

# MicroRNA-421-targeted PDCD4 regulates breast cancer cell proliferation

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**Abstract.** MicroRNAs (miRNAs) are expressed aberrantly in various types of cancer, and negatively regulate the expression of target genes which may be useful in therapeutic strategies in several biological processes. In the present study, the expression levels and the effects of miRNA (miR)-421 in breast cancer tissues and MCF-7 and MDA-MB-231 cells were evaluated to elucidate therapeutic targets in breast cancer cells. The putative targets of miR-421 were predicted by bioinformatics approaches, and the expression levels of miR-421 were measured in MCF-7 and MDA-MB-231 cells by reverse transcription-quantitative polymerase chain reaction analysis following miR-421 knockdown. The rates of cell proliferation, migration capacity, invasiveness and apoptosis were determined in miR-421 inhibitor-transfected MCF-7 and MDA-MB-231 cells. The expression levels of target proteins regulated by miR-421 in MCF-7 and MDA-MB-231 cells were analyzed by western blot analysis. miR-421 was increased significantly in breast cancer tissues and cells, and was regulated by miR-421 antisense oligonucleotides. The knockdown of miR-421 in MCF-7 and MDA-MB-231 cells decreased cell proliferation, migration capacity and invasiveness, and promoted apoptosis compared with control groups. The expression of target protein programmed cell death 4 (PDCD4) were decreased in MCF-7 and MDA-MB-231 cells transfected with miR-421 inhibitors. These results suggested a correlation between miR-421 and PDCD4, and physiological functions of breast cancer cells, suggesting that miR-421 may be a potential strategy in the therapy of breast cancer.

## Introduction

Breast cancer (BC) is one of the most common types of cancer and main causes of cancer-associated mortality in women worldwide (1). The onset of BC typically occurs between 20 and

60 years of age, and the incidence of this disease has markedly increased in China in recent years (2). Due to improvements in diagnostic and therapeutic methods, including surgery, radiotherapy, chemotherapy, hormone therapy, immunotherapy, stem cell therapy and gene silencing, the mortality rate of BC has markedly decreased (3-5). However, the molecular mechanisms of BC remain to be fully elucidated. It is well known that an accumulation of hereditary and epigenetic changes, including oncogene activation, tumor suppressor gene inactivation (6), changes in intercellular complex signal networks, microenvironment (7,8) and epigenetic changes (9,10) contribute to the formation of malignant tumors. An increasing number of studies have indicated that epigenetic changes may represent important events in the pathogenesis of BC (11-13), and that gene silencing at the post-transcriptional level may be an important epigenetic change (14,15).

Although prognostic microRNAs (miRNAs), including miRNA (miR)-21, miR-489 and miR125b, have been identified in BC (16), the underlying pathways that regulate the invasiveness of BC remain to be elucidated. miRNAs are able to bind to their target mRNAs to induce degradation or suppress translation (17). miRNAs are involved in cellular processes, including cell differentiation, cell proliferation, apoptosis and tumor inhibition (18-20). Previous studies have shown that miRNAs are critical in the development and progression of BC, for example, by functioning as tumor suppressors or oncogenes.

miR-421 is a tumor suppressor that is aberrantly expressed in several types of cancer. For example, miR-421 inhibits the proliferation and metabolism of prostate cancer cells by targeting Cullin 4B (21) and suppresses BC metastasis by targeting metastasis associated 1 (22). Furthermore, the higher positive detection rate of miR-421 compared with serum carcino-embryonic antigen in gastric cancer indicates that miR-421 may serve as a superior diagnostic marker (23). A number of studies have reported that miR-421 acts as an oncogene, however, its underlying mechanisms in BC remain to be fully elucidated.

The aim of the present study was to investigate the regulatory role of miR-421 in BC and the underlying molecular mechanism responsible.

## Materials and methods

**Patients.** A total of 52 BC tissue samples were collected from 52 patients (52 females; 43-68 years old; mean age

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55.6±12.5 years) histologically diagnosed with BC between 2015 and 2017 at the Second Affiliated Hospital of Xi'an Medical University (Xi'an, China). In addition, 52 normal tissue samples (52 females; 44-74 years old; mean age 50.2±10.4 years) were obtained from the same hospital. All protocols were approved by the Ethics Committee of the Second Affiliated Hospital of Xi'an Medical University and written informed consent was provided by all participants. The clinical characteristics of the patients with BC are summarized in Table I.

**Cell culture.** The MCF-7 and MDA-MB-231 BC cells, in addition to the normal Hs578bst cell line, were purchased from the Cell Resource Center of the Shanghai Academy of Sciences (Shanghai, China). The cells were cultured in an atmosphere containing 5% CO<sub>2</sub> at 37°C in DMEM supplemented with 10% FBS (both from Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted from the BC tissues, MCF-7 cells and MDA-MB-231 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and was quantified using a photometer. cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. All PCR primers were designed and synthesized by Invitrogen; Thermo Fisher Scientific, Inc. qPCR analysis was performed according to the manufacturer's protocol. The reaction mixture comprised the following: cDNA (0.5 µl), forward primer (0.5 µl), reverse primer (0.5 µl), 2.5 mM dNTPs (2 µl), 10 U/µl DNA polymerase (0.5 µl), 5X buffer (4 µl) and ddH<sub>2</sub>O (12 µl). The thermocycling conditions were as follows: 35 cycles at 95°C for 5 min, 95°C for 15 sec and 56°C for 40 sec. U6 was used as an internal reference. The primers used were as follows: miR-421, forward, 5'-ACACTC CAGCTGGGATCAACAGACATTAATTG-3' and reverse, 5'-TGGTGTCTCGTGGAGTCG-3'; U6, forward, 5'-CTCGCT TCGGCAGGACA-3' and reverse, 5'-AACGCTTCACGA ATTTGCGT-3'; PDCD4, forward, 5'-TCTGGGAAAGGA AGGGGACTAC-3' and reverse, 5'-TTCATAAACACAGTT CTCCTGGTCAT-3'; U6 forward, 5'-CTCGCTTCGGCAGCA CA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The results were quantified using the 2<sup>-ΔΔC<sub>q</sub></sup> method (24).

