RSK4 forward, 5'-TGA GAGGGTTTGTTGAGTATGTGTG-3' and reverse, 5'-CCT CTATACTACCTCTCCAAAAACTAC-3'. The PCR products were cloned into the pMD19-T vector (Takara Biotechnology Co., Ltd.) according to manufacturer's protocols. The ligation product (10 μl) was transformed into DH5α competent cells (Invitrogen; Thermo Fisher Scientific, Inc.) using the conversion method. The cells were subsequently plated onto Luria broth (LB; Thermo Fisher Scientific, Inc.) plates containing 0.1 mg/ml ampicillin and cultured overnight at 37˚C. Eight

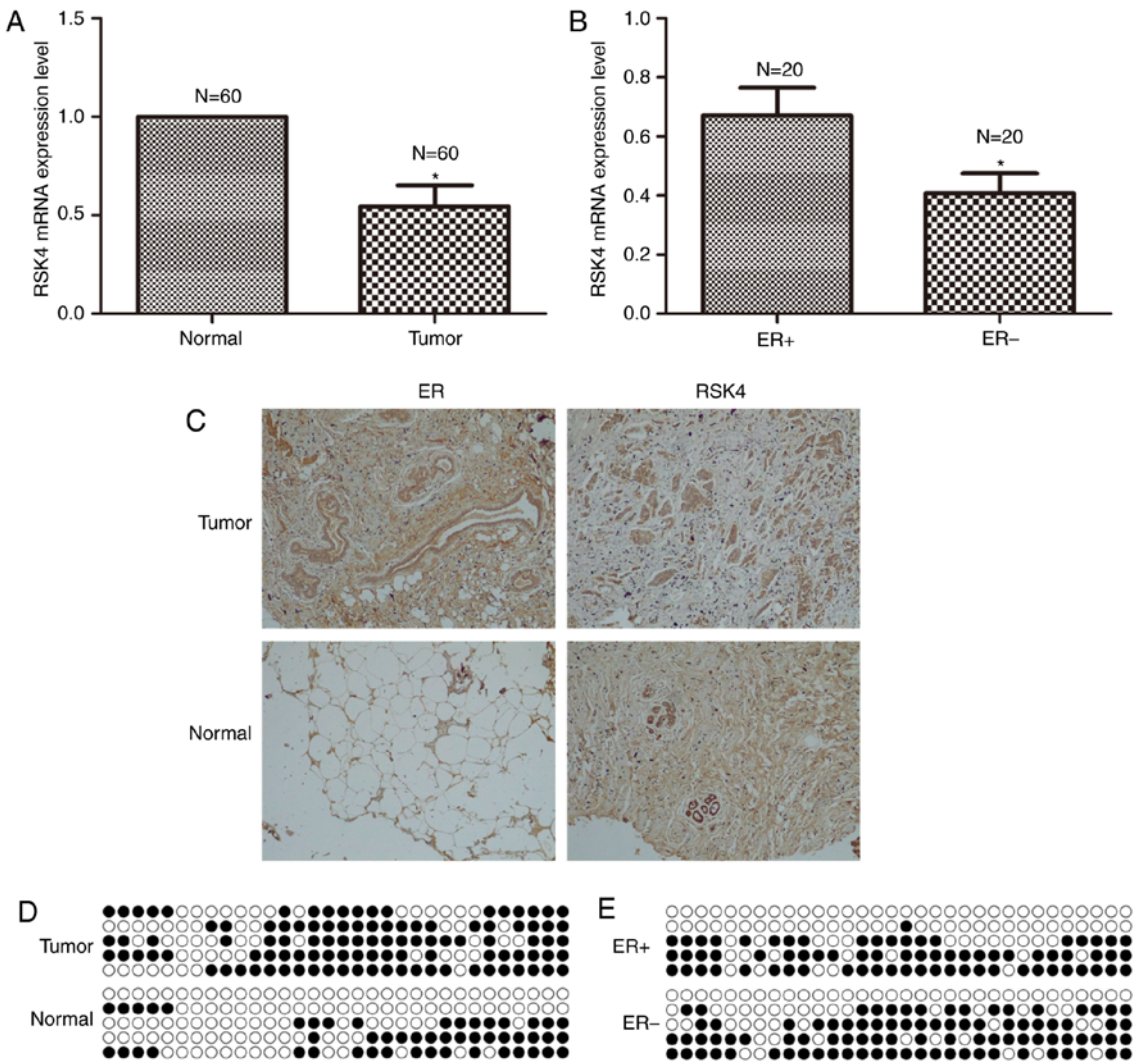

Figure 1. Expression of RSK4 in breast cancer tissue. Levels of RSK4 mRNA, as measured by reverse-transcription quantitative PCR, were compared (A) between breast cancer and paired adjacent normal tissues, as well as (B) between ER^+ and ER^- breast cancer tissues. (C) and RSK4 protein expression in breast cancer and paired adjacent normal tissues, as assessed by immunohistochemistry. Magnification, x400. (D) Bisulfite map showing RSK4 promoter methylation in breast cancer and paired adjacent normal tissues. (E) RSK4 promoter methylation levels in ER^+ and ER^- tumor tissue. Black circles indicated methylated CpGs and white circles presented unmethylated CpGs. Five clones were sequenced per locus. $^*P<0.05$ vs. Normal or ER^+. RSK4, X-linked ribosomal S6 kinase 4; ER, estrogen receptor.

bacterial colonies were selected from each plate and the presence of the insert was confirmed by PCR as aforementioned. Colonies containing the cloned inserts (at least 5 per plate) were inoculated in 3 ml LB containing 0.1 mg/ml ampicillin and cultured overnight at 37˚C. Plasmids were extracted using the AxyPerp Plasmid Miniprep kit (Axygen; Corning, Inc.) and analyzed using the Quantification Tool For Methylation Analysis (quma.cdb.riken.jp). Five clones were sequenced per locus.

