

# ARHGEF19 promotes the growth of breast cancer *in vitro* and *in vivo* by the MAPK pathway

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Received: July 16, 2021 • Accepted: October 13, 2021

Published online: November 23, 2021

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## ABSTRACT

**Objective:** To assess the expression of ARHGEF19 in human breast cancer, investigate its role in breast cancer, and clarify the mechanism. **Methods:** Bioinformatics analysis, immunoblot, quantitative PCR, and immunohistochemical (IHC) assays were performed to assess ARHGEF19 expression in breast cancer. CCK-8 and Edu assays were conducted to reveal its role in breast cancer cell proliferation. Flow cytometry (FCM) assays and immunoblot were performed to confirm its effects on breast cancer apoptosis. Immunoblot was also performed to clarify the mechanism. Finally, tumor growth assays were aimed to confirm the role of ARHGEF19 in mice. **Results:** We observed that ARHGEF19 was highly expressed in human breast cancer. ARHGEF19 promoted breast cancer cell growth *in vitro*, and suppressed apoptosis. In addition, we found that ARHGEF19 could activate the MAPK pathway in breast cancer cells. Our findings further confirmed that ARHGEF19 contributed to breast cancer growth in mice. **Conclusion:** We observed that ARHGEF19 promoted the growth of breast cancer *in vitro* and *in vivo* via MAPK pathway, and presume it could serve as a breast cancer therapeutic target.

## KEYWORDS

ARHGEF19, proliferation, apoptosis, MAPK pathway, therapeutic target

## INTRODUCTION

Breast cancer is the most common malignancy in women worldwide [1]. The mortality among breast cancer patients has been declining because of early detection and improved treatment

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outcomes [2]. However, metastasis remains the leading cause of breast cancer-related death [3]. Due to the high metastatic nature of advanced breast cancer, current treatments have little effect [4, 5]. Targeted therapy of breast cancer has made a series of outstanding achievements, many targeted therapy drugs are used in clinical practice and clinical trials [6, 7]. To improve the prognosis of patients in advanced stage, new and more effective therapeutic targets are still needed.

Rho GTPases are highly expressed in numerous tumors and are associated with survival as well as metastasis [8, 9]. Rho guanine nucleotide exchange factors (RHOGEFs) are proteins which could regulate Rho GTPases, which can be involved in a variety of physiological processes, such as survival, development, differentiation, and motility [10]. ARHGEF19, a member of the RhoGEF family, has a variety of biological functions, affecting both development and tumor development [11]. ARHGEF19 promotes the formation of renal cilia in *Xenopus* fetuses by activating RhoA through the Wnt-PCP axis [12]. ARHGEF19 may be associated with the complications of coronary artery disease and chronic kidney disease [13].

The effects of ARHGEF19 on tumor growth and development have been widely revealed [11, 14, 15]. ARHGEF19 was overexpressed in renal carcinoma and associated with poor prognosis [16]. ARHGEF19 was also overexpressed in lung cancer, and through its interaction with HRAS or BRAF, it activates the ERK/MAPK signaling pathway, thereby promoting the growth and motility of lung cancer cells [15]. However, the possible role of ARHGEF19 in breast cancer progression is still unclear.

TCGA analysis showed that ARHGEF19 was highly expressed in breast cancer patients, suggesting that this protein may be involved in the process of breast cancer. We therefore investigate its expression in breast cancer and its role in breast cancer progression, and explore the mechanism in this study.

## MATERIALS AND METHODS

### Bioinformatic analysis

UALCAN was used to analyze ARHGEF19 expression in breast cancer tissues (<http://ualcan.path.uab.edu/index.html>).

### Antibodies, plasmids, and drugs

Anti-ARHGEF19 antibody (1:100 dilution for IHC, 1:500 dilution for immunoblot, PA5-66978, Invitrogen), anti-Ki67 antibody (1:200 dilution for IHC, ab15580, abcam), anti-Bax antibody (1:500 dilution, ab32503, abcam), anti-Bcl-2 antibody (1:500 dilution, ab182858, abcam), anti-cleaved-PRAP antibody (1:500 dilution, #9544S, CST), anti-cleaved-Caspase-3 antibody (1:1000 dilution, ab32042, abcam) anti-ERK1/2 antibody (1:500 dilution, ab184699, abcam), anti-pERK1/2 antibody (1:100 dilution for IHC, 1:500 dilution for immunoblot, ab278538, abcam), anti- $\beta$ -actin antibody (1:3000 dilution, ab8226, abcam).

The vector plasmid of shRNA was bought from Addgene, pLKO.1-vector (#10878). The ARHGEF1# 2# shRNA plasmids were constructed based on the pLKO.1-vector plasmids according to the previous study ([3]). U0126 (MEK inhibitor) was bought from Sigma.