**Immunohistochemistry.** Tissues slides (1.2x1.2 cm) were incubated with primary PDCD4 antibody (dilution 1:600; cat. no. PA5-20309; Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 30 min and were washed twice with cold PBS. Subsequently, the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) secondary antibody (dilution 1:100; cat. no. 31460; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. The slides were developed with 3,3'-diaminobenzidine and the nuclei were lightly stained with hematoxylin. The expression of PDCD4 was evaluated by observing at least five fields of the slides under an inverted non-confocal microscope (CKX53 type, 4000K LED light; Olympus Corporation, Tokyo, Japan), with at least 100 cells per field assessed.

**Cell transfection.** miR-421 mimics, miR-421 inhibitors, mock miRNAs and the luciferase reporter plasmid were designed and synthesized by Invitrogen; Thermo Fisher Scientific, Inc. The MCF-7 and MDA-MB-231 cells were transfected with miR-421 mimics, miR-421 inhibitors or mock miRNAs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

**Western blot analysis.** The cells were lysed and proteins were quantified using a BCA assay. The proteins (25 µg/lane) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were incubated with primary antibodies against PDCD4 (dilution 1:600; cat. no. PA5-20309; Invitrogen; Thermo Fisher Scientific, Inc.) and GAPDH (dilution 1:800; cat. no. MA5-15738; Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 30 min. The membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) secondary antibody. The band intensities were evaluated using ImageJ 1.47 software (National Institutes of Health, Bethesda, MD, USA).

**Luciferase reporter assay.** A search for putative targets of miR-421 was performed with TargetScan Human7.2 software. Target genes of miR-421 were predicted by TargetScan ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)). To determine whether PDCD4 is a direct target gene of miR-421, luciferase reported assays were performed. The luciferase reporter plasmids pGL3-PDCD4 3'UTR wild-type (WT) and pGL3-PDCD4 3'UTR mutant type (Mut) were designed and synthesized by Invitrogen; Thermo Fisher Scientific, Inc. The MCF-7 and MDA-MB-231 cells were seeded in 24-well plates and cultured until they reached 40% confluence. The cells were then transfected with miR-421 mimics, inhibitors or mock miRNA using Lipofectamine® 2000 according to the manufacturer's protocol. After 24 h incubation at 37°C, the cells were harvested and luciferase activity was assessed using the Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA). Each experiment was performed in triplicate.

**Cell proliferation assay.** Following transfection, the proliferation of the MCF-7, MDA-MB-231 and Hs578bst cells was determined using Cell Counting Kit-8 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's protocol. For each experimental group, six duplicate wells were assessed.

**Cell migration and invasion assays.** The MCF-7 and MDA-MB-231 cells were cultured and treated as described above, following which cell migration and invasion were assessed using Transwell assays. The filters (Corning Incorporated, Corning, NY, USA) were washed in serum-free DMEM and placed into 24-well plates. The lower Transwell chamber contained DMEM supplemented with 10% FBS. A total of 3x10<sup>4</sup> BC cells were seeded in the upper chamber with 200 µl DMEM supplemented with 0.1% bovine serum albumin (BD Biosciences, Franklin Lakes, NJ, USA), with a 2 mg/ml Matrigel-coated membrane containing 8-µm pores. The Transwell chamber was subsequently incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub> for 24 h. Following incubation, any cells

Table I. Association between clinicopathological factors and miR-421 levels.

Clinicopathological factor	No. of patients (n=52)	Level of miR-421		P-value
		High, n (%) (n=28)	Low, n (%) (n=24)	
Age (years)				<0.001
<55	20	11 (39.28)	9 (37.50)	
≥55	32	17 (60.71)	15 (62.50)	
Pathological stage				<0.001
I+II	38	20 (71.4)	18 (75.00)	
III+IV	14	8 (28.57)	6 (25.00)	
Histological grade				<0.001
I+II	34	18 (64.28)	16 (66.66)	
III+IV	18	10 (35.71)	8 (33.33)	
Lymph node status				<0.001
Negative	32	16 (57.14)	16 (66.66)	
Positive	20	12 (42.85)	8 (33.33)	
ER status				0.069
Negative	26	15 (53.57)	11 (45.83)	
Positive	26	13 (46.42)	13 (54.16)	
PR status				0.053
Negative	30	17 (60.71)	13 (54.16)	
Positive	22	11 (39.28)	11 (45.83)	
HER-2 status				0.074
Negative	40	21 (75.00)	19 (79.16)	
Positive	12	7 (25.00)	5 (20.83)	
Ki67				0.081
<15%	29	17 (60.71)	12 (50.00)	
≥15%	23	11 (39.28)	12 (50.00)	

P-values were calculated by  $\chi^2$  test, Fisher's exact test, Student's t-test or Mann-Whitney U tests. P<0.05 was considered statistically significant. miR, microRNA; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor-2.

remaining on the upper membrane surface were removed with a cotton swab, whereas cells on the lower surface of the membrane were fixed in 10% formalin at room temperature for 30 min and stained with 0.5% crystal violet. Images were captured of six randomly selected fields of view using an inverted microscope (Nikon Corporation, Otsu, Japan) at x200 magnification. For the migration assay, transfected cells ( $2 \times 10^4$  cells/chamber) were seeded in the top chamber, as above, without Matrigel. After 24 h, the migrated cells were lysed in glacial acetic acid and the solution was transferred to a 96-well culture plate. Colorimetry was performed at 560 nm to determine the optical density. Each experiment was performed in triplicate.

**Statistical analysis.** All results were analyzed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).  $\chi^2$  or Fisher's exact test was used to compare categorical variables as appropriate. Student's t-test, Mann-Whitney U test and one-way analysis of variance with Tukey's post hoc test were used to compare continuous data. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Patient characteristics.** To investigate the expression of miR-421 in patients with BC with different clinicopathological features, 52 patients with BC were divided into two groups (28 in the miR-421 high expression group and 24 in the low expression group). Patients' characteristics are summarized in Table I. No significant differences were observed in clinicopathological factors, including estrogen receptor status, progesterone receptor status, human epidermal growth factor receptor-2 status, and Ki-67 status, between the miR-421 high expression group and miR-421 low expression group. However, an older age at diagnosis was associated with a high expression of miR-421 in patients with BC (P<0.001). The pathological stage, histological grade and lymph node status were also closely associated with the expression of miR-421 (P<0.001).

**Protein expression of PDCD4 in BC tissues.** The protein expression of PDCD4 in tissues of patients with BC were analyzed by immunohistochemistry, although only 15 pairs of

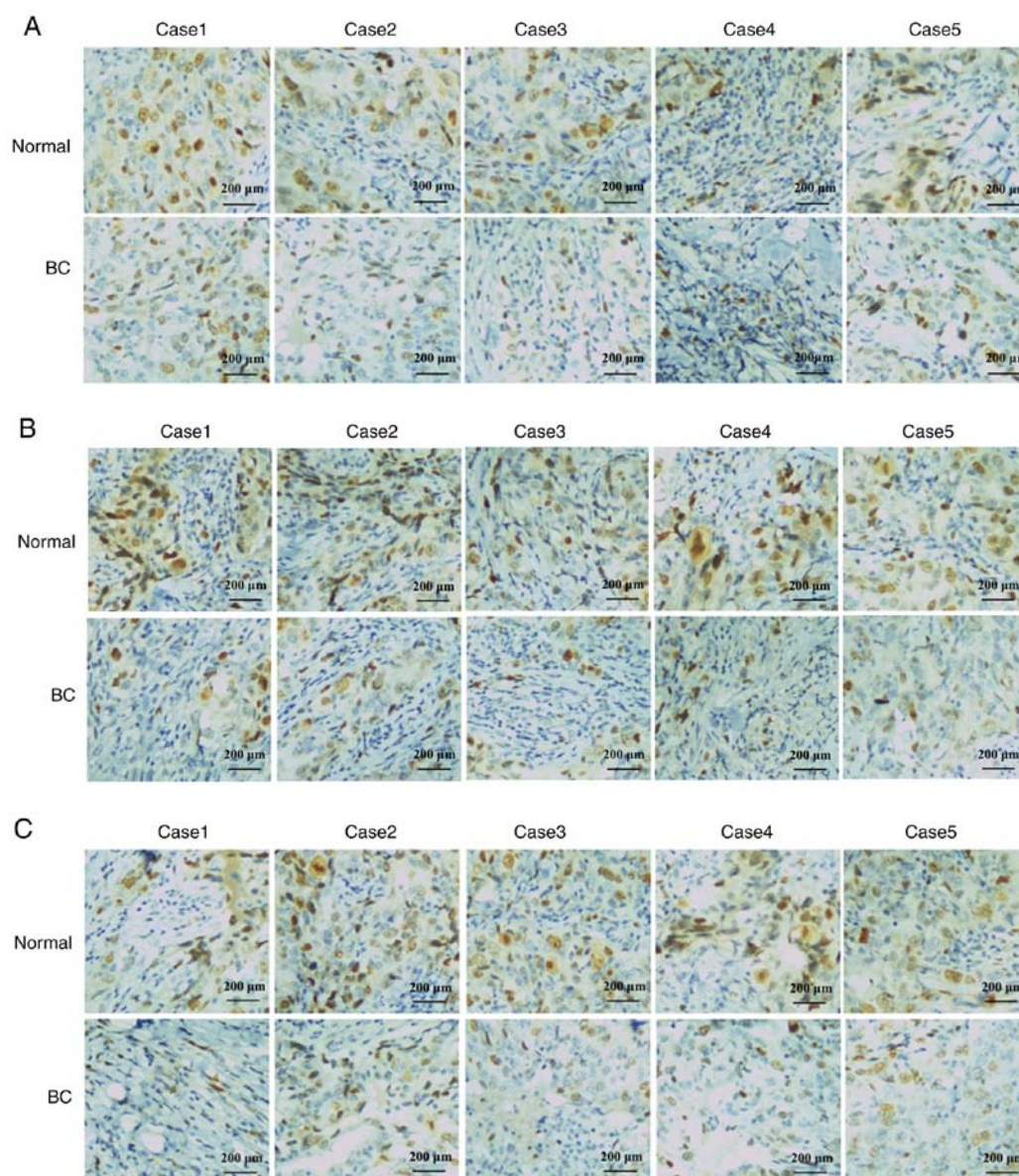


Figure 1. PDCD4 protein was detected in three sets of normal and breast cancer tissues using immunohistochemistry. (A) PDCD4 protein expression was detected in five normal and five BC tissues using immunohistochemistry, and decreased substantially in BC tissues compared with normal control tissues. (B) PDCD4 protein expression was determined by immunohistochemistry in five normal and five BC tissues, and was notably decreased in BC tissues. (C) PDCD4 protein expression was analyzed in five normal and five BC tissues by immunohistochemistry, but was downexpressed in BC tissues. BC, breast cancer; PDCD4, programmed cell death 4.

immunohistochemistry results are presented. PDCD4 protein expression was detected in three sets of five normal and five BC tissues in each set, using immunohistochemistry (Fig. 1A-C), and was decreased substantially in BC tissues compared with normal control tissues.

**Expression of miR-421 in BC tissues and cell lines.** RT-qPCR analysis was performed to assess the expression of miR-421 in BC tissues, Hs578bst cells, MCF-7 cells and MDA-MB-231 cells (Fig. 2A and B). The expression of miR-421 was significantly increased in the BC tissues and cells compared with the normal controls. By contrast, the protein expression of PDCD4 was markedly downregulated in BC cells compared with the controls (Fig. 2C and D). These results suggested that the overexpression of miR-421 was associated with the downregulation of PDCD4 in BC.

*PDCD4 is a direct target gene of miR-421.* PDCD4 was found as a potential target gene of miR-421. Luciferase reporter assays were performed to further confirm whether PDCD4 is a direct target of miR-421. The 3'-UTR of PDCD4 mRNAs, including putative binding sites of miR-421 together with their corresponding mutated sequences, were cloned into vectors and co-transfected into BC cells with miR-421 mimics, inhibitors or mock miRNA (Fig. 3A). Transfection with miR-421 mimics suppressed the luciferase activity significantly in MCF-7 cells (Fig. 3B) and MDA-MB-231 cells (Fig. 3C); by contrast, miR-421 inhibitors significantly enhanced the luciferase activity in MCF-7 cells (Fig. 3B) and MDA-MB-231 cells (Fig. 3C). The functions of the miR-421 mimics and inhibitors were abrogated when mutated 3'-UTR PDCD4-vector constructs were used, suggesting that miR-421 directly targets the 3'-UTRs of PDCD4.



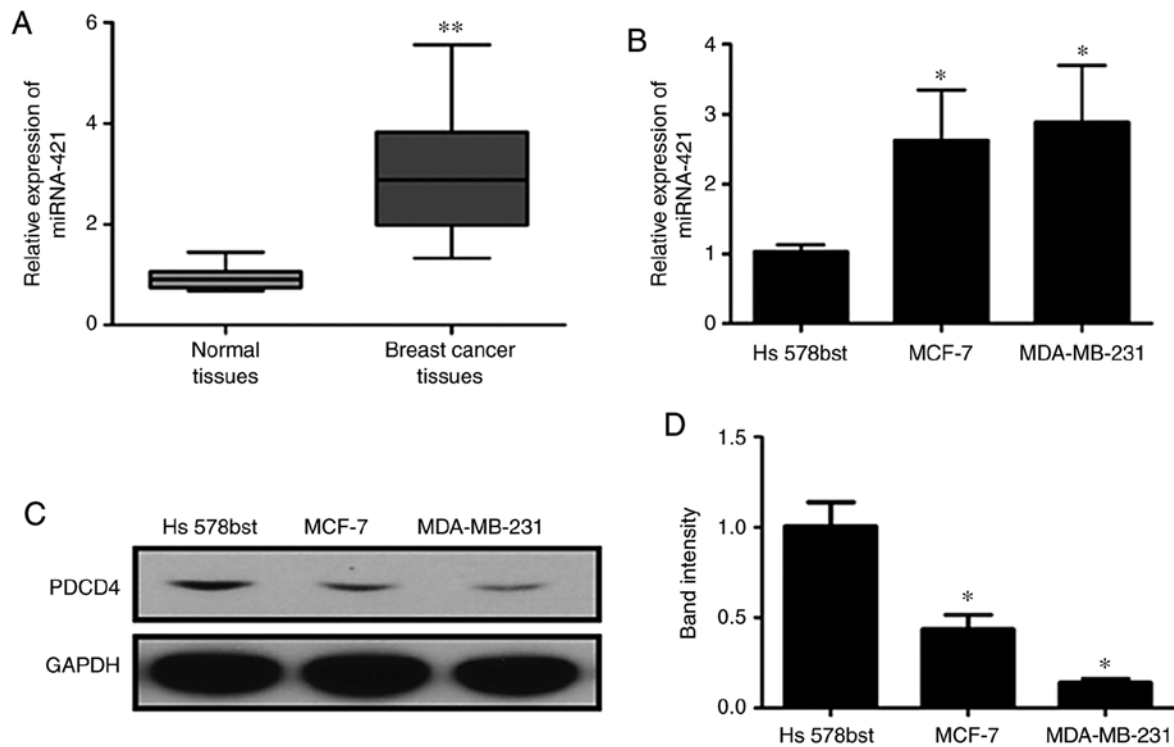


Figure 2. Levels of miR-421 and expression of PDCD4 in BC. miR-421 levels were determined in BC tissues, MCF-7 cells and MDA-MB-231 cells, and the expression of PDCD4 was determined in MCF-7 cells and MDA-MB-231 cells. (A) Relative expression of miR-421 in 52 paired BC and normal tissues. (B) Relative expression of miR-421 in Hs578bst, MCF-7 and MDA-MB-231 cells. (C) Protein expression and PDCD4 in Hs578bst, MCF-7 and MDA-MB-231 cells was assessed using western blot analysis and (D) quantified. U6 was used as an endogenous control. \* $P < 0.05$  and \*\* $P < 0.01$  vs. normal/control groups. BC, breast cancer; miR, microRNA; PDCD4, programmed cell death 4.

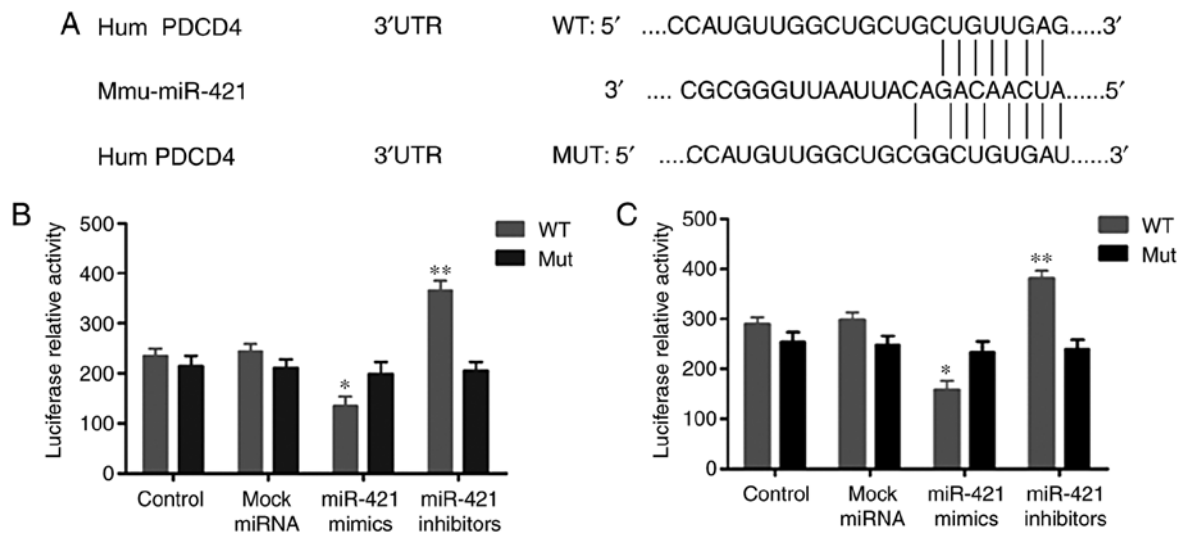


Figure 3. PDCD4 is a direct target of miR-421. (A) A luciferase activity assay confirmed that miR-421 directly binds to PDCD4, as predicted by TargetScan. (B) Overexpression of miR-421 significantly inhibited the luciferase activity of PDCD4 in MCF-7 cells. (C) Luciferase activity of PDCD4 in MDA-MB-231 cells transfected with miR-421 inhibitors was significantly increased. \* $P < 0.05$  and \*\* $P < 0.01$  vs. normal/control groups. miR, microRNA; Hum, human; PDCD4, programmed cell death 4; WT, wild-type; Mut, mutant.

*miR-421 promotes BC cell proliferation in vitro.* The results revealed that transfection with miR-421 mimics enhanced the proliferation of Hs578bst (Fig. 4A), MCF-7 (Fig. 4B) and MDA-MB-231 (Fig. 4C) cells; however, transfection with miR-421 inhibitors had the opposite effect. These results suggested that the overexpression of miR-421 accelerates BC cell proliferation *in vitro*.

*miR-421 promotes the migration and invasion of BC cells.* The Transwell results revealed that the overexpression of miR-421 significantly promoted the migration of MCF-7 and MDA-MB-231 cells, whereas miR-421 knockdown inhibited cell migration (Fig. 5A). The Matrigel assay revealed that the invasive abilities of the MCF-7 and MDA-MB-231 cells were enhanced following transfection with miR-421 mimics

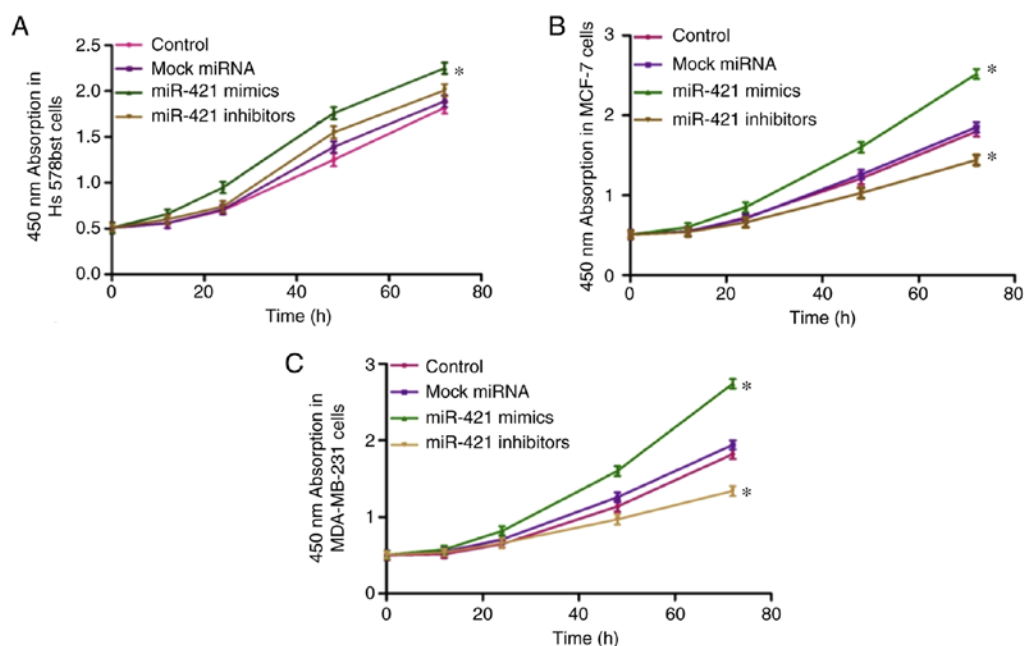


Figure 4. miR-421 promotes the proliferation of MCF-7 and MDA-MB-231 cells *in vitro* as analyzed using a Cell Counting Kit-8 assay. (A) miR-421 inhibitors promoted the proliferation of normal Hs578bst cells compared with other groups. (B) MCF-7 proliferation was enhanced following transfection with miRNA-421 mimics compared with the control groups. (C) MDA-MB-231 cell growth was regulated by miR-421. \* $P < 0.05$  vs. normal/control groups. miR, microRNA.

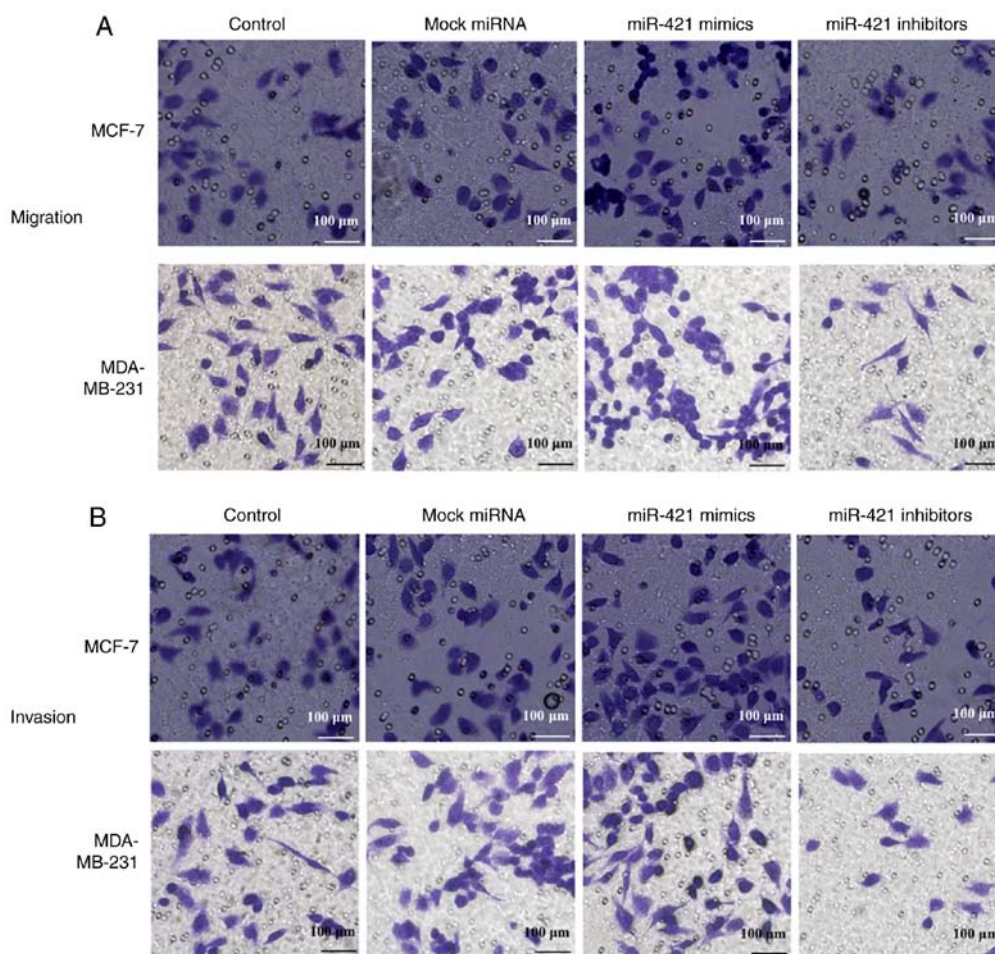


Figure 5. Transwell results show miR-421 promotes the migration and the invasion of breast cancer cells. (A) MCF-7 and MDA-MB-231 cell migration increased following transfection with miR-421 mimics, but was suppressed in the miR-421 inhibitors group compared with the control or mock miRNA groups. (B) MCF-7 and MDA-MB-231 cell invasion was enhanced following transfection with miR-421 mimics, whereas the opposite was observed following transfection with miR-421 inhibitors. Images were captured using an inverted microscope (magnification, x200). miR, microRNA.

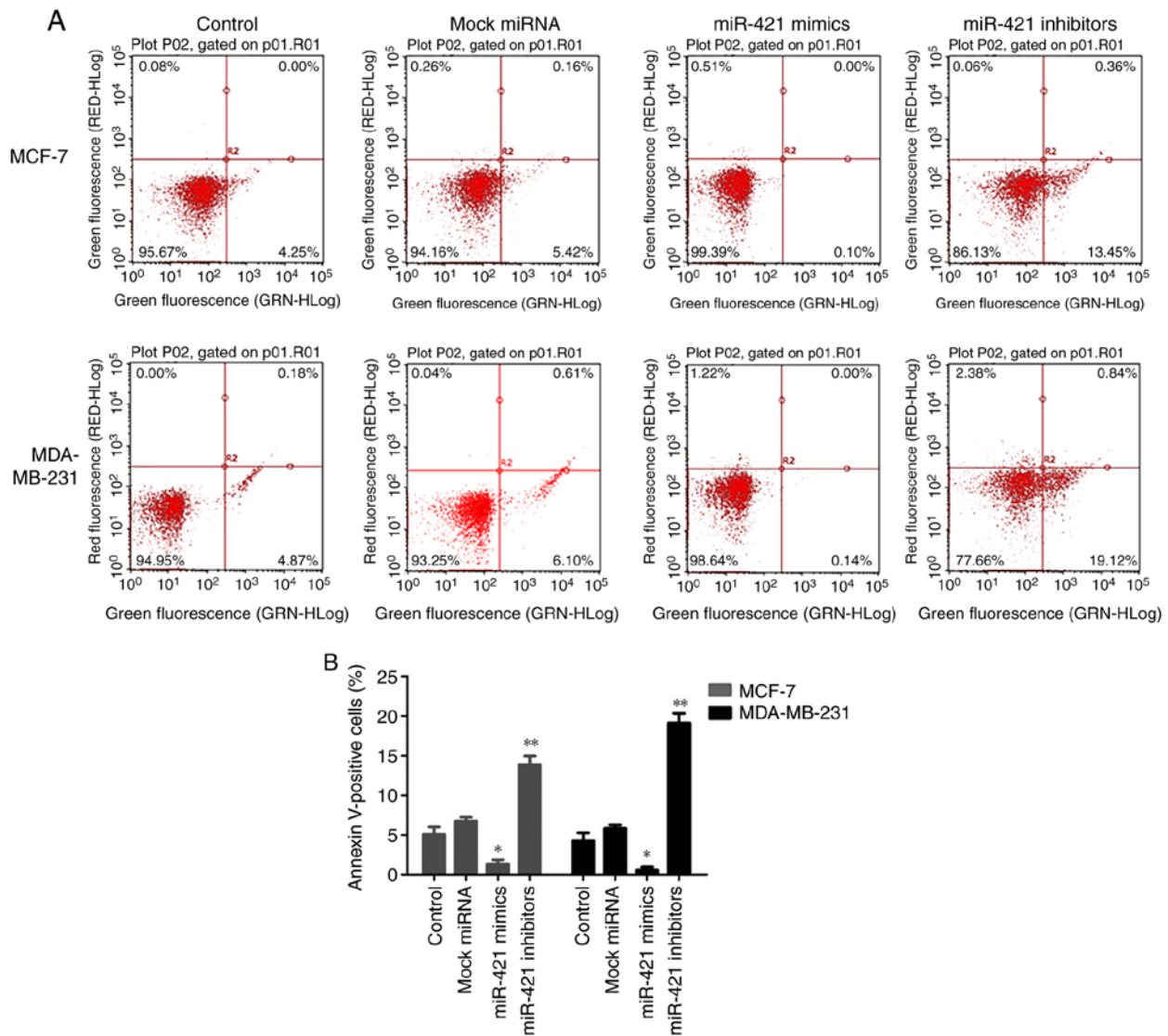


Figure 6. Effects of expression of miR-421 on breast cancer cell apoptosis. Apoptosis was assessed using flow cytometry. (A) MCF-7 and MDA-MB-231 cell apoptosis was assessed following transfection with miR-421 mimics and inhibitors. (B) Statistical analysis of MCF-7 and MDA-MB-231 cell apoptosis. \*P<0.05 and \*\*P<0.01 vs. control groups. miR, microRNA.

and downregulated by miR-421 inhibitors (Fig. 5B). These results suggested that the expression of miR-421 increases the motility of MCF-7 and MDA-MB-231 BC cells.

**miR-421 influences BC cell apoptosis.** The flow cytometry results suggested that the number of apoptotic MCF-7 and MDA-MB-231 cells (Fig. 6A) was significantly increased following transfection with miR-421 inhibitors compared with the control group (Fig. 6B), suggesting that miR-421 affects the apoptosis of MCF-7, MDA-MB-231 cells via regulating PDCD4.

**miR-421 regulates the protein expression of PDCD4.** The RT-qPCR and western blot analyses revealed that the expression of miR-421 was increased in MCF-7 and MDA-MB-231 cells transfected with miR-421 mimics, but decreased in cells transfected with miR-421 inhibitors (Fig. 7A). Furthermore, the overexpression of miRNA-421 suppressed the expression of PDCD4 at the mRNA (Fig. 7B) and protein (Fig. 7C and D) levels in MCF-7 and MDA-MB-231 cells. By contrast, miR-421 knockdown upregulated the mRNA and protein

levels of PDCD4, suggesting that PDCD4 is directly regulated by miR-421 and may function as an anticancer gene.

## Discussion

miRNAs have previously been reported to be associated with key developmental pathways, and various miRNA disorders contribute to a number of human ailments, including inflammatory diseases, infection, developmental disabilities and cancer (25). Abnormal miRNA expression has been reported in a number of types of cancer, with miRNAs serving tumor-suppressive or oncogenic roles to modulate tumor cell growth, cell cycle progression, migration and metastasis (26). miR-421, which is located in Xq13.2, has been reported to be involved in the post-transcriptional regulation of gene expression by affecting the stability and translation of genes in multicellular organisms, while regulating the expression of multiple tumor-promoting genes (27). Previous studies have shown that miR-421 is significantly overexpressed in gastric cancer tissues and promotes gastric cancer cell proliferation by

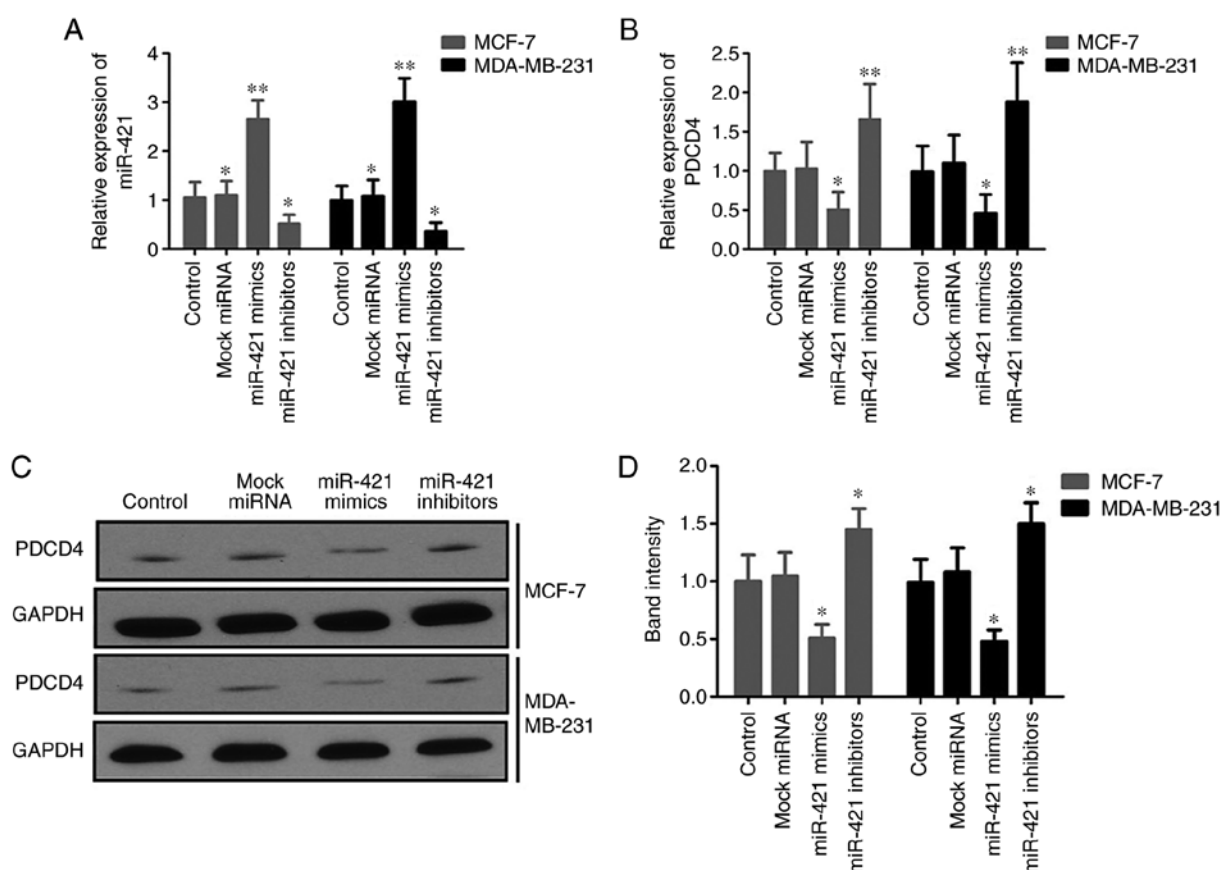


Figure 7. Assessment of the expression of PDCD4 in breast cancer cell lines using RT-qPCR and western blot analysis. (A) miR-421 and (B) PDCD4 gene expression were measured in MCF-7 and MDA-MB-231 cells transfected with mock miRNA, miR-421 mimics and miR-421 inhibitors using RT-qPCR analysis. (C) Protein expression of PDCD4 was assessed in MCF-7 and MDA-MB-231 cells transfected with miR-421 mimics and miR-421 inhibitors using western blot analysis with (D) quantification of results. \* $P < 0.05$  and \*\* $P < 0.01$  vs. normal/control groups. miR, microRNA; PDCD4, programmed cell death 4; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

downregulating the expression of caspase-3 (28). Furthermore, miR-421 may act as a tumor promoter in pancreatic cancer by targeting DPC4/Smad4 (29).

In the present study, it was found that miR-421 was significantly upregulated in BC tissues and cells compared with normal controls. However, miR-421 knockdown inhibited the proliferation, migration and invasion abilities of BC cells, and promoted apoptosis. PDCD4 was predicted as a target gene of miR-421 using TargetScan software. PDCD4 is a tumor suppressor protein, which is able to induce apoptosis in tumor cells (30,32) by binding to the translation initiation factor eukaryotic initiation factor-4A, inhibiting RNA-helicase activity and in turn suppressing protein translation (33). Furthermore, PDCD4 is able to inhibit activator protein-1-mediated transactivation (34) and induce the expression of cyclin-dependent kinase inhibitor p21 (35). PDCD4 is involved in tumorigenesis and tumor progression (36,37) and is downregulated in BC (38). These results suggest that miRNA-421 may function as an onco-miRNA in BC cells by regulating PDCD4.

The results of the present study indicated that miR-421 regulates the proliferation, migration, invasion and apoptosis of BC cells by targeting PDCD4. PDCD4 is important in a number of cellular physiological activities. In conclusion, PDCD4 may represent a novel therapeutic target for BC, and miR-421 may be used as a potential treatment for BC.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

YW and ZL completed all experiments and wrote the manuscript. JS designed and guided all experiments in the paper. In addition, all authors revised the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All protocols were approved by the Ethics Committee of the Second Affiliated Hospital of Xi'an Medical University (Xi'an, China) and written informed consent was provided by all participants.



## Patient consent for publication

All patients involved in the present study consented to the publication of their data.

## Competing interests

The authors declare that they have no competing interests.

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