Cell proliferation assay. The proliferation of transduced cells was determined using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). Cells were seeded in a 96-well plate at a density of $3x10^3$ cells per well and cultured at 37˚C. A total of 10 μl CCK-8 solution was added following 1, 2, 3, 4 and 5 days of incubation, and cells were incubated at 37˚C for a further 2 h. Cell proliferation was subsequently measure at a wavelength of 450 nm a microplate reader (Thermo Fisher Scientific, Inc.). Samples were analyzed in triplicate.

Clone formation assay. A total of 2 ml transfected cell suspension ($1x10^3$ cells/ml) were added to each well of a 6-well plate and cultured until 30-50 cell clusters had formed, ~5-7 days later. The spent cell culture medium was discarded and the cells were fixed in methanol (0.1%, 37˚C, 15 min) and stained with crystal violet (0.1%, 37˚C, 20 min). Clones were counted using an inverted fluorescence microscope (Olympus Corporation; magnification, x100). Clone formation was calculated as the number of clones formed/number of cells seeded x100%.

Cell migration assay. A total of 100 μl transfected cell suspension was pipetted into the upper chamber of 24-well Transwell inserts at a density of $4x10^4$ cells per well. The lower chamber was filled with DMEM supplemented with 10% FBS. Cells were incubated at 37˚C and 5% CO_2 for 24 h, after which the cells in the upper chamber that had not migrated through the polycarbonate membrane were wiped off. Cells in the lower chamber were fixed with 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at 37˚C

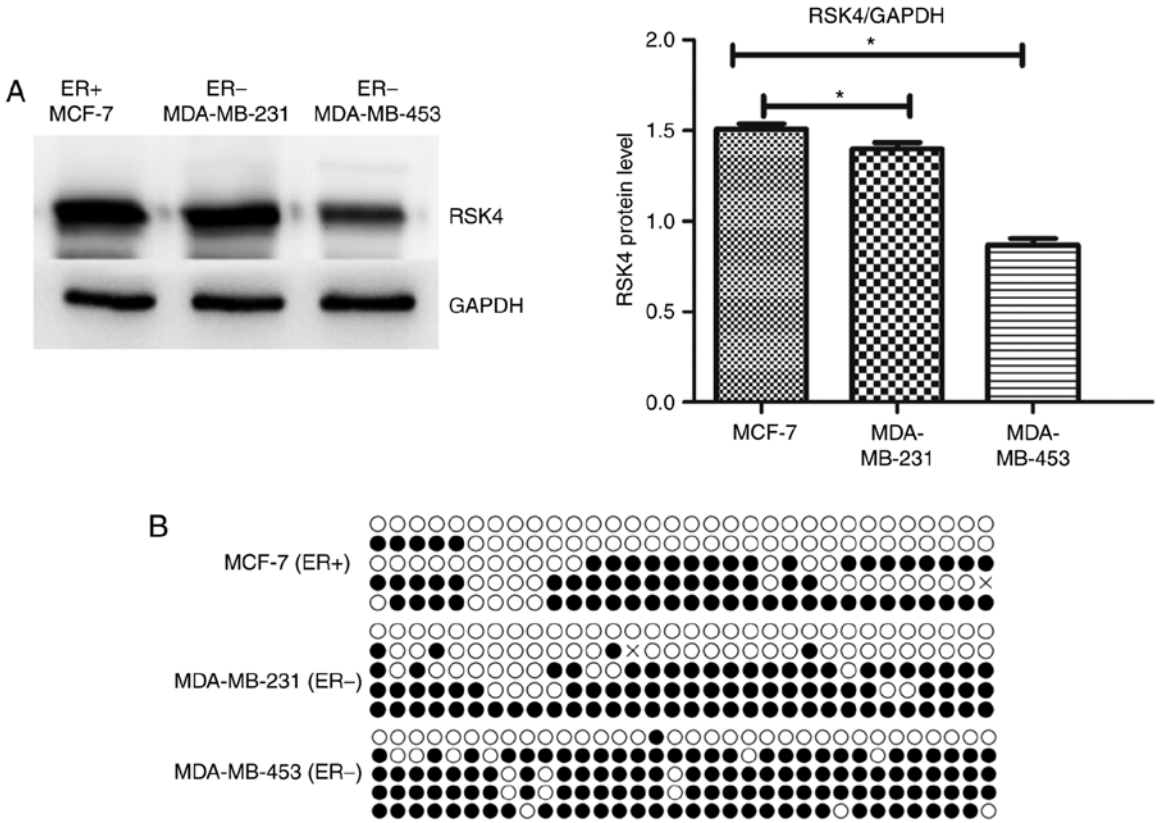

Figure 2. Expression of RSK4 in breast cancer cell lines. (A) Western blot analysis of RSK4 protein levels in MCF-7, MDA-MB-231 and MDA-MB-453 cells. (B) Bisulfite map showing RSK4 promoter methylation in MCF-7, MDA-MB-231 and MDA-MB-453 cells. Black circles indicated methylated CpGs and white circles presented unmethylated CpGs. Five clones per locus were sequenced. *P<0.05. RSK4, X-linked ribosomal S6 kinase 4; X, no available data.

and stained with 0.1% crystal violet (Beijing Solarbio Science & Technology Co., Ltd.) for 20 min at 37˚C. The cells were subsequently washed three times with PBS. Stained cells were counted in five randomly selected fields using an inverted fluorescence microscope (Olympus Corporation; magnification, x100). Each well was counted in triplicate.

