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Ras GTPase-Activating-Like Protein IQGAP1 (IQGAP1) Promotes Breast Cancer Proliferation and Invasion and Correlates with Poor Clinical Outcomes

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Statistical Analysis C
Data Interpretation D
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Background: Breast cancer is one of the most common female cancers in the world. As a key integrator of cell signaling pathways, IQGAP1 contributes to the development and progression of several cancers. However, the exact effects and molecular mechanisms of IQGAP1 in breast cancer progression remain poorly understood.

Material/Methods: In the present study, IQGAP1 expression was measured in 96 paired breast cancer samples and the corresponding adjacent non-cancerous tissues by immunohistochemistry and quantitative polymerase chain reaction. To further explore the biological function of IQGAP1 in breast cancer cells, we knocked down IQGAP1 expression in MCF-7 cells and overexpressed it in SK-BR-3 cells.

Results: IQGAP1 was specifically upregulated in breast cancer tissues compared with the corresponding adjacent non-cancerous tissues. Moreover, IQGAP1 expression was positively correlated with breast cancer survival rate. IQGAP1 also promoted breast cancer cell proliferation and cell cycle progression and suppressed apoptosis.

Conclusions: In conclusion, our results suggest that IQGAP1 plays an important role in the cell proliferation and invasion of human breast cancer cells, thus indicating that IQGAP1 may be a potential therapeutic target for the treatment of human breast cancer.

MeSH Keywords: Cell Proliferation • Clinical Medicine • Neoplasm Invasiveness

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Background

Breast cancer (BC) is one of the most common female cancers in the world. In 2015, the incidence of BC in the United States ranked first in female malignancies and second in cancer-related mortality, which seriously threatens women's lives and health [1,2]. The incidence of breast cancer is estimated to be 15% in China and has the highest incidence of cancer in women, and the incidence and mortality rates are rising [3]. In recent years, although the combined treatment regimens of surgery, radiotherapy, chemotherapy, endocrine therapy, and targeted therapy have made important progress in the treatment of BC, the long-term prognosis of the patients is still poor. The molecular mechanism of BC pathogenesis and disease development remain unclear.

IQ motif containing GTPase acting proteins (IQGAPs) are scaffold proteins that comprise multiple domains and are so named because they contain RasGAP catalytic domains and 4 IQ motifs that are capable of interacting with calmodulin [4]. IQGAPs are widely expressed in eukaryotes, from yeast to mammals. The IQGAP family has the same IQ motif, which can regulate many signaling pathways and affect biological functions of cells, including cell adhesion, cell migration, and extracellular secretion [5–7]. In mammals, there are 3 isoforms of IQGAP: IQGAP1, IQGAP2, and IQGAP3. The function, tissue expression, and subcellular localization of these 3 isoforms are different [8]. IQGAP1 is widely expressed in human tissues, whereas IQGAP2 and IQGAP3 are expressed only in a few tissues, including liver, small intestine, brain, testis, lung, and stomach [9,10].

Unlimited cell proliferation is a characteristic of tumor growth, and IQGAP1 plays a critical role in cell proliferation and apoptosis. In umbilical vein and aortic endothelial cells, IQGAP1 is an important molecular transmitter and is necessary for VEGF to promote cell proliferation. However, the VEGF-regulated proliferation disappears when siRNA-knockdown is used to reduce the expression of IQGAP1 [11].

E-cadherin, as a single transmembrane glycoprotein, maintains the polarity and integrity of epithelial cells in normal tissues and is involved in the invasive and metastatic abilities of tumor cells [12]. Both *in vivo* and *in vitro* experiments confirmed that deleted or decreased E-cadherin expression weakened tumor cell adhesion, loosened tight junctions between cells, affected cell polarity, and increased cell motility [13,14]. E-cadherin is also involved in the occurrence of epithelial-to-mesenchymal transition (EMT) and is related to recurrence and metastasis, as well as resistance of tumors to chemotherapy [15–17].

As the key integrator of cell signaling pathways, IQGAP1 plays an integral role in regulating cell adhesion, cell migration, and cell signaling [18–21]. Many studies have found that IQGAP1 has a potent tumor-promoting activity in many malignancies, including lung cancer, colorectal cancer, pancreatic cancer, and gastric cancer [22–25]. In squamous cell carcinoma tissues, overexpression of IQGAP1 is strongly associated with local recurrence and distant metastasis [26]. These findings suggest that overexpression of IQGAP1 may be importantly correlated with tumor proliferation and metastasis. Additionally, in a mouse cancer model, overexpressed IQGAP1 can activate the Wnt/β-catenin signaling pathway and promote the nuclear translocation of β-catenin and the level of cyclin D1, thereby promoting the proliferation and growth of hepatocellular carcinoma cells [27].

In the present study, the functional role and expression levels of IQGAP1 were measured in human BC tissues and cell lines. Our findings demonstrate that IQGAP1 expression is increased in BC tissues and is positively correlated with prognosis. Furthermore, IQGAP1 expression affects cell proliferation, cell cycle, apoptosis, and invasiveness of cancer cells.

Material and Methods

Patients, tissue specimens, and cell lines

Ninety-six pairs of BC tissues and corresponding non-cancerous adjacent tissues were obtained from the Second Department of Breast Surgery of the Oncological Hospital affiliated to Xinjiang Medical University. Ethics approval was granted from the Clinical Research Ethics Committee of Xinjiang Medical University.

Three breast cancer cell lines (MDA-MB-231, SK-BR-3, and MCF-7) and 1 normal mammary cell line (MCF-10A) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were plated in dishes with RPMI-1640 medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and were incubated in a 5% CO_2 incubator at 37°C.

Immunohistochemistry

IQGAP1 expression levels were analyzed by immunohistochemistry (IHC) as described previously [28]. The prepared sections were incubated overnight with antibody against IQGAP1 (1: 100, Santa Cruz, USA). Finally, all sections were observed and evaluated by 2 independent senior pathologists without prior knowledge of the clinical data. The evaluation of immunoreactivity was scored as described previously [28].

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RNA isolation and quantitative reverse transcription polymerase chain reaction

Total RNA was extracted with Trizol reagent (Thermo Fisher Scientific) from BC tissues and cells following the manufacturer's protocol. Extracted RNA was used for cDNA synthesis with SuperScript II (Thermo Fisher Scientific). Quantitative reverse transcription polymerase chain reaction (QRT-PCR) analyses were then performed using SYBR Premix Ex Taq (Takara Biotechnology, China) under the following conditions: denaturation for 30 s at 95°C and then 40 cycles at 95°C for 5 s and 60°C for 30 s. The primer sequences used were: IQGAP1 forward 5'-ATGGATGGGATGAAGCACAGAG-3' and reverse 5'-CAGGACAGAGCCATAGTGCG-3'; β-actin forward 5'-AGCGAGCATCCCCCAAAGTT-3' and reverse 5'-GGGCACGAAGGCTCATCATT-3'. The expression of β-actin was used as an internal control. IQGAP1 mRNA levels were calculated using the $2^{-\Delta\Delta CT}$method.

Cell transfection

An oligonucleotide sequence of siRNA for targeting human IQGAP1 gene and a scrambled sequence were synthesized by RiboBio (Guangzhou, China). The target sequence for IQGAP1 was 5'-UUAUCGCCCAGAAACAUCUUGUUGG-3'. For overexpression of genes, full-length IQGAP1 cDNA were obtained and then subcloned into the pcDNA3.1 vector (Invitrogen, USA). Cells were incubated and transfected with si-IQGAP1, si-control, pcDNA-IQGAP1 (overexpresses IQGAP1), and pcDNA-control with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell proliferation assay

To perform proliferation assays, transfected cells were plated in 96-well plates at a density of 5×10^3 cells in each well. After 24 h, the cell counting kit-8(CCK-8, Dojindo, Japan) was used and added to each well in accordance with the manufacturer's instructions. Finally, the absorbance of each well was measured at 450 nm.

Western blot assay

Transfected cells were lysed using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) with a mixture of protease inhibitors. The total protein concentration was determined using the Bradford method. We separated 20 μg of total protein using SDS-PAGE, and then transferred it onto PVDF membranes (Millipore, USA). Immunoblots were blocked with 5% non-fat dried milk for 1 h at room temperature. After blocking, membranes were incubated with antibodies for IQGAP1 (1: 1000; Abcam, USA), E-cadherin (1: 1000; GeneTex, USA), N-cadherin (1: 1000; Abcam), and β-actin (1: 2000, Santa Cruz) separately overnight at 4°C. Secondary antibodies (1: 2000, Cell Signaling Technology, USA) were then used for incubation for 2 h at room temperature. Results were visualized using an ECL kit (Tanon, Shanghai, China).

Cell cycle and apoptosis analysis

Cells were seeded into 6-wells plates and transfected for 48 h. Transfected cells were then harvested and fixed with 70% ethanol overnight at 4°C. After washing with cold PBS, the fixed cells were treated with 50 μg/ml propidium iodide for 30 min in the dark. Cells were analyzed immediately by flow cytometry with FACSCanto (BD Pharmingen, USA) and calculated with ModFit software (Becton-Dickinson, USA). For apoptosis assay, a FITC AnnexinV Apoptosis Detection Kit (Sigma, St. Louis, MO, USA) was used according to the manufacturer's instructions.

Transwell invasion assay

For the invasion assay, 24-well Transwell chambers (Costar, Corning, MA) were used and the upper chamber was precoated with Matrigel (BD Bioscience, CA, USA). Transfected cells were seeded into upper chambers in serum-free RPMI-1640 and the complete medium (containing 10% FBS) was added into the lower chamber as a chemoattractant. After 24-h incubation, invasive cells were fixed with 4% formaldehyde and were stained with 0.1% crystal violet. The number of invasive cells was counted under a microscope.

Statistical analyses

The data are presented as mean ±SD. The *t* test was used to evaluate data between 2 groups. Survival curves were analyzed using the Kaplan-Meier method and log-rank method. $P<0.05$ was considered statistically significant. All data analyses were performed with SPSS 16.0 software.

Results

IQGAP1 is overexpressed in BC tissues and correlates with prognosis

We used IHC and QRT-PCR to evaluate IQGAP1 expression in 96 pairs of BC and non-cancerous adjacent tissues. IQGAP1 staining was markedly stronger in the BC tissues than in the non-cancerous adjacent tissues and the immunohistochemical indices were 5.13±1.519 for BC tissues and 1.34±1.164 for non-cancerous adjacent tissues (Figure 1A). QRT-PCR results showed that mRNA expression levels for IQGAP1 in BC tissues were significantly higher than in corresponding non-cancerous adjacent tissues ($P=0.0005$, Figure 1B). We next investigated the protein levels of IQGAP1 expression in different

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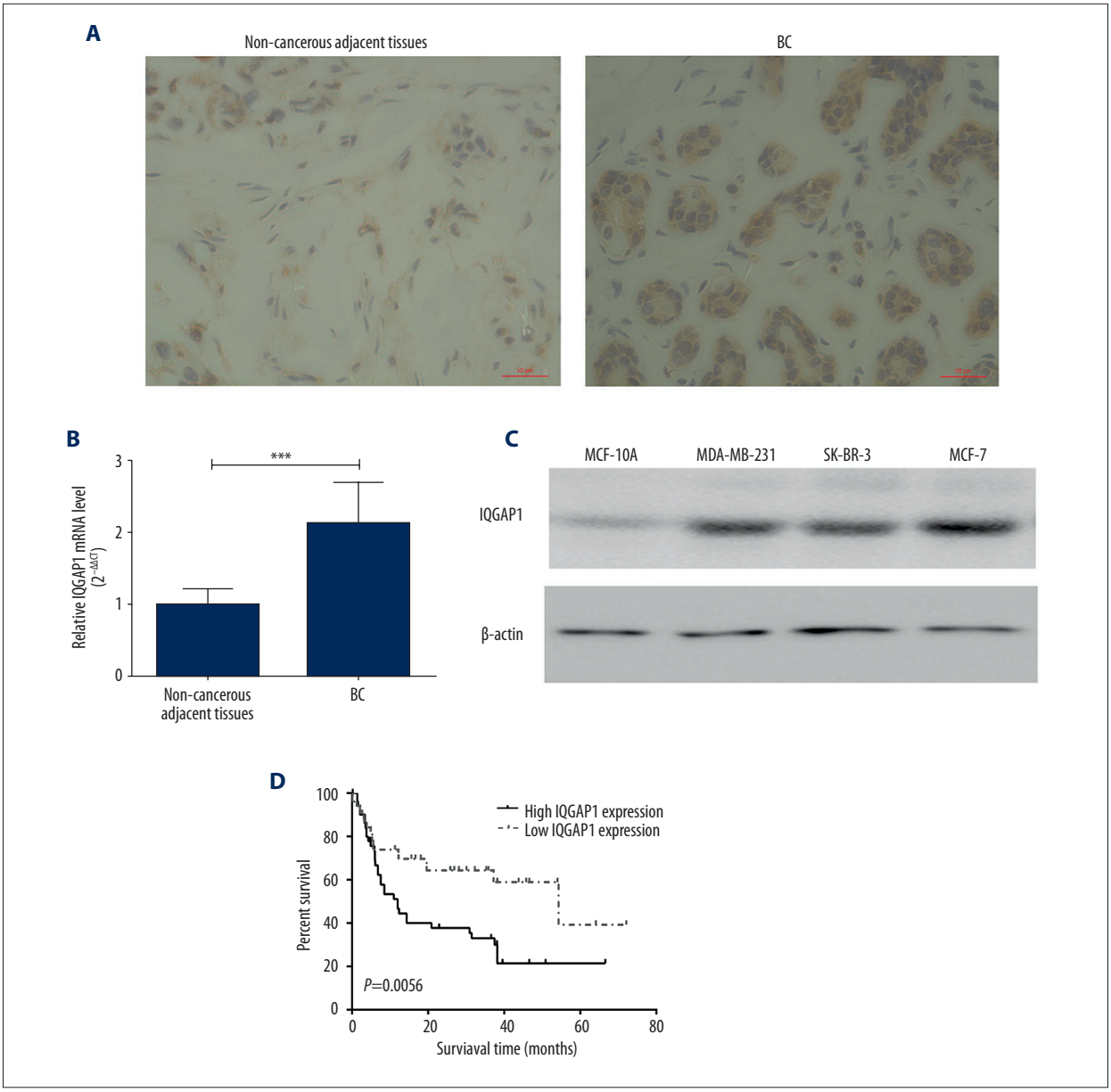

Figure 1. IQGAP1 overexpression in BC tissues correlates with prognosis. (**A**) Expression of IQGAP1 in BC tissues and the corresponding adjacent non-cancerous tissues was analyzed using immunohistochemistry. (**B**) Relative mRNA expression levels of IQGAP1 in BC tissues and the corresponding adjacent non-cancerous tissues. (**C**) Protein levels of IQGAP1 expression in different BC cell lines (MCF-7, SK-BR-3, and MDA-MB-231) and the normal mammary epithelial cell line MCF-10A. (**D**) Correlation between IQGAP1 expression levels and survival rates was analyzed by the log-rank test. ***, $P<0.001$

BC cell lines (MCF-7, SK-BR-3, and MDA-MB-231) and a normal mammary epithelial cell line (MCF-10A) by Western blot assay. Protein levels of IQGAP1 were much higher in the BC cell lines than in MCF-10A cells. Moreover, IQGAP1 expression was increased in MCF-7 cells compared with the other BC cell lines and was lowest among the BC cell lines in the SK-BR-3 cells (Figure 1C). Therefore, we used MCF-7 cells and SK-BR-3 cells for further experiments. As shown in Figure 1D, the log-rank test demonstrated that patients with lower expression of IQGAP1 survived significantly longer than those with higher expression of IQGAP1 ($P=0.0056$, Figure 1D). These results indicate that IQGAP1 may be involved in the pathogenesis of human breast cancer.

Knockdown of IQGAP1 inhibited BC cell proliferation

To further explore the biological function of IQGAP1 in BC cells, we knocked down IQGAP1 expression in MCF-7 cells by RNA

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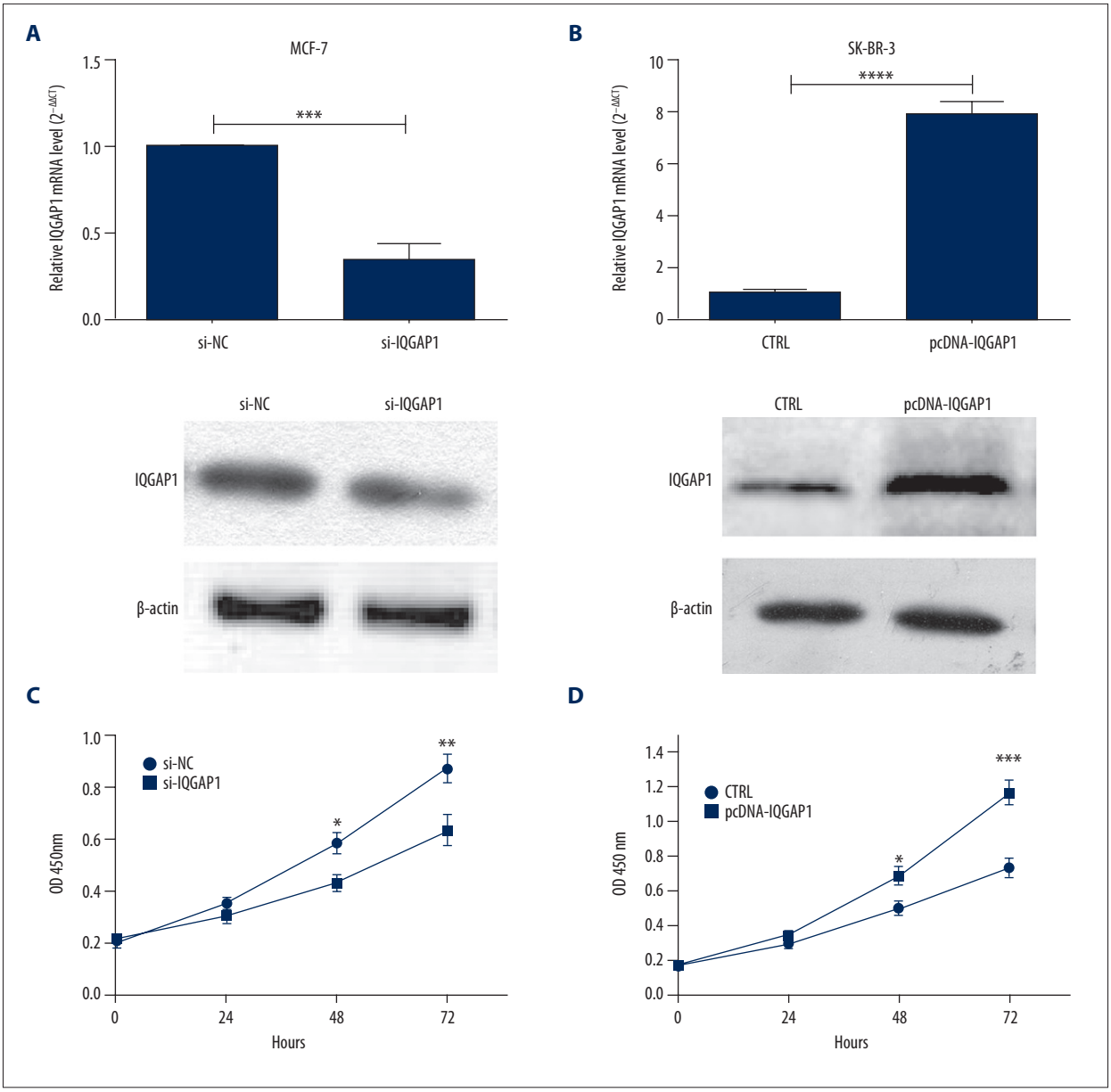

Figure 2. Knockdown of IQGAP1 inhibits BC cell proliferation. IQGAP1 expression was evaluated in IQGAP1-knockdown MCF-7 cells (**A**) and IQGAP1-overexpressing SK-BR-3 cells (**B**) using QRT-PCR and Western blot. Cell proliferation assay was performed in IQGAP1-knockdown MCF-7 cells (**C**) and IQGAP1-overexpressing SK-BR-3 cells (**D**). *** $P<0.001$; ** $P<0.01$; * $P<0.05$

interference and overexpressed it in SK-BR-3 cells by transfecting plasmid pcDNA-IQGAP1 (Figure 2A, 2B). Cell proliferation was determined by a CCK-8 assay. IQGAP1 silencing inhibited the proliferation of MCF-7 cells (Figure 2C). In contrast, IQGAP1 overexpressing in SK-BR-3 cells promoted cell proliferation compared with the control group (Figure 2D).

IQGAP1 promoted BC cell cycle progression and suppress apoptosis

Cell cycle progression and apoptosis were detected in IQGAP1-knockdown and IQGAP1-overexpressing BC cells separately by flow cytometry analysis. Cell cycle analysis showed that IQGAP1-knockdown cells were significantly arrested at the G0/G1 phase and had fewer S-phase cells than in the si-control group (Figure 3A). A significant higher rate of apoptosis was observed in the IQGAP1-knockdown MCF-7 cells compared with the controls (Figure 3B). Furthermore, overexpression of

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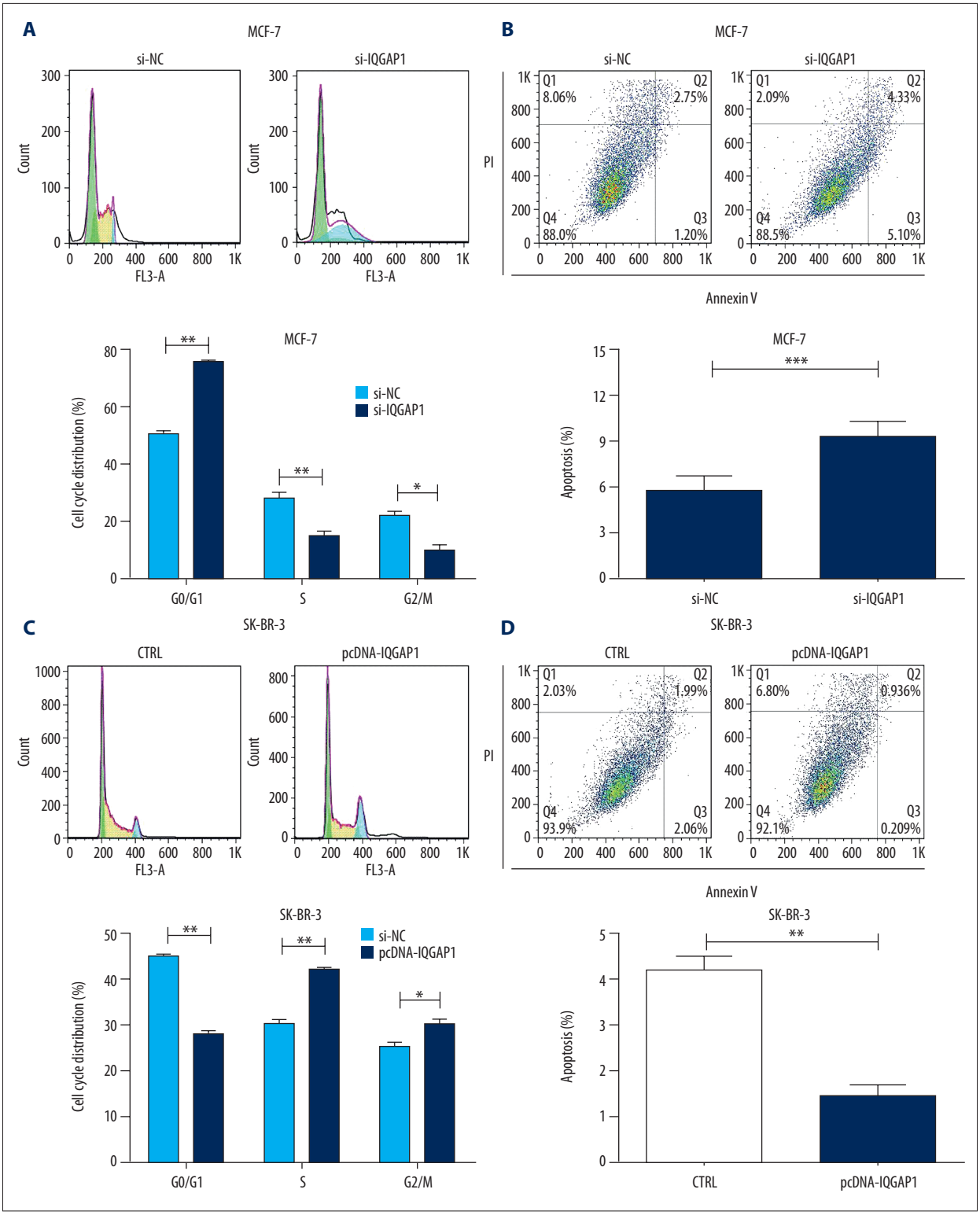

Figure 3. IQGAP1 promotes BC cell cycle progression and suppresses apoptosis. Cell cycle progression was analyzed in IQGAP1-knockdown MCF-7 cells (**A**) and IQGAP1-overexpressing SK-BR-3 cells (**C**). Apoptosis analysis was performed in IQGAP1-knockdown MCF-7 cells (B) and IQGAP1-overexpressing SK-BR-3 cells (**D**). *** $P<0.001$; ** $P<0.01$; * $P<0.05$

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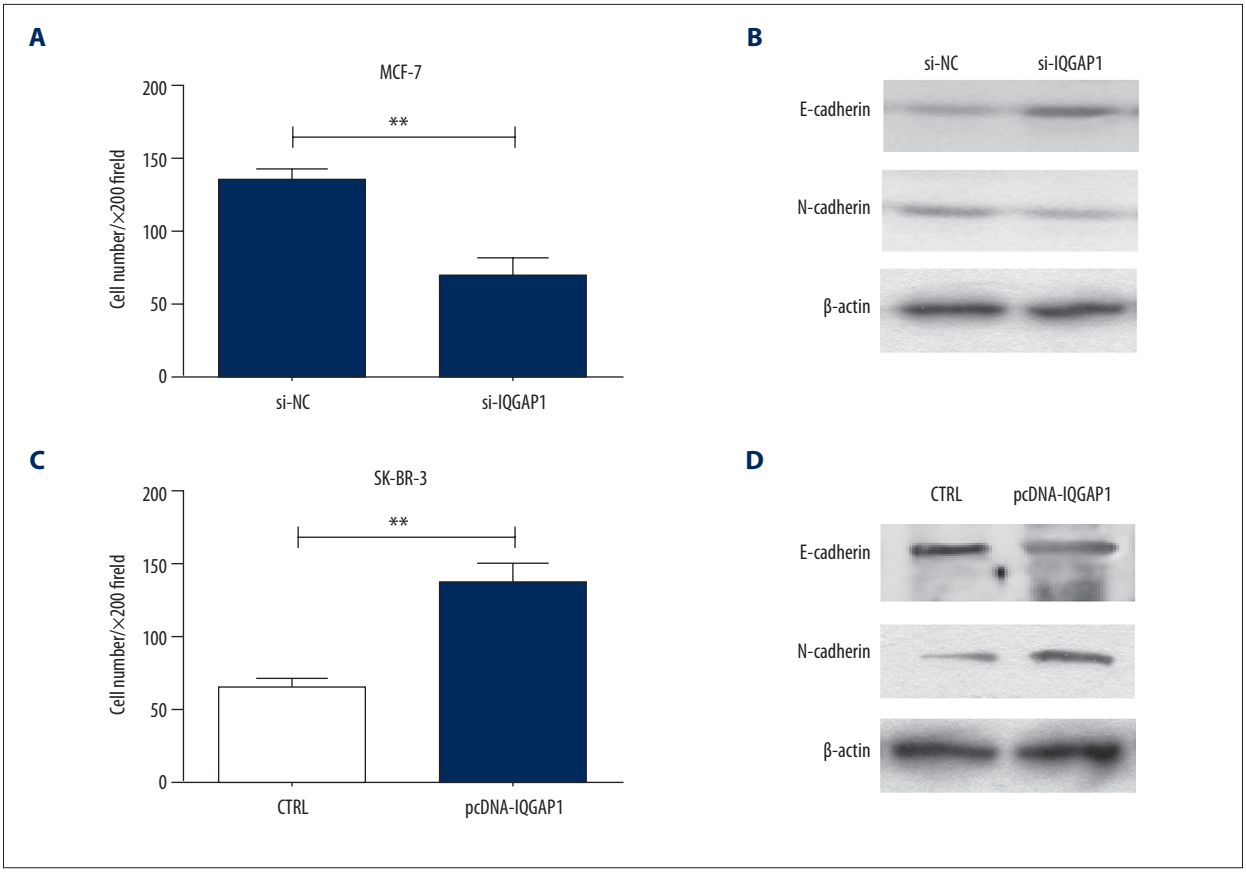

Figure 4. Knockdown of IQGAP1 inhibits BC cell invasion. The effect of IQGAP1 on BC cell invasion was evaluated in IQGAP1-knockdown MCF-7 cells (**A**) and IQGAP1-overexpressing SK-BR-3 cells (**C**). EMT-related proteins (E-cadherin and N-cadherin) were detected in IQGAP1-knockdown MCF-7 cells (**B**) and IQGAP1-overexpressing SK-BR-3 cells (**D**) by Western blot. ** $P<0.01$

IQGAP1 in SK-BR-3 cells promoted cell cycle progression and inhibited apoptosis (Figure 3C, 3D). Taken together, the results indicate that knockdown of IQGAP1 inhibited cell cycle progression and promoted apoptosis in BC.

Knockdown of IQGAP1 inhibited BC cell invasion

A Transwell invasion assay was performed to further investigate the effect of IQGAP1 on BC cell invasion. Compared with si-control-transfected cells, the number of invasive cells was significantly decreased in the MCF-7 cell line due to si-IQGAP1 transfection (Figure 4A). In addition, the expression levels of EMT-related proteins were detected using Western blot analysis. As shown in Figure 4B, knockdown of IQGAP1 significantly upregulated the expression of E-cadherin and downregulated N-cadherin compared with the control group (Figure 4B). Furthermore, we found that transfection of pcDNA-IQGAP1 markedly increased cell invasion capacity, and the results for protein levels of E-cadherin and N-cadherin were reversed as well (Figure 4C, 4D). All these alterations demonstrate that knockdown of IQGAP1 inhibited cell invasion and affected EMT in BC cells.

Discussion

Breast cancer is the most prevalent cancer in women. Common sites of distant metastasis include bone, brain, liver, and other tissues and organs [1]. Distant metastasis has been suggested to be the predominant reason for reduced quality of life and cause of death for breast cancer patients. Therefore, it is of great importance to elucidate the underlying mechanisms of BC and to search for effective molecular targets to improve disease therapy and prognosis.

It has been reported that IQGAP1 can bind directly to estrogen receptor α (ERα) in breast cancer cells. This function is regulated by estradiol. The inhibited expression of endogenous IQGAP1 in breast cancer cells may therefore weaken estradiol-induced transcription [7]. In the present study, we investigated the expression level of IQGAP1 in BC tissues and non-cancerous adjacent tissues. QRT-PCR and IHC results confirmed that expression levels for IQGAP1 in BC tissues were significantly higher than in corresponding non-cancerous adjacent tissues. Consistent with this, IQGAP1 expression was higher in BC cell lines than in the normal mammary epithelial

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cell line MCF-10A. As a multifunctional tumor-associated protein, IQGAP1 has been shown to act as an oncogene in the development of various types of tumors. Liu, et al. found that in benign thyroid tumors, papillary thyroid carcinoma (PTC), thyroid follicular carcinoma, tall-cell PTC, and undifferentiated carcinoma, the expression level of IQGAP1 was directly correlated to worse prognosis of malignant tumors [23]. IQGAP1 was also suggested to have an effect on the success rate of triple-negative breast cancer (TNBC) treatment. For example, by binding disulfiram and a motif of IQGAP1, the proliferation of a TNBC cell line could be inhibited, and apoptosis was promoted when doxorubicin was added to this combination. The effective inhibitory rates of disulfiram/doxorubicin, doxorubicin, and disulfiram were 65%, 57%, and 5%, respectively, in cancer stem cells in TNBC [29].

Mounting evidence confirms that IQGAP1 can be regarded as a biomarker for the diagnosis, recurrence, and prognosis of a variety of malignant tumors. A clinical study of thyroid carcinoma of follicular epithelial origin discovered that the expression of IQGAP1 increased gradually with the escalation of aggressiveness and malignancy of different subtypes. Furthermore, the expression of IQGAP1 was strongly associated with invasion and metastasis in the blood, and increased IQGAP1 expression indicated a higher recurrence rate and a poorer prognosis [23]. In addition, an analysis of gene expression profiles of oligodendrocytes documented that among 176 abnormally expressed genes, the expression of IQGAP1 was significantly correlated with poor prognosis and chemotherapy resistance in patients [30]. To identify the potential association between IQGAP1 expression and tumor clinical pathological features in BC, the patients were classified into low IQGAP1 or high IQGAP1 groups according to mRNA expression levels. The log-rank test showed that patients with low IQGAP1 expression had significantly higher survival rates than those with high levels of IQGAP1 expression. Similar results have also been reported in the study of other tumors. For example, high expression of IQGAP1 can predict the recurrence of colon cancer [31].

To further explore the biological function of IQGAP1 in BC cells, we knocked down IQGAP1 expression in the highly-expressing MCF-7 cells by RNA interference and overexpressed it in the relatively lower-expressing SK-BR-3 cells. The cell proliferation assay indicated that silencing IQGAP1 inhibited proliferation of BC cells. High expression of RhoC and IQGAP1 has been previously observed in gastric cancer tissues and cell lines [32]. Immunofluorescence showed that both proteins were localized on the cell membrane, and co-immunoprecipitation confirmed that they interact. *In vitro* studies further indicated that down-regulation of IQGAP1 can decrease the proliferation and migration ability of gastric cancer cells. However, down-regulation of RhoC expression had little effect on the proliferation and migration of gastric cancer cells. Importantly, there was no interference between RhoC and IQGAP1 [33], and its role in cell proliferation was mainly reflected in the up-regulation of cyclin E and cyclin D1 expression so that more cells could enter S-phase [32]. Additionally, we investigated whether IQGAP1 mediates tumor cell cycle progression and apoptosis in BC cells. Flow cytometry analysis showed that IQGAP1-knockdown cells were largely arrested at the G0/G1 phase and showed fewer S-phase cells than in the si-control group. The percentage of apoptotic cells in the IQGAP1-knockdown MCF-7 cells was much higher than in the controls. The role of IQGAP1 in regulating cellular apoptosis has gradually attracted increased research attention [34–37]. A recent report demonstrated that IQGAP1 contributes to AngII-induced apoptosis of podocytes by interacting with the ERK1/2 signaling protein [37]. Sato et al. found that RNase L-IQGAP1 association may regulate JNK phosphorylation in RNase L-mediated apoptosis and IQGAP1 may act as a regulator in apoptosis [36]. Our results suggest that IQGAP1silencing in cancer cells suppresses cancer cell proliferation and cell cycle progression, together with the enhancement of apoptosis.

Previous studies on the mechanisms of IQGAP1 found that the degree of ERK and Akt phosphorylation in undifferentiated carcinoma cell lines SW1736 and KAT18 and follicular carcinoma cell line FTC133 with knockdown of IQGAP1 gene expression was lower than in those cells without knockdown, suggesting that the MAPK signaling pathway and the PI3K/AKT signaling pathway may not play a critical role. Furthermore, co-immunoprecipitation revealed that IQGAP1 could directly bind with E-cadherin, and IQGAP1 might promote the invasion and metastasis of thyroid carcinoma by inhibiting the function of E-cadherin [23]. In the present study, an *in vitro* Transwell invasion assay revealed that the cell-invasive capacity of the MCF-7 cells treated with IQGAP1 siRNA was significantly lower than that of the controls, indicating an important potential role of IQGAP1 in the metastasis of BC cells. The E-cadherin-β-catenin-IQGAP1 complex is formed when IQGAP1 binds to E-cadherin, which breaks the α-catenin/β-catenin interaction. This weakens cell–cell adhesion and is involved in EMT. Moreover, the migration and invasion of epithelial-derived tumors are achieved by EMT [8,38]. In addition, Western blot analysis was performed to detect the expression levels of the EMT-related proteins E-cadherin and N-cadherin. Compared with the control group, knockdown of IQGAP1 significantly elevated the epithelial marker E-cadherin and decreased the mesenchymal marker N-cadherin. In malignant tumors, IQGAP1 can also indirectly suppress E-cadherin-mediated adhesion. Also, in malignant tumors, activated Rac 1 binds with IQGAP 1 and forms a complex with β-catenin, which may contribute to inhibiting the formation of the E-cadherin/β-catenin complex, inducing the migration of β-catenin from the cell membrane to the nucleus, and activating the downstream Wnt signaling pathway. These changes result in decreased cell adhesion and enhanced

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motility [39]. Therefore, our data indicate that knockdown of IQGAP1 inhibits cell invasion and might affect EMT in BC cells.

Conclusions

Taken together, our study demonstrated that IQGAP1 is up-regulated in breast cancer tissues and cells. The expression of IQGAP1 is positively correlated with the survival rate in BC. Moreover, our results illustrated the role of IQGAP1 in BC cell proliferation, cell cycle, apoptosis, and invasive capacity. These data suggest that the functions of IQGAP1 in BC might play a key role in tumor progression and could be a promising predictive biomarker and potential therapeutic target for BC.

Conflict of interest

None.

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