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ORIGINAL RESEARCH

LncRNA PTPRG-AS1 Promotes Breast Cancer Progression by Modulating the miR-4659a-3p/QPCT Axis

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Background: Overwhelming evidence has suggested that dysregulated long noncoding RNAs (lncRNAs) play a critical modulating effect in the evolution of breast cancer (BRCA). Nevertheless, the roles of lncRNA PTPRG antisense RNA 1 (PTPRG-AS1) in BRCA and the underlying mechanisms have not been experimentally validated and functionally annotated.

Methods: The expression of lncRNA PTPRG-AS1 in BRCA tissues and cell lines was evaluated by reverse transcription-quantitative PCR (RT-qPCR), and by using public databases. The proliferation of BRCA cells was detected using Cell Counting Kit-8 and colony formation assays. Wound healing assay, and Transwell migration and invasion assays were carried out to explore the migratory and invasive abilities of BRCA cells. The interaction between lncRNA PTPRG-AS1, microRNA (miR)-4659a-3p and glutaminyl-peptide cyclotransferase (QPCT) was verified using RT-qPCR, dual-luciferase reporter assay and Western blotting.

Results: The results showed that LncRNA PTPRG-AS1 was markedly upregulated in BRCA tissues and cell lines. Knocking down lncRNA PTPRG-AS1 significantly inhibited the proliferation, migration and invasion of BRCA cells, while overexpression of lncRNA PTPRG-AS1 enhanced the aforementioned properties of BRCA cells. Further analyses revealed that PTPRG-AS1 may act as a molecular sponge for miR-4659a-3p, thus regulating QPCT expression, therefore, acting as an oncogene in BRCA.

Conclusion: Collectively, the study demonstrates that lncRNA PTPRG-AS1 may act as a competing endogenous RNA by regulating the miR-4659a-3p/QPCT axis in BRCA progression. This lncRNA could potentially be a biomarker and therapeutic target for BRCA.

Keywords: long noncoding RNA PTPRG-AS1, microRNA-4659a-3p, QPCT, progression, breast cancer

Introduction

Breast cancer (BRCA) is the most frequently occurring malignancy and the primary cause of cancer-associated mortality among women, accounting for almost one-third of all newly diagnosed cancers in female patients in 2022.[1,2] Despite tremendous efforts, the underlying molecular mechanisms of BRCA progression have remained elusive in recent years. The development of novel biomarkers for cancer diagnosis and prognosis is urgently required.

Long noncoding RNAs (lncRNAs) are RNA molecules with a minimum length of 200 nucleotides, which lack coding abilities. LncRNAs have been reported to participate in pathological tumor progression via various cellular biological processes.[3–7]

The competing endogenous RNA (ceRNA) theory proposes that lncRNAs can act as sponges to sequester microRNAs (miRNAs/miRs) and control their levels in the cytoplasm of cells, thereby reducing the regulatory effects of miRNAs on targeted mRNA.[8–11] MiRNAs are a class of small noncoding RNA molecules that hybridize to 3'-untranslated regions (3'-UTRs) and post-transcriptionally regulate mRNA expression; therefore, they may be involved in various pathogeneses.[12–16]

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Previous bioinformatics analysis and research revealed that the lncRNA PTPRG antisense RNA 1 (PTPRG-AS1) is an essential modulator of cancer progression.[17] LncRNA PTPRG-AS1 has been reported to participate in the advancement of various types of cancer, such as BRCA, pancreatic adenocarcinoma, nasopharyngeal carcinoma, epithelial ovarian cancer, lung cancer and gastric cancer.[18–22] Nevertheless, the regulatory mechanism of PTPRG-AS1 and its role in BRCA remain unclear.

This study sought to investigate the role of lncRNA PTPRG-AS1 in the oncogenic development of BRCA. Furthermore, we attempted to elucidate the role of the novel lncRNA PTPRG-AS1/miR-4659a-3p/QPCT axis in BRCA, which might provide promising biomarkers and targets for treatment protocols.

Materials and Methods

Patients and Specimens

A total of 73 pairs of BRCA tissues and matched adjacent normal breast tissues were collected from patients who had provided written informed consent prior to tissue collection. The samples were stored at −80°C until RNA extraction. Each clinical sample was obtained from The Fourth Hospital of Hebei Medical University from January 2022 to December 2022, and the study was approved by the Ethical Committee of this hospital. All 73 female participants (age, 28–73 years) were first diagnosed with BRCA and none of them underwent any treatment prior to tissue collection, including surgery, chemotherapy or radiotherapy. All research protocols were consistent with the Declaration of Helsinki. The clinical information of the patients is provided in Table 1.

Table 1 Clinicopathological Characteristics of Patients with Breast Cancer (n=73) in the Present Study

Characteristics	Patients, n (%)	PTPRG-AS1 level		P value
		Low	High	
Age				0.780
≤50	27 (37.0)	12	15	
>50	46 (63.0)	22	24	
Tumor size				0.239
<3cm	46 (63.0)	19	27	
≥3cm	27 (37.0)	15	12	
TNM stage				0.963
I,IIA	44 (60.3)	21	23	
IIB,III,IV	29 (39.7)	14	15	
Histological grade				0.538
I,II	54 (74.0)	24	30	
III	19 (26.0)	10	9	
Lymph nodes status				0.334
Negative	30 (41.1)	14	16	
Positive	43 (58.9)	25	18	
HER-2 status				0.537
0,1+	38 (52.1)	18	20	
2+	14 (19.2)	8	6	
3+	21 (28.7)	8	13	
ER status				0.287
Negative	17 (23.3)	6	11	
Positive	56 (76.7)	28	28	
PR status				0.098
Negative	25 (34.2)	8	17	
<20%	5 (6.8)	3	2	
≥20%	43 (58.9)	23	20	

(*Continued*)

Table 1 (Continued).

Characteristics	Patients, n (%)	PTPRG-AS1 level		P value
		Low	High	
Ki-67				0.624
<30%	43 (58.9)	19	24	
≥30%	30 (41.1)	15	15	

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.

Bioinformatics Analysis

The GEPIA 2.0 database (http://gepia.cancer-pku.cn/),starBase database (http://starbase.sysu.edu.cn/), GEO database (https://www.ncbi.nlm.nih.gov/geo/) and the Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/) were used for the expression analysis of PTPRG-AS1 in breast cancer tissues. The TargetScan database (www.targetscan.org) and miRDB database (http://mirdb.org) were used to predict lncRNA-binding miRNAs, and the relationships between lncRNAs-miRNAs and miRNAs-mRNAs. TargetScan, mirPathDB (https://mpd.bioinf.uni-sb.de/overview.html), miRWalk (http://mirwalk.umm.uni-heidelberg.de) and mirDIP (https://ophid.utoronto.ca/mirDIP/) databases were employed to predict the target genes of miRNAs.

Cell Culture and Transfection

Human BRCA cell lines, including MDA-MB-231, MDA-MB-453, BT-549, MCF-7, SK-BR-3 cells and the normal human epithelial cell line MCF-10A, as well as the human embryonic kidney 293T cells (HEK293T cells), were obtained from Procell Life Science and Technology Co., Ltd. (Wuhan, China). All of the cells were cultivated under conditions recommended by the supplier and prior literature. Plasmids for knockdown of PTPRG-AS1 and their negative control (NC), and for overexpression and knockdown of QPCT and their NC were constructed by the Jikai Gene Company (Xu Hui, Shanghai, China). Plasmids for overexpression of PTPRG-AS1 and their NC were purchased from Shanghai GenePharma Co., Ltd. The miR-4659a-3p mimics, miR-NC mimics, miR-4659a-3p inhibitor and miR-NC inhibitor were constructed by iGene Biotechnology Company (Guangzhou, China). Their sequences are shown in Supplemental Tables S1-S3. Transfection was performed using FuGENE® HD Transfection Reagent (Promega, Roche, Boulogne, France) according to the manufacturer's protocol. The transfection efficiency was determined by reverse transcription-quantitative PCR (RT-qPCR) and Western blot. Follow-up experiments were conducted using cells transfected for 48–72 h.

Subcellular Fractionation

The cellular localization of lncRNA PTPRG-AS1 was detected using the Minute™ Cytoplasmic and Nuclear Extraction Kit (Invent Biotechnologies, Inc). according to the manufacturer's protocol. The extracted cytoplasmic and nuclear RNA were isolated and purified, and were then reverse-transcribed and measured by RT-qPCR. GAPDH mRNA was used as a cytoplasmic control, while U6 mRNA was used as a nuclear control.

RNA Extraction and RT-qPCR

According to the manufacturer's specifications, we extracted total RNA from BRCA tissues and cells using an RNAiso Plus reagent (iGene Biotechnology Co). Next, Transcriptor First Strand cDNA Synthesis Kit [TIANGEN BIOTECH (BEIJING) CO., LTD.] was used to reverse transcribe RNA into complementary DNA (cDNA). Finally, qPCR was performed to examine gene expression using the GoTaq® Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). First-strand cDNA synthesis and PCR of miR-4659a-3p were carried out using the All-in-One™ miRNA RT-qPCR Detection System 2.09 (iGene Biotechnology Company). In the aforementioned experiments, GAPDH or β-actin served as the internal control for lncRNA and mRNA, and U6 served as the internal control for miRNA. Relative expression levels of target genes were assessed using the 2-ΔΔCt method. The sequences of primers used for RT-qPCR are listed in Supplemental Table S4.

Western Blotting Assay

RIPA lysis buffer (Solarbio, Beijing, China) supplemented with PMSF (Beyotime Institute of Biotechnology) was used for cell lysis and total protein isolation, and the BCA kit (Boster, Wuhan, China) was used to quantify protein concentration. Western blotting was performed according to a common protocol. Details of the primary antibodies and secondary antibodies are displayed in Supplemental Table S5. Ultimately, the experimental results were detected using an enhanced chemiluminescence reagent (Thermo, Rockford, USA) and the final protein bands were captured using a Chemidoc XRS imaging system (Bio-Rad, Hercules, CA, USA).

Dual-Luciferase Reporter Assay

The wild-type (WT) fragments of PTPRGAS1 (WT-PTPRG-AS1) or QPCT (WT-QPCT) and the mutant (MUT) fragments of PTPRG-AS1 (MUT-PTPRG-AS1) or QPCT (MUT-QPCT) were amplified and subcloned into the pGL3 vector (GenePharma, Shanghai, China). MiR-4659a-3p or miR-NC mimics was then cotransfected with WT or MUT vectors into HEK293T cells using FuGENE® HD Transfection Reagent (Promega, Roche, Boulogne, France). A total of 48 h after transfection, luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega Corporation).

Cell Counting Kit-8 (CCK-8) Assay

The transfected MCF-7 cells and SK-BR-3 cells in the experimental and control groups were inoculated into 96-well plates at a density of 2×103 cells/well. The cell proliferation assay was performed using CCK-8 reagent (Boster, Wuhan, Hubei, China) at 0, 24, 48 and 72 h after cell adhesion. Briefly, 10 μL CCK8 reagent was added to each well and cultured for 2 h. Finally, we used the Tecan Infinite F50 (Tecan, Perkin-Elmer, Waltham, UK) to detect the absorbance of each well at a wavelength of 450 nm.

Colony Formation Assay

The transfected MCF-7 and SK-BR3 cells were inoculated at 1×103 cells/well into a 6-well plate and were incubated with 4 mL complete culture medium at 37°C and 5% CO2. After 15 days, colonies could be seen with the naked eye. The colonies were fixed with 4% paraformaldehyde at room temperature for 20 min and stained with 1% crystal violet (Solarbio, Beijing, China) at room temperature for 30 min. Finally the number of colonies (containing >50 cells) was counted under a microscope.

Wound Healing Assay

The experimental and control groups of transfected MCF-7 cells and SK-BR-3 cells were inoculated into a 6-well plate. After reaching 100% cell confluence, a wound was scratched into the cell layer using the end of a 10-μL pipette tip. Subsequently, the cells were cultured in medium without FBS at 37°C. Finally, cell images were under at least five random microscope fields of view at 0 and 24 h after wound generation.

Transwell Assays

We assessed cell migration and invasion using an empty Transwell chamber (Corning, New York, USA). MCF-7 cells and SK-BR-3 cells transfected with different plasmids were seeded in the upper chamber at a density of 2×105 cells/well. The upper chamber contained serum-free medium and was coated with Matrigel (Solarbio, Beijing, China) for 1 h at 37°C or without Matrigel, and the lower chamber contained 600 μL 10% serum-containing medium. The cells were incubated at 37°C for 24–48 h, after which, the chamber was removed, the Matrigel and cells were gently wiped with a cotton swab, and washed with PBS three times. Next, the chamber was incubated in 4% paraformaldehyde for 20 min and washed three times with PBS. After air drying, the chamber was stained with crystal violet solution for 20 min. Finally, the number of cells in five random fields was counted using a microscope.

Statistical Analysis

All experiments were repeated at least three times. Using GraphPad Prism 9 for statistical analysis, the data are presented as the mean ± SD. Differences between the two groups were analysed using the independent samples *t*-test. One-way ANOVA and χ2 test were performed to compared the differences among multiple groups. Spearman correlation analysis was used to determine correlation between PTPRG-AS1, miR-4659a-3p and QPCT expression levels. P<0.05 was considered to indicate a statistically significant difference.

Results

LncRNA PTPRG-AS1 Expression is Upregulated in BRCA Tissues and Cell Lines

We analyzed lncRNA expression profiles using the public GEPIA2 dataset (http://gepia.cancer-pku.cn/) and starBase database (http://starbase.sysu.edu.cn/). The results showed that lncRNA PTPRG-AS1 was markedly upregulated in BRCA tissues compared with in normal tissues (fold change, ≥2 and P<0.05; Figure 1A and B). Simultaneously, differential expression analyses of PTPRG-AS1 conducted within the GEO (https://www.ncbi.nlm.nih.gov/geo/) and TCGA (https://portal.gdc.cancer.gov/) databases both yielded consistently significant results(Supplemental Figure S3 and S4). In order to further investigate the relationship between lncRNA PTPRG-AS1 and BRCA progression, the expression of PTPRG-AS was detected in 73 pairs of BRCA tissues and adjacent normal breast tissues using RT-qPCR. It was found that the expression of PTPRG-AS1 in BRCA tissues was markedly increased compared with that in normal breast tissues (P<0.001; Figure 1C). Furthermore, RT-qPCR analyses showed that the expression levels of PTPRG-AS1 were markedly

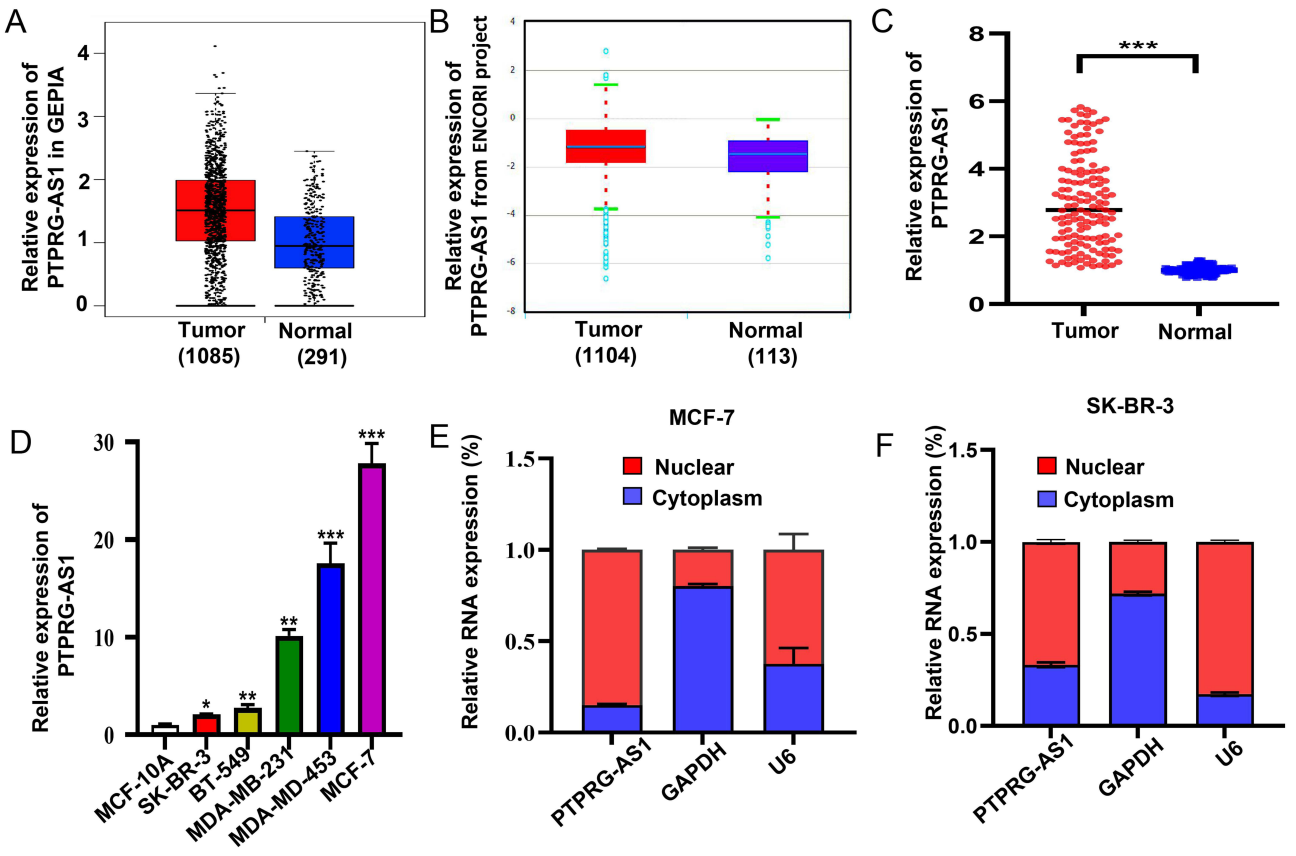

Figure 1 LncRNA PTPRG-AS1 expression is upregulated in BRCA tissues and cell lines. (**A** and **B**) LncRNA PTPRG-AS1 was upregulated in breast cancer tumor tissues compared with normal tissues in GEPIA 2.0 database and starBase database. (**C**) The expression level of PTPRG-AS1 in 73 pairs of breast cancer and adjacent normal tissues was detected by RT-qPCR. GAPDH was used as the internal control. (**D**) The expression level of PTPRG-AS1 in six breast cancer cell lines was detected by RT-qPCR respectively. GAPDH was used as the internal control. (**E** and **F**) The expression levels of lncRNA PTPRG-AS1 in the nuclear and cytoplasmic fractions of MCF-7 cells and SK-BR-3 cells were detected by RT-qPCR. U6 and GAPDH were used as the nuclear and cytoplasmic markers, respectively. Experiments were performed in triplicate. *p<0.05, **p<0.01, ***p<0.001.

elevated in BRCA cells (MDA-MB-231, MDA-MB-453, BT-549, MCF-7 and SK-BR-3) compared with those in MCF-10A cells (P<0.05; Figure 1D), suggesting that lncRNA PTPRG-AS1 may be involved in the regulation of BRCA development. Moreover, the nucleocytoplasmic separation experiment suggested that PTPRG-AS1 was expressed in both the nucleus and in the cytoplasm (P<0.05; Figure 1E and F).

The aforementioned evidence confirmed that the expression of PTPRG-AS1 was significantly increased in BRCA and partially localized in the cytoplasm, which indicates that PTPRG-AS1 may act as a potential miRNA sponge.

LncRNA PTPRG-AS1 Promotes the Proliferation, Migration and Invasion of BRCA Cells

To investigate the biological roles of PTPRG-AS1 in BRCA progression, MCF-7 cells were transfected with human PTPRG-AS1 knockdown plasmid and SK-BR-3 cells were transfected with PTPRG-AS1 overexpression plasmid. The transfection

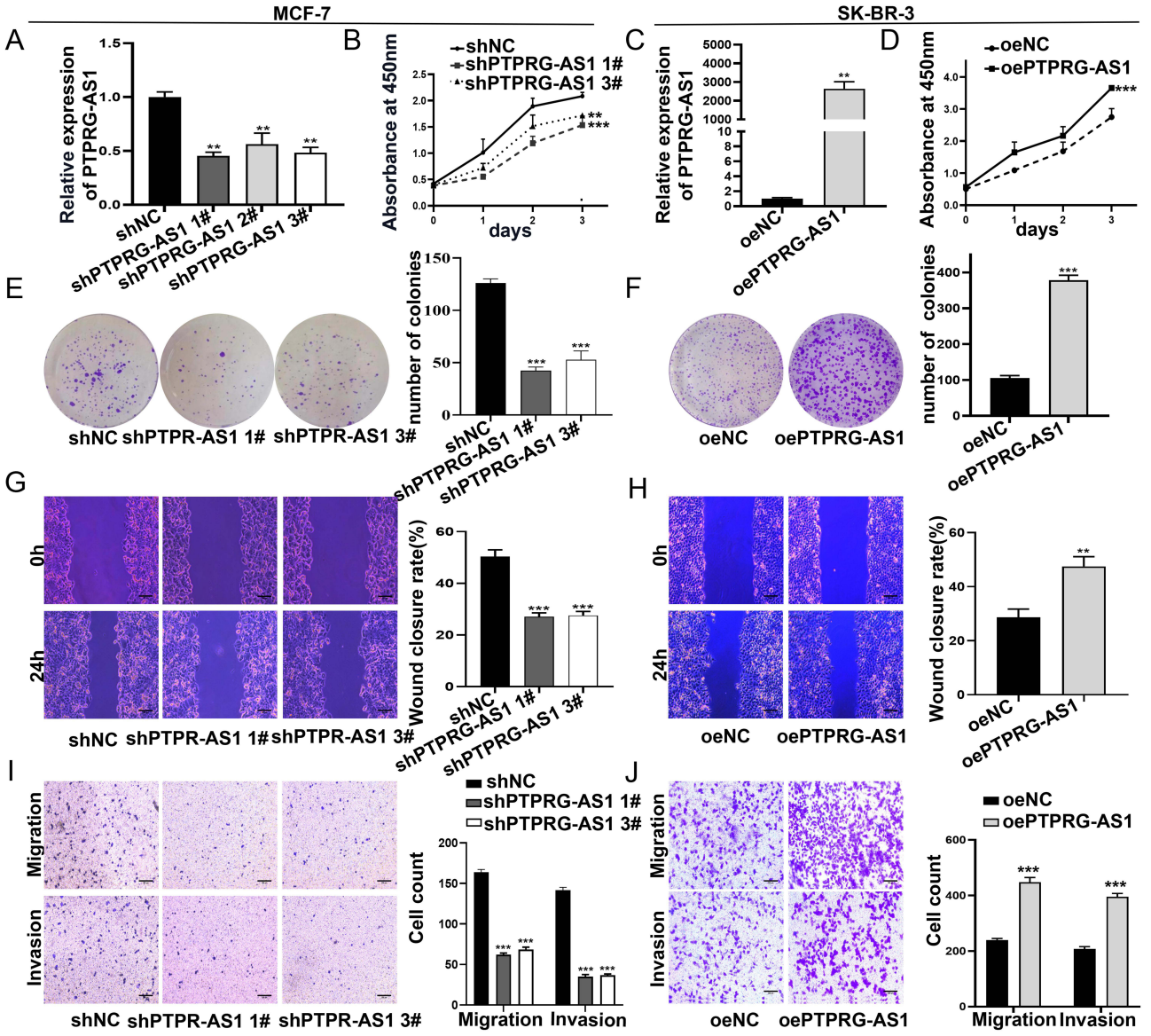

Figure 2 LncRNA PTPRG-AS1 promotes the proliferation, migration and invasion of BRCA cells. (**A**) The transfection efficiency of PTPRG-AS1 was detected by RT-qPCR when MCF-7 cells were transfected with three different sequences of human PTPRG-AS1 knockdown plasmid. (**B**) Cell proliferation by CCK-8 assay in MCF-7 cells after transfection. (**C**) The transfection efficiency of PTPRG-AS1 was detected by RT-qPCR when SK-BR-3 cells were transfected with PTPRG-AS1 overexpression plasmid. (**D**) Cell proliferation by CCK-8 assay in SK-BR3 cells after transfection. (**E** and **F**) Colony formation assay was used to evaluated the proliferation of MCF-7 cells and SK-BR-3 cells. (**G-J**) Wound healing assay and Transwell assay were used to analyze the migration and invasion abilities of MCF-7 cells and SK-BR-3 cells. Scale bar, 200 μm. **p<0.01, ***p<0.001.

efficiency of three different sequences in MCF-7 cells was detected by RT-qPCR. The results are shown in Figure 2A; the expression of PTPRG-AS1 was markedly lower in cells transfected with short hairpin RNA (sh)PTPRG-AS1 1# and shPTPRG-AS1 3# compared with that in cells transfected with negative control group (shNC) (P<0.01). The CCK-8 assay results showed that knocking down lncPTPRG-AS1 significantly reduced the proliferation of MCF-7 cells (P<0.01, Figure 2B). Moreover, after transfection with the overexpression PTPRG-AS1 plasmid, the expression levels of PTPRG-AS1 were markedly increased in SK-BR-3 cells compared with that in cells transfected with empty plasmid (oeNC) (P<0.01, Figure 2C). The CCK-8 assay showed that overexpression of PTPRG-AS1 significantly enhanced the proliferation of SK-BR-3 cells (P<0.001, Figure 2D). Subsequently, the colony formation assay showed that the proliferation of MCF-7 cells transfected with shPTPRG-AS1 1# and shPTPRG-AS1 3# was obviously inhibited, while overexpression of PTPRG-AS1 in SK-BR-3 cells promoted proliferation (P<0.001; Figure 2E and F). Wound healing assays showed that PTPRG-AS1 knockdown restrained the migration of MCF-7 cells (P<0.001; Figure 2G), and overexpression of PTPRG-AS1 facilitated the migration of SK-BR-3 cells (P<0.01; Figure 2H). In addition, Transwell migration and invasion assays showed that PTPRG-AS1 knockdown suppressed the migration and invasion of MCF-7 cells (P<0.001; Figure 2I), while overexpression of PTPRG-AS1 in SK-BR-3 cells showed the opposite effect (P<0.001; Figure 2J).

The aforementioned findings indicated that PTPRG-AS1 could facilitate the proliferation, migration and invasion of BRCA cells.

LncRNA PTPRG-AS1 Acts as a Sponge of miR-4659a-3p in BRCA Cells

Since lncRNA PTPRG-AS1 is partly localized in the cytoplasm, we hypothesized that it may fulfil a role as a ceRNA, and could regulate the expression of target genes by binding and sequestering specific miRNAs. We identified eight candidate miRNAs (miR-4659a-3p, miR-587, miR-3148, miR-376-3p, miR-519a-2-5p, miR-520b-5p, miR-8067 and miR-924) by screening the latent target miRNAs of PTPRG-AS1 using the miRDB and TargetScan databases. Furthermore, RT-qPCR was carried out to show that knockdown or overexpression of PTPRG-AS1 could negatively regulate the expression levels of most of these miRNAs. MiR-4659a-3p demonstrated the highest increased expression levels compared with the control group after knocking down PTPRG-AS1 in MCF-7 cells (P<0.05; Figure 3A). Meanwhile, when PTPRG-AS1 was overexpressed in SK-BR-3 cells, the expression levels of miR-4659a-3p were the lowest among the eight miRNAs aforementioned (P<0.05; Figure 3B). Therefore, we focused on miR-4659a-3p in the follow-up research. In addition, we further studied the expression of miR-4659a-3p in 30 pairs of BRCA and normal breast samples using RT-qPCR analysis; the results showed that compared with in adjacent normal tissues, the expression levels of miR-4659a-3p in BRCA tissues were significantly reduced (P<0.001; Figure 3C). Meanwhile, there was a negative correlation between miR-4659a-3p and PTPRG-AS1 ($r = -0.5859$, $P < 0.001$, Figure 3D). Moreover, the miRDB database was applied to predict the binding sites between lncRNA PTPRG-AS1 and miR-4659a-3p (Figure 3E). Next, dual-luciferase reporter assay was performed in HEK293T cells to further confirm whether miR-4659a-3p was a direct target of PTPRG-AS1. The results showed that when cotransfected with WT-PTPRG-AS1 and miR-4659a-3p mimics, luciferase activity was markedly reduced. However, no statistical changes in luciferase activity were observed when cells were cotransfected with MUT-PTPRG-AS1 and miR-4659a-3p mimics (P<0.001; Figure 3F). These data indicated that PTPRG-AS1 may act as a miR-4659a-3p sponge in BRCA cells.

PTPRG-AS1 Exerts an Aggressive Effect on BRCA via miR-4659a-3p

In order to study the biological effects of miR-4659a-3p and to determine whether PTPRG-AS1 affects the biological function of BRCA cells by targeting miR-4659a-3p, we subsequently conducted rescue experiments. Since shPTPRG-AS1 1# showed better transfection efficiency and had more obvious effects on biological properties than shPTPRG-AS1 3#, we selected the shPTPRG-AS1 1# plasmid as shPTPRG-AS1 for subsequent experiments. The CCK-8 and colony formation assay indicated that when miR-4659a-3p was inhibited alone the proliferation and viability of MCF-7 cells was increased. Simultaneously, transfection with the miR-4659a-3p inhibitor could abrogate the suppressive effect of shPTPRG-AS1 (P<0.01; Figure 4A and B). Coinciding with these data, the wound healing assay and Transwell assay displayed the same results (P<0.01; Figure 4C and D). As expected, overexpression of miR-4659a-3p in SK-BR-3 cells exhibited the opposite results. When transfected with miR-4659a-3p mimics alone, the proliferation, migration and

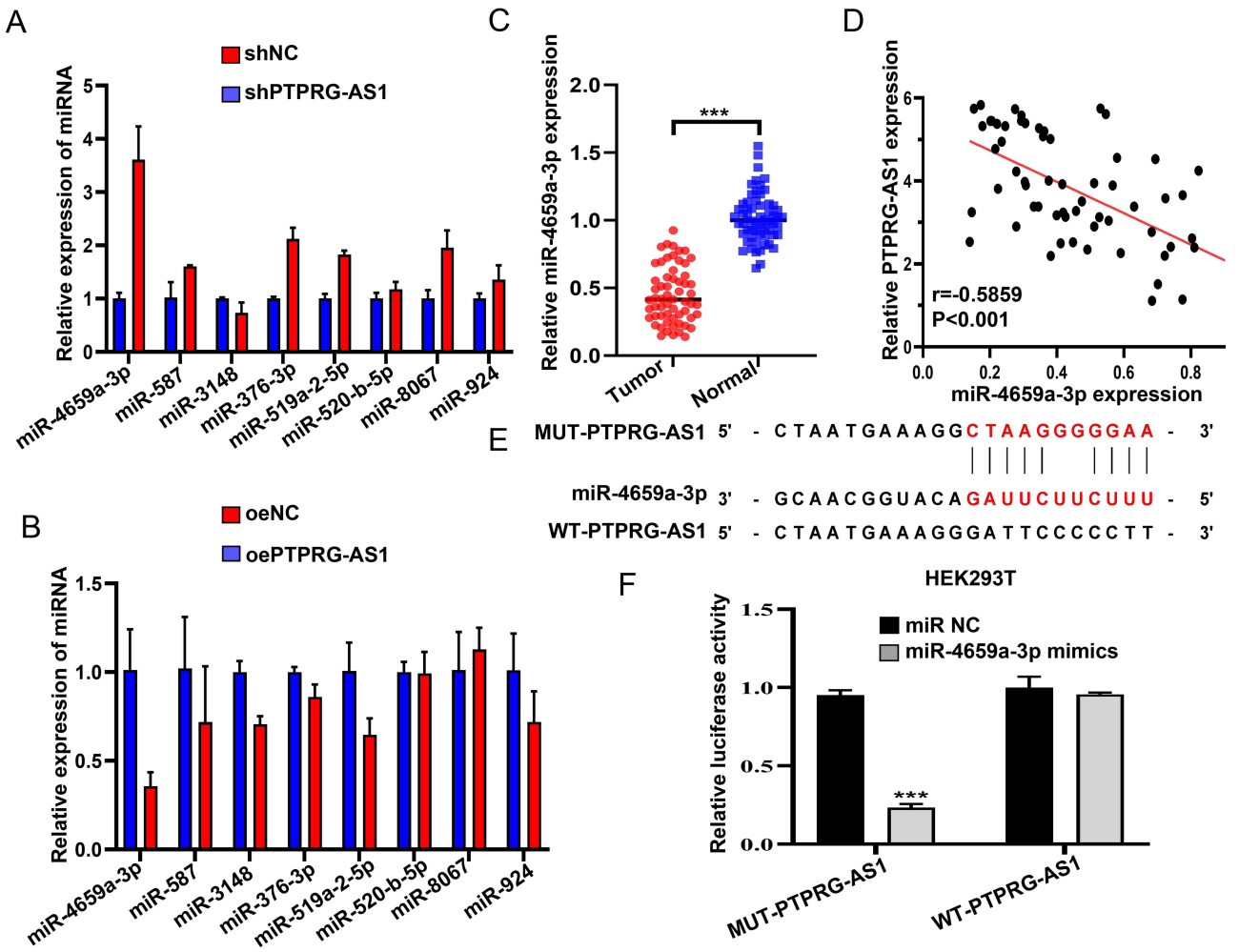

Figure 3 LncRNA PTPRG-AS1 acts as a sponge of miR-4659a-3p in BRCA cells. (**A** and **B**) RT-qPCR was used to detect the expression levels of eight candidate miRNAs after knocking down PTPRG-AS1 in MCF-7 cells and overexpressing PTPRG-AS1 in SK-BR-3 cells. (**C**) RT-qPCR analysis showed the expression levels of miR-4659a-3p in the breast cancer tissues and normal breast tissues (n=30). U6 was used as the internal control. (**D**) Pearson's correlation analysis was used to determine the relationship between expression of miR-4659a-3p and PTPRG-AS1. (**E**) The predicted binding sites for miR-4659a-3p in PTPRGAS1 and mutant fragments of the potential miR-4659a-3p binding sites. (**F**) The dual luciferase assay in HEK293T cells showed that miR-4659a-3p mimics significantly reduced the luciferase activity of WT-PTPRG-AS1 but not MUT-PTPRG-AS1. Experiments were performed in triplicate. ***p<0.001.

invasion of SK-BR-3 cells was decreased. Cotransfection with PTPRG-AS1 overexpression plasmid and miR-4659a-3p mimics offset the enhancing effects of lncRNA PTPRG-AS1 (P<0.01; Figure 4E-H).

The aforementioned results revealed that miR-4659a-3p exerted a tumor suppressive role in BRCA cells. LncRNA PTPRG-AS1 could enhance the proliferation, migration and invasion of BRCA cells by targeting miR-4659a-3p, and these effects could be inhibited by miR-4659a-3p.

QPCT Was Regulated by PTPRG-AS1 and miR-4659a-3p

Next, by analyzing online databases, such as TargetScan, miRWalk, miRPathDB and mirDIP, we identified QPCT as a downstream signaling pathway of miR-4659a-3p (Figure 5A). The binding sites between QPCT and miR-4659a-3p were predicted via the TargetScan database and are demonstrated in Figure 5B. Subsequently, a dual-luciferase reporter gene assay was performed to evaluate the potential interaction between QPCT mRNA and miR-4659a-3p. The results showed that the relative firefly luciferase activity in HEK293T cells was significantly reduced following cotransfection with the WT-QPCT plasmid and miR-4659a-3p mimics, but the relative firefly luciferase activity in HEK293T cells cotransfected with the MUT-QPCT plasmid and miR-4659a-3p mimics was unaffected (P<0.001; Figure 5C). RT-qPCR analysis showed that QPCT was significantly upregulated in 30 of the BRCA tissues analysed compared to normal tissues

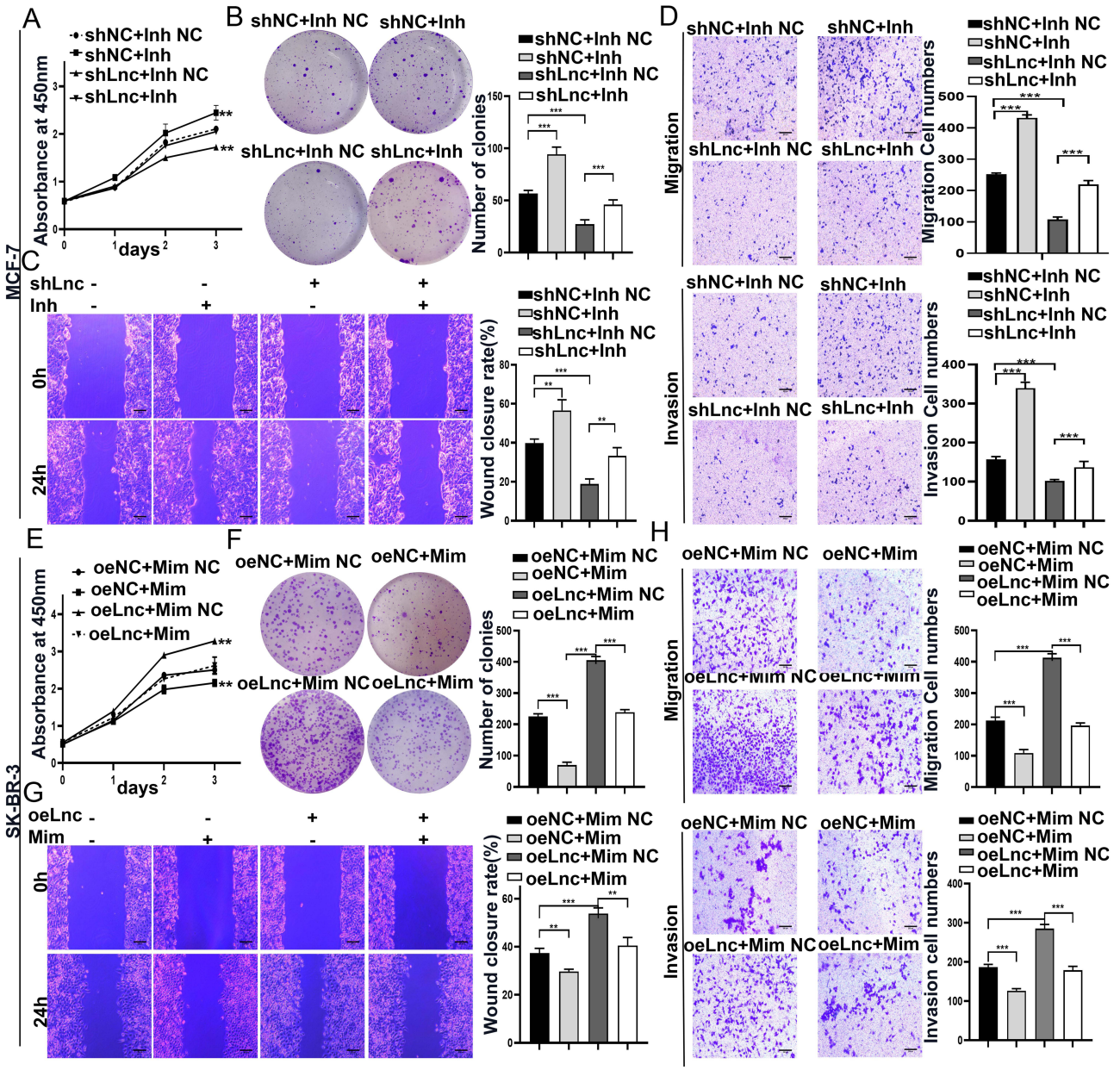

Figure 4 PTPRG-AS1 exerts an aggressive effect on BRCA via miR-4659a-3p. (**A** and **B**) CCK8 and Colony formation assay was used to evaluated the proliferation of MCF-7 cells after cotransfection. (**C** and **D**) Wound healing assay and Transwell assay were used to analyze the migration and invasion ability of MCF-7 cells after cotransfection. Scale bar, 200μm. (**E** and **F**) CCK8 and Colony formation assay was used to evaluated the proliferation of SK-BR-3 cells after cotransfection. (**G** and **H**) Wound healing assay and Transwell assay were used to analyze the migration and invasion ability of SK-BR-3 cells. Scale bar, 200μm. Experiments were performed in triplicate. **p<0.01, ***p<0.001. shLnc, knock down of PTPRG-AS1; Inh NC, miRNA inhibitor NC; Inh, miR-4659a-3p inhibitor; oeLnc, overexpression of PTPRG-AS1; oeNC, empty plasmid of PTPRG-AS1; Mim NC, miRNA mimics NC; Mim, miR-4659a-3p mimics.

(P<0.001; Figure 5D). We then investigated the expression relationship between LncRNA PTPRG-AS1, miR-4659a-3p, and QPCT in these 30 clinical BRCA tissues. Our findings demonstrated that there was a negative correlation between miR-4659a-3p and QPCT (r=−0.4051, P<0.01; Figure 5E). Meanwhile, QPCT showed a positive correlation with PTPRG-AS1 (r=0.6609, P<0.001; Figure 5F). Similarly, the GTEx database also demonstrated a positive correlation between QPCT and PTPRG-AS1 (r=0.27, P<0.001; Supplementary Figure S5). Besides, RT-qPCR results showed a positive association between QPCT mRNA and PTPRG-AS1 levels after knocking down or overexpressing PTPRG-AS1 (P<0.05; Figure 5G and H). Western blotting showed that knocking down or overexpressing of PTPRG-AS1 significantly decreased or increased the protein expression levels of QPCT (Supplementary Figure S2), respectively, but

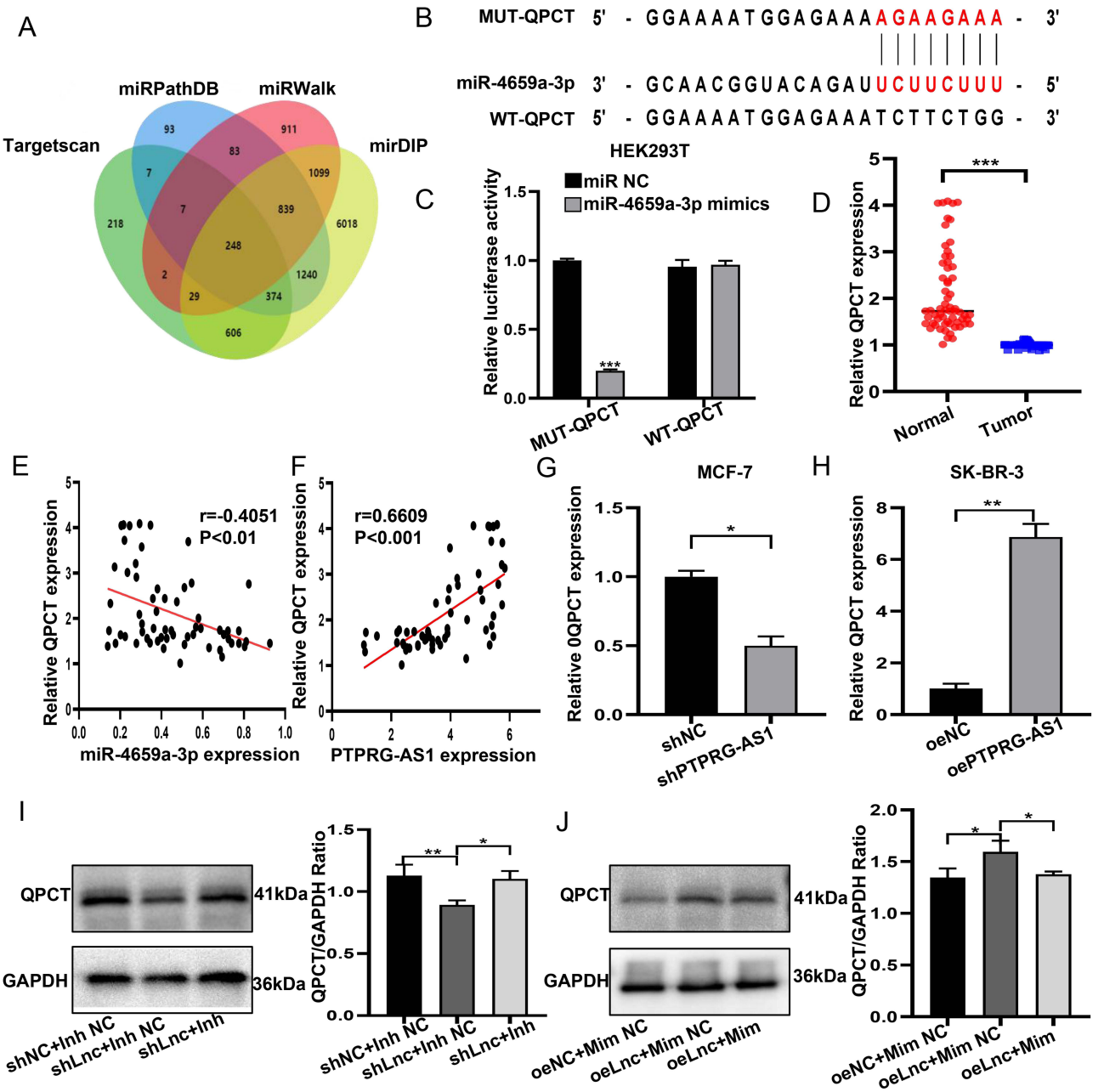

Figure 5 QPCT was regulated by PTPRG-AS1 and miR-4659a-3p. (**A**) Schematic illustration showing the overlapping target genes of miR-4659a-3p predicted by Targetscan, miRPathDB, miRWalk and mirDIP databases. (**B**) The predicted binding sites for miR-4659a-3p in QPCT and mutant fragments of the potential miR-4659a-3p binding sites. (**C**) The dual luciferase assay in HEK293T cells showed that miR-4659a-3p mimics significantly reduced the luciferase activity of WT-QPCT but not MUT-QPCT. (**D**) The expression level of QPCT in 30 pairs of breast cancer and adjacent normal tissues was detected by RT-qPCR. GAPDH was used as the internal control. (**E** and **F**) Pearson's correlation analysis was used to determine the relationship between expression of miR-4659a-3p and QPCT, as well as PTPRG-AS1 and QPCT, respectively. (**G** and **H**) RT-qPCR was used to detect the effect of knocking down or overexpressing lncRNA PTPRG-AS1 on QPCT expression in MCF-7 cells and SK-BR-3 cells respectively. (**I** and **J**) Western blot analysis was used to detect the protein expression levels of QPCT in MCF-7 cells and SK-BR-3 cells cotransfected with shPTPRG-AS1 plasmid and miR-4659a-3p inhibitor or PTPRG-AS1 overexpression plasmid and miR-4659a-3p mimics respectively. Experiments were performed in triplicate. *p<0.05, **p<0.01, ***p<0.001.

these effects were abrogated when MCF-7 cells were cotransfected with shPTPRG-AS1 and miR-4659a-3p inhibitor or when SK-BR-3 cells were cotransfected with oePTPRG-AS1 and miR-4659a-3p mimics ($P < 0.05$, Figure 5I and J).

To summarize these results, QPCT was determined to be a candidate target for miR-4659a-3p, and was regulated by PTPRG-AS1 and miR-4659a-3p.

The PTPRG-AS1/miR-4659a-3p/QPCT Axis Accelerates the Progression of BRCA Cells

To further establish the potential function of the lncRNA PTPRG-AS1/miR-4659a-3p/QPCT axis in BRCA cells, MCF-7 cells were transfected and subdivided with three groups: cotransfected with negative control of inhibitor (inhibitor NC) and negative control of shQPCT (shNC), the miR-4659a-3p inhibitor cotransfected with shNC, the miR-4659a-3p inhibitor cotransfected with QPCT knockdown plasmid (shQPCT). Meanwhile, SK-BR-3 cells were transfected with negative control of mimics (mimics NC) and empty plasmid of QPCT (oeNC), miR-4659a-3p mimics cotransfected with oeNC, miR-4659a-3p mimics cotransfected with QPCT overexpression plasmid (oeQPCT). The transfection efficiency of the miR-4659a-3p inhibitor in MCF-7 cells and miR-4659a-3p mimics in SK-BR-3 cells is shown in Figure 6A and C. Subsequently, rescue experiments were conducted for further exploration. The results of the CCK-8 and colony formation assays indicated that knocking down QPCT partially offset the effects

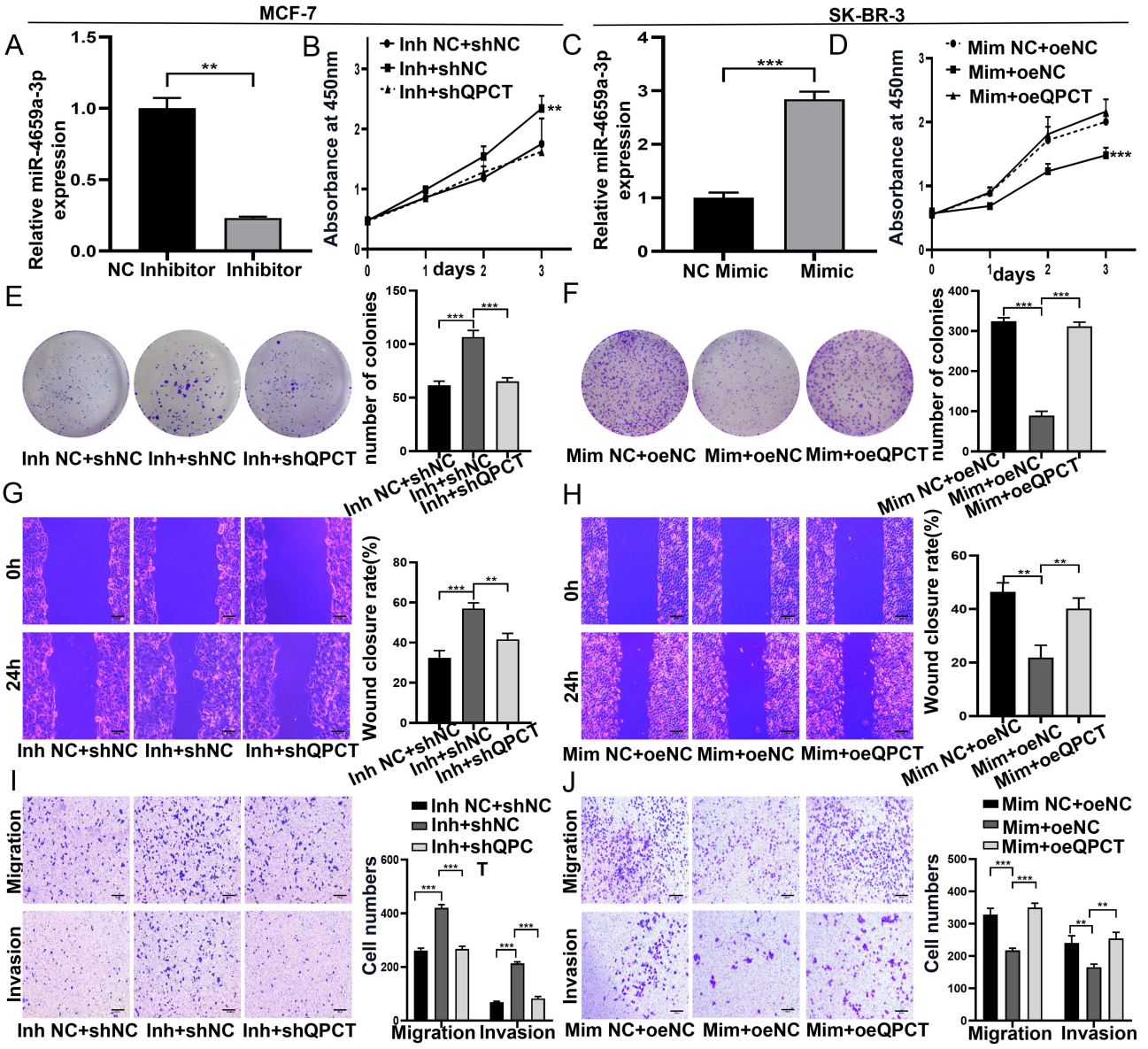

Figure 6 The PTPRG-AS1/miR-4659a-3p/QPCT axis accelerates the progression of BRCA cells. (**A** and **C**) Transfection efficiency of miR-4659a-3p inhibitor and miR-4659a-3p mimics was confirmed by RT-qPCR assay in MCF-7 and SK-BR-3 cells. (**B, D, E** and **F**) The proliferation viability and colony formation ability of MCF-7 and SK-BR-3 cells were evaluated by CCK-8 assay and colony formation assay. (**G-J**) The migration and invasion abilities of MCF-7 and SK-BR-3 cells were evaluated by wound healing assay and Transwell assay. Scale bar, 200 μm. Experiments were performed in triplicate. **p<0.01, ***p<0.001. Inh NC, miRNA inhibitor NC; shQPCT, knock down of QPCT; Inh, miR-4659a-3p inhibitor; oeNC, empty plasmid of QPCT; oeQPCT, overexpression of QPCT; Mim NC, miRNA mimics NC; Mim, miR-4659a-3p mimics.

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of the miR-4659a-3p inhibitor on proliferation, thereby avoiding the tumor-promoting effects of the miR-4659a-3p inhibitor on MCF-7 cells. Moreover, overexpression of QPCT in SK-BR-3 cells could attenuate the inhibitory effect of miR-4659a-3p mimics on BRCA proliferation (P<0.01; Figure 6B, D, E and F). In addition, migration and invasion assays indicated that, in MCF-7 cells and SK-BR-3 cells, the miR-4659a-3p inhibitor enhanced the migration and invasion of BRCA cells, and miR-4659a-3p mimics weakened the aforementioned processes, whereas overexpression or knockdown of QPCT eliminated these effects, respectively (P<0.01; Figure 6G-J).

Discussion

Herein, lncRNA PTPRG-AS1 was found to be overexpressed in BRCA tissues and cells, and to contribute to the pathogenesis of BRCA, by affecting cell proliferation, migration and invasion. Specifically, we confirmed that lncRNA PTPRG-AS1 mediated its effects via miR-4659a-3p/QPCT in BRCA, and may be considered as a novel biomarker and potential therapeutic target for BRCA treatment.

Increasing evidence has demonstrated that abnormal expression of lncRNAs can affect the expression of tumor suppressor genes or oncogenes through the lncRNA-miRNA-mRNA axis.[8,23–26] Previous evidence has suggested that lncRNA PTPRG-AS1 is not only highly expressed in various types of cancer, but also plays an important role in the carcinogenesis of different tumors.[18–22] In our current study, PTPRG-AS1 was not only significantly upregulated in BRCA tissues and cells, but was also demonstrated to be involved in the regulation of proliferation, migration and invasion of tumor cells. But the correlation between PTPRG-AS1 and clinicopathological parameters of breast cancer remains controversial, as previous studies reported conflicting results. We attempted to investigate this issues in these 73 BRCA patients and the results were shown in Table 1. Our study showed that no significant association was found between gene expressions and clinical parameters such as age, tumour size, histological grade, TNM stage, ER status, PR status, lymph node status and Ki-67. Meanwhile, the analysis of the expression level of PTPRG-AS1 and different TNM stages and lymph node status in TCGA database also showed the same results (Supplemental Figure S1). These results seem to be in agreement with Iranpour et al[17] but contrary to another research.[27] Therefore, the relationship between PTPRG-AS1 expression levels across different populations still requires further validation and elaboration through larger-scale and more comprehensive studies.

A previous study of 306 patients[27] indicated that the patients with statistically low expression of PTPRG-AS1 were associated with the long survival times. However, since we used samples from newly diagnosed patients, it is temporarily impossible to evaluate the role of PTPRG-AS1 as a prognostic biomarker. Concurrently, we investigated the role of PTPRG-AS1 in cancer prognosis utilizing the GEPIA and TCGA databases (Supplementary Figure S6). Although the statistical significance of PTPRG-AS1 in terms of overall survival (OS) and disease-free survival (DFS) was not prominent within these databases, this does not diminish the value or importance of real-world studies in this context. Undoubtedly, this represents a direction for our future research endeavors.

Nuclear and cytoplasmic separation experiments revealed that lncRNA PTPRG-AS1 was partly localized in the cytoplasm, indicating that it may function as a miRNA sponge protecting target mRNAs from suppression.[28,29] Evidence has suggested that miRNAs can play roles as oncogenes or tumor suppressors, and could be used as diagnostic and prognostic biomarkers in the cell.[30,31] MiR-4659a-3p has been reported to inhibit the proliferation and metastasis of gastric cancer cells,[32] but its role in BRCA or other tumors has not been reported. Bioinformatics analysis and dual-luciferase reporter assay indicated that binding sites existed for miR-4659a-3p in the lncRNA PTPRG-AS1 sequence. Furthermore, a negative association existed between PTPRG-AS1 and miR-4659a-3p in clinical tissues, and a significant reciprocal suppression feedback loop was present in BRCA cells. In summary, miR-4659a-3p acted as a tumor suppressor in BRCA, and miR-4659a-3p overexpression partially reversed the lncRNA PTPRG-AS1 overexpression-mediated promotion of proliferation, migration and invasion of BRCA cells.

Furthermore, the results of the dual-luciferase reporter assay combined with the bioinformatics analysis showed that QPCT was a direct target of miR-4659a-3p. We also revealed a significant positive relationship between the expression of lncRNA PTPRG-AS1 and QPCT in BRCA cells and tissues. Moreover, lncRNA PTPRG-AS1 overexpression led to increased protein expression of QPCT, which could be partially reversed by miR-4659a-3p overexpression, indicating the presence of a lncRNA PTPRG-AS1/miR-4659a-3p/QPCT axis in BRCA.

QPCT, a secreted protein implicated in the biosynthesis of pyroglutamyl peptides, has been reported to be an important oncogene in the development of numerous malignancies, including thyroid cancer,[33] malignant melanoma[34] and renal cell carcinoma.[35] Our previous study also confirmed that QPCT could promote the progression of BRCA cells and tissues, and that it was positively associated with poor disease-free survival and could reduce the sensitivity of doxorubicin treatment by being positively regulated by the MTDH/NF-κB(p65) axis.[36,37] Therefore, it also provides a research direction for us to further investigate the relationship between PTPRG-AS1 and doxorubicin resistance.

However, this study has certain limitations. Firstly, the experiments involved only two cell lines, and the sample size was insufficient, which may affect the accuracy of the results. Secondly, the small sample size may be too small to show the expression significance of the LncRNA PTPRG-AS1, miR-4659a-3p, and QPCT in breast cancer patients and the relationship between lncRNA PTPRG-AS1 and other potentially targeted miRNAs requires further investigation and attention. Therefore, more research is required to fully understand how PTPRG-AS1 operates in BRCA and the sample size will be expanded and relevant studies on the clinical significance of LncRNA PTPRG-AS1, miR-4659a-3p and QPCT in breast cancer will be the main focus.

In conclusion, lncRNA PTPRG-AS1 expression was upregulated in BRCA tissues and cells. LncRNA PTPRG-AS1 could serve as a molecular sponge of miR-4659a-3p, and significantly promoted the development of BRCA and its tumorigenesis by activating QPCT protein expression. The discovery of the lncRNA PTPRG-AS1/miR-4659a-3p/QPCT axis might provide a more effective clinical therapeutic strategy for patients with BRCA.

Data Sharing Statement

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

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (approval no. 2021KY056). All patients agreed and provided signed informed consent.

Patient Consent for Publication

The authors confirm that written informed consent for publication of this article was obtained from all individual participants.

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Disclosure

The authors declare that they have no competing interests.

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