Flow cytometry. After 48 h following transduction, cells were washed with PBS, trypsinized and suspended in 500 μl binding buffer which was included in the kit. Cells were subsequently incubated with 5 μl Annexin V-allophycocyanin (APC) and 5 μl propidium iodide (PI; Annexin V-APC-PI Apoptosis Analysis kit; Sungene) in the dark at room temperature for 10-15 min. Annexin V fluorescence was measured using a FACSAria Cell Sorter c6 flow cytometer (Accuri C6; BD Biosciences). The experiment was performed in triplicate.

Angiogenesis assay. Matrigel (BD Biosciences) was pipetted into a 96-well plate (50 μl/well), incubated for 30 min at 4˚C and allowed to set for 30 min at 37˚C. A total of $5x10^4$ HUVECs suspended in 50 μl serum-free conditioned medium taken from MCF-7 cells cultured for 24 h were seeded on Matrigel-coated wells. Endothelial tube formation was assessed following 5 h of culture and the mean number of tubes was counted in three randomly selected fields per well using an inverted fluorescence microscope (Olympus Corporation; magnification, x100).

E2 induction of MCF-7 cells. MCF-7 cells were plated into a 6-well plate at a density of $3x10^5$ cells/well and incubate at 37˚C and 5% CO_2. E2 (Sigma-Aldrich; Merck KGaA) was diluted in dimethyl sulfoxide (Beijing Solarbio Science & Technology Co., Ltd.) to prepare a stock solution of 10 mM and added to MCF-7 cells at final concentrations of 1, 5 or 10 nM. Cells were subsequently incubated with E2 for 48 h at 37˚C. Untreated MCF-7 cells served as controls. Cells were imaged prior and following treatment using an inverted fluorescence microscope (Olympus Corporation; magnification, x100; three random fields were selected).

Disease-free survival and overall survival. The 60 patients with breast cancer enrolled in the current study were followed-up by telephone or by electronic medical records until November 30, 2018. The median follow-up time was 52 months (range, 10-66 months). Local recurrence was defined as recurrence in the ipsilateral chest wall or regional lymph nodes detected by imaging or histology. Distant metastasis was defined as distant metastatic lesions detected by imaging. Disease-free survival was defined as time from the date of surgery to recurrence or metastasis. Overall survival was defined as time from the date of surgery to death. Patients with RSK4 methylation levels above or equal to the mean methylation value of 60 patients were defined as hypermethylated, while those with RSK4 methylation below the mean were defined as hypomethylated. Patient characteristics are presented in Table I.

Statistical analysis. Data were presented as mean ± standard deviation. Data were analyzed using SPSS software (version 19; IBM Corp.). A Student's t-test was used for the

