miR-455-5p promotes cell invasion and migration in breast cancer

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Abstract. MicroRNA (miR)-455-5p has been identified as a biomarker for various types of cancer and may therefore be involved in the regulation of cancer development and progression. However, the specific role and function of miR-455-5p in breast cancer remains unclear. The present study explored the expression levels and function of miR-455-5p in breast cancer. The results from reverse transcription-quantitative polymerase chain reaction analysis revealed that miR-455-5p was significantly upregulated in breast cancer. Clinically, increased expression of miR-455-5p predicted a poor survival rate and miR-455-5p was identified as one of the independent prognostic factors for breast cancer patients. Furthermore, results from wound healing and Transwell assays revealed that miR-455-5p accelerated invasiveness and migration capabilities of breast cancer cells. In addition, programmed cell death 4 was identified as a downstream target of miR-455-5p and its expression was observed to be negatively regulated by miR-455-5p. Overall, miR-455-5p may function as an oncogene in breast cancer, and may therefore be used as a prognostic marker for breast cancer patients.

Introduction

Progression has previously been made regarding the diagnosis and treatment of various cancers associated with mortality in women, however, breast cancer is still considered to be of primary concern worldwide (1). In order to diagnose and treat breast cancer during an earlier progressive stage, the underlying molecular mechanisms of its development and progression need to be elucidated (2).

It has been reported that microRNAs (miRNAs), small RNAs composed of 18-25 nucleotides, are incapable of coding protein, and dysregulated miRNA expression is present in multiple cancers, including breast cancer (3). miRNA is important in cancer progression via an influence on diverse cellular activities, including cell growth (4), apoptosis (5), metastasis (6), invasion (7) and the cell cycle (8). MiR-455-5p as an oncogene or tumor suppressor has been verified to be associated with several cancers, including hepatocellular adenoma (9), head and neck squamous cell carcinoma (10) and oral squamous cell carcinoma (11). It has previously been demonstrated that in hepatocellular adenoma tissues and cell lines, miR-455-5p expression levels are deregulated (9). In oral cancer, transforming growth factor (TGF)-β-mediated highly expressed miR-455-5p promotes cancer progression via decreasing the expression of ubiquitin conjugating enzyme E2 B (UBE2B) (11). Currently, there are not many studies that have been conducted regarding the expression and role of miR-455-5p. The present study demonstrated that miR-455-5p was upregulated in breast cancer. Further statistical analysis revealed that miR-455-5p was notably associated with breast cancer patient prognosis. Cytological experimental results demonstrated that miR-455-5p exerted a positive influence on breast cancer cell migratory and invasive abilities.

Previous studies have reported that miRNAs directly interact with their targets to regulate tumor progression (12). In gastric cancer, miR-455-5p acts as a tumor suppressor via targeting and downregulating RAB18, member RAS oncogene family RAB18 (13). The present study demonstrated that miR-455-5p targeted and negatively regulated programmed cell death (PDCD)4, which has been identified as a tumor suppressor in breast cancer, and inhibits breast cancer cell migration, invasion and growth. Overall, the results of the present study suggest that miR-455-5p acts as a biomarker that may be used in the prediction of breast cancer patient prognosis, in addition to aiding in the development of novel molecular targets and therapeutic strategies to treat the disease.

Patients and methods

Patients and tissue samples. A total of 70 paired breast cancer tissue samples and adjacent normal tissues were collected from patients (age range, 24-76 years) following confirmation of written informed consent. All the patients underwent surgical resections at the Affiliated Tumor Hospital of Xinjiang Medical University (Urumqi, China), during the period of January 2010-March 2016. The present study gained the approval of the Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University. Prior to mastectomy, the 70 patients had not received radiotherapy or chemotherapy. The extracted specimens were verified as breast cancer tissue with pathological

