

miR-520e regulates cell proliferation, apoptosis and migration in breast cancer

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Abstract. Previous studies have indicated that the deregulation of microRNAs contributes to tumorigenesis. Misregulation of microRNA-520e (miR-520e) has been observed in various types of cancer. However, the expression profile and biological function of miR-520e in breast cancer remains largely unknown. The present study demonstrated that miR-520e expression was significantly increased in breast cancer tissues compared with adjacent non-cancerous breast tissues in 21 patients, as revealed by reverse transcription-quantitative polymerase chain reaction. Furthermore, the proliferation capacity of breast cancer cells was markedly enhanced by the introduction of miR-520e *in vitro* using a cell counting kit-8 assay. The present study also revealed that the overexpression of miR-520e could suppress breast cancer cell apoptosis, revealed using Annexin V/propidium iodide double staining and flow cytometry analysis. In addition, the ectopic expression of miR-520e promoted the migration of breast cancer cells *in vitro*, as demonstrated by a Transwell assay. Overall, the findings of the present study highlight an important role for miR-520e in breast cancer development and in the molecular etiology of breast cancer, which indicates the potential application of miR-520e in cancer therapy.

Introduction

Previous studies have demonstrated that alterations in protein-coding genes are critical for the development of human cancer (1,2). Other studies have indicated that deregulation of

non-coding genes, particularly microRNAs (miRNAs), also contribute to tumorigenesis (3-6).

miRNAs belong to a class of phylogenetically conserved non-coding RNAs that regulate an abundance of cellular processes by binding to specific target messenger RNAs (7). Numerous studies indicate that miRNAs are important in various biological processes, including the cell cycle, development, cell proliferation, differentiation and apoptosis (8-12). More than half of the miRNA genes are located in chromosomes that have been observed to become amplified, deleted or translocated during the development of cancer (13). In addition, deregulation of specific miRNAs occurs in certain cancer types (14,15), and this has been suggested to be associated with the prognosis of cancer patients (16). Furthermore, a previous study proposed that miRNAs may act as oncogenes or tumor suppressor genes (6). These findings highlight the importance of miRNAs in cancer development and provide insight into the molecular mechanisms that cause tumorigenesis.

Globally, breast cancer is the most common cause of cancer-associated mortality in women, and accounted for ~1.38 million novel cases and 458,000 mortalities in 2008 (17). As with other solid tumors, multiple genetic and epigenetic alterations in protein-coding genes have been observed in breast cancer (18). However, previous findings alone cannot explain the complexity of breast cancer. Previous studies have demonstrated that dysfunction of miRNAs contributes to breast cancer development (11,14). Zhang *et al* reported that microRNA-520e (miR-520e) was associated with hepatocarcinogenesis (19); however, the biological significance of miR-520e in breast cancer remains largely unknown. The present study demonstrated that miR-520e is upregulated in breast cancer tissues and promotes proliferation, invasion and migration, and inhibits the apoptosis of cancer cells. The results of the present study suggest that miR-520e may be an attractive target for cancer therapy.

Materials and methods

Human tissue specimens. The present study was approved by the Institutional Review Board and Ethical Committee of Peking University Shenzhen Hospital (Shenzhen, Guangdong,

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China). All patients provided written informed consent prior to the study. In total, 21 paired breast cancer and adjacent non-cancerous breast tissues were obtained from patients that had undergone resection for breast cancer. The patient characteristics are reported in Table I. The patients had no previous local or systemic treatment prior to resection. No anticancer therapy was administered to the patients prior to relapse. The tissue samples were collected between 2012 and 2013 at the Peking University Shenzhen Hospital, and were snap frozen in liquid nitrogen and stored at -80°C until required. Tumor and non-cancerous tissue samples were confirmed by histology. The histological grade was determined based on the modified Scarff-Bloom-Richardson grading system (20).