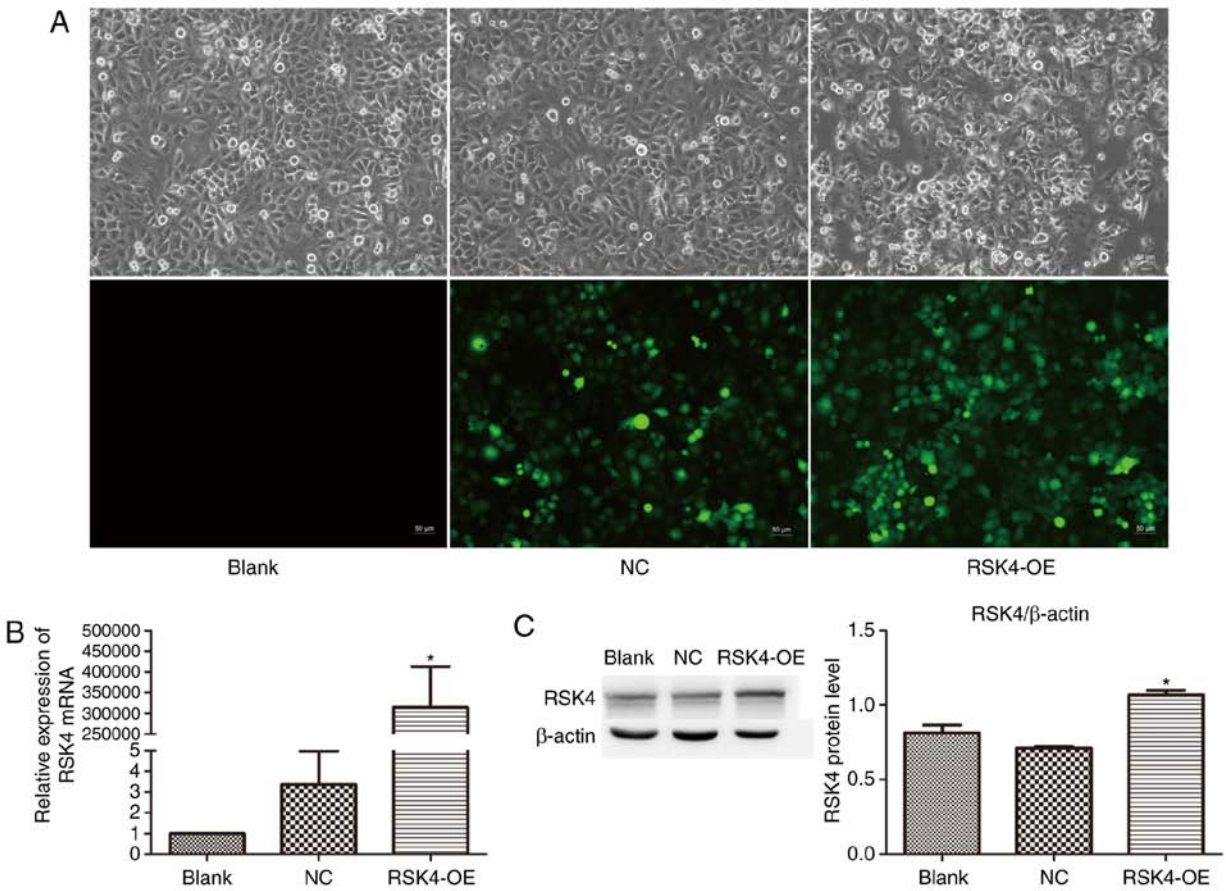

Figure 3. RSK4 OE in MCF-7 cells 72 h following transfection. (A) Bright field (upper row) and fluorescence (lower row) micrographs of MCF-7 cells transfected with RSK4-OE or NC. Magnification, x400. (B) RSK4 mRNA expression levels in MCF-7 cells transfected with RSK4-OE or NC, and in nontransfected controls (blank). Expression values were normalized to the blank group. (C) RSK4 protein levels in MCF-7 cells transfected with lenti-RSK4-EGFP or NC, and in nontransfected controls (blank). β-actin was used as a loading control. *P<0.05 vs. NC and blank. RSK4, X-linked ribosomal S6 kinase 4; NC, negative control; OE, overexpression.

comparison of two groups while one-way ANOVA was used for the comparison of three or more groups followed by a Least Significant Difference post-hoc test. Pearson's correlation analysis was used to assess the correlation of ER status with RSK4 expression. Patient survival was assessed using the Kaplan-Meier method, and survival curves were compared using the log-rank test. R x C bidirectional disordered count data were analyzed via a Pearson's χ^2 test. Grade data were analyzed via a Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference. Three independent experiments were performed.

Results

ER⁺ status is associated with higher RSK4 expression and lower promoter methylation in breast cancer tissue compared with ER⁻ status. The current study examined whether there was an association between RSK4 expression and breast cancer by comparing breast cancer and adjacent normal tissues. RSK4 mRNA expression was significantly decreased in breast cancer tissues compared with adjacent normal tissues (P=0.003; Fig. 1A). Similarly, immunohistochemical analysis revealed that RSK4 protein expression was markedly decreased in breast cancer tissues compared with adjacent normal tissues (Fig. 1C). ER protein expression was notably increased in breast cancer tissues compared with adjacent normal tissues (Fig. 1C). Bisulfite sequencing revealed that RSK4 promoter methylation was increased in breast cancer tissues compared with adjacent normal tissues (66.2 vs. 31.2%; Fig. 1D).

The association between RSK4 expression and ER status in breast cancer was investigated by analyzing ER⁺ and ER⁻ tumor tissues. RSK4 mRNA expression was significantly decreased in ER⁻ breast cancer tissues compared with ER⁺ breast cancer tissues (P=0.037; Fig. 1B).Bisulfite sequencing demonstrated that RSK4 promoter methylation was decreased in ER⁺ breast cancer tissues compared with ER⁻ breast cancer tissues (45 vs. 56.9%; Fig. 1E).

ER⁺ status is associated with increased RSK4 protein levels and decreased promoter methylation in breast cancer cell lines compared with ER⁻ status. The RSK4 protein levels in breast cancer cell lines were assessed using western blotting. Similarly to the mRNA data obtained from patient tissue samples, RSK4 protein expression was significantly increased in the ER⁺ breast cancer cell line MCF-7 compared with the ER⁻ breast cancer cell lines MDA-MB-231 (P=0.037) and MDA-MB-453 (P<0.001; Fig. 2A). The aforementioned results suggested that RSK4 expression decreased with increasing tumor malignancy as MDA-MB-231 and MDA-MB-453 cells have a higher degree of malignancy than MCF-7 cells (24).

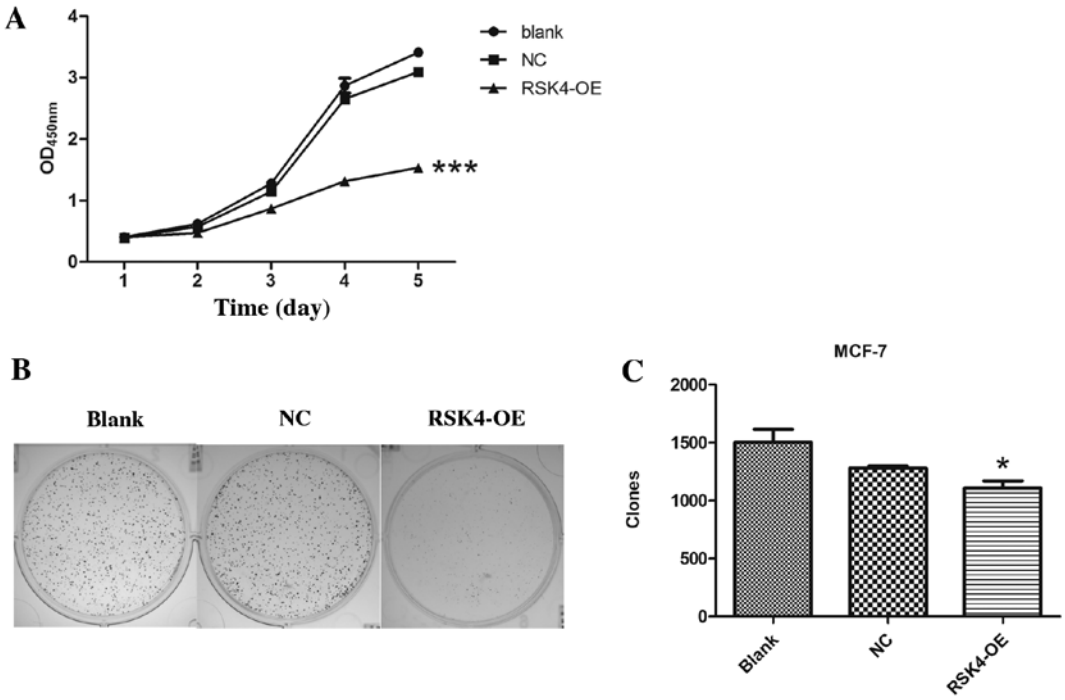

Figure 4. Effects of RSK4 OE on the proliferation of MCF-7 breast cancer cells. (A) Growth curves of MCF-7 cells transfected with RSK4-OE or NC, and nontransfected controls (blank), as measured by a Cell Counting Kit-8 assay. (B) Plates of clone formation in MCF-7 cells transfected with RSK4-OE or NC, and blank cells. (C) Quantitation of the experiments. Results are presented as the mean ± standard deviation. *P<0.05 and ***P<0.001 vs. NC and blank. RSK4, X-linked ribosomal S6 kinase 4; NC, negative control; OE, overexpression.

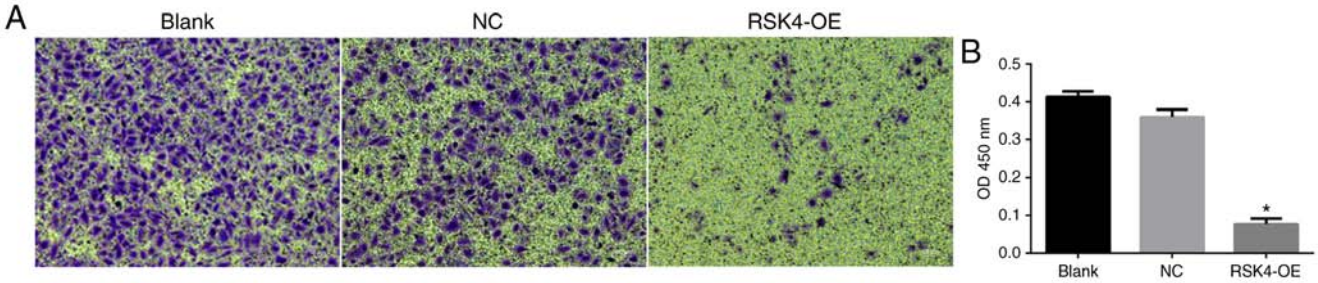

Figure 5. Effects of RSK4 OE on the migration of MCF-7 breast cancer cells. (A) images of migratory MCF-7 cells transfected with RSK4-OE or NC, and nontransfected controls (blank). Magnification, x100. (B) Quantitation of the experiments. Results are presented as the mean ± standard deviation. *P<0.05 vs. NC and blank. RSK4, X-linked ribosomal S6 kinase 4; NC, negative control; OE, overexpression.

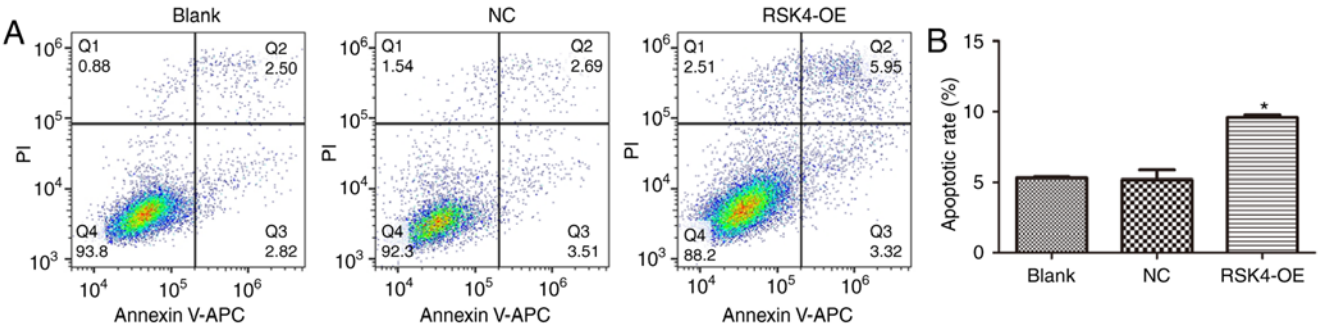

Figure 6. Effects of RSK4 OE on apoptosis level in MCF-7 breast cancer cells. (A) Annexin V-APC fluorescence in MCF-7 cells transfected with RSK4-OE or NC, and nontransfected controls (blank). (B) Quantification of the experiments. Results are presented as the mean ± SD. *P<0.05 vs. NC and blank. APC, allophycocyanin; RSK4, X-linked ribosomal S6 kinase 4; NC, negative control; OE, overexpression; PI, propidium iodide.

Furthermore, the ER+ breast cancer cell line MCF-7 exhibited decreased RSK4 promoter methylation (compared with the ER- breast cancer cell lines MDA-MB-231 and MDA-MB-453 (42.8 vs. 52.8% and 71.8%, respectively; Fig. 2B).

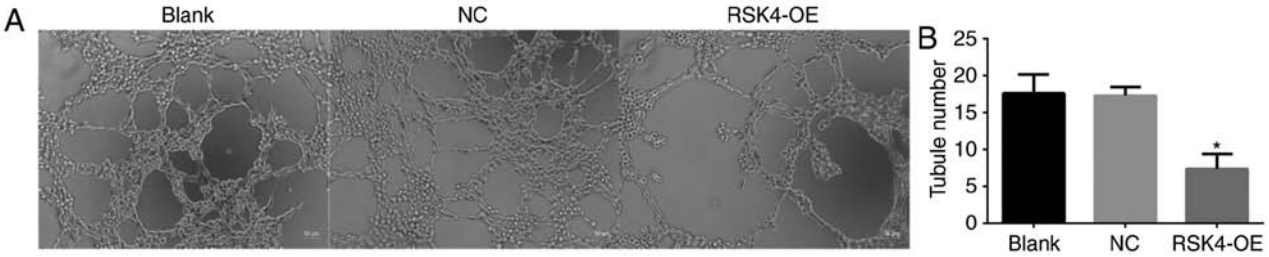

Figure 7. Effects of conditioned medium from MCF-7 overexpressing RSK4 on HUVEC tube formation. HUVECs were exposed to conditioned medium from MCF-7 cells transfected with RSK4-OE or NC, and nontransfected controls (blank). (A) Representative photomicrographs. Magnification, x400. (B) Quantification of tubule number from the experiments (A) Results are presented as the mean ± standard deviation. *P<0.05 vs. NC and blank. RSK4, X-linked ribosomal S6 kinase 4; HUVECs, human umbilical vein endothelial cells; NC, negative control; OE, overexpression.

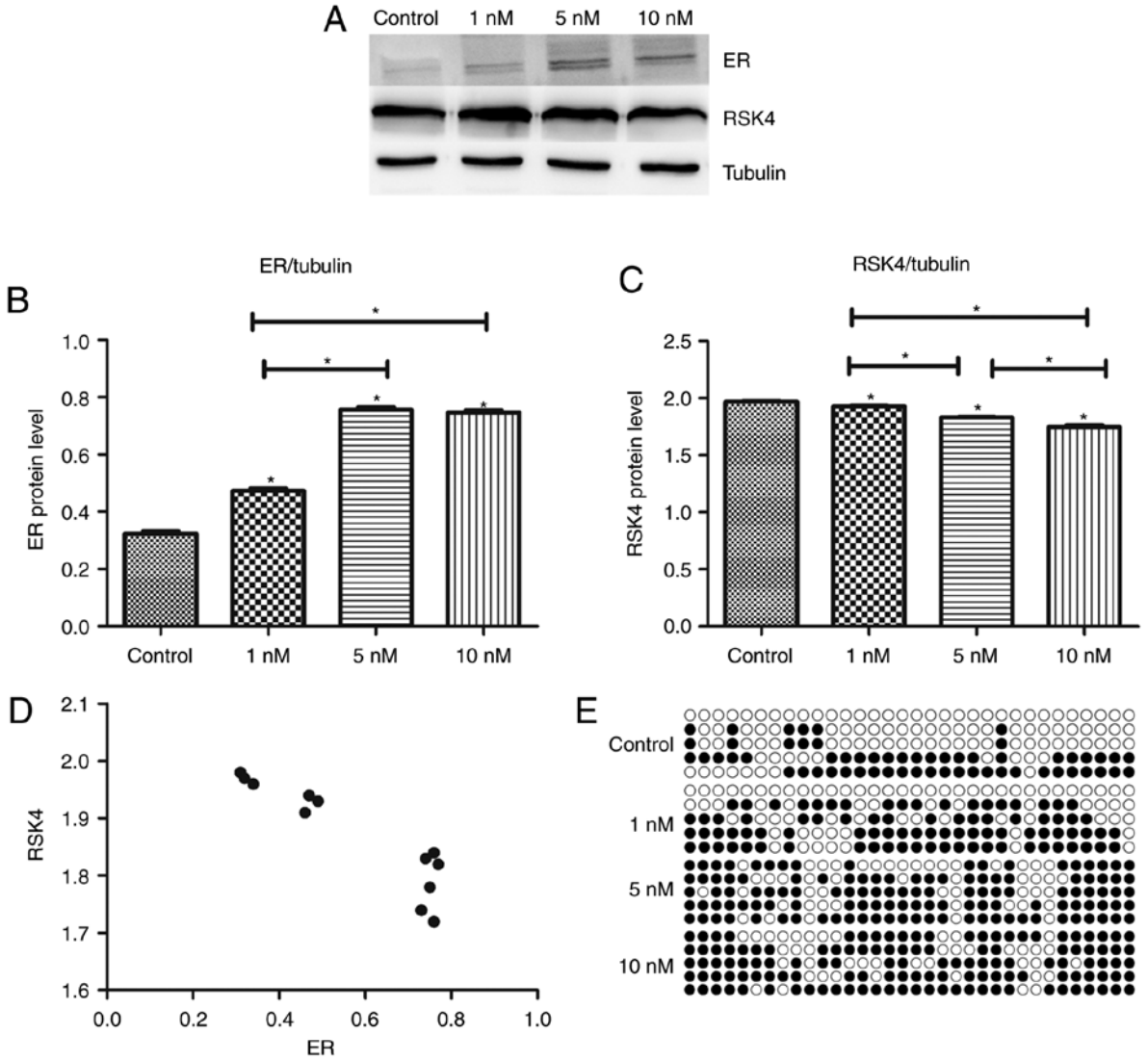

Figure 8. Effects of estrogen stimulation on ER and RSK4 expression and RSK4 promoter methylation in MCF-7 cells. (A-C) ER and RSK4 protein levels in MCF-7 cells treated with the indicated estrogen concentrations for 48 h. (D) Correlation analysis between ER and RSK4. (E) Bisulfite map showing RSK4 promoter methylation in MCF-7 cells treated with the indicated estrogen concentrations for 48 h. Black circles indicate methylated CpGs and white circles unmethylated CpGs. Five clones per locus were sequenced. *P<0.05. ER, estrogen receptor; RSK4, X-linked ribosomal S6 kinase 4.

RSK4 overexpression inhibits the proliferation and clone formation in ER+ breast cancer cells. To investigate the effects of RSK4 overexpression on breast cancer cells, MCF-7 breast cancer cells were transfected with RSK4-OE or Lenti-EGFP (NC) vectors. MCF-7 cells were selected since they are ER+ breast cancer cells. Cells transfected with RSK4-OE exhibited a notable increase in EGFP fluorescence over 72 h; the transfection efficiency was over 80% (Fig. 3A). Quantitation of RSK4 mRNA and protein by RT-qPCR and western blotting revealed that RSK4-OE cells exhibited a significant increase

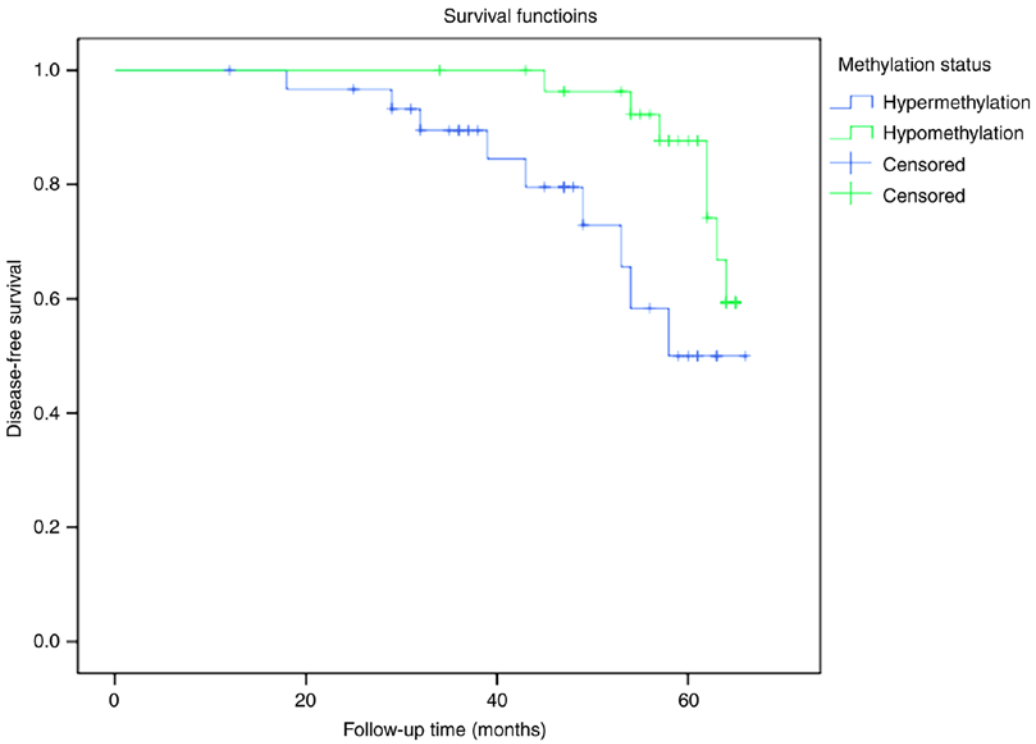

Figure 9. Association between RSK4 promoter methylation level and disease-free survival in 60 patients with breast cancer. Patients were stratified based on the mean methylation level. RSK4, X-linked ribosomal S6 kinase 4.

in RSK4 expression compared with NC cells (RSK4 mRNA, P=0.008; RSK4 protein, P=0.002) and nontransfected cells (RSK4 mRNA, P=0.008; RSK4 protein, P<0.001; Fig. 3B and C). No significant differences were observed between NC and nontransfected cells.

The proliferation of transfected cells was assessed using a CCK-8 assay. RSK4-OE cells exhibited significant decreases in the proliferation compared with NC (P<0.001) and nontransfected groups (P<0.001; Fig. 4A). Furthermore, RSK4-OE cells formed significantly fewer clones than NC cells (P=0.001) and nontransfected cells (P<0.001; Fig. 4B and C). There was no difference between NC cells and nontransfected cells.

RSK4 overexpression reduces the migration of ER⁺ breast cancer cells. The effects of RSK4 overexpression on the migration of transfected MCF-7 cells was assessed using a Transwell assay. RSK4-OE cells exhibited significantly reduced migration compared with NC cells (P<0.001) and nontransfected cells (P<0.001; Fig. 5A and B). There was no significant difference in migration between NC and nontransfected cells.

RSK4 overexpression promotes the apoptosis in ER⁺ breast cancer cells. Flow cytometry analysis of cells stained with Annexin V-APC and PI revealed that RSK4-OE cells exhibited a significant increase in the number of apoptotic cells compared with the NC (P<0.001) and nontransfected cells (P<0.001; Fig. 6). There was no significant difference in apoptosis between NC cells and nontransfected cells (P=0.822).

RSK4 overexpression reduces HUVEC tubule formation in vitro. The effects of RSK4 on the ability of HUVECs to form tubes *in vitro* was assessed. HUVECs were incubated with conditioned medium from nontransfected MCF-7 cells, and those transfected with RSK4-OE or NC cultured for 24 h. Cultured medium from RSK4-OE cells induced significantly fewer tubules compared with NC (P=0.001) and nontransfected controls (P=0.001; Fig. 7). There was no significant difference in tubule formation between conditioned medium from NC and nontransfected cells.

E2 stimulation of MCF-7 cells increases the expression of ER and RSK4 promoter methylation, but decreases the expression of RSK4. To investigate the effects of E2 signaling on ER and RSK4 expression, MCF-7 cells were treated with E2 for 48 h. ER protein levels were significantly increased in treated cells compared with untreated controls (P<0.001). Furthermore, ER protein levels were increased in the 5 and 10 nM groups compared with the 1 nM-treated group (P<0.001). There was no significant difference between the 5 and 10 nM groups (P=0.446; Fig. 8A and B). RSK4 protein levels were decreased in the treated cells compared with the untreated controls (P<0.05). RSK4 protein levels were significantly decreased in the 5 and 10 nM groups compared with the 1 nM group (P<0.001), and it was lower in the 10 nM group compared with the 5 nM-treated group (P=0.001; Fig. 8A and C). ER and RSK4 expression exhibited a negative correlation (r=-0.914; P<0.001; Fig. 8D). Bisulfite sequencing demonstrated that RSK4 promoter methylation was markedly increased in the 10 nM-treated group (75.3%) compared with the 5 nM (71.2%) and 1 nM-treated groups, (53.8%) and the control group (36.9%; Fig. 8E).

RSK4 hypomethylation correlates with longer disease-free survival in patients. The correlation between RSK4 promoter

methylation and survival was investigated in the 60 patients with breast cancer enrolled in the current study. Patients with RSK4 methylation levels above or equal to the mean methylation value (n=31) were defined as hypermethylated, while those with RSK4 methylation below the mean (n=29) were defined as hypomethylated. The disease-free survival rate was significantly increased in the hypomethylated group compared with the hypermethylated group (P=0.026; Fig. 9). No patient succumbed due to breast cancer during the follow-up period, so the overall survival could not be compared.

Discussion

The current study revealed that breast cancer tissues expressed significantly decreased levels of RSK4 and increased RSK4 promoter methylation compared with adjacent normal tissues. Furthermore, RSK4 expression was negatively correlated with ER expression in breast cancer cell lines. In addition, ER^+ status was associated with increased RSK4 expression and decreased promoter methylation compared with ER^- status. RSK4 overexpression inhibited ER^+ breast cancer cell proliferation, migration and clone formation, and promoted apoptosis. Additionally, conditioned medium from an ER^+ cell line overexpressing RSK4 inhibited HUVEC tubule formation *in vitro*. The aforementioned results suggested that RSK4 acts as a tumor suppressor, and its downregulation through the hypermethylation of the RSK4 promoter may be associated with breast cancer. Consistent with this hypothesis, the disease-free survival in the current study was significantly longer among patients with RSK4 promoter hypomethylation than among those with hypermethylation.

Previous studies have revealed that the E2-ER signaling pathway regulates mammary gland growth, development and apoptosis through genomic and non-genomic effects, which alter the expression of genes in normal breast epithelial cells (25,26). Dysregulation of the E2-ER signaling pathway alters the expression of genes, including cell surface heparan sulfate proteoglycans, and function of proteins such as the receptors for insulin-like growth factor and epidermal growth factor (27-31), which in turn dysregulates cell proliferation and mammary gland apoptosis (32-35). Additionally, alterations of the E2-ER signaling pathway may promote the conversion of E2-dependent tumors to non-dependent tumors, which are much more resistant to therapy (36-38). However, few studies have investigated the regulatory kinases and upstream/downstream molecules of the estrogen-ER signaling pathway. The results of the present study suggested a mechanistic link between ER signaling and RSK4 in breast cancer.

The present study revealed that RSK4 expression was reduced in breast cancer tissues compared with adjacent normal tissues, and that RSK4 expression decreased with increasing tumor malignancy. A previous study using a smaller number of breast cancer tissues reported a negative correlation between RSK4 mRNA expression and breast cancer tumor size, and clinical stage (8).

The results obtained in the present study revealed that RSK4 expression may correlate with ER upregulation in breast cancer tissues and cell lines, suggesting a mechanistic link between ER signaling and RSK4. The results revealed that the expression of ER was enhanced with increasing E2 concentration. Additionally, RSK4 protein levels decreased and the methylation of the RSK4 promoter increased with increasing E2 concentration. Furthermore, the expression of the was negatively correlated with the expression of RSK4, suggesting that the E2/ER signaling pathway may regulate the methylation of the RSK4 promoter. Inducing E2/ER signaling in breast cancer cells may therefore increase the methylation of the RSK4 promoter, thereby reducing the expression of RSK4 and affecting the development of breast cancer.

In the present study, patients with breast cancer and RSK4 hypomethylation had longer disease-free survival than patients with RSK4 hypermethylation. RSK4 methylation status may thus serve as an independent prognostic marker in breast cancer. However, an association between methylation status and overall survival was not observed in the current study. In summary, our results suggested that altered estrogen-ER signaling may be associated with decreased RSK4 expression and increased RSK4 methylation in breast cancer, leading to enhanced cell proliferation that may accelerate tumor development.

Acknowledgements

Not applicable.

Funding

The present study was supported by a grant from the National Natural Science Foundation of China (grant no. 81560431).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

QL designed, analyzed and revised the experiment. YJ designed and analyzed the experiment. HH conducted experiments, data analysis and article writing. XY conducted data processing and collected specimens. HY carried out specimen collection and follow-up. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the Guangxi Medical University Cancer Hospital and patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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