## Immunohistochemical (IHC) assays

The operations in the assays were all approved by the Institutional Human Use Committee of The First Affiliated Hospital of Xi'an Jiaotong University. The expression of proteins in tumor and normal tissues were detected through IHC assays. The sections were fixed using 4% paraformaldehyde (PFA) for 30 min. The sections were subsequently blocked through 2% BSA treatment for another 20 min. The sections were subsequently incubated with antibodies for 2 h, and after washing, sections were incubated with secondary antibodies for 1 h. Then the substrate was applied to the sections to detect the expression of proteins.

## Cell culture and transfection

The human breast cancer cell lines, including MCF-7, MDA-MB-361, T-47D, and MDA-MB-436, and the normal breast cell line, HMEC, were all bought from ATCC and maintained in DMEM supplemented with 10% of FBS in a 5% CO<sub>2</sub> incubator.

The plasmids in this study were transfected into breast cancer cells by Lipofectamine 3000 (Invitrogen). The cells were transfected using 10 µL Lipofectamine<sup>®</sup> 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) in each well. After the incubation for 20 min at 20 °C, the transfection was completed.

## Quantitative PCR assays

Total RNA was extracted from tumor cells by the use of Trizol (Invitrogen; Thermo Fisher). The RNA was then reverse-transcribed through using reverse transcriptase kit (Promega). After obtaining cDNA, quantitative PCR was performed through using SYBR mixture (Takara). The relative mRNA levels of ARHGEF19 were normalized to GAPDH. The ARHGEF19 quantitative PCR primer sequences were: forward, 5'-GGAGGCTCGAAGTGTAGAGAT-3' and reverse, 5'-CCAATCGCCCTCTCGTGAG-3'; GAPDH primer sequences were: 5'-CGACCACTTTGTCAAGCTCA-3' and reverse, 5'-GGTTGAGCACAGGGTACTTTATT-3'.

## Immunoblot assay

Tissues or cells were lysed using RIPA buffer (Cell Signaling). All samples were used to extract the total proteins and separated them by SDS-PAGE. Then, the total proteins were transferred onto PVDF membranes, followed by blocking using 5% milk (without fat) in TBST buffer. All PVDF membranes were incubated with primary antibodies for 2 h. Then they were incubated with the corresponding secondary antibodies for another 1 h. Blots in the membranes were detected through ECL kit.

## CCK-8 assay

Cells were seeded into plates (96-well, 3 repeat) at a density of 1000 cells per well and then cultured for 48 h after transfection. Breast cancer cells were then incubated with CCK-8 kit for 4 h and the OD 450 value was measured.

## Edu assay

Cells were seeded into plates (24-well, 3 repeat) at a density of 10,000 cells per well and cultured for 48 h after transfection. Cells were then incubated with the Edu kit (abcam). Then Edu-positive cells were detected.



## Cell apoptosis

For apoptosis detection, Annexin V-FITC Apoptosis Detection Kit (Abcam) was used. MCF-7 cells and MDA-MB-361 cells at the density of  $1 \times 10^6$  cells/ml, were seeded in 6-well plates. Cells were digested, washed with PBS buffer and collected at 1000 rpm. Cells were stained in 200  $\mu$ l of binding buffer with 20  $\mu$ l FITC-labelled Annexin V and cultured in the dark for 20 min. Then cells were cultured with 5  $\mu$ l propidium iodide in the dark and analyzed in a flow cytometer (Becton-Dickinson, USA).

## Tumor growth *in vivo* assay

The tumor growth *in vivo* assays were approved by the Institutional Animal Use Committee of The First Affiliated Hospital of Xi'an Jiaotong University. The BALB/c nude mice (20–24 g, female, 8-week-old) were bought from Vital River Company. 10 nude mice were used (5 for each group). MCF-7 cells were transfected with ARHGEF19 shRNA plasmids to stably decrease its expression and then cells were injected into nude mice. The tumor volume was measured every 7 days. After 28 days, the tumors were analyzed and volume was compared between control and ARHGEF19-depleted groups.