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diagnosis according to the International Union against Cancer. All fresh samples were immediately placed in a liquid nitrogen container and then stored at -80˚C for further analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Invitrogen TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to isolate total RNA samples from tissues or cells. In order to obtain cDNA, the All-in-One miRNA qRT-PCR kit (GeneCopoeia, Inc., Rockville, MD, USA) was used for conduction of reverse transcription and the PCR reaction, according to the manufacturer's protocol. For reverse transcription, the reaction was incubated at 42˚C for 15 min; heated to 95˚C for 5 min and finally incubated at 5˚C for 5 min. Stem-loop primers used for reverse transcription of miR-455-5p and U6 were 5'-GTCGTATCGAGTGGAGCG TCGAGCTATACGCACTCGATACGACACAAA-3' and 5'-GTCCTATCCAGTGCAGGGTCCGAGGTGCACTGGATA CGACAAAATATGGAAC-3', respectively. For RT-qPCR, the following thermocycling conditions were used: Initial denaturation at 95˚C for 5 min; followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing/elongation at 60˚C for 30 sec. The PCR primers for U6 and miR-455-5p were obtained from GeneCopoeia, Inc. The PCR primer sequences were as follows: U6 forward, 5'-CGCTTCGGCAGCACATAT ACTA-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTC A-3'; miR-455-5p forward, 5'-CGAGCTTCCTTCTGCAGGT-3' and reverse, 5'-CACCACTGCCATCCCACA-3'. The $2^{-\Delta\Delta Cq}$ method was used to quantify the level of miRNA (14).

Cell culture and transfection. The breast cancer cell lines, including MDA-MB-453, MCF-7, SK-BR-3, MDA-MB-231 and the normal breast cell line MCF-10A were purchased from American Type Culture collection (Manassas, VA, USA), then cultured with Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (GE Healthcare Life Sciences, Logan, UT, USA). All of the cells were maintained in an incubator at 37˚C, in an environment containing 5% CO_2. The miR-455-5p inhibitors (5'-CGAUGUAGUCCAAAGGCACAUA-3') and negative control (NC) (5'-UUCUCCGAACGUGUCACGUTT-3') were obtained from Shanghai GenePharma Co., Ltd., (Shanghai, China). Cells underwent the transfection procedure when the cell confluence reached 50-70%, using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The final concentrations of the mimics and its corresponding NC were adjusted to 50 nM (6-well plate), while inhibitors and its NC were modulated to 200 nM (6-well plate). Total RNA and protein was extracted 24 h following transfection, and the cells were harvested 48 h following transfection for the subsequent experiments.

Wound-healing assay. The 6-well plates were used to culture cells ($5x10^5$) and scratch wounds were created using a 200 μl pipette tip. Following a culture period of 0 and 48 h, cells were washed three times using sterile PBS to remove cellular debris, then were imaged under an inverted microscope.

Transwell assay. The cell migratory and invasive abilities were detected using non-Matrigel-coated/Matrigel-coated chambers, respectively (BD Biosciences, Franklin Lakes, NJ, CA, USA). Dulbecco's modified Eagle's medium supplemented with 10% FBS was added into the lower chambers. MDA-MB-231 and MCF-7 cells ($1x10^5$ cells/well), which had been transfected with miR-455-5p inhibitors or NC, were plated into the upper chambers. Following incubation for 24 or 48 h, the cells that remained in the upper chambers were removed by PBS and cotton swabs. Following this, 4% paraformaldehyde was used to fix cells at room temperature for 30 min, which had passed through the filters, and cells were stained with 0.1% crystal violet at room temperature for 5 min. Cells were observed and imaged using a fluorescence microscope (Olympus Corporation, Tokyo, Japan; magnification, x100) and 5 separate fields were used for every filter to obtain an average.

Western blotting. Total protein was extracted from breast cancer cells using a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. The protein concentration was determined using the bicinchonininc protein assay kit (Thermo Fisher Scientific, Inc.). Following separation by 10% SDS-PAGE (25 μg/lane), the proteins were transferred to a polyvinlyidene membrane. Then, the membrane was blocked using 5% non-fat milk at room temperature for 2 h and incubated with PDCD4 (Cell Signaling Technology, Inc., Danvers, MA, USA) or β-actin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) primary antibodies, overnight at 4˚C. The primary antibodies included anti-PDCD4 (1:1,000; cat. no. ab51495; Abcam, Cambridge, MA, USA) and anti-β-actin (1:1,000; cat. no. ab8226; Abcam). Following incubation with their respective secondary antibodies at room temperature for 1 h the proteins were visualized using an enhanced chemiluminescence chromogenic substrate [Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China]. The secondary antibodies included goat anti-mouse IgG2b (1:8,000; cat. no. sc-2062; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and goat anti-rabbit IgG2b (1:8,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.). The immune complexes were quantified using Image J (version 1.46; National Institutes of Health, Bethesda, MA, USA).

Bioinformatics analysis. Bioinformatics tools TargetScan (www.targetscan.org), miRNA.org (www.microrna.org) and miRbase (www.mirdb.org) were used to predict the targets of miR-455-5p. Then the data from the three databases were combined to obtain the most popular candidates in the three databases using Microsoft Excel (version 2017; Microsoft Corporation, Redmond, Washington) and SPSS software (version 22.0; IBM SPSS, Armonk, NY, USA).

Statistical analysis. Data was analyzed using the Kaplan-Meier method, unpaired Student's t-test, χ^2 test, analysis of variance followed by the least significant difference test and univariate and multivariate Cox regression analysis. SPSS software, version 22.0 (IBM SPSS, Armonk, NY, USA) was used to analyze data. All the presented data were expressed as the mean ± standard deviation. Images were created using GraphPad Prism software (version 6; GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Association between miR-455-5p expression level and clinical characteristics.

Clinical characteristics	Cases (n=70)	miR455-5p expression		P-value
		High (n=36)	Low (n=34)	
Age, years				
<45	29	13	16	0.467
≥45	41	23	18	
Tumor size, cm				
<2	23	13	10	0.616
≥2	47	23	24	
Tumor location				
Left	34	16	18	0.633
Right	36	20	16	
Differentiation				
Moderate/High	26	7	19	0.003[b]
Low	44	29	15	
Lymph node metastasis				
Negative	15	12	3	0.019[a]
Positive	55	24	31	
TNM stage				
I/II	38	31	17	0.002[b]
III/IV	32	5	17	
ER status				
Negative	37	16	22	0.100
Positive	33	20	12	
HER2 status				
Negative	28	17	20	0.350
Positive	42	19	14	
PR status				
Negative		16	12	0.473
Positive		20	22	

[a]P<0.05, [b]P<0.01; TNM, tumor node metastasis; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

Results

Expression of miR-455-5p in breast cancer tissues increases. The present study explored miR-455-5p expression levels in 70 paired breast cancer and adjacent non-cancerous tissue samples. Results from RT-qPCR revealed that compared with control group, miR-455-5p expression levels in breast cancer tissues were notably increased (Fig. 1; P<0.01). Therefore, miR-455-5p may be considered a valuable oncogene in the progression of breast cancer.

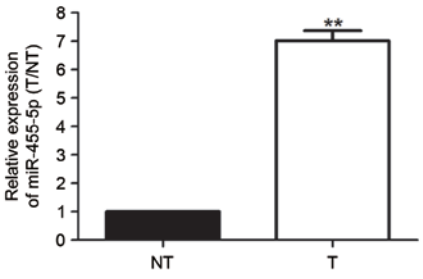

Figure 1. Expression of miR-455-5p in breast cancer tissues. Reverse transcription-quantitative polymerase chain reaction results revealed that the mean expression of miR-455-5p in cancerous tissues was significantly increased compared with control group. **P<0.01 vs. NT. miR, microRNA; T; tumor tissues; NT, non-tumor tissues.

Clinical significance of miR-455-5p expression in breast cancer. Based on miR-455-5p average expression level (7.02; range, 6.87-7.11), the 70 breast cancer patients were categorized into two groups, low miR-455-5p and high miR-455-5p groups. Then, the correlations between clinical characteristics and miR-455-5p expression were assessed. The analysis revealed that miR-455-5p expression was notably correlated with TNM stage, lymph node metastasis and tumor differentiation (P<0.05; Table I). Additionally, univariate Cox regression analysis (Table II) and multivariate Cox regression

Table II. Univariate Cox regression analysis for the identification of prognostic factors for overall survival and disease-free survival time in patients with breast cancer.

	Overall survival		Disease-free survival	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years(<45 vs. ≥45)	1.853 (1.251-2.104)	0.568	1.532 (1.463-1.604)	0.845
Tumor size, cm (<2 vs. ≥2)	1.332 (1.238-1.457)	0.697	1.792 (1.659-1.846)	0.446
Tumor location (left vs. right)	1.643 (1.549-1.784)	0.297	1.406 (1.379-1.523)	0.684
Differentiation (moderate/high vs. low)	2.729 (2.654-2.803)	0.042	2.912 (2.878-3.041)	0.004
Lymph node metastasis (negative vs. positive)	1.478 (1.238-1.739)	0.007	1.649 (1.587-1.882)	0.015
TNM stage (I/II vs. III/IV)	1.193 (1.046-1.254)	0.026	1.264 (1.206-1.321)	0.043
ER status (negative vs. positive)	1.774 (1.536-1.862)	0.922	2.012 (1.897-2.145)	0.238
HER2 status (negative vs. positive)	1.547 (1.502-1.621)	0.218	1.708 (1.654-1.783)	0.465
PR status (negative vs. positive)	2.745 (2.133-3.027)	0.589	1.965 (1.457-2.541)	0.628
miR-455-5p relative expression (<7.02 vs. ≥7.02)	2.612 (2.544-2.764)	0.024	2.832 (2.478-3.007)	0.017

HR, hazard ratio; CI, confidence interval; OS, overall survival; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; TNM, tumor node metastasis. n=70.

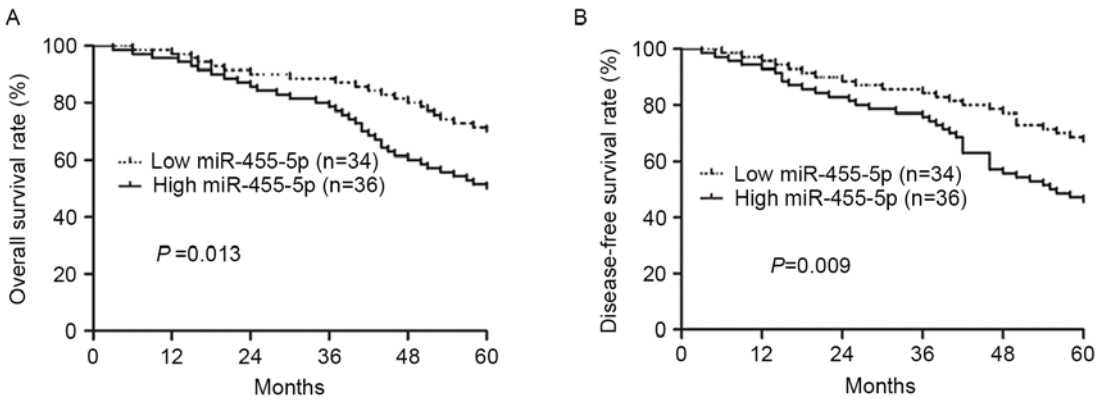

Figure 2. Effects of miR-455-5p expression on breast cancer patient prognosis. Patients in the high miR-455-5p expression group exhibited a significantly poorer (A) overall survival rate and (B) disease-free survival rate. P<0.05. miR, microRNA.

analysis (Table III) were conducted for the identification of prognostic factors for overall survival (OS) and disease-free survival (DFS) time in patients with breast cancer. Results revealed that TNM stage, lymph node metastasis, tumor differentiation and miR-255-5p were prognostic factors of patients with breast cancer. Therefore, it was hypothesized that miR-455-5p may be associated with the survival rate of breast cancer and may be used to predict the prognosis of breast cancer patients.

Elevated miR-455-5p is associated with poor prognosis of breast cancer. The present study compared the survival rate of two groups of patients using Kaplan-Meier survival analysis, and it was revealed that patients with increased expression of miR-455-5p exhibited a poorer OS and DFS rate compared with lower miR-455-5p expression (P=0.013, P=0.009, respectively; Fig. 2A and B). Furthermore, univariate and multivariate Cox proportional hazard regression analysis were conducted to explore whether miR-455-5p acts as an independent prognostic factor in breast cancer. The data demonstrated that miR-455-5p expression, TNM stage and lymph node metastasis were independent prognostic factors of breast cancer patients, which may be used to predict their prognosis (P<0.05; Table II). The results demonstrated that miR-455-5p may act as an independent prognostic factor for breast cancer patients.

Table III. Multivariate Cox regression analysis for the identification of prognostic factors for overall survival and disease-free survival time in patients with breast cancer.

	Overall survival		Disease-free survival	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Differentiation (moderate/high vs. low)	1.894 (1.761-2.327)	0.918	1.664 (1.498-1.863)	0.089
Lymph node metastasis (negative vs. positive)	2.003 (1.847-2.214)	0.028	2.784 (2.516-2.943)	0.003
TNM stage (negative vs. positive)	1.339 (1.235-1.456)	0.026	1.649 (1.468-1.892)	0.011
miR-455-5p relative expression (<7.02 vs. ≥7.02)	2.334 (2.178-2.619)	0.003	2.568 (2.511-2.597)	0.019

HR, hazard ratio; CI, confidence interval; OS, overall survival; TNM, tumor node metastasis. n=70.

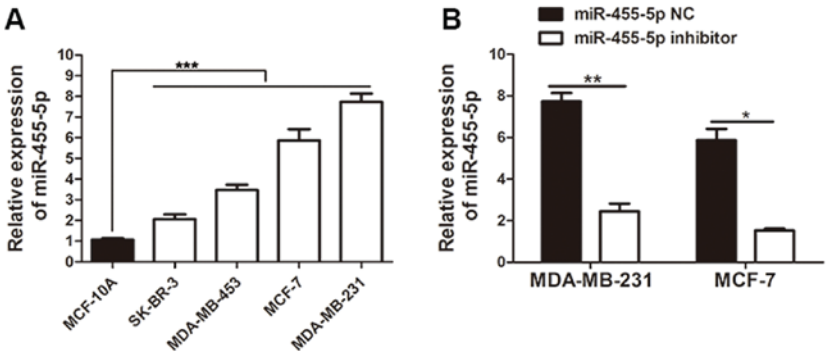

Figure 3. Expression of miR-455-5p in breast cancer cell lines. Reverse transcription-quantitative polymerase chain reaction data revealed that (A) mean expression levels of miR-455-5p in cancer cell lines were significantly increased compared with normal cell MCF-10A. (B) Expression levels of miR-455-5p in MDA-MB-231 and MCF-7 cells that were transfected with miR-455-5p inhibitor and negative control were measured. *P<0.05, **P<0.01, ***P<0.001. miR, microRNA; NC, negative analysis.

MiR-455-5p is overexpressed in breast cancer cell lines. RT-qPCR was used to measure the expression of miR-455-5p in breast cancer cell lines, including MDA-MB-453, MCF-7, SK-BR-3 and MDA-MB-231, in addition to the normal cell line MCF-10A. Consistent with the clinical results, the experiment revealed that when compared with MCF-10A cells, miR-455-5p expression in all breast cancer cell lines was markedly upregulated (P<0.001; Fig. 3A). MDA-MB-231 and MCF-7 cells exhibited a relatively increased expression of miR-455-5p, and the two cell types were therefore selected to be used in further experiments. To explore the function of miR-455-5p in breast cancer progression, the miR-455-5p expression in MCF-7 cells and MDA-MB-231 cells was manipulated via transfection with miR-455-5p inhibitor or NC into cells. The results of the RT-qPCR demonstrated that the miR-455-5p inhibitor markedly repressed miR-455-5p expression in these cell lines (Fig. 3B; P<0.05).

MiR-455-5p promotes cell invasion and migration. Furthermore, the present study investigated whether miR-711 promotes invasion and migration abilities of breast cancer cells, using wound healing and Transwell assays. The results indicated that compared with the NC, the groups with downregulated miR-455-5p via miR-455-5p inhibitor transfection, exhibited suppressed invasion and migration abilities (Figs. 4 and 5; P<0.05). Therefore, the findings demonstrated that miR-455-5p facilitated breast cancer cell migratory and invasive abilities.

PDCD4 is a downstream target of miR-455-5p in breast cancer cells. In order to uncover the functional mechanism of miR-455-5p in breast cancer, the present study explored the potential targets of miR-455-5p using bioinformatic tools (TargetScan, miRNA.org and miRbase). The results suggested that PDCD4 acted as a downstream target of miR-455-5p (Fig. 6A). Following this, the expression levels of PDCD4 were detected by western blotting in MCF-7 and MDA-MB-231 cells, which had been transfected with the miR-455-5p inhibitor. The results demonstrated that the expression of PDCD4 was significantly increased in these two breast cancer cell lines transfected with the miR-455-5p inhibitor, compared with the NC group (P<0.05; Fig. 6B and C). Therefore, PDCD4 may be negatively regulated by miR-455-5p in breast cancer cells. Overall, the findings demonstrated that PDCD4 may act as a downstream target of miR-455-5p in breast cancer cells.

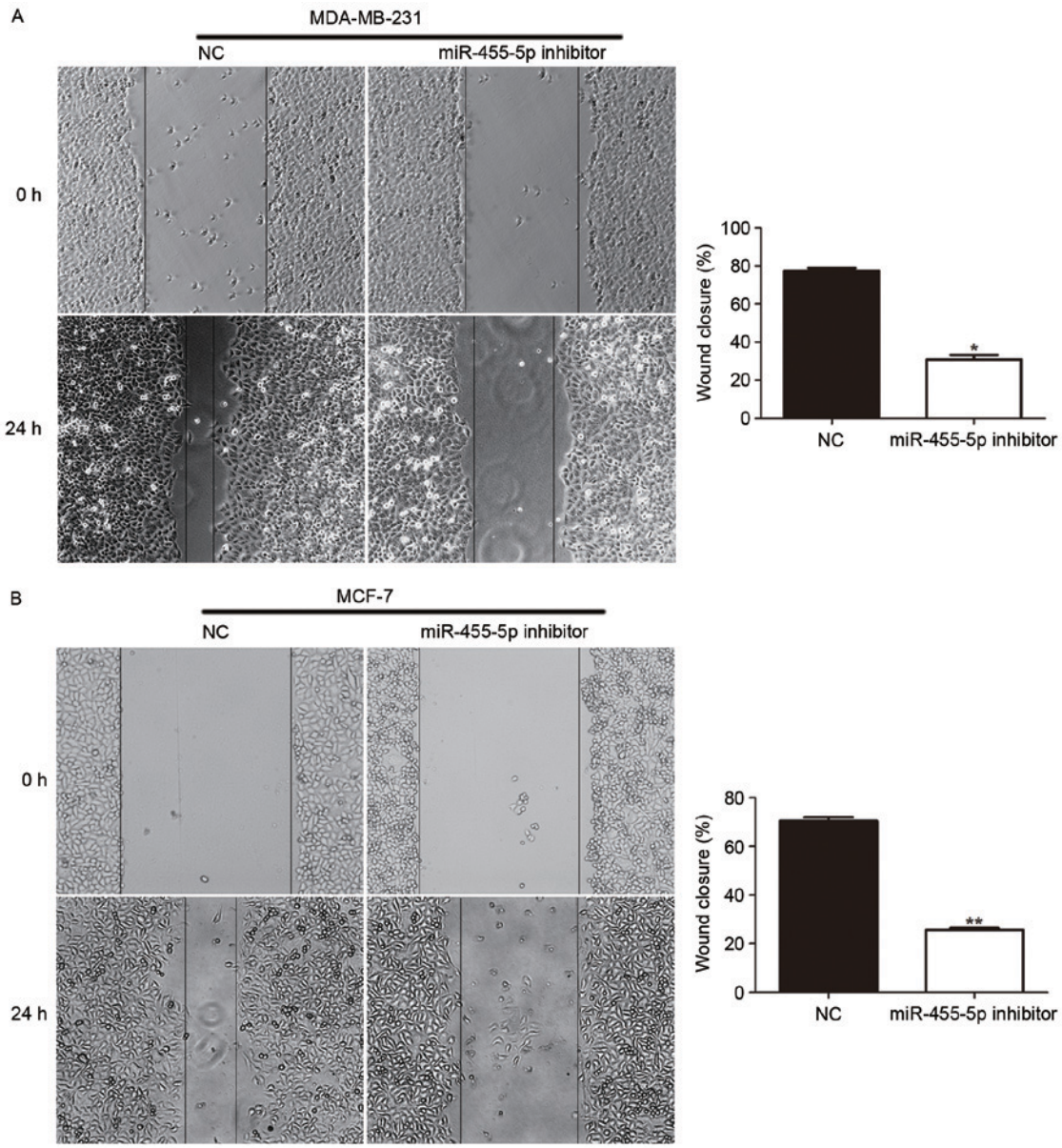

Figure 4. MiR-455-5p promotes breast cancer cell mobility. Downregulated miR-455-5p suppressed cell mobility as measured by wound healing assay in (A) MDA-MB-231 cells and (B) MCF-7 cells. *P<0.05, **P<0.01 vs. NC. miR, microRNA; NC, negative control.

Discussion

The diagnosis and treatment strategies regarding breast cancer have markedly improved over previous years, however patients currently still exhibit a poor prognosis (15). There are >1 million newly diagnosed cases of male breast cancer each year, and breast cancer results in ~520,000 mortalities each year worldwide (16). Therefore, development of novel treatment strategies for breast cancer is of primary concern.

miRNAs have been extensively investigated as effective prognostic biomarkers for cancer, including breast cancer (17,18). It has previously been reported that miR-145 exhibits a suppressive role in the regulation of breast cancer cell migration through specifically inhibiting the expression of fascin and epithelial to mesenchymal transition progression (19). Zhang *et al* (20) demonstrated that low expression of miR-124-3p promotes breast cancer cell development, primarily by increasing beclin-1 expression. Wu *et al* (21) suggested that in breast cancer, miR-613 negatively regulates vascular endothelial growth factor expression and restrains the cell proliferation and invasion. Notably, miR-455-5p has been identified as an oncogene or anti-oncogene in previous studies (11,13,22). In gastric cancer, miR-455-5p has been identified as a tumor suppressor, which decreases the expression of RAB18 (13). Conversely, TGF-β-induced miR-455-5p is overexpressed, which results in low expression of UBE2B and promotes cancer progression (11). In the present study, the data indicated that in breast cancer tissues and cell lines, miR-455-5p expression levels were significantly upregulated, which suggested that miR-455-5p may act as an oncogene in breast cancer.

Malignant tumors with poor tumor differentiation tend to present more lymph node metastasis and an advanced TNM stage, which results in an increased tendency of tumor metastasis and invasion (23,24). The present study demonstrated that miR-455-5p was closely correlated with lymph node metastasis, TNM stage and tumor differentiation.

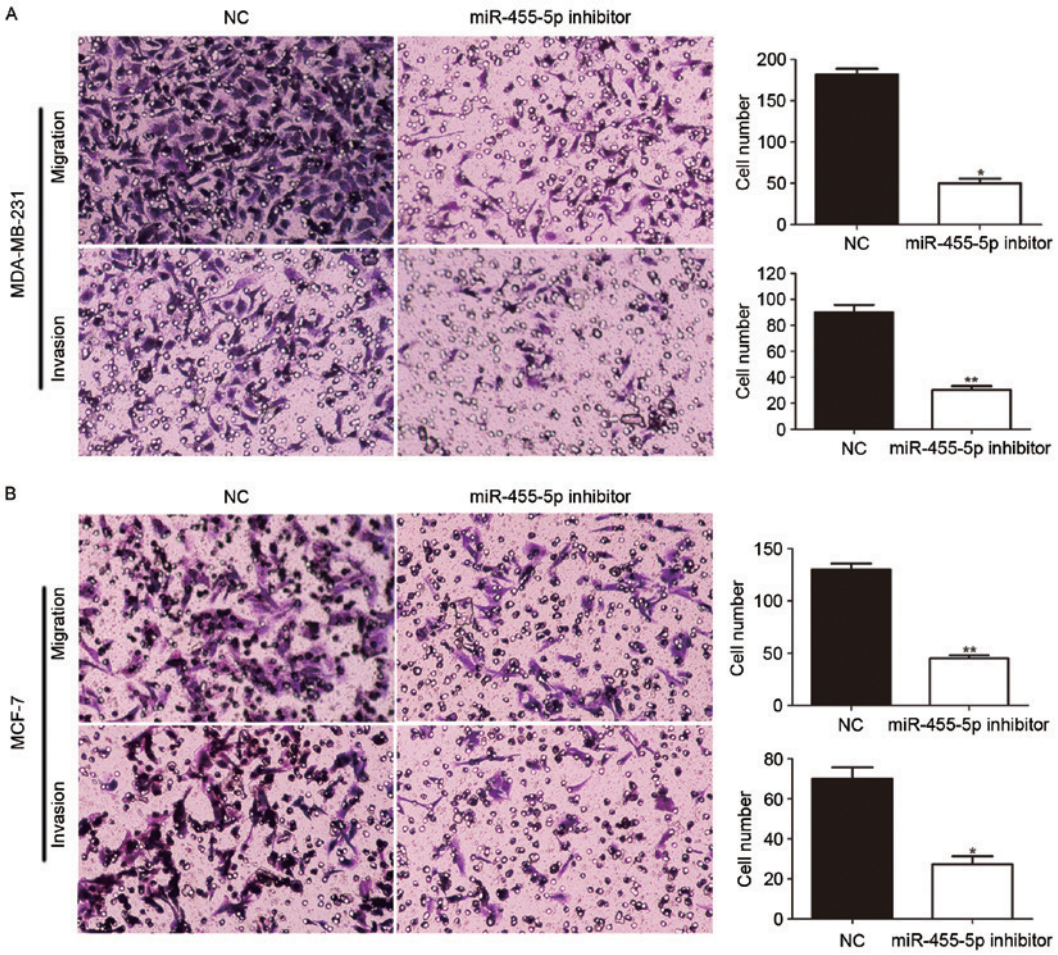

Figure 5. MiR-455-5p promotes breast cancer cell migration and invasion. Downregulated miR-455-5p suppressed cell mobility as measured by Transwell assays in (A) MDA-MB-231 and (B) MCF-7 cells. *P<0.05, **P<0.01 vs. NC. miR, microRNA; NC, negative control.

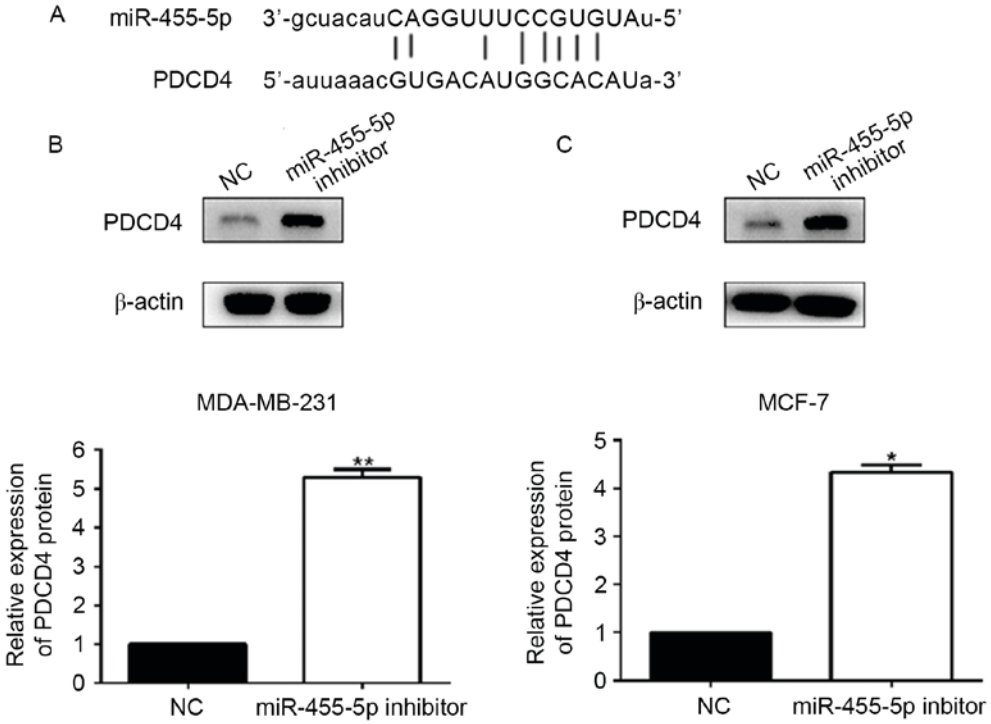

Figure 6. PDCD4 is a downstream target of miR-455-5p. (A) Bioinformatic analysis suggested that miR-455-5p directly targeted the 3'-untranslated region of PDCD4. (B) Western blotting demonstrated that the expression of PDCD4 was significantly increased in (B) MDA-MB-231 and (C) MCF-7 cells transfected with the miR-455-5p inhibitor. *P<0.05, **P<0.01 vs. NC. miR, microRNA; NC, negative control; PDCD4, programmed cell death 4.

Further analysis indicated that upregulated miR-455-5p was additionally associated with poorer OS and DFS survival rates. The results additionally revealed that TNM stage, lymph node metastasis, tumor differentiation and miR-255-5p acted as prognostic factors for patients with breast cancer. It was therefore hypothesized that miR-455-5p acts as an oncogene to promote migration and invasion of breast cancer.

Next, cytological experiments indicated that silencing miR-455-5p had a suppressive effect in terms of the invasive and migratory abilities of breast cancer cells. This indicated that miR-455-5p may promote breast cancer progression by accelerating cell migration and invasion.

MiRNAs exert various functions in cancer cells via interaction with specific targets (11,12). In oral squamous cell carcinoma, UBE2B has been identified as a target of miR-455-5p (11). In gastric cancer, miR-455-5p may directly target RAB18 (13). Therefore, in order to identify a target of miR-455-5p in breast cancer, the present study applied bioinformatics tools to analyze potential targets. PDCD4 was subsequently identified as a potential target, and has previously been verified to act as a tumor suppressor in multiple cancers, including breast cancer (25-27). PDCD4 is regulated by various miRNAs in breast cancer, including miR-183-5p and miR-21 (28,29). In order to verify PDCD4 as a target of miR-455-5p, the protein expression of PDCD4 was detected in breast cancer cells, where the expression of miR-455-5p had been decreased. It was revealed that miR-455-5p inversely regulated PDCD4 expression levels in breast cancer cells.

In conclusion, the results of the present study identified PDCD4 as a downstream target of miR-455-5p. However, further studies are required in order to validate this finding, and explore the underlying molecular mechanism of the role of miR-455-5p in breast cancer. The findings demonstrated that miR-455-5p was highly expressed in breast cancer, and therefore may facilitate cancer development, acting as an oncogene and biomarker for the prognosis of breast cancer patients.

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