

ORIGINAL RESEARCH

RTKN2, a potential target of breast cancer (BCa), can promote the growth and migration of breast cancer cells

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Abstract

Breast cancer (BCa) is a type of malignancy; however, the exact mechanisms responsible for breast cancer have yet to be elucidated. Rhotekin 2 (RTKN2) is a member of the Rhotekin family. Previous studies demonstrated that RTKN2 is highly expressed in numerous tumors. Previous analysis of the The cancer genome atlas (TCGA) database demonstrated that PRKN2 was overexpressed in BCa. However, RTKN2 is rarely reported in BCa and the mechanisms remain unclear. The aim of this study was to elucidate the effect of RTKN2 on the growth of breast cancer cells and investigate the mechanisms involved. Our analysis revealed high expression of RTKN2 in human BCa. The depletion of RTKN2 suppressed the growth of breast cancer cells. In addition, the knockout of RTKN2 restrained the progression of BCa. We also revealed that the knockdown of RTKN2 stimulated cell cycle arrest in BCa. Mechanistically, RTKN2 mediated the Wnt/ β -catenin axis to affect the progression of breast cancer. In conclusion, RTKN2 promotes the growth and migration of breast cancer cells by mediating the Wnt/ β -catenin axis.

Keywords

Rhotekin 2 (RTKN2); Breast cancer (BCa); Motility; Cell cycle; Wnt/ β -catenin axis

1. Introduction

Breast cancer (BCa) is a common type of malignancy in women although the death rate of patients with BCa has been declining over recent years. However, metastasis remains the leading cause of death in patients with BCa [1]. Although researchers have focused on the mechanisms underlying carcinogenesis and metastasis in BCa, the specific mechanisms involved have yet to be fully elucidated [2]. Targeted therapy for breast cancer is a relatively new treatment method. Compared with traditional chemotherapy and radiotherapy, this method is more accurate and effective, and has important significance for the treatment of BCa. However, novel targets are required if we are to fully combat this disease.

Rhotekin 2 (RTKN2) is a member of the Rhotekin family [3]. Two types of rubinoid proteins, RTKN1 and RTKN2, possess a Rho-GTPase binding domain as well as a pleckstrin homology domain [4]. Rho proteins are essential for the growth and transformation of cells [5]. The dysregulation of Rho pathways is known to be associated with many types of cancer [6]; therefore, it follows that RTKN2 also plays a very important role in cancer.

RTKN2 is expressed at high levels in several tumors. For example, RTKN2 was shown to be highly expressed in bladder cancer tissues [7]. Furthermore, the knockout of RTKN2 was

shown to restrain the growth of bladder cancer and lead to cell cycle arrest. Moreover, overexpression of the *RTKN2* gene is known to promote the proliferation of colon cancer cells and block apoptosis [4]. Similarly, RTKN2 has been shown to be overexpressed in osteosarcoma tissues and promote apoptosis in osteosarcoma cells [8]. RTKN2 enhances resistance to radiotherapy in gastric cancer by regulating the Wnt/ β -Catenin axis [9]. In addition, sequencing analysis has shown that the mRNA levels of *RTKN2* showed a notable trend for upregulation in ovarian cancer [10]. Another study showed that increasing the expression of RTKN2 can also increase the expression of proliferating cell nuclear antigen (PCNA) in liver cancer, thus promoting the proliferation as well as metastasis of liver cancer cells [11]. However, RTKN2 is rarely reported in BCa, and the mechanisms involved have yet to be fully elucidated is unclear. TCGA analysis also showed that PRKN2 was overexpressed in BCa.

This study was to investigate the effect of RTKN2 on the growth of breast cancer cells and explore the mechanisms involved. We found that RTKN2 promoted the growth and motility of BCa cells by regulating the Wnt/ β -catenin axis.

2. Materials and methods

2.1 Bioinformatics

Transcriptomic and survival data were acquired from the TCGA database. In addition, the expression levels of RTKN2 in breast cancer were analyzed using the TCGA.

2.2 Cell culture and transfection

Human mammary epithelial cells (HMECs) and two types of BCa cells were purchased from the Cell Bank of Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% Fetal Bovine Serum (FBS). After 12 h of culture, Lipofectamine 3000 reagent (L3000015, Invitrogen, Carlsbad, CA, USA) was co-transfected with sh-negative control (NC), sh-RTKN2#1 and sh-RTKN2#2, respectively; 100 μ L of the mixture was slowly added 6-well plates containing the cells.

2.3 Western blotting

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and further transferred to a Poly (vinylidene fluoride) (PVDF) membrane. Separated proteins were then blocked with 5% skimmed milk for 1 h and then incubated at 4 °C overnight with several antibodies: RTKN2 (Abcam, ab154954; 1:500), β -catenin (Abcam, ab32572; 1:1000), c-Myc (Abcam, ab32072; 1:2000), β -tubulin (Abcam, ab7291; 1:2000), histone H3 (Abcam, ab1791; 1:2000) and β -actin (Abcam, ab8226; 1:3000). The following morning, membranes were washed and incubated in an appropriate secondary antibody for 1 h; then, the membranes were analyzed for chemiluminescence and typical images were acquired.

2.4 CCK-8 assays

Cells were subsequently incubated with Cell counting kit-8 (CCK-8) solution (Beyotime, Shanghai, China) for 4 h. Then, we measured the optical density (OD) at 450 nm.

2.5 Colony formation assays

Cells were plated and cultured in 10% FBS for 14 days at 37 °C. Cells were then fixed with paraformaldehyde (PFA) for 15 min and stained with 0.1% crystal violet. Then, representative images were acquired.

2.6 Cell cycle assays

Cells were fixed in 70% ethanol at -20 °C for 1 h. Subsequently cells were stained with propyl iodide (PI) and cell cycle analysis was performed with a flow cytometer (FACSCalibur, BD, Franklin Lake, NJ, USA).

2.7 Transwell assay

Cells were allowed to move into a Transwell chamber to detect migration (without Matrigel) or invasion (with 20% Matrigel, BD). Then, cells in the upper chamber were fixed, stained with 2% crystal violet, and images were captured. Cell invasion was observed by the analysis of stained cells.

2.8 Statistics

GraphPad 8.0 software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Data are represented as mean \pm standard deviation (SD) and $p < 0.05$ represented statistical significance.

3. Results

3.1 RTKN2 was expressed at high levels in BCa

To investigate the role of RTKN2 in the progression of BCa, we detected its expression levels in the TCGA database by performing bioinformation analysis. Analysis of the TCGA database revealed a high transcript per million (TPM) of RTKN2 in BCa tissues (Fig. 1A). Furthermore, data revealed high expression levels of RTKN2 in BCa tissues (Fig. 1B). We also detected the levels of RTKN2 protein in HMEC cells (a human breast cell line), and two types of BCa cells (MCF7 and MDA-MB-231). Analysis confirmed that RTKN2 was expressed at high levels in BCa cells (Fig. 1C). Therefore, we had demonstrated that RTKN2 was highly expressed in human BCa.

3.2 The knockdown of RTKN2 inhibited the growth of BCa cells

The RTKN2 shRNA sh-RTKN2#1 and sh-RTKN2#2 constructs were transfected into the MCF7 and MDA-MB-231 BCa cell lines. The transfection of RTKN2 shRNA led to clear reduction in expression when compared to the control and the sh-negative control (sh-NC, Fig. 2A). The depletion of RTKN2 suppressed cell growth and led to a lower OD450 value (Fig. 2B). Furthermore, the depletion of RTKN2 reduced the colony numbers of both MCF7 and MDA-MB-231 cells (Fig. 2C). Our data indicate that the depletion of RTKN2 inhibited the proliferation of BCa cells.

3.3 RTKN2 depletion induced cell cycle arrest in BCa cells

Next, we investigated whether RTKN2 could influence the cell cycle in BCa cells. Interestingly, the depletion of RTKN2 reduced the proportions (%) of MCF7 and MDA-MB-231 cells (Fig. 3). Furthermore, we found that the ablation of RTKN2 reduced the migration of cells at the S phase, with an increased proportion of cells (Fig. 3). Therefore, our data indicate that the depletion of RTKN2 induced cell cycle arrest in BCa cells.

3.4 RTKN2 depletion restrained breast cancer cell motility

Next, we investigated whether RTKN2 affected the motility of BCa cells. We observed that the ablation of RTKN2 reduced the migration of both MCF7 and MDA-MB-231 cells (Fig. 4A). Furthermore, it was evident that the ablation of RTKN2 also reduced the invasion of cells, with reduced cell numbers (Fig. 4B). Therefore, our data indicate that the depletion of RTKN2 suppressed the motility of BCa cells.

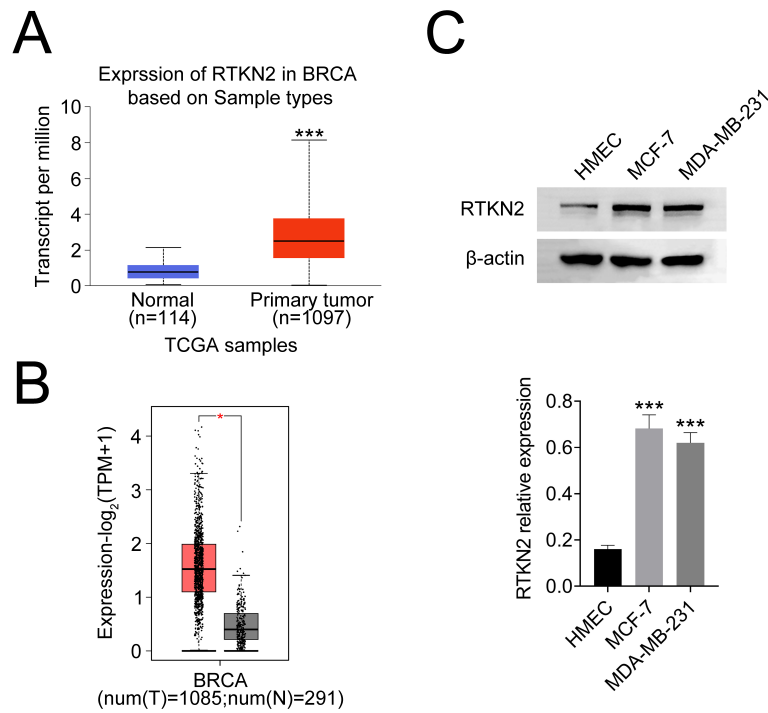


FIGURE 1. RTKN2 is expressed at high levels in BCa. (A) Analysis of the GEPIA database revealed the expression of RTKN2 in 114 normal and 1097 primary tumors (in transcripts per million). (B) The expression levels of RTKN2 in normal and primary tumors. (C) The expression of RTKN2 in HMEC, MCF-7 and MDA-MB-231 cells. Error bars indicate SD. *** $p < 0.001$. RTKN2: Rhotekin 2; BRCA: breast cancer; TCGA: The Cancer Genome Atlas; HMEC: Human mammary epithelial cell.

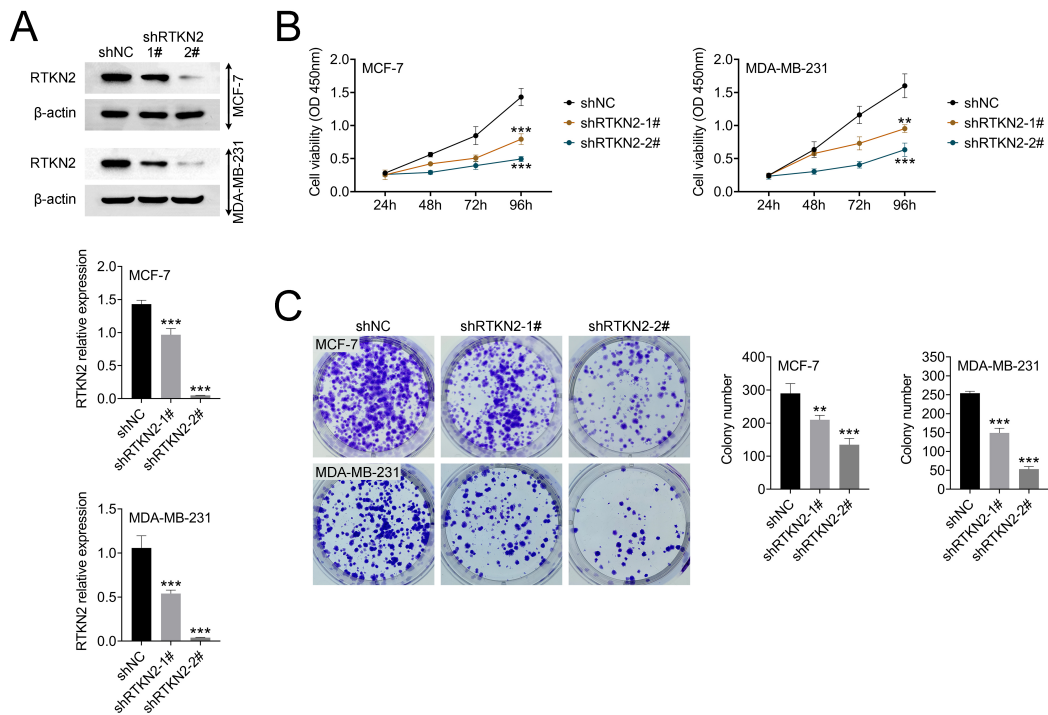


FIGURE 2. The knockdown of RTKN2 inhibited the growth of breast cancer cells. (A) Immunoblot assays showing the expression of RTKN2 in cells upon transfection with the indicated constructs. (B) CCK-8 assays showing the viability of cells upon transfection with the indicated constructs; OD values at 450 nm were measured. (C) Colony formation assays showing the proliferation of cells upon transfection with the indicated constructs. Colony numbers were counted. Error bars indicate SD. ** $p < 0.01$, *** $p < 0.001$. NC: negative control; CCK-8: cell counting kit-8.

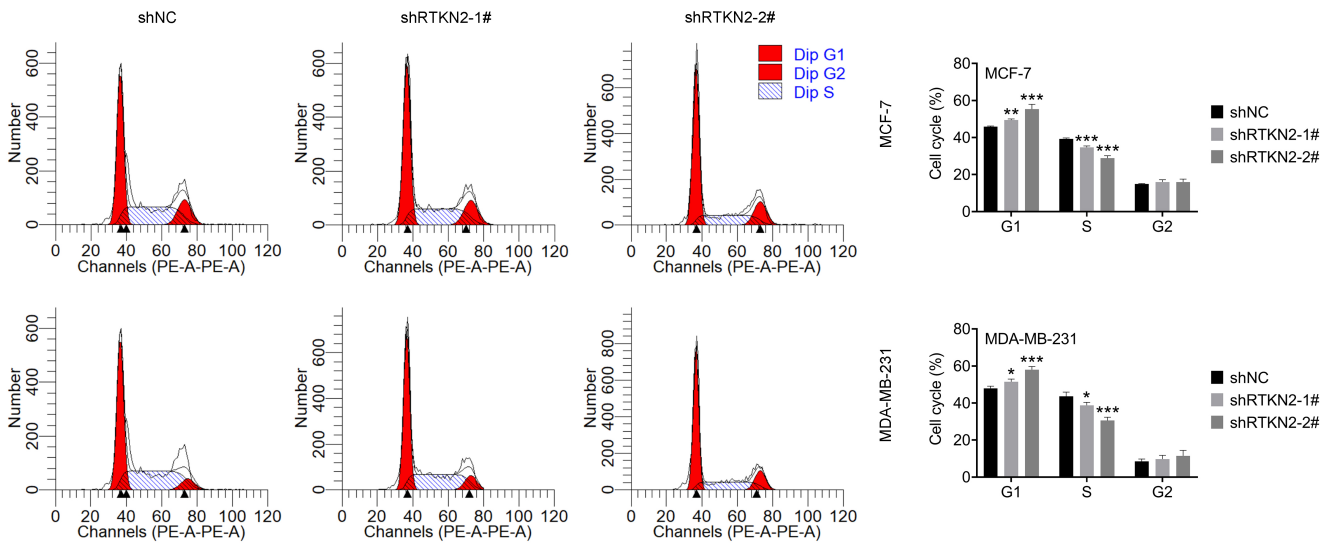


FIGURE 3. The depletion of RTKN2 induced cell cycle arrest in breast cancer cells. FCM assays were used to investigate the cell cycles of cells upon transfection with the indicated constructs. The percentage of cells in each stage of the cell cycle was quantified. Error bars indicate SD. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$. NC: negative control; FCM: flow cytometry.

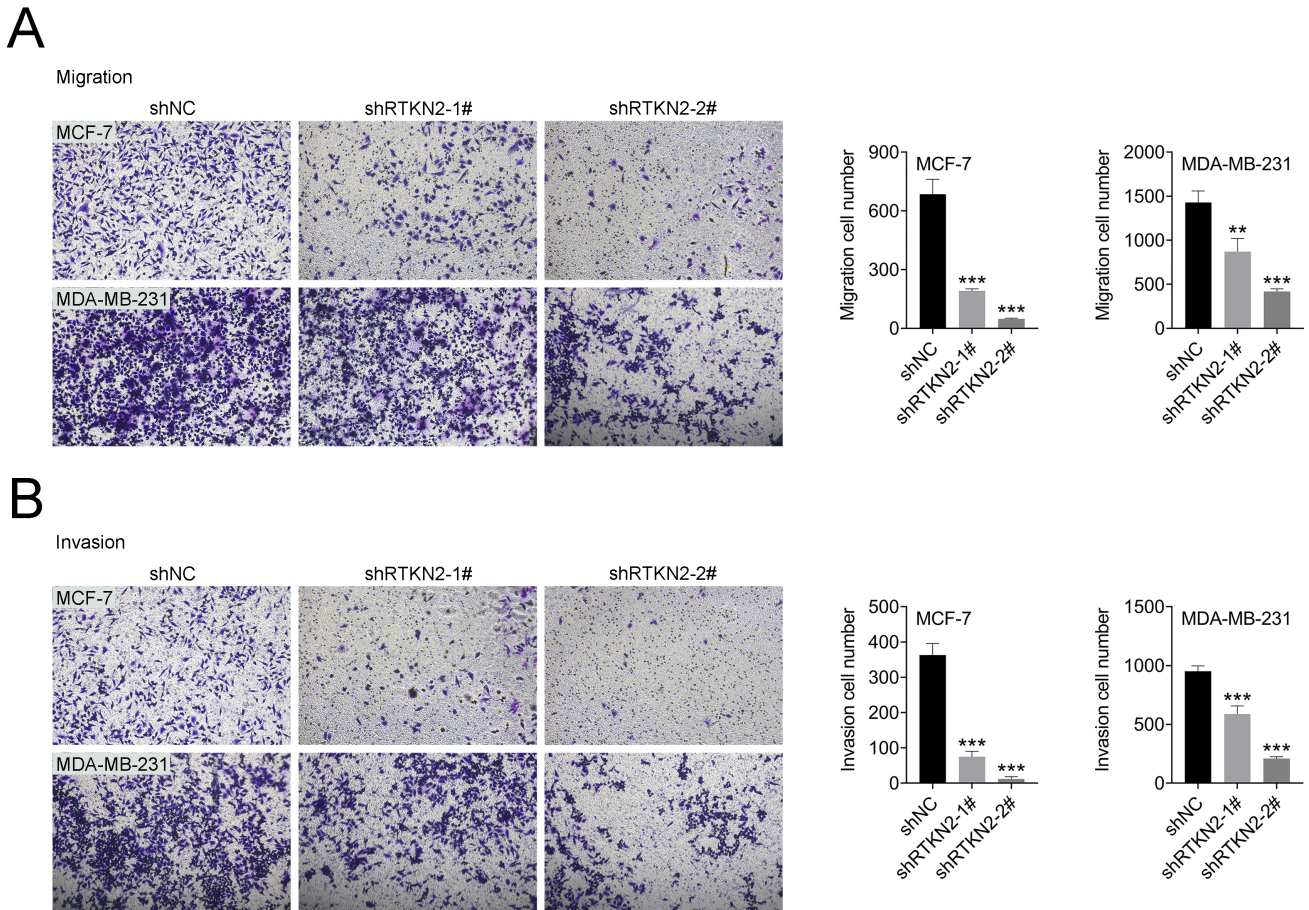


FIGURE 4. The depletion of RTKN2 reduced the migration of breast cancer cells. (A) The results of Transwell assays showing the migration of cells upon transfection with the indicated construct. We determined the numbers of invasive cells. (B) The results of Transwell assays showing the migration of cells upon transfection with the indicated construct. We determined the relative number of migrating cells. Error bars indicate SD. ** $p < 0.01$, *** $p < 0.001$. NC: negative control; RTKN2: Rhotekin 2.

3.5 The ablation of RTKN2 restrained the WNT/ β -catenin pathway in BCa cells

Next, we investigated the mechanisms responsible for how RTKN2 promotes the progression of BCa. Immunoblot analysis showed that the knockdown of RTKN2 reduced the phosphorylation of β -catenin as well as c-Myc cells, thus suggesting the suppression of the WNT/ β -catenin pathway in both MCF7 and MDA-MB-231 cells (Fig. 5A). In addition, the depletion of RTKN2 suppressed the nuclear localization of β -catenin (Fig. 5B), further confirming that the WNT/ β -catenin pathway had been suppressed. Therefore, the ablation of RTKN2 restrained the WNT/ β -catenin axis in BCa cells.

4. Discussion

BCa has a high recurrence rate and a lower survival rate [12]. This form of tumor is associated with a poor prognosis; thus, new therapeutic targets are urgently required. The existing therapeutic drugs for BCa are mainly targeted at PIK3CA, mTOR and other targets; trastuzumab and pertuzumab are commonly used for patients with early breast cancer [13]. Currently, there are several anti-angiogenic agents that can be used to treat BCa, including bevacizumab and remolunab, and sorafenib and sunitinib, both of which have multiple targets [13]. However, current therapeutic drugs are insufficient in terms of improving prognosis, and further research on the pathogenesis of BCa is still needed. In this study, we identified member of the Rhotekin family of proteins, RTKN2, which is expressed at high levels in BCa. We further confirmed that RTKN2 contributes to the progression of BCa.

As reported previously, RTKN2 is a Rho-GTPase binding protein that mediates multiple cellular processes, such as differentiation, and microtubule organization; all of these processes can influence the development and progression of tumors [3, 9]. The effects of RTKN2 on tumor progression have also been investigated. Previous research showed that RTKN2 can regulate cell proliferation, as well as the motility of multiple types of cancers, including osteosarcoma. The depletion of RTKN2 was previously shown to lead to cell cycle arrest in several types of tumors [14]. Similarly, in the present study, we also revealed that RTKN2 plays a role in the cell cycle of BCa. In addition, RTKN2 is known to contribute to the progression of endometrial carcinoma *via* the /Protein kinase B/Glycogen synthase kinase-3 beta (Akt/GSK3 β) pathway [10]. A previous study indicated that in hepatocellular carcinoma (HCC) cells, high expression levels of RTKN2 can lead to the upregulation of PCNA, thus promoting both proliferation and metastasis [11]. Phosphorylated PCNA can activate the telangiectasia ataxia mutation factor (ATM)/Akt/GSK3 β /Snail axis [15], thereby increasing the proliferation and motility of tumor cells. In the present study, we observed similar effects on the proliferation and motility of BCa cells.

RTKN2 is also known to influence the progression of BCa *via* the Wnt/ β -catenin axis [3]. This pathway is over-activated in BCa; this is a signal transduction pathway that is closely related to the growth and apoptosis of tumor cells. The Wnt/ β -catenin axis is an axis related to growth as well as development and features multiple regulators and links; this pathway exhibits low levels of activation in mature cells [16]. Intracellular β -catenin exists on the cell membrane in the form of

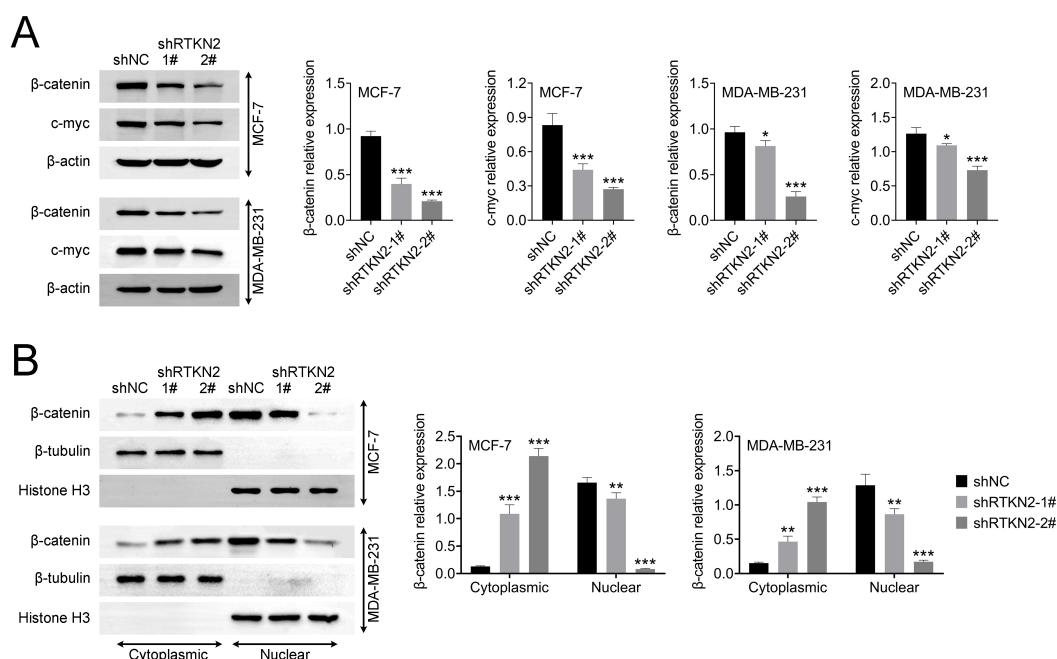


FIGURE 5. The ablation of RTKN2 restrained the WNT/ β -catenin pathway in breast cancer cells. (A) Immunoblot assays showing the expression and phosphorylation levels of β -catenin and c-myc in cells upon transfection with the indicated constructs. (B) Immunoblot assays showing the expression and phosphorylation levels of β -catenin, β -tubulin, and histone H3 in cells upon transfection with the indicated construct. Error bars indicate SD. ** $p < 0.01$, *** $p < 0.001$. NC: negative control; RTKN2: Rhotekin 2.

complexes [17]. Once a cell is stimulated by relevant signals, the degradation of β -catenin is inhibited, thus resulting in the accumulation of a large amount of intracellular β -catenin which can enter the nucleus and induce the transcription of downstream target genes such as *c-myc* and *cyclinD1* which are both responsible for tumor growth and metastasis [18]. The Wnt/ β -catenin axis is closely associated with BCa; it plays a role in the differentiation, proliferation, growth and apoptosis of [19]. Our data further confirmed the crucial role of this pathway in the progression of BCa.

This study has certain limitations that need to be considered. For example, we did not perform *in vivo* assays. Future work will include tumor growth assays using shRNAs of RTKN2 to confirm its role in tumor growth in a mouse model.

5. Conclusions

In summary, RTKN2 promotes the growth and migration of BCa cells by mediating the Wnt/ β -catenin axis.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

YTC, WTB and BLZ—designed the study and carried them out, supervised the data collection, analyzed the data, interpreted the data, prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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