## Statistics

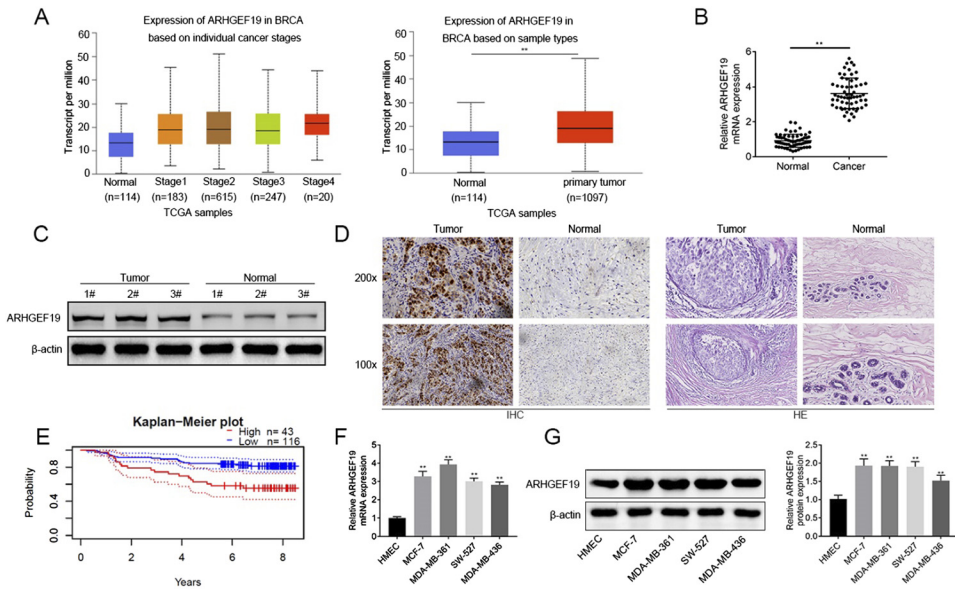
GraphPad 6.0 was used in this study for all statistical analysis. Data were represented as mean  $\pm$  SD. Student's *t*-test was used for comparing the results of *in vitro* and *in vivo* experiments in this study. \* or #,  $P < 0.05$  and considered as significant, \*\* or ##,  $P < 0.01$  and \*\*\* or ###,  $P < 0.001$ .

# RESULTS

## ARHGEF19 has high expression in human breast cancer

To clarify the role of ARHGEF19 in breast cancer, we first detected its transcript per million (TPM) in human breast cancer tissues through bioinformation analysis. Interestingly, we found that the TPM value was not different between normal and tumor tissues at different stages (Fig. 1A, left), and high TPM value in tumor tissues compared to normal tissues, suggesting the high expression of ARHGEF19 in breast cancer tissues (Fig. 1A, right). The mRNA levels of ARHGEF19 in normal and breast cancer tissues were detected. We observed high ARHGEF19 mRNA levels in breast cancer tissues (Fig. 1B). Similarly, high protein levels of ARHGEF19 in 3 representative breast cancer tissues were also detected, through immunoblot assays (Fig. 1C). In addition, IHC assays further confirmed high ARHGEF19 expression in breast cancer tissues (Fig. 1D). H&E staining (Fig. 1D) showed that in tumor tissues the cells are compact and large in size, with diverse nuclei, obvious nucleoli and deep staining, further confirmed the tumor tissues (Fig. 1D). We further observed that ARHGEF19 expression was correlated with the prognosis of patients with breast cancer via PrognosScan database ( $P = 0.000744$ , Fig. 1E). Subsequently, we detected the mRNA levels and protein levels of ARHGEF19 in human breast cancer cell lines, including MCF-7, MDA-MB-361, T-47D, and MDA-MB-436, and normal breast cell line, HMEC, through qPCR and immunoblot assays, respectively. We found that both mRNA levels and protein levels of ARHGEF19 were higher in breast cancer cells than in normal cells (Fig. 1F, G).





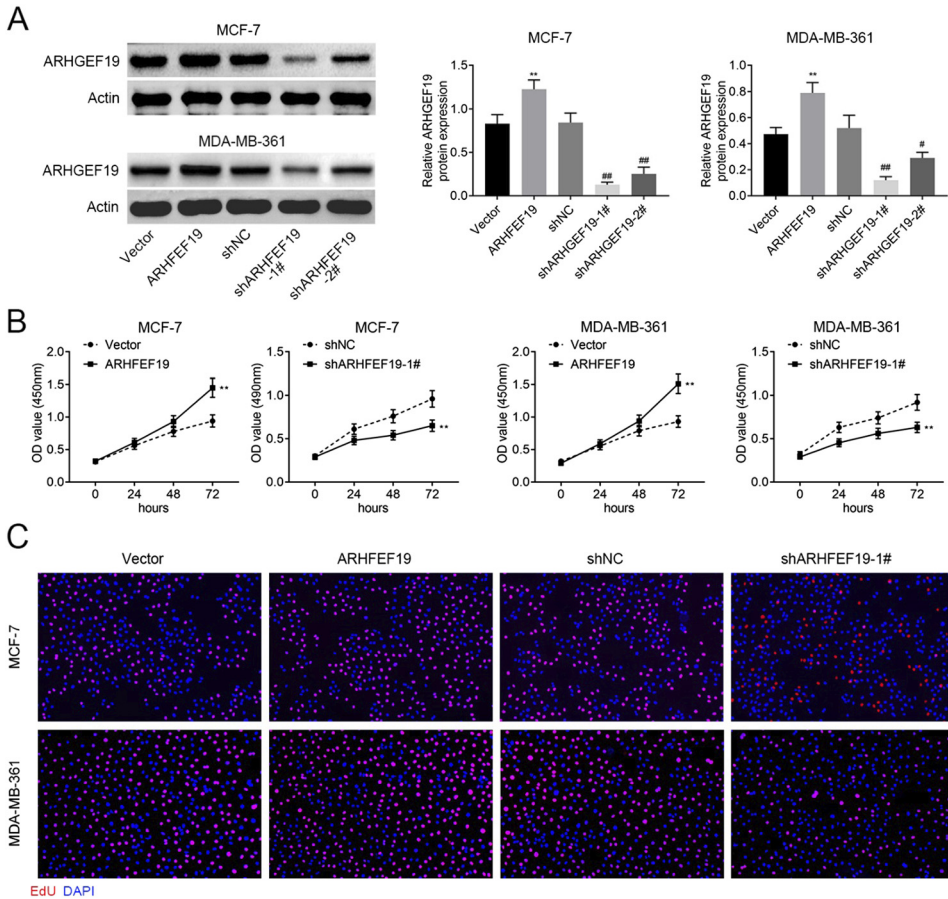
**Fig. 1. ARHGEF19 had high expression in human breast cancer tissues and cell lines.**

(A). TCGA database showed the transcript per million of ARHGEF19 in breast cancer (BRCA) tumor tissues at different tumor stages (left) and the different mRNA levels of tumor tissues and normal tissues (right). (B). qPCR assays showed the mRNA levels of ARHGEF19 in breast cancer tissues and normal tissues from our hospital. (C). Immunoblot assays showed the expression of ARHGEF19 in 3 representative breast cancer primary tissues and normal tissues. (D). IHC assays showed the expression of ARHGEF19 in breast cancer primary tissues and adjacent tissues (100 and 200 magnification, respectively). (E). The effects of ARHGEF19 on the prognosis of patients with breast cancer was revealed via PrognScan database ( $P = 0.000744$ ). (F). Quantitative PCR assays showed the expression of ARHGEF19 in HMEC, MDA-MB-361, T-47D, and MDA-MB-436 cells. (G). Immunoblot assays showed the expression of ARHGEF19 in HMEC, MDA-MB-361, T-47D, and MDA-MB-436 cells. All experiments were repeated three times. Data are presented as mean  $\pm$  SD. \*\*  $P < 0.01$

### ARHGEF19 contributes to the growth of breast cancer cells *in vitro*

Subsequently, we aimed to study the effects of ARHGEF19 on the growth of breast cancer cells. ARHGEF19 shRNA and overexpression plasmids and the corresponding control plasmids were transfected into MCF-7 cells and MDA-MB-361 cells, respectively, to alter their expression levels. We found that the transfection of two types of its shRNA plasmids (1# and 2#) all decreased the expression of ARHGEF19, whereas 1# had the more obvious silencing efficiency, and transfection of ARHGEF19 overexpression plasmids increased its expression levels in MCF-7 cells and MDA-MB-361 cells (Fig. 2A). We therefore used shRNA1# in the next assays. Performing CCK-8 assays, we observed that ARHGEF19 overexpression increased the proliferation of MCF-7 cells, whereas its depletion suppressed MCF-7 cell and MDA-MB-361 cell proliferation (Fig. 2B). Further through Edu assays, we also found ARHGEF19 overexpression increased the Edu-positive cell numbers, and its depletion decreased the cell numbers,





**Fig. 2. ARHGEF19 contributes to the survival and proliferation of breast cancer cells *in vitro*.**

(A). Immunoblot showed the expression of ARHGEF19 in MCF-7 cells and MDA-MB-361 cells transfected with the indicated plasmids. (B). CCK-8 assays showed the proliferation capacity of MCF-7 cells and MDA-MB-361 cells transfected with the indicated plasmids. (C). EdU assays showed the percentage of EdU-positive MCF-7 cells and MDA-MB-361 cells transfected with the indicated plasmids. All experiments were repeated three times. Data are presented as mean  $\pm$  SD. pcDNA3.1-ARHGEF19 vs pcDNA3.1, \*\*  $P < 0.01$ , sh-ARHGEF19 vs sh-NC, ##  $P < 0.01$ . NC, negative control

suggesting ARHGEF19 promoted MCF-7 and MDA-MB-361 cell growth (Fig. 2C). Therefore, we assumed ARHGEF19 contributed to the growth of breast cancer cells *in vitro*.

### ARHGEF19 suppressed the apoptosis of breast cancer cells

Since ARHGEF19 promoted breast cancer cell growth, we next observed its effects on breast cancer cell apoptosis. Through FCM assays, we demonstrated that ARHGEF19 overexpression suppressed the apoptosis of MCF-7 and MDA-MB-361 cells, and the knockdown of ARHGEF19 stimulated the apoptosis (Fig. 3A). Similarly, the immunoblot assays showed that ARHGEF19

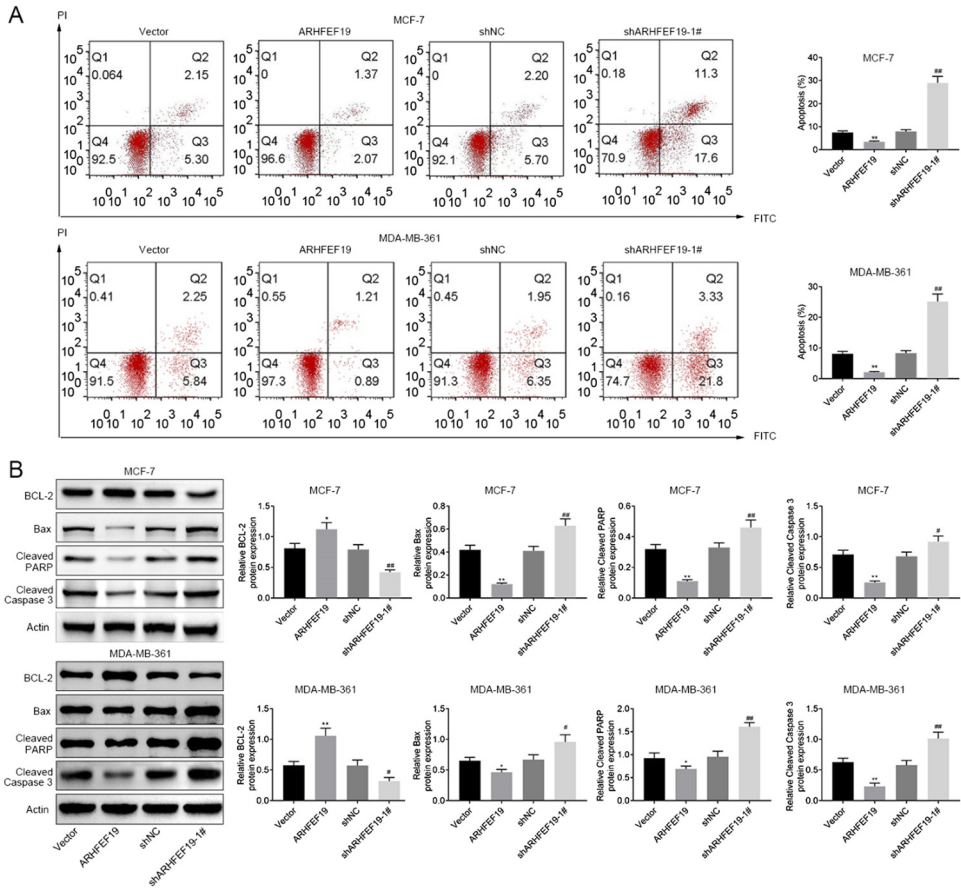


Fig. 3. ARHGEF19 suppressed the apoptosis of breast cancer cells.

(A). FCM assays showed the apoptosis degree of MCF-7 cells and MDA-MB-361 cells transfected with the indicated plasmids. (B). Immunoblot showed the expression of Bcl-2, Bax, cleaved-PARP, and cleaved-caspase-3 in MCF-7 cells and MDA-MB-361 cells transfected with the indicated plasmids. All experiments were repeated three times. Data are presented as mean ± SD. pcDNA3.1-ARHGEF19 vs pcDNA3.1, \*  $P < 0.05$ , \*\*  $P < 0.01$ , sh-ARHGEF19 vs sh-NC, #  $P < 0.05$ , ##  $P < 0.01$ . NC, negative control

overexpression increased the protein levels of Bcl-2, and decreased the expression of Bax, Cleaved-PARP, and Cleaved-Caspase-3 (Fig. 3B). However, its depletion decreased Bcl-2 protein levels, and increased the expression of Bax, Cleaved-PARP, and Cleaved-Caspase-3 in MCF-7 and MDA-MB-361 cells (Fig. 3B). These data confirmed that ARHGEF19 suppressed the apoptosis of breast cancer cells.

### ARHGEF19 activates MAPK pathway in breast cancer cells

Previous data showed that ARHGEF19 affected both proliferation and apoptosis of MCF-7 and MDA-MB-361 cells. We then investigated the possible molecular mechanism. Previous studies



showed ARHGEF19 could regulate the MAPK pathway and affect cancer progression, and we wished to clarify whether ARHGEF19 affects the proliferation and apoptosis of MCF-7 and MDA-MB-361 cells via this pathway [15]. ARHGEF19 overexpression increased the phosphorylation levels of ERK1/2, a regulator in MAPK pathway, in MCF-7 and MDA-MB-361 cells (Fig. 4). In addition, knockdown of ARHGEF19 decreased the phosphorylation levels of ERK1/2 in MCF-7 and MDA-MB-361 cells (Fig. 4).

We then performed the rescue assays using the MEK inhibitor (10 $\mu$ M U0126) and found that ARHFEF19 overexpression promoted the proliferation of MCF7 cells, confirmed by CCK-8 assays (Fig. 5A). However, the treatment of U0126 at the concentration of 10  $\mu$ M obviously reversed the promotion of MCF-7 cells after ARHFEF19 overexpression (Fig. 5A). We further performed Edu assays and found ARHFEF19 overexpression promoted MCF-7 cell proliferation, whereas U0126 treatment suppressed the proliferation of MCF-7 cells after ARHFEF19 overexpression (Fig. 5B). FCM assays further confirmed that inhibition of the MEK pathway by U0126 treatment reversed the suppression of MCF-7 cell apoptosis caused by ARHFEF19

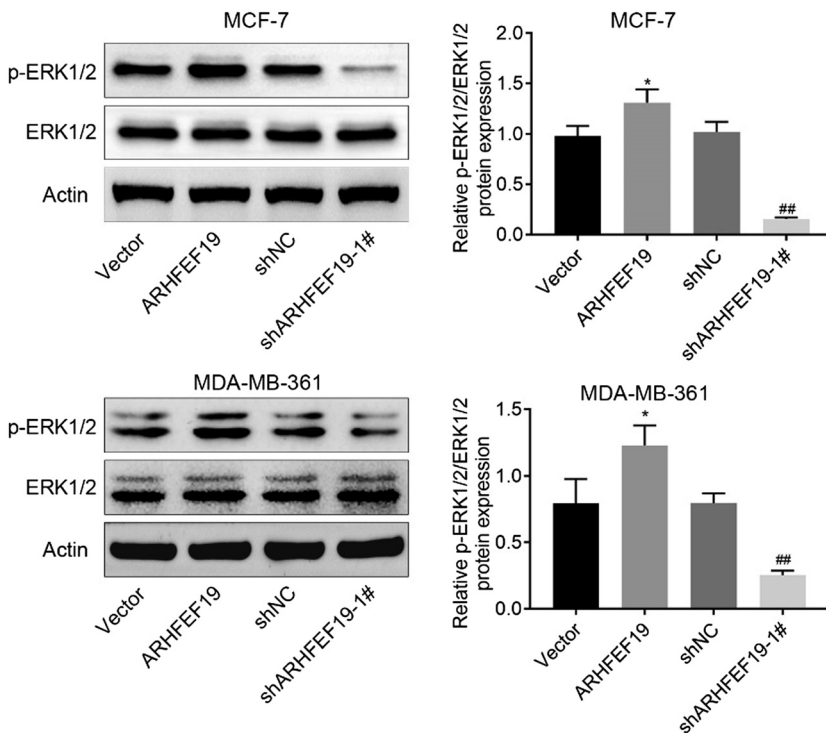
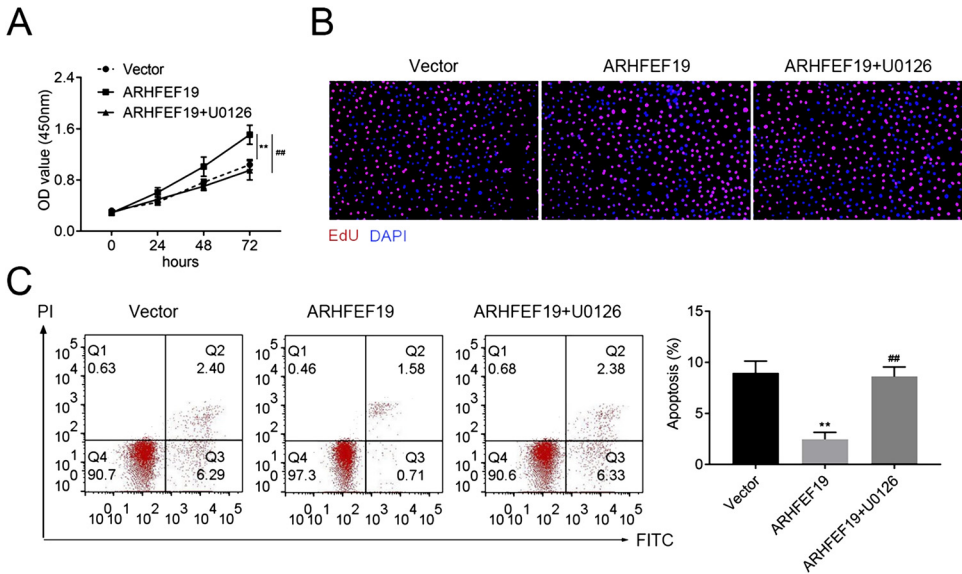


Fig. 4. ARHGEF19 activates MAPK pathway in breast cancer cells.

Immunoblot showed the expression of p-ERK-1/2 and ERK1/2 in MCF-7 cells and MDA-MB-361 cells transfected with the indicated plasmids. Data are presented as mean  $\pm$  SD. pcDNA3.1-ARHGEF19 vs pcDNA3.1, \*  $P < 0.05$ , sh-ARHGEF19 vs sh-NC, ##  $P < 0.01$ . NC, negative control







**Fig. 5. Inhibition of the MEK pathway reversed the effects of ARHGEF19 on the proliferation and apoptosis of breast cancer cells.**

(A). CCK-8 assays showed the proliferation capacity of MCF-7 cells treated with the indicated plasmids or drugs. (B). Edu assays showed the percentage of Edu-positive MCF-7 cells treated with the indicated plasmids or drugs. (C). FCM assays showed the percentage of apoptosis of MCF-7 cells treated with the indicated plasmids or drugs. Data are presented as mean  $\pm$  SD. pcDNA3.1-ARHGEF19 vs pcDNA3.1, \*\*  $P < 0.01$ , sh-ARHGEF19 vs sh-NC, ##  $P < 0.01$ , sh-ARHGEF19 + U0126 vs sh-ARHGEF19 + control

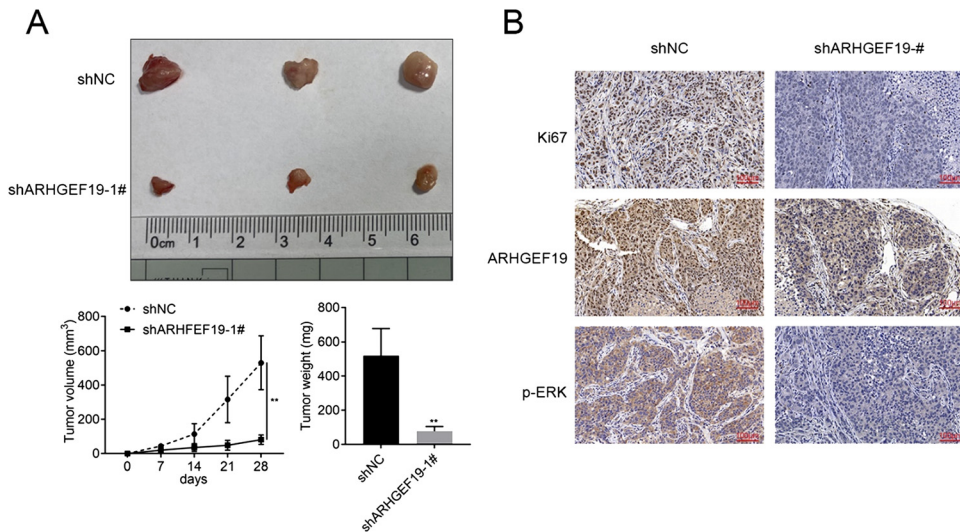
overexpression (Fig. 5C). These data confirmed that ARHFEF19 affected the proliferation and apoptosis of breast cancer cells via activating the MEK pathway.

Therefore, we assumed that ARHGEF19 activated the MAPK pathway in breast cancer cells.

### ARHGEF19 stimulates tumor growth of breast cancer cells in mice

Since ARHGEF19 affected breast cancer cell growth *in vitro*, we then investigated its effects on tumor growth in mice. We further explored its effects on breast cancer growth *in vivo*. MCF-7 cells with ARHGEF9 stably ablated were constructed, and then  $10^5$  MCF-7 cells (control or ARHGEF19 stably depleted) were injected subcutaneously into nude mice. The tumor volume was assessed every 7 days and until 28 days. Interestingly, ARHGEF19 ablation dramatically restrained breast cancer growth (Fig. 6A). We further found that the expression levels of ARHGEF19 were obviously decreased in ARHGEF19-depleted tumor tissues (Fig. 5B). In addition, the protein levels of Ki67 in ARHGEF19-depleted tissues were decreased, suggesting the inhibition of cell proliferation (Fig. 6B). We also observed that ARHGEF19 depletion suppressed the phosphorylation levels of ERK1/2 in tumor tissues (Fig. 6C). Therefore, we assumed ARHGEF19 stimulated breast cancer growth in mice.





**Fig. 6. ARHGEF19 stimulates tumor growth of breast cancer cells in mice.**

(A). MCF-7 cells stably transfected with the sh-Control (sh-NC), ARHGEF19 shRNA plasmids were injected subcutaneously into the abdomen of nude mice ( $n = 5$  for each group) to induce tumor growth. The tumor growth curves were recorded according to the measure of tumor volume every 7 days, and the tumor volume was measured. Three representative tumors are shown (B). IHC assays show the expression of ARHGEF19, Ki67, and p-ERK1/2 in sh-NC or ARHGEF19-depleted tumor tissues. Data are presented as mean  $\pm$  SD. \*\*  $P < 0.01$

## DISCUSSION

Breast cancer is known as the most common malignancy in women [4]. Early breast cancer is curable, but patients with early breast cancer have no symptoms [17]. Therefore, patients are often in a progressive stage when they seek medical attention, when lymph node metastasis is prone to have occurred [18]. Advanced breast cancer does not respond well to current major treatments, including surgical resection, radiotherapy and chemotherapy [19]. Therefore, targeted therapy for breast cancer is still the best choice [20]. In this study, we found a Rho guanine nucleotide exchange factor protein, ARHGEF19 with high expression in human breast cancer. ARHGEF19 affects the proliferation and apoptosis of breast cancer cells, and has the potential to serve as a target of breast cancer.

Performing CCK-8 and Edu assays, we observed the effects of ARHGEF19 on breast cancer growth. Furthermore, through FCM and immunoblot assays, we confirmed that ARHGEF19 affected the apoptosis of breast cancer cells. In fact, the effects of ARHGEF19 on cancer progression have been widely revealed in previous studies [11, 15]. ARHGEF19 was overexpressed in renal carcinoma and associated with the prognosis of patients [16]. ARHGEF19 also promoted the growth and motility of lung cancer cells [11]. In the TCGA database, ARHGEF19 had high expression in human breast cancer tissues, consistently with our *in vitro* and *in vivo* data. These studies all confirmed the potential of ARHGEF19 as a promising target for cancer treatment.



In addition, most RhoGEFs have both DH and PH domains, both of which have critical roles [10]. Therefore, we presume that in breast cancer cells, ARHGEF19 activate the MAPK pathway through these domains. Similarly, a previous study showed ARHGEF19 was associated with the binding to BRAF in lung cancer cells [15].

In this study, we also observed that ARHGEF19 affected breast cancer progression via targeting the MAPK pathway. Previous studies have confirmed that ARHGEF19 could interact with HRAS or BRAF to activate the MAPK pathway, thus promoting the growth and motility of lung cancer cells [15]. ERK1/2 are critical in mediating cell growth [21]. Activation of the MAPK pathway plays an important role in the occurrence of breast cancer [22]. It is involved in the process of breast cancer growth, motility, apoptosis and other processes, and regulates the malignant progression of tumors [23]. For example, F1012-2 induced DNA damage response via targeting the MAPK pathway in breast cancer [24]. Another study showed that EGFL7 regulated the growth of breast cancer cells via the MAPK pathway [25]. These studies, together with our findings, confirmed that the MAPK pathway could serve as a promising target of breast cancer.

In conclusion, we found ARHGEF19 was highly expressed in human breast cancer. ARHGEF19 promoted breast cancer cell growth *in vitro*, and suppressed apoptosis. Our findings further confirmed that ARHGEF19 contributed to breast cancer growth in mice. Regarding the mechanism, we found that ARHGEF19 promoted the growth of breast cancer via targeting the MAPK pathway. We assume that ARHGEF19 could serve as a promising breast cancer target.

**Funding:** This work was supported by the Shaanxi Provincial Key Research and Development Program (Grant No. 2021SF-203).

**Competing interests:** The authors state that there are no conflicts of interest to disclose.

**Ethics approval:** All procedures performed in studies involving human participants were in accordance with the standards upheld by the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University and with those of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects.

All animal experiments were approved by the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University for the use of animals and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

**Statement of informed consent:** Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

**Contribution of authors:** Ligang Niu and Yuhui Zhou designed the study, supervised the data collection, Wei Zhang analyzed the data, interpreted the data, Yu Yan and Yu Ren prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

## ACKNOWLEDGMENTS

Not applicable.



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