RNA oligoribonucleotides. All RNA oligoribonucleotides were purchased from Genepharma, Ltd. (Shanghai, China). miRNA duplexes corresponding to mature miR-520e were designed as previously described (21). The negative control (NC) RNA duplex for the miRNA mimic was non-homologous to any human genome sequence. The sequences were as follows: hsa-miR-520e mimic, 5'-AAAGUGCUUCCUUUUUAGGG-3'; and the hsa-miR-520e NC, 5'-UUCUCCGAACGUGACGUTT-3'.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and primers. Total RNA was extracted from tissue cells using Invitrogen TRIzol reagent (catalog no. 15596026; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. cDNA was synthesized from the extracted RNA using the SYBR PrimeScript miRNA RT-PCR kit A (catalog no. RR716; Takara Biotechnology, Co., Ltd., Dalian, China). qPCR was performed with the SYBR PrimeScript miRNA RT-PCR kit B (catalog no. RR716; Takara Biotechnology, Co., Ltd.) using a LightCycler® 480 Fluorescent Quantitative PCR system (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, single-stranded cDNA was synthesized from 500 ng of total RNA in a 10 µl reaction volume using the SYBR PrimeScript miRNA RT-PCR kit A. The reactions were incubated at 37°C for 60 min and were then inactivated by incubation at 85°C for 5 sec. RT-qPCR analysis for the expression of miR-520e and the reference gene RNU6B was performed using SYBR PrimeScript miRNA RT-PCR kit B. The temperature cycle profile for the RT-qPCR reactions was as follows: 95°C for 30 sec; 40 cycles of 95°C for 5 sec; and 60°C for 20 sec. To verify the specificity of the PCR product, a melting curve analysis was performed immediately subsequent to amplification, as follows: Heating to 95°C for 20 sec; cooling to 60°C for 20 sec; and heating to 95°C with a transition rate of 0.11°C/sec, while continuously collecting the fluorescent signal. All reactions were performed on a LightCycler 480 and were run in triplicate. The cycle quantification (Cq) values did not differ by more than 0.5 among the triplicates. The levels of target genes were normalized to the levels of the internal control genes to permit the calculation of the $2^{-\Delta\Delta Cq}$ value. Cq is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The following primers were used: miR-520e forward, 5'-GAAAGTGCTTCCTTTTGGG-3' and reverse, SYBR PrimeScript miRNA RT-PCR kit universal primer; U6 forward, 5'-CTC

GCTTCGGCAGCACA-3' and reverse, 5'-ACGCTTCACGAA TTTGCGT-3' (Thermo Fisher Scientific, Inc.).

Cell culture and transfection. The human breast cancer MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells lines were maintained in HyClone Dulbecco's modified Eagle's medium (DMEM; catalog no. SH30022.01B; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% HyClone fetal bovine serum (FBS; catalog no. 10270-106; GE Healthcare Life Sciences) and HyClone 100 U penicillin-streptomycin (catalog no. SV30010; GE Healthcare Life Sciences) at 37°C in a humidified atmosphere containing 5% CO₂. RNA oligoribonucleotides were reverse transfected using Invitrogen Lipofectamine® 2000 (catalog no. 11668-019; Thermo Fisher Scientific, Inc.). A final concentration of 100 nM RNA duplex was used for each transfection. The RNA transfection efficiency using this method is ~90%. All transfections were performed according to the manufacturer's protocol.

Assessment of cell proliferation by cell counting kit-8 (CCK-8) assay. CCK-8 assay was used to assess the proliferation of MDA-MB-231 and MCF-7 subsequent to transfection with miR-520e mimics. Briefly, MDA-MB-231 and MCF-7 cells were plated at 3.0×10^3 cells/well in a 96-well plate and transfected with NC or miR-520e mimics at a final concentration of 100 nM. Prior to harvesting the cells, 100 µl of spent medium was replaced with an equal volume of fresh medium containing 10 µl CCK-8 (catalog no. CK04-500; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and incubated for 1 h at 37°C. The light absorbance of each well was measured at a wavelength of 450 nm using an Enzyme Immunoassay Instrument (model no. 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each assay was repeated in triplicate.

Flow cytometric analysis of cell apoptosis. The apoptotic rate of cells was determined using Invitrogen Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining (catalog no. V13241; Thermo Fisher Scientific, Inc.). In total, ~10,000 MCF-7 and MDA-MB-231 cells were cultured in 5% CO₂ in 6-well plates at 37°C. The cells were transfected with the miR-520e mimic or NC when the cells had reached ~50% confluency. Subsequent to 72 h, floating and adherent cells were collected, washed twice with PBS (pH 7.4), resuspended in 100 µl Annexin-binding buffer and incubated with 5 µl Annexin V-FITC and 3 µl PI (50 µg/ml). Subsequent to 20 min incubation in the dark at room temperature, 400 µl Annexin-binding buffer was added prior to flow cytometric analysis. The samples were analyzed within 30 min of staining. Flow cytometry was performed using the FACSCalibur and Cell Quest software (catalog no. Navil05; Beckman Coulter, Inc., Miami, FL, USA).

Transwell migration assay. A 24-well Boyden chamber with a 8 µm pore polycarbonate membrane (catalog no. 3422; Corning, Inc., Corning, NY, USA) was used to analyze the migration of MCF-7 and MDA-MB-231 cells. In total, 24 h subsequent to transfection with the RNA duplex, tumor cells (4×10^4 MCF-7 cells or 3×10^4 MDA-MB-231 cells in 200 µl

Table I. Clinical characteristics of 21 patients with breast cancer.

Characteristics	Cases, n (%)
Age, years	
>50	10 (47.6)
≤50	11 (52.4)
Histological grade	
>I	10 (47.6)
I	11 (52.4)
Tumor size	
>5 cm	6 (28.6)
≤5 cm	15 (71.4)
Tumor number	
>1	10 (47.6)
1	11 (52.4)

The histological grade was determined based on the modified Scarff-Bloom-Richardson grading system.

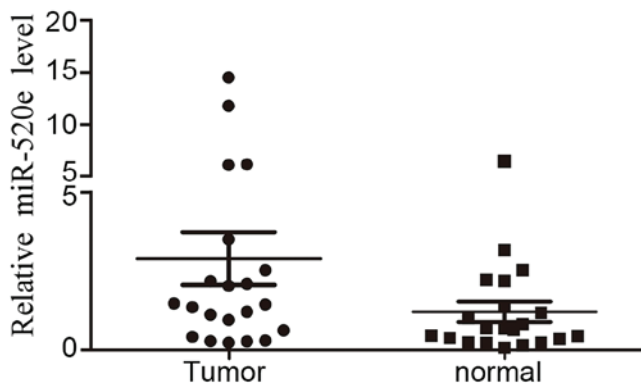


Figure 1. miR-520e expression was upregulated in BC tissues compared with adjacent non-cancerous tissues. Expression of miR-520e in 21 BC patients was examined by reverse transcription-quantitative polymerase chain reaction and the data was normalized to the expression of a reference gene (RNU6B) to yield the relative miR-520e levels for each sample. The median level of miR-520e in all BC tissues was set as relative 1. Cases were divided into two groups on the x axis the BC (Tumor) and matched adjacent non-cancerous tissue (normal) groups. Mann-Whitney U-test was applied to compare the differences in the miR-520e expression between BC tissues and adjacent non-cancerous tissues. The horizontal line indicates the median and interquartile range (25 to 75th percentiles). Data are expressed as the mean \pm standard error of the mean of 1 representative experiment (n=3). Similar results were obtained in ≥ 3 independent experiments. *P<0.05. BC, breast cancer.

serum-free DMEM) were added to the upper compartment of the Boyden chamber, while the lower compartment was filled with 600 μ l DMEM containing 10% FBS. Subsequent to incubation at 37°C for indicated times (24 h for MDA-MB-231, 48 h for MCF-7), cells that had not migrated and remained on the upper surface of the polycarbonate membrane were removed with cotton swabs. The cells on the lower surface of the membrane (those that had migrated) were fixed with formaldehyde, stained with crystal violet and counted in 5 randomly selected fields of view per well, under a light microscope at a

magnification of x150. The cells were transfected in 3 separate transfections and each was analyzed in triplicate.

Statistical analysis. The data was expressed as the mean \pm standard error of the mean for ≥ 3 independent experiments. Student's *t*-test was employed to analyze the significance of differences between the cancerous and non-cancerous tissue cells. For the comparison of miR-520e expression levels between the matched breast cancer and normal tissue samples, a Mann-Whitney U-test was used. All statistical tests were two-tailed and P<0.05 was considered to indicate a statistically significant difference. All analyses were performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

miR-520e expression is upregulated in human breast cancer tissues. A RT-qPCR array was performed to analyze the expression level of miR-520e in 21 paired breast cancer and adjacent non-cancerous breast tissues. miR-520e was significantly upregulated in breast cancer tissues compared with normal tissues (Fig. 1), suggesting that miR-520e overexpression in breast cancer cells is a frequent event and that there is an association between miR-520e upregulation and breast cancer tumorigenesis.

miR-520e promotes breast cancer cell growth in vitro. The upregulation of miR-520e in breast cancer tissue samples lead the present study to investigate whether miR-520e functions as an oncogene. The effect of miR-520e restoration on the proliferation of breast cancer cells was evaluated using a CCK-8 assay on the human breast cancer MCF-7 and MDA-MB-231 cell lines, which were transfected with a control RNA duplex (NC) or miR-520e duplex. miR-520e-transfected cells exhibited an increased proliferation compared with NC transfectants (Fig. 2). These findings indicate a cell growth-promoting role for miR-520e.

miR-520e promotes breast cancer cell migration in vitro. The effect of miR-520e on breast cancer cell migration was investigated using a Transwell assay. The restoration of miR-520e substantially increased the number of MCF-7 and MDA-MB-231 cells that invaded the lower chamber in the Boyden chamber (Fig. 3).

miR-520e suppresses breast cancer cell apoptosis in vitro. The effect that miR-520e exerts on the apoptotic rate of breast cancer cells was assessed using Annexin V-FITC and PI double staining, followed by flow cytometric analysis. Fig. 4 demonstrates that the restoration of miR-520e substantially decreased the percentage of MCF-7 and MDA-MB-231 cells that underwent apoptosis. These results indicate that miR-520e inhibits the apoptosis of breast cancer cells.

Discussion

Abnormal and uncontrolled cell proliferation is a hallmark of cancer and is caused by the misregulation of various crucial proteins and alterations in miRNA expression (22). miRNAs regulate numerous biological processes, including disease

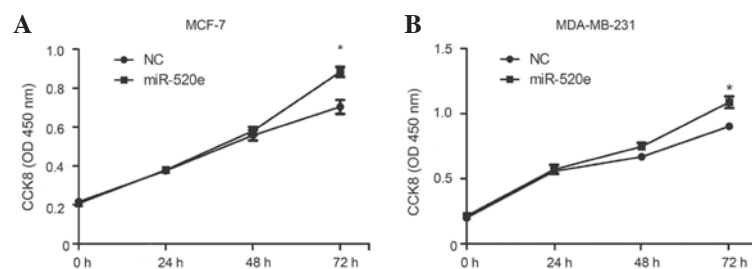


Figure 2. Overexpression of miR-520e promotes breast cancer cell proliferation *in vitro*. Human cell lines (A) MCF-7 and (B) MDA-MB-231 were transfected with miR-520e or NC mimics. The proliferating capacity of transfected cells was evaluated using a CCK8 assay at 0, 24, 48 and 72 h subsequent to transfection. Data are presented as the mean \pm standard error from the mean. In 1 representative experiment $n=3$. Similar results were obtained in ≥ 3 independent experiments. * $P < 0.05$. miR, microRNA; NC, negative control; CCK, cell counting kit; OD, optical density.

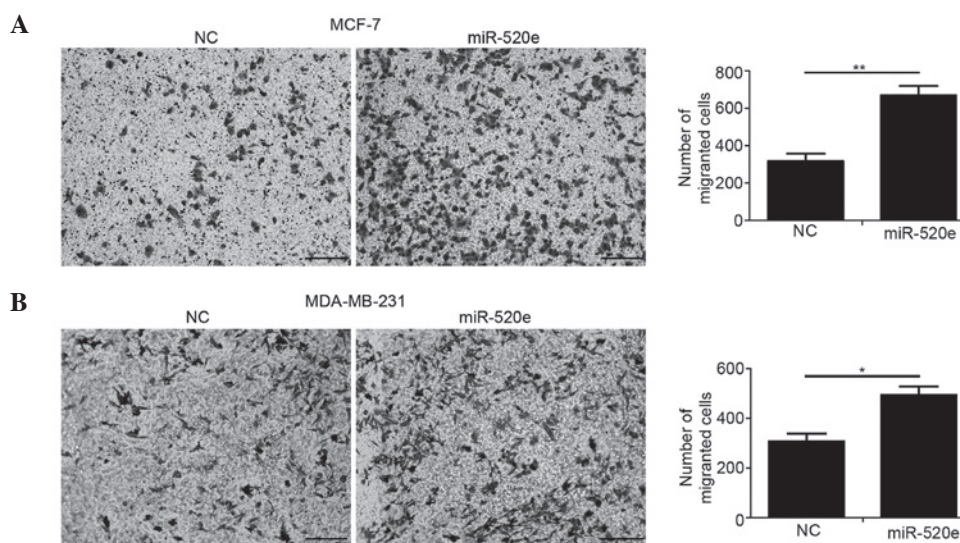


Figure 3. Restoration of miR-520e promotes breast cancer cell migration *in vitro*. Human cell lines (A) MCF-7 and (B) MDA-MB-231 transfected with NC or a miR-520e duplex were applied to a Boyden chamber and incubated for 24 h and 48 h, respectively, followed by staining with crystal violet. The 8 μ m pores of the polycarbonate membranes are visible. Representative images and the number of migrated cells are shown. Similar results were obtained in ≥ 3 independent experiments. Scale bar = 100 μ m. * $P < 0.05$, ** $P < 0.01$. miR, microRNA; NC, negative control.

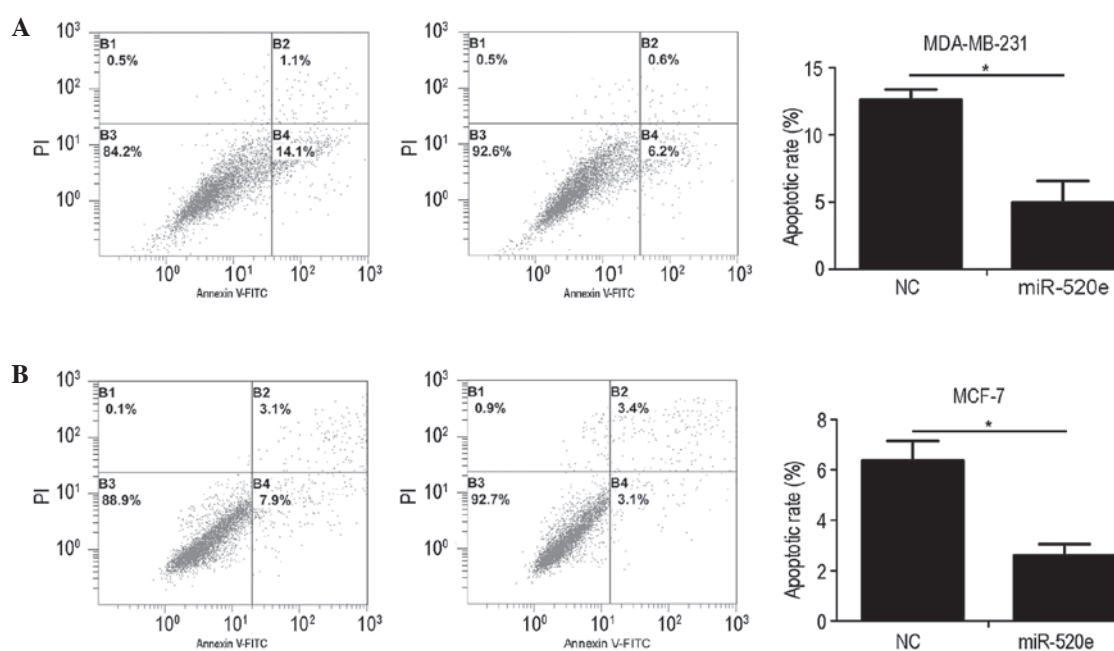


Figure 4. Ectopic expression of miR-520e inhibits breast cancer cell apoptosis. Human cell lines (A) MDA-MB-231 and (B) MCF-7 were transfected with NC or a miR-520e duplex for 72 h. Apoptosis was analyzed using Annexin V-fluorescein isothiocyanate and propidium iodide double staining and a flow cytometer. Representative images and the apoptotic rates (%) are shown. Similar results were obtained in ≤ 3 independent experiments. * $P < 0.05$. NC, negative control.

progression and cell development, proliferation, apoptosis and differentiation (8-12). Due to the numerous biological processes that miRNAs are involved in, