PRAF2 is an oncogene acting to promote the proliferation and invasion of breast cancer cells

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Abstract. Prenylated rab acceptor 1 domain family member 2 (PRAF2) acts as an oncogene and is closely related to the occurrence and development of various tumors. The present study aimed to clarify the functional relevance of PRAF2 in the biological behaviors of breast cancer by determining the expression of PRAF2 in breast cancer tissues and the corresponding adjacent tissues. The gene phenotypes of PRAF2 in patients with breast cancer in The Cancer Genome Atlas database were predicted using a cancer data online analysis website: The University of Alabama at Birmingham Cancer Data Analaysis Portal (UALCAN). The mRNA and protein expression of PRAF2 was further examined in 37 pairs of fresh frozen breast cancer tissues and adjacent non-tumor tissues by reverse transcription-quantitative PCR (RT-qPCR) and western blotting. High expression of PRAF2 was verified by RT-qPCR in the breast cancer cell line, MCF-7, and small interfering RNA (siRNA) technology was used to silence PRAF2. In the *in vitro* cell functional experiment, three groups were used: Negative control (NC) group, siRNA-NC group and siRNA-PRAF2 group. Cell Counting Kit-8 (CCK-8) and colony formation assays were conducted to analyze the effect of downregulation of PRAF2 on the proliferation of breast cancer cells. Transwell invasion and cell scratch assays were performed to examine the effect of downregulation of PRAF2 on the invasion and migration of breast cancer cells. UALCAN analysis results indicated that PRAF2 expression was upregulated in breast cancer compared with normal tissue samples (P<0.001). High expression of PRAF2 in breast cancer was associated with TNM stage and regional lymph node metastasis. RT-qPCR results showed increased mRNA expression of PRAF2 in clinical tissue samples from 37 patients with breast cancer, compared with normal adjacent tissues (P<0.001). Protein expression of PRAF2 was also shown to be higher in the breast cancer MCF-7 cells than in the MDA-MB-231 cells. Western blotting analysis combined with ImageJ software quantification showed that the relative expression of PRAF2 protein was significantly higher in clinical tissue samples from 37 patients with breast cancer (1.9750±0.0103) than that in normal adjacent tissues (0.9818±0.0140) (P<0.001). Western blotting analysis results indicated that transfection with siRNA PRAF2 in MCF-7 cells decreased PRAF2 expression (P<0.001). The results of CCK-8 and colony formation assays revealed that downregulation of PRAF2 expression suppressed the proliferation of MCF-7 cells (P<0.05 and P<0.001, respectively). In addition, Transwell invasion and cell scratch assay results showed that downregulation of PRAF2 expression in MCF-7 cells repressed invasion and migration of cancer cells (P<0.001). Overall, PRAF2 expression was significantly higher in breast cancer tissues than normal adjacent tissues, and was closely related to TNM stage and regional lymph node metastasis in breast cancer. PRAF2 was found to act as an oncogene that is able to promote breast cancer cell proliferation and invasion. Thus, PRAF2 may be a potential prognostic factor in patients with breast cancer and a potential target for the treatment of breast cancer metastasis.

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Introduction

The three most common cancers worldwide are breast, lung and colon, of which, breast cancer is the most prevalent malignant tumor in women (1). In 2012, ~1.7 million individuals were diagnosed with breast cancer worldwide, and ~500,000 individuals succumbed to the disease (2). Global cancer statistics in 2018 showed ~2.1 million new female breast cancer cases worldwide, accounting for nearly a quarter of the total number of women with cancer (3). However, the mechanisms underlying breast cancer cell migration, invasion and metastasis are still poorly understood. Targeted therapy has become a popular research field, and has demonstrated efficacy in breast cancer treatment; for example, trastuzumab combined with paclitaxel after doxorubicin and cyclophosphamide treatment

improves outcomes among women with surgically removed HER2-positive breast cancer (4). Therefore, it is imperative to seek novel molecular targeted therapies of breast cancer at the genomic level and to dissect the mechanisms underlying breast cancer cell invasion and metastasis.

Prenylated rab acceptor 1 domain family member 2 (PRAF2), originally known as JM4, is considered to be a novel endoplasmic reticulum (ER) protein involved in protein transport and vesicle transport from ER to Golgi (5-7). The PRAF2 gene encodes a 178-residue protein, with a molecular weight of 19.3 kDa, an isoelectric point of 9.21 and a net charge of +7.4 at pH 7.0 (8). The PRAF2 protein has four transmembrane domains, including a large PRA1 domain. The three members of the PRAF family, PRAF1, PRAF2 and PRAF3, are functionally and structurally related proteins, representing a new family of membrane transport-related proteins (8). PRAF2 was found to be expressed in most human tissues, showing strong expression in the brain, small intestine, lung, spleen and pancreas, but not detected in testicular tissues (8). A previous study has reported that PRAF1 regulates colon cancer cell proliferation and tumor progression by regulating T-cell factor (TCF)/β-catenin signaling pathway (9). Multiple research groups have demonstrated that PRAF3 acts as a tumor suppressor and regulates the migration and angiogenesis of gastric cancer, melanoma and cervical cancer cells through a variety of downstream signalling pathways (10-12). Downregulation of PRAF3 expression can be used as a poor prognostic indicator for a variety of cancers.

Existing studies have shown that PRAF2 is closely associated with the clinical pathology and poor prognosis of neuroblastoma, malignant glioma and liver cancer, and greatly promotes tumor cell proliferation, migration and metastasis. Geerts *et al* (13) found that the mRNA expression of PRAF2 was significantly upregulated in neuroblastoma, which is associated with many genetic and clinical features, such as age, survival, International Neuroblastoma Staging System stage and MYCN (neuroblastoma derived homolog gene) amplification of patients. The amplification of genomic MYCN plays a key oncogenic role in the pathogenesis of neuroblastoma (14). In *in vitro* experiments, researchers have found that silencing PRAF2 in SK-N-SH cells (human neuroblastoma cells) inhibits PRAF2 protein expression, resulting in a significant decrease in cell proliferation, migration and cell matrix adhesion capacity (15). Borsics *et al* (16) highlighted the prevalent PRAF2 expression in brain tissues, while PRAF2 expression was significantly higher in glioma tissue samples than in normal adjacent brain tissues. PRAF2 may render malignant gliomas highly aggressive through its involvement in vesicle transport or its interaction with chemokine receptors. In addition, Wang *et al* demonstrated that PRAF2 is an independent factor that negatively affects the overall survival of patients with hepatocellular carcinoma based on a study of 518 individuals. PRAF2 may also serve an oncogenic role in hepatocellular carcinoma progression (17). Another study has shown that the mRNA expression of PRAF2 is significantly increased in esophageal squamous cell carcinoma (ESCC) tissues compared with non-tumor tissues (18). Survival analysis results revealed that elevated expression of PRAF2 was associated with poor overall survival in patients with ESCC. Downregulation of PRAF2 expression inhibited the proliferation, cell cycle progression and invasion of ESCC cells, and induced cell apoptosis. Results suggest that PRAF2 may serve as a potential prognostic biomarker and therapeutic target for ESCC (18).

The aforementioned data have suggested the involvement of PRAF2 in the progression of multiple tumors. However, the underlying mechanisms of PRAF2 in tumor development and progression have, to the best of our knowledge, not yet been reported. In addition, PRAF3 has been confirmed to be an important regulatory protein of the p38 signaling pathway in breast cancer MDA-MB-231 cells (19). It can also inhibit the migration and invasion of breast cancer cells by downregulating the expression of C-X-C chemokine receptor type 4 (20). Therefore, we hypothesized that PRAF2 may also play a role as an oncogene in the development of breast cancer.The present study aimed to confirm that PRAF2 can act as a potential prognostic biomarker and theraptic target for patients with breast cancer.

Materials and methods

Cell culture and transfection. The breast cancer cell line MDA-MB-231 and the human normal mammary epithelial cell line MCF-10A were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences, while MCF-7 (cat. no. CL-0149) was obtained from Procell Life Science & Technology Co., Ltd. All cell lines were cultured with high-glucose DMEM (cat. no. SH30022.01; Cytiva) supplemented with 10% FBS (cat. no. S711-001S; Shanghai Shuangru Biotechnology Co., Ltd.) at 37˚C and 5% CO_2. Small interfering RNA (siRNA) sequences were designed and synthesized by TsingKe Biotechnology Co., Ltd., comprising three siRNA sequences targeting PRAF2 and a negative control (NC) sequence: siRNA1, siRNA2, siRNA3 and siRNA-NC. The siRNA sequences are provided in Table I. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cell transfection. The MDA-MB-231, MCF-10A and MCF-7 cells were seeded in 24-well plates at an optimized concentration of ~1x10^5 cells/well, 24 h before transfection. On the following day, when cell confluence had reached 60-70%, PRAF2 siRNA (10 μl/well) or siRNA-NC (10 μl/well) were transfected using 1 μl Lipofectamine 2000 reagent at room temperature for 48 h at a final concentration of 100 nM. Complete media was added to each well 6 h after transfection as per the manufacturer's protocol. Subsequent cellular function assays were performed on cells transfected with the siRNA that possessed the highest transfection efficiency, which was determined by PRAF2 protein expression analysis 48 h after transfection.

Breast cancer samples. The present study enrolled 37 patients diagnosed with breast cancer and also receiving modified radical mastectomy at the Department of Thyroid and Breast Surgery, Yijishan Hospital of Wannan Medical College (Wuhu, China) from October 2017 to October 2019. All patients had not received neoadjuvant chemotherapy, radiotherapy or endocrine treatment before surgery. Patients were female, aged between 45-76 years, with a median age of 58 years. Cases of invasive ductal carcinoma, intraductal carcinoma and other types of carcinoma were included in the present study. Cases with multiple primary cancers and a postoperative positive margin were excluded. The postoperative immunohistochemical

Table I. siRNA sequences.

Product cat. no.	Product name	Position	Target sequence (5'-3')
stB0009893A	siRNA1	471	AAGAUCGAGAGCAUUGGUCUCdTdT
stB0009893B	siRNA2	532	AAGAGCAGGAGGCUGGAUCCUdTdT
stB0009893C	siRNA3	916	AAGGCACUCUCAAAUCUUGAAdTdT
stB0009893D	siRNA-NC	0	GCCUGUGCGAAGGAAUCUUAAdTdT

NC, negative control; siRNA, small interfering RNA.

staining results of the patients for the markers estrogen receptor, progesterone receptor, HER2 and Ki67 were recorded, and clinical and pathological data, including age, histopathological grade, number of axillary lymph node dissections and sites of metastasis, were also collected. The patient characteristics are presented in Table II. Once the breast tissue specimens were surgically resected, the fresh breast cancer tissues and normal adjacent tissues were separated, quickly frozen in liquid nitrogen tanks and then stored in a -80˚C freezer. All patients were informed in writing and signed an informed consent form before sample collection. The study protocol was approved by the Ethics Committee of the Yijishan Hospital of Wannan Medical College (Wuhu, China).

Reverse transcription-quantitative PCR (RT-qPCR). QuantiNova™ SYBR Green PCR kit was used for this assay (cat. no. 208252; Qiagen AB). The primers for PRAF2 and GAPDH genes were synthesized by Guangzhou RiboBio Co. Ltd., and their sequences are detailed in Table III.

Total RNA from tissue samples and transfected cells was extracted by using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (cat. no. RR047A; Takara Bio, Inc.), according to the manufacturer's protocols. qPCR was performed with the QuantiNova™ SYBR Green PCR kit (cat. no. 208252; Qiagen AB) according to the manufacturer's protocols, using the ABI PRISM 7000 fluorescent quantitative PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: 1 cycle at 94˚C for 3 min; followed by 35 cycles at 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 45 sec. Relative expression was calculated based on the $2^{-\Delta\Delta Cq}$ method (21). GAPDH served as the internal control. This experiment was repeated three times.

Western blotting. MCF-7 cells were collected, washed with cold PBS, and lysed with PhosphoSafe™ Extraction Reagent (cat. no. P0013; Beyotime Institute of Biotechnology). The culture medium was aspirated from cells, before cells were rinsed once with PBS. The recommended amount of Phospho Safe™ Extraction Reagent was added, cultures were incubated at room temperature for 5 min, cells were dislodged using a cell scraper, and the lysate was transferred to a 1.5 ml tube before centrifugation for 5 min at 16,000 x g at 4˚C. Finally, the supernatant was transferred to a new tube and protein content was quantified using BCA assay (cat. no. AS1086; Wuhan Aspen Biotechnology Co., Ltd.). The total protein loading of each sample was normalized to 30 μg, then samples were electrophoresed using 15% SDS-PAGE and transferred to a PVDF membrane. Non-specific binding in membranes was blocked using PBS containing 5% non-fat milk for 2 h at room temperature. Subsequently, the membranes were incubated with primary antibodies at 4˚C overnight. The membranes were then incubated with an HRP-conjugated goat anti-rabbit IgG [1:5,000; cat. no. BL103A; Micro Biotechnology (Shanghai) Co., Ltd.] secondary antibody for 2 h at room temperature.

Table II. Clinicopathological patient characteristics.

Patients characteristics (n=37)	Absolute number	Ratio (%)
Age, years		
≤60	25	67.57
>60	12	32.43
Tumor size, cm		
≤2	23	62.17
>2	14	37.83
Lymph node status		
No metastasis (N0)	29	78.38
Metastasis (N1-3)	8	21.62
Histological type		
Invasive	32	86.30
DCIS	2	5.60
Others	3	8.10
Estrogen receptor status		
Negative	8	21.62
Positive	29	78.38
Progesterone receptor status		
Negative	14	37.83
Positive	23	62.17
HER-2 status		
Negative	16	43.24
Equivocal	16	43.24
Positive	5	13.52
PRAF2 status		
Negative	9	24.32
Positive	28	75.68

Table III. Reverse transcription-qPCR primer sequences.

Product cat. no.	Product name	Primer sequence (5'-3')
GQP0005157	geneDETECTTM h-PRAF2_qPCR_91bp_F1	CCCAGGTCAAGACATTGCC
GQP0005158	geneDETECTTM h-PRAF2_qPCR_91bp_R1	GGTCCAACAGTCAGGATACCC
ssD1021	GAPDH_2_forward primer (h,m,r)	GAACGGGAAGCTCACTGG
ssD1022	GAPDH_2_reverse primer (h,m,r)	GCCTGCTTCACCACCTTCT
B6612	TCF4 forward primer	CCTGGCTATGCAGGAATGTT
B6612	TCF4 reverse primer	CAGGAGGCGTACAGGAAGAG
B6611	β-catenin forward primer	AACAGGGTCTGGGACATTAGTC
B6611	β-catenin reverse primer	CGAAAGCCAATCAAACACAAAC

PRAF2, prenylated rab acceptor 1 domain family member 2; qPCR, quantitative PCR; F, forward; R, reverse; h, human; m, mouse; r, rat.

Finally, protein bands were detected using an enhanced chemiluminescence kit (cat. no. A38555; Thermo Fisher Scientific, Inc.). The following antibodies were used in the present study: β-actin (1:5,000; cat. no. AF7018; Affinity Biosciences, Ltd.), GAPDH (1:5,000; cat. no. ab37168; Abcam), PRAF2 (1:3,000; cat. no. ab230420; Abcam), β-catenin (1:3,000; cat. no. ab223075; Abcam) and TCF4 (1:3,000; cat. no. ab217668; Abcam). β-actin and GAPDH were used for normalization. ImageJ software (version 4.1; National Institutes of Health) was used to evaluate and quantify the gray value of protein bands. This experiment was repeated three times.

Cell function assays. The following three groups were set for the subsequent experiments in MCF-7 cell lines: NC group (with complete medium only), siRNA-NC group and siRNA-PRAF2 group.

Cell Counting Kit-8 (CCK-8) assay. The CCK-8 assay (Beyotime Institute of Biotechnology) was used to determine the cell proliferation, according to the manufacturer's instructions. Cells in the logarithmic growth phase (100 μl) were seeded on a 96-well plate (10^3 cells/well), then the culture medium was discarded after the cells had adhered. The cells were subsequently incubated with 10 μl CCK-8 reagent for 72 h. Culture medium without cells was used as the negative control, which was then incubated with CCK-8 reagent. The absorbtion of each well at 450 nm was measured using a microplate reader to calculate an average value of each group of cells and the negative controls, which allowed the calculation of the MCF-7 cell proliferation rate. MCF-7 cell proliferation rate (%)=Value of each experimental group/NC control group x100. This experiment was repeated three times.

Colony formation assay. For each experimental group, cells were resuspended with 1 ml complete culture medium and diluted to $1x10^4$/ml for counting. Each well of a 6-well plate was seeded with 300 cells, with each group seeded in triplicate. The 6-well plate was incubated at 37˚C with 5% CO_2 for 2 weeks. After being fixed with 4% paraformaldehyde for 30 min at 25˚C, the cell colonies were stained for 30 min at room temperature using 0.01% crystal violet solution. To observe stained colonies (>50 cells), samples were imaged under a light microscope (IX71; Olympus Corporation). The average value of the number of colonies for MCF-7 cells in each group was then calculated manually. The experiment was repeated three times.

Scratch wound healing assay. Using a marker pen, evenly spaced parallel lines, ~0.5 cm apart, were drawn on the bottom of a 6-well plate, with 5 lines per well. In each well, ~$5x10^5$ cells suspended in 2 ml high-glucose DMEM (cat. no. SH30022.01; Cytiva) supplemented with 10% FBS (cat. no. S711-001S; Shanghai Shuangru Biotechnology Co., Ltd.). Once the cells reached 100% confluence, a pipette tip was used to mark the cell monolayer perpendicular to the horizontal lines created by the marker pen. The scratched cells were rinsed gently with PBS, before adding fresh culture medium. Scratched cultures were imaged under a light microscope, and this was recorded as the 0 h time point. siRNA solution was prepared by adding 10 μl of the stock solution to 250 μl of Opti-MEM serum-free medium (cat. no. SH30022.01; Cytiva). Once siRNA solutions were added, the plate was left at room temperature for 5 min before it was returned to the incubator. After 24 h of incubation at 37˚C, each well was imaged at the same location under a light microscope, which was recorded as the 24 h time point. Calculation of the scratch healing rate used the following formula: Scratch healing rate (%)=(0 h scratch width-24 h scratch width)/0 h scratch width x100. This experiment was repeated three times.

Transwell invasion assay. The Transwell chamber (Corning, Inc.) with inserts was prepared by diluting 50 μl of Matrigel solution with complete culture medium at a dilution ratio of 1:3. The dilution was then added to the Transwell chamber and allowed to dry at 37˚C for 2 h. To each Transwell chamber, 200 μl of cell suspension at 10^5 cells/ml in serum-free culture medium was added, before each chamber was inserted into the wells of a 24-well plate with 500 μl high-glucose DMEM (cat. no. SH30022.01; Cytiva) supplemented with 10% FBS (cat. no. S711-001S; Shanghai Shuangru Biotechnology Co., Ltd.) per well in the lower chamber. The plate was then incubated at 37˚C for 48 h. The Transwell chambers were removed from the wells and the culture medium was discarded. Using a cotton swab, the upper portion of the chamber was wiped clean to remove any non-migratory cells. The remaining cells on the bottom side of the chamber were then stained with 0.01% crystal violet stain solution in PBS at room temperature for 10 min. The chambers

were rinsed to remove excess crystal violet stain, before being imaged using an inverted light microscope. To quantify the invasion in each chamber, three regions of interest were randomly identified at x40 magnification, before capturing corresponding triplicate x100 magnification images. From these images, the average number of invaded cells was calculated manually. This experiment was repeated three times.

The Cancer Genome Atlas (TCGA) database validation. The University of Alambama in Birmingham Cancer Data Analysis Portal (UALCAN; http://ualcan.path.uab.edu/index.html) is an effective website for online analysis and mining of cancer data, mainly based on relevant cancer data from TCGA database (22). Through this tool the expression of PRAF2 in breast cancer samples in TCGA database was verified. The database of Genotypes and Phenotypes accession no. for the analyzed data is phs000178 (23).

Statistical analysis. Statistical analysis was performed using SPSS 22.0 software (IBM Corp.) and GraphPad Prism 7.0 (GraphPad Software, Inc.) Data are presented as the mean ± SD. Comparisons between two groups were made using paired or unpaired Student's t-test, while comparisons among multiple groups were performed by one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PRAF2 expression in clinical samples measured by RT-qPCR and western blotting. In the present study, PRAF2 gene expression was firstly analyzed in patients with breast cancer in TCGA database using the cancer data accessed through UALCAN. PRAF2 was found to be upregulated in breast cancer tissue samples compared with normal tissue samples (Fig. 1A). Additionally, with the increase of the clinical stage of tumors, PRAF2 expression in breast cancer tissues showed an upward trend (Fig. 1B). The most common form of metastasis in breast cancer is lymphatic metastasis (24). The data showed that the high expression of PRAF2 in breast cancer samples was closely associated with metastasis to the regional lymph nodes in each stage (Fig. 1C). Subsequently, PRAF2 expression in cancer and normal adjacent tissues from 37 patients with breast cancer was examined by RT-qPCR. The results showed that the mRNA expression of PRAF2 was increased in cancer tissues compared with normal adjacent tissues (Fig. 1D). Based on the results of western blot analysis, the protein expression of PRAF2 was also increased in breast cancer tissues of the 37 patients. Gray value analysis of the PRAF2 and β-actin bands showed that the relative expression intensity of PRAF2 protein in cancer tissues of 37 patients with breast cancer (1.9750±0.0103) was higher than that in normal adjacent tissues (0.9818±0.0140); the expression levels were determined in all tissue samples and six representative samples are shown (Fig. 1E and F).

PRAF2 expression in breast cancer cell lines and silencing in MCF-7 cells through siRNA transfection. The expression of PRAF2 in breast cancer cell lines MCF-7 and MDA-MB-231 and the human normal mammary epithelial cell line MCF-10A was detected by RT-qPCR (Fig. 2A) and western blotting (Fig. 2B and 2C). The results showed significantly higher PRAF2 mRNA and protein expression in MCF-7 cells compared with MDA-MB-231 and MCF-10A cells. MCF-7 cells were subsequently used for siRNA transfection to knock down the expression of PRAF2 protein. The following three groups were used: NC group, siRNA-NC group and siRNA-PRAF2 group, divided into siRNA-1, siRNA-2 and siRNA-3. Total protein from cells of each group was extracted after transfection, and the gene knockdown rate in the control group was detected by RT-qPCR (Fig. 2D) and western blotting (Fig. 2E and 2F). The results showed that PRAF2 expression was decreased in MCF-7 cells transfected with siRNA-PRAF2 compared with siRNA-NC, with siRNA-1 showing superior knockdown efficiency. Therefore, siRNA-1 was selected for subsequent experiments.

Effects of PRAF2 on the proliferation of MCF-7 cells.
Effect of PRAF2 on the colony formation capacity of MCF-7 cells. The tumorigenic ability of MCF-7 cells was examined by colony formation assay. The results indicated that transfection of siRNA-PRAF2 reduced the number of colonies in MCF-7 cells compared with the siRNA-NC group (Fig. 3A and B), indicating that decrease of PRAF2 can reduce the colony formation of breast cancer cells.

Effect of PRAF2 on the viability of MCF-7 cells as examined by CCK-8 assay. The effect of PRAF2 on the viability of MCF-7 cells was examined using the CCK-8 assay. The results showed that transfection of siRNA-PRAF2 led to lower proliferation rate of MCF-7 cells after 72 h compared with siRNA-NC cells (Fig. 3C). This suggested that decrease of PRAF2 expression can diminish the viability of MCF-7 cells.

Invasion of MCF-7 cells assessed by Transwell assay. The Transwell invasion assay was used to investigate the effect of PRAF2 on the invasive ability of breast cancer cells. The results showed that the number of cells crossing the basement membrane, counted at x100 magnification, was 104.67±7.14 cells/high power (HP) in the NC group, 103.33±6.84 cells/HP in the siRNA-NC group and 60.89±5.06 cells/HP in the siRNA-PRAF2 group. There was no significant difference in the number of cells crossing the basement membrane between the siRNA-NC group and the NC group. However, invasion of MCF-7 cells was reduced in the siRNA-PRAF2 group when compared with the siRNA-NC and NC groups (Fig. 4A and B). These data showed that decrease of PRAF2 in MCF-7 cells significantly reduced cell invasion *in vitro*.

Effect of PRAF2 on the migration of MCF-7 cells. A scratch test was used to investigate the effect of PRAF2 on the migration of breast cancer cells. The results revealed that the healing rate (%) of MCF-7 cells after 24 h was 23.09±0.78 in the NC group, 22.90±0.96 in the siRNA-NC group and 13.94±0.79 in the siRNA-PRAF2 group. No significant difference was found between the siRNA-NC group and the NC group; however, the migration of the siRNA-PRAF2 group was significantly reduced compared with that of the siRNA-NC group (Fig. 5A and B). This indicated that downregulation of PRAF2 in MCF-7 cells significantly suppressed cell migration *in vitro*.

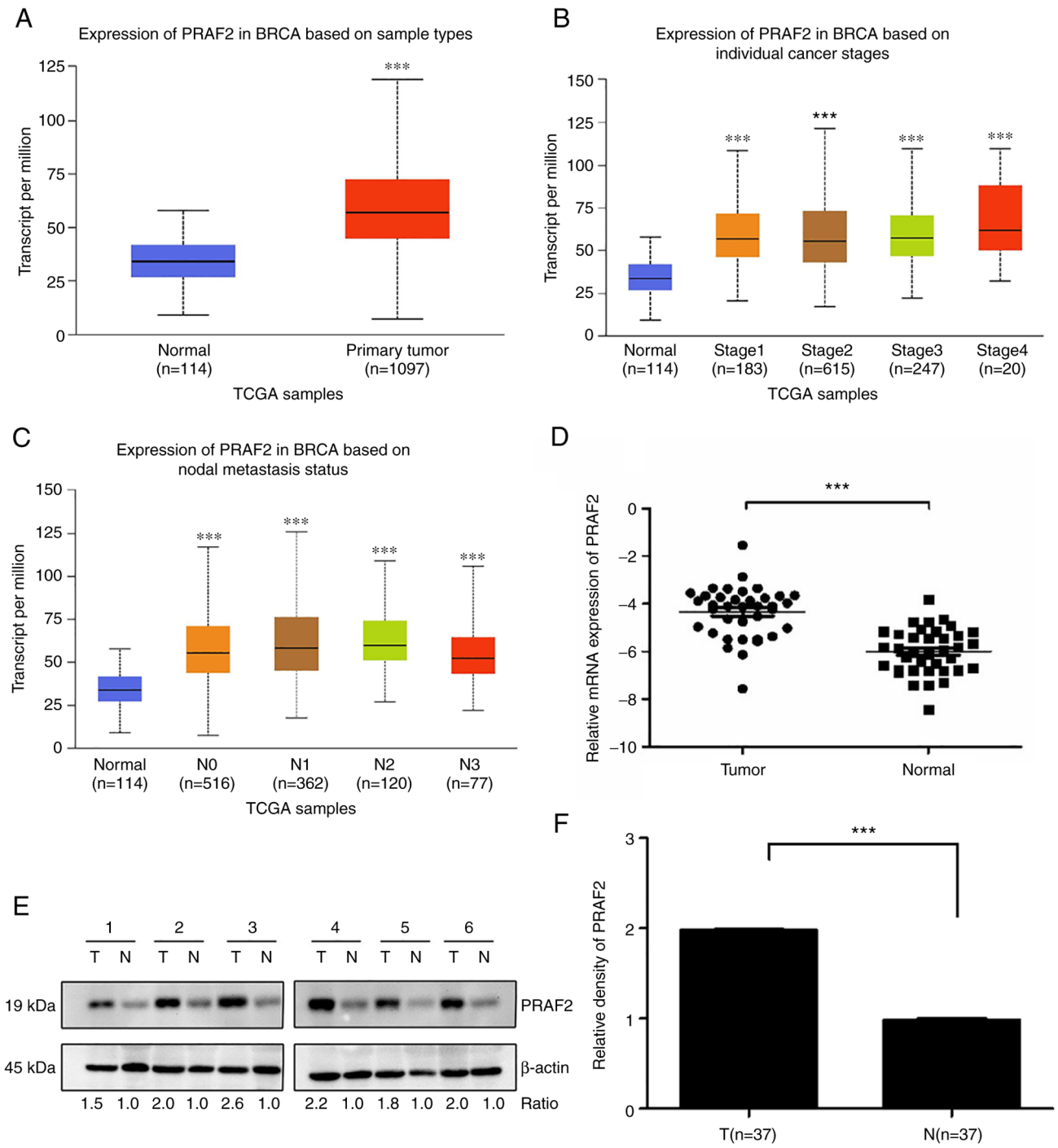

Figure 1. PRAF2 expression in breast cancer and normal adjacent tissues. Expression of PRAF2 in (A) BRCA samples and normal breast tissues, (B) BRCA samples at different cancer stages and (C) BRCA samples with different conditions of nodal metastasis, based on data from TCGA database. (D) Quantitative analysis of the mRNA expression of PRAF2 in T and N tissues as detected by reverse transcription-quantitative PCR. (E) Representative western blots of the protein expression of PRAF2 in T and N tissues, normalized to β-actin. (F) Semi-quantitative analysis of protein expression of PRAF2 in T and N tissues by western blotting (n=37). ***P<0.001 compared with normal. BRCA, breast cancer; N, normal; T, tumor; PRAF2, prenylated rab acceptor 1 domain family member 2; TCGA, The Cancer Genome Atlas.

Western blot and RT-qPCR analysis determines the mRNA and protein expression levels of β-catenin and TCF4. MCF-7 cells were transfected with either siRNA-NC or siRNA-PRAF2. Cells transfected with siRNA-PRAF2 showed decreased β-catenin and TCF4 expression compared with those transfected with siRNA-NC (Fig. 6A-C). This indicated that downregulation of PRAF2 in MCF-7 cells may affect the Wnt/β-catenin signalling pathway.

Discussion

Breast cancer is the most common cancer in women worldwide, seriously threatening their health (1-3). The current major treatment options for breast cancer include surgery, radiotherapy, endocrine therapy, targeted therapy and chemotherapy (25). Among them, targeted therapy has achieved remarkable efficacy in the treatment of HER-2-positive breast

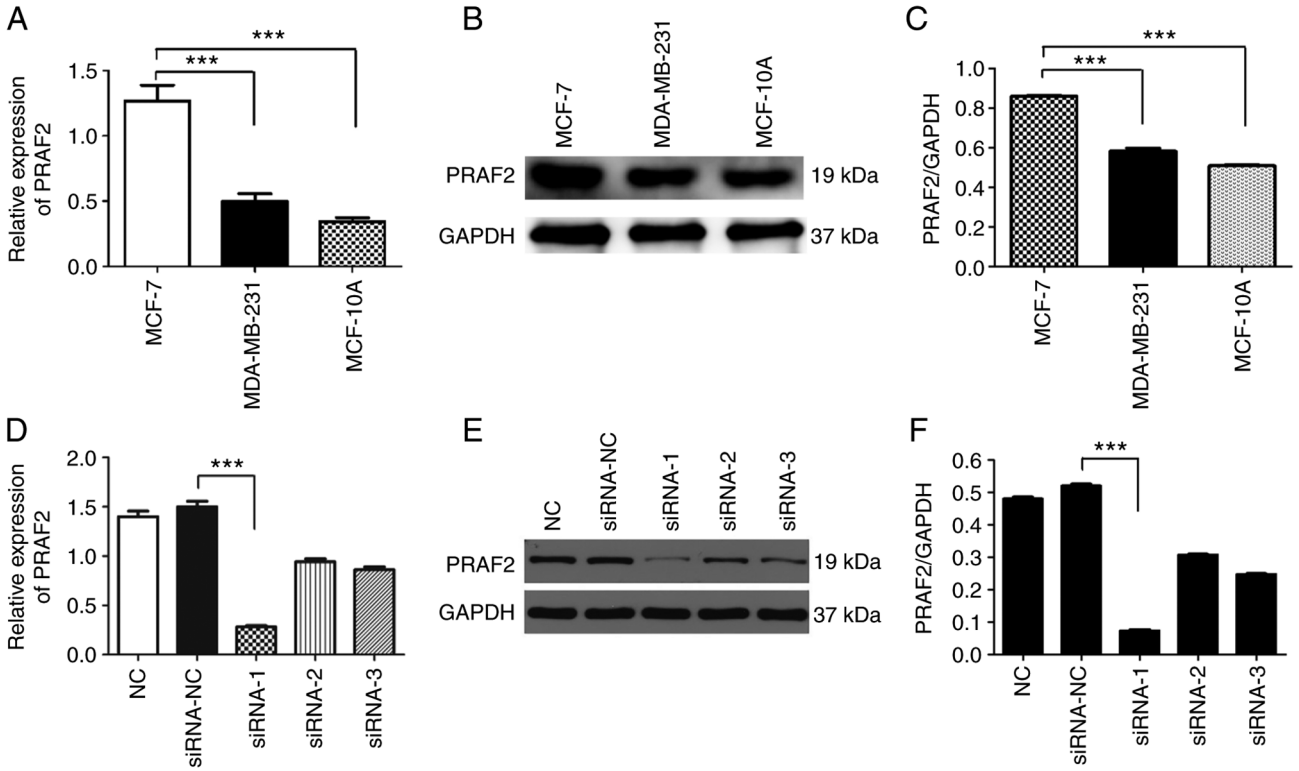

Figure 2. Western blot analysis detection of the siRNA knockdown efficiency of PRAF2. (A) Expression of PRAF2 mRNA in breast cancer cell lines MCF-7 and MDA-MB-231 and normal mammary epithelial cell line MCF-10A detected by RT-qPCR. (B) Expression of PRAF2 protein in MCF-7, MDA-MB-231 and MCF-10A cell lines detected by western blotting and (C) semi-quantitative analysis of the protein expression of PRAF2. (D) PRAF2 expression in MCF-7 cells transfected with siRNA-PRAF2 detected by RT-qPCR. (E) PRAF2 expression in MCF-7 cells transfected with siRNA-PRAF2 detected by western blotting and (F) semi-quantitative analysis of the protein expression of PRAF2. ***P<0.001. PRAF2, prenylated rab acceptor 1 domain family member 2; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; NC, negative control.

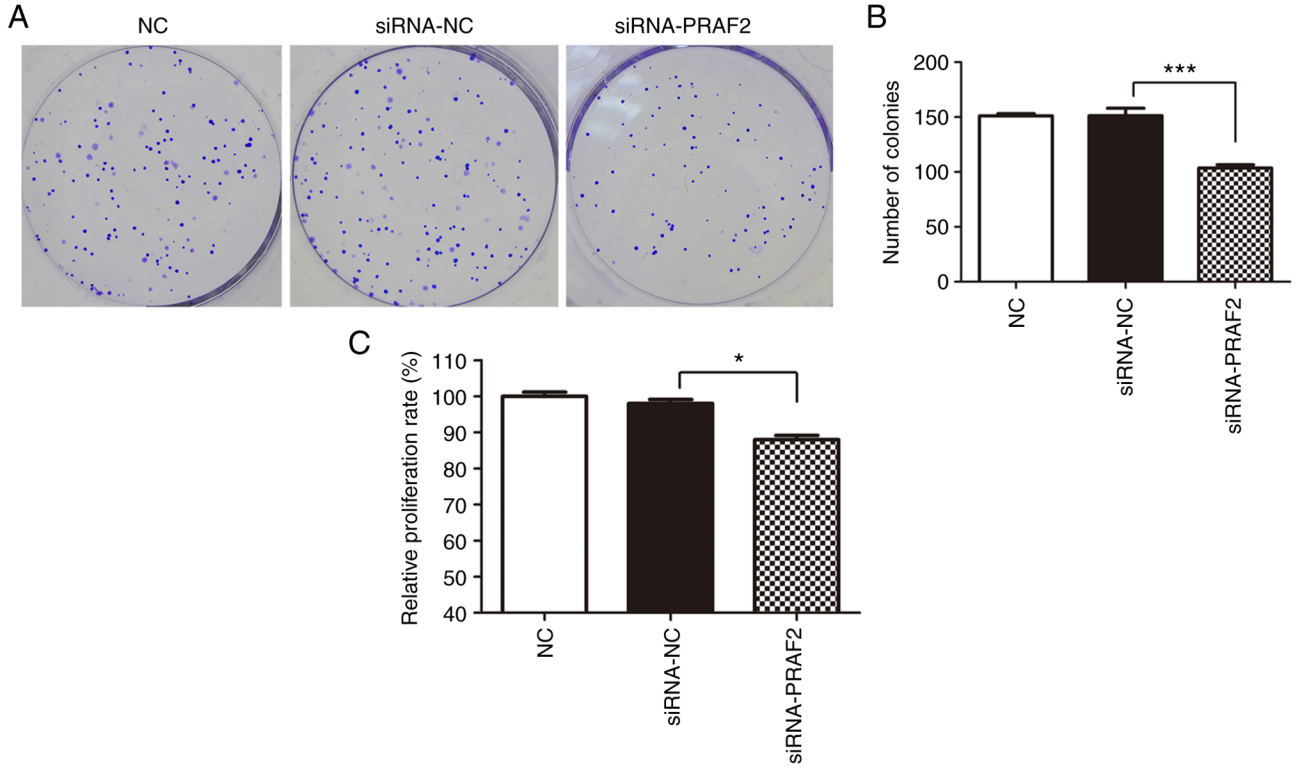

Figure 3. Effect of PRAF2 on the proliferation of MCF-7 cells as assessed by Cell Counting Kit-8 and colony formation assays. (A) Representative images from colony formation assay in MCF-7 cells. (B) A statistically significant difference in the colony number was found between the siRNA-NC and siRNA-PRAF2 groups. (C) A statistically significant difference in the proliferation rate was found between siRNA-NC and siRNA-PRAF2 MCF-7 cells. *P<0.05; ***P<0.001. PRAF2, prenylated rab acceptor 1 domain family member 2; si, small interfering; NC, negative control.

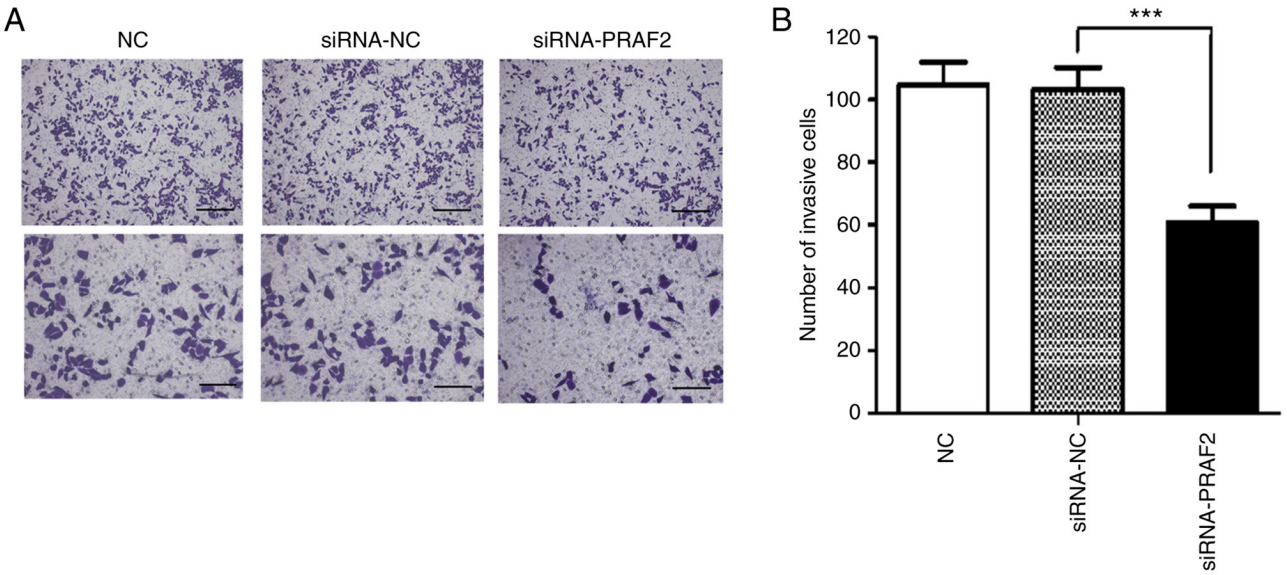

Figure 4. Effect of PRAF2 on the invasion of MCF-7 cells as examined by Transwell invasion assay. (A) Representative images showing differences observed in the number of invasive MCF-7 cells. Scale bars, 100 μm. (B) A statistically significant difference in cell invasion was found between siRNA-NC and siRNA-PRAF2 MCF-7 cells. ***P<0.001. PRAF2, prenylated rab acceptor 1 domain family member 2; si, small interfering; NC, negative control.

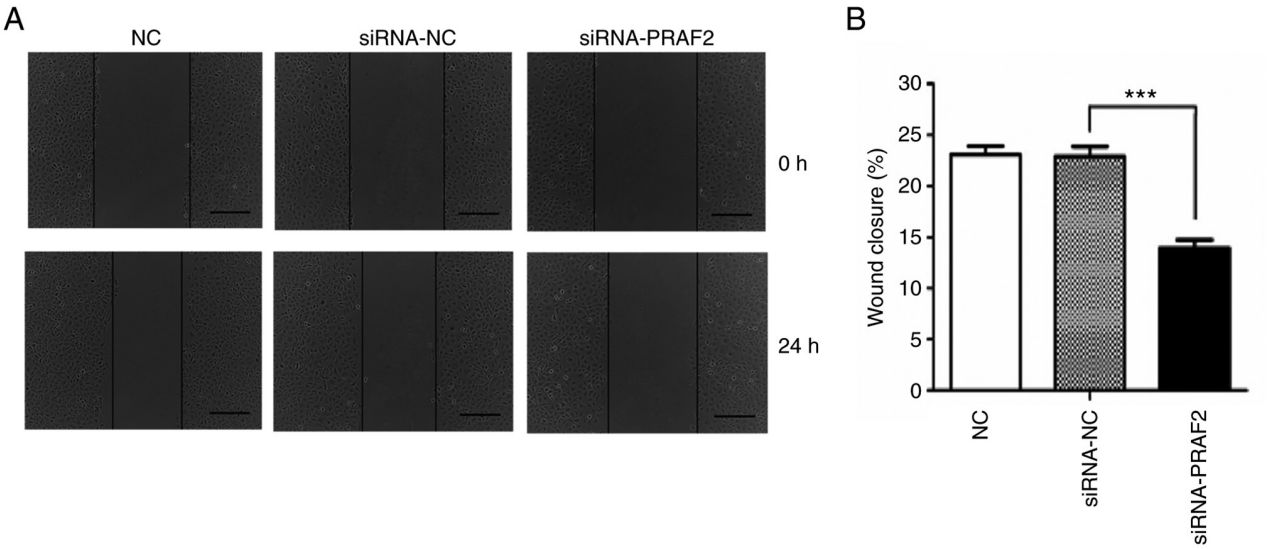

Figure 5. Effect of PRAF2 on the migration of MCF-7 cells as detected by scratch assay. (A) Representative images of wound healing assay of MCF-7 cells at 0 and 24 h after transfection. Scale bars, 500 μm. (B) A statistically significant difference in cell migration was found between siRNA-NC and siRNA-PRAF2 MCF-7 cells. ***P<0.001. PRAF2, prenylated rab acceptor 1 domain family member 2; si, small interfering; NC, negative control.

cancer, and has become the basic treatment regimen for this type of breast cancer (4). In recent years, targeted therapy has become a popular field of research, with targeted drug therapy and clinical treatment developing rapidly. The current perspective of diagnosis and treatment indicates that early breast cancer can be cured. However, due to the invasion and metastasis of breast cancer cells, current treatment methods fail to cure the patients with advanced breast cancer, who have developed metastases to bone, lung, liver and other organs (26). The primary goal of treatment for such patients is to prolong the survival and maintain the quality of life. Therefore, the early detection, diagnosis and treatment of patients with breast cancer has become even more important (27). Obvious genomic and genetic regulatory abnormalities occur in breast cancer cells, which may play an important role in development of breast cancer. For example, in carriers of BRCA1 or BRCA2 mutations, the risk of developing breast cancer by 80 years of age is as high as 70%, compared with a 10% risk for women in the general population (28). Therefore, the present study aimed to investigate the occurrence and development of breast cancer at the molecular level by analyzing gene expression differences in breast cancer tissues and cells with a view to provide earlier and more accurate diagnosis and targeted treatment for patients with breast cancer, in addition to effectively evaluating patient prognosis to develop better personalized treatment methods. Moreover, the investigation of the invasion and metastasis of breast cancer cells also aimed to exploit new anti-metastatic therapeutic targets.

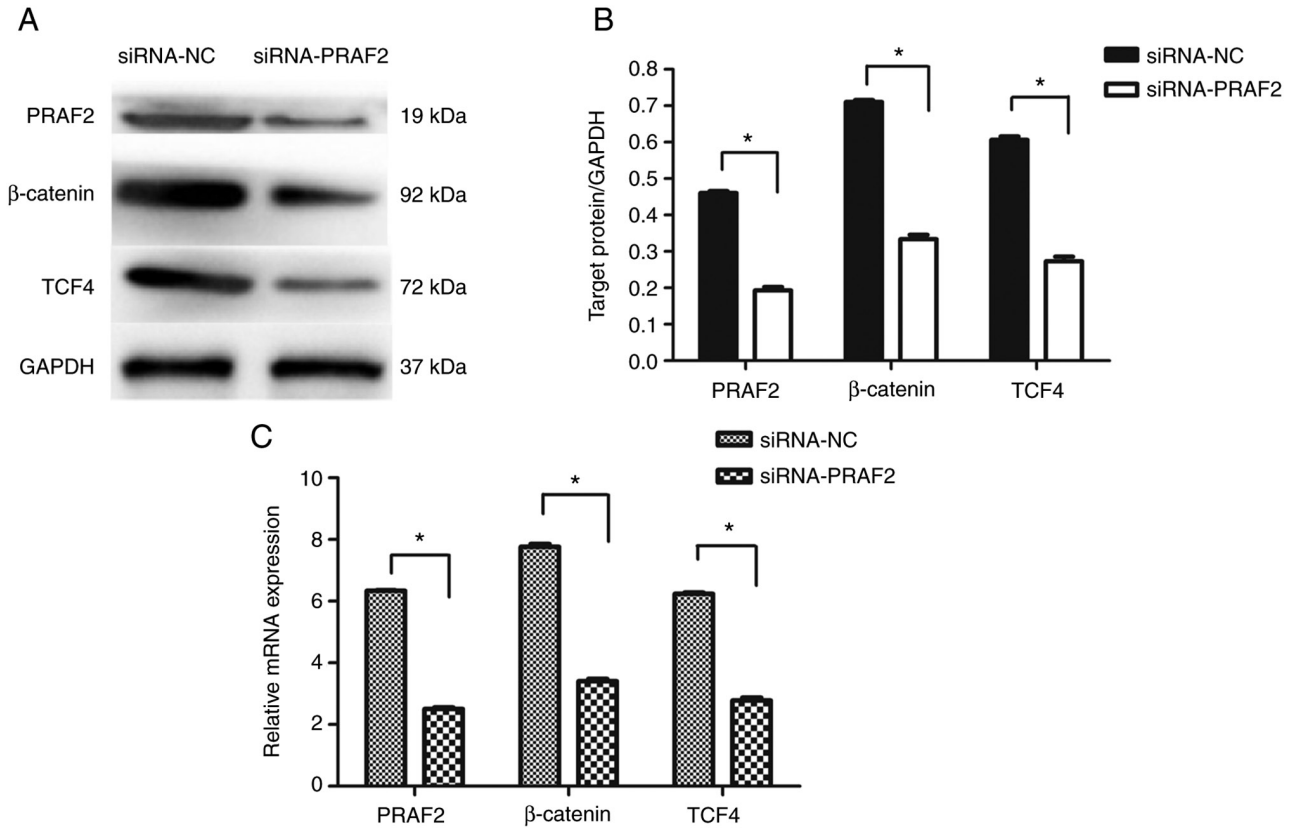

Figure 6. Effect of PRAF2 on the Wnt/β-catenin signaling pathway. (A) Western blotting of PRAF2, β-catenin and TCF4 proteins in MCF-7 cells. (B) Semi-quantitative analysis of protein expression of PRAF2, β-catenin and TCF4 in MCF-7 cells. (C) mRNA expression of PRAF2, β-catenin and TCF4 in MCF-7 cells detected by reverse transcription-quantitative PCR. *P<0.05. PRAF2, prenylated rab acceptor 1 domain family member 2; si, small interfering; NC, negative control; TCF4, T-cell factor 4.

Firstly, the PRAF2 gene phenotype in patients with breast cancer was analyzed in TCGA database using the cancer data online analysis website UALCAN. It was found that PRAF2 expression was significantly upregulated in breast cancer tissue samples when compared with normal tissue samples. It was also demonstrated that PRAF2 was strongly expressed in tumor tissues of the breast, colon, lung and ovary, as well as in tissues immediately adjacent to the corresponding tumor tissue, while expression of PRAF2 in corresponding normal tissues was significantly weaker (8). However, there are few reports on the PRAF2 expression in breast cancer cells and its effects on the proliferation, invasion and migration (29). Therefore, in order to clarify the expression of PRAF2 in breast cancer tissues and its impact on cellular functions, breast cancer tissue samples and corresponding normal adjacent tissues were collected from 37 patients with breast cancer undergoing modified radical mastectomy. The expression of PRAF2 in fresh breast cancer tissues was determined by RT-qPCR and western blot analysis. The results revealed that the mRNA expression of PRAF2 was increased in the cancer tissues compared with normal adjacent tissues. Similarly, the protein expression of PRAF2 was also found to be upregulated in cancer tissues of the 37 patients with breast cancer compared with normal adjacent tissues, consistent with the outcome of RT-qPCR. These results indicated that PRAF2 expression is significantly upregulated in breast cancer tissues, suggesting that high PRAF2 expression may be associated with the occurrence and development of breast cancer. During *in vitro* cell functional experiments, the expression of PRAF2 was firstly examined in the breast cancer cell lines MCF-7 and MDA-MB-231 by RT-qPCR, and it was found that PRAF2 gene expression was significantly higher in MCF-7 cells than in MDA-MB-231 cells. Therefore, MCF-7 cells were used in further *in vitro* cell functional experiments.

A key mechanism of tumorigenesis is the imbalance between cell proliferation and apoptosis (30). PRAF2 has been reported to be involved in the development of multiple cancer types, such as hepatocellular carcinoma and esophageal carcinoma (17,18). In the present study, the results of colony formation and CCK-8 assays demonstrated that silencing of PRAF2 in MCF-7 cells suppressed the proliferation of MCF-7 cells, which is consistent with relevant findings on PRAF2 in hepatocellular carcinoma and neuroblastoma (15,17).

Tumor metastasis is a complex cascade of events involving interactions between cancer cells and the surrounding microenvironment, including mesenchymal cells, immune cells and the extracellular matrix (31). The first stage of breast cancer metastasis is the invasion of primary tumor cells into the basement membrane and the subsequent development of disseminated tumor cells (32). These cells consequently promote abnormal angiogenesis, enter the circulatory or lymphatic system, migrate to distant organs and form secondary tumors, ultimately leading to poor prognosis in patients with metastasized breast cancer (33). TCGA database analysis revealed that high PRAF2 expression in breast cancer was closely associated with the the TNM clinical stage as well as metastasis to regional

lymph nodes at each stage, suggesting that PRAF2 is critical for breast cancer progression. Subsequent results of scratch and Transwell assays indicated that the decrease of PRAF2 significantly reduced the invasion and migration of MCF-7 cells. A previous study has shown that PRAF2 interacts with C-C chemokine receptor type 5 (CCR5) (34), with increased CCR5 expression being involved in brain tumorigenesis, especially in glioblastoma progression (29). This may partially explain the mechanism of metastasis associated with PRAF2. Another member of the PRAF family, PRAF3, acts as a tumor suppressor to regulate cancer cell migration, apoptosis and angiogenesis through a variety of downstream signaling pathways, such as the MAPK signalling cascade, integrin-linked kinase, integrin αvβ3 pathway and SP1/MMP2 signaling pathway (10-12,35). Therefore, we hypothesized that PRAF2 may reversely regulate these pathways and this should be analyzed in further studies in the future. Previous research has successively revealed that PRAF3 is an important regulatory protein of p38 signaling pathway in MDA-MB-231 cells (19), and can inhibit cancer cell migration and invasion by downregulating the expression of C-X-C chemokine recptor type 4 (20).

β-catenin signalling has been broadly implicated in human cancer. Aberrant activation of β-catenin signalling has been implicated in malignant progression and poor patient prognosis (36). The β-catenin pathway involves the nuclear translocation of β-catenin and activation of target genes via TCF/LEF transcription factors, which control cell proliferation and migration (37). In the present study, inhibition of PRAF2 expression could reduce β-catenin and TCF4 expression. The study first found that PRAF2 regulated the β-catenin pathway in MCF7 cells. Results indicated that PRAF2 may enhance the activation of the β-catenin signaling pathway to promote breast cancer progression. He *et al* (38) revealed that PRAF2 overexpression could increase the secretion of colon cancer cell exosomes, and promote the migration and invasion of tumor cells through Notch signal transduction. Therefore, PRAF2 expression may also affect the malignant behavior of breast cancer through other signalling pathways. The present study only investigated the β-catenin signalling pathway and more in-depth research on the specific mechanisms is required.

The present studiy provided solid evidence for further investigation of the mechanisms through which PRAF2 regulates the proliferation, invasion and migration of breast cancer. In conclusion, PRAF2 expression was significantly higher in breast cancer tissues compared with normal adjacent tissues, and was closely associated with TNM stage and regional lymph node metastasis in breast cancer. Furthermore, PRAF2 was indicated to function as an oncogene capable of promoting breast cancer cell growth and invasion, providing novel insights into the metastasis in breast cancer. However, the effect of PRAF2 on the apoptosis and cell cycle of MCF-7 cells and the mechanism of PRAF2 regulation of the proliferation, invasion and migration of MCF-7 cells have not been investigated in the present study, and additional studies are needed to validate the findings of these investigations and expand the translational potential of this direction. In the current study, the effect of PRAF2 was investigated in only one breast cancer cell line, MCF-7, which has certain limitations, as it is a luminal A subtype breast cancer cell line. In conclusion, PRAF2 may be a potential prognostic factor in patients with breast cancer and may become a potential target for the prevention and treatment of breast cancer metastasis. PRAF2 may promote breast cancer cell proliferation and invasion through activation of the β-catenin signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW, ZW, ZY and BC substantially contributed to the conception and the design of the study. ZZ, HB, WJ and ZB performed the experiments. ZZ, WJ, ZY, WZ, BD and BC analyzed and interpreted the data. ZW, HB, ZB, BD and WZ revised the article. YW, ZW and BC supervised the present study and wrote the manuscript. YW and ZZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Yijishan Hospital of Wannan Medical College (Wuhu, China) and written informed consent was obtained from all patients before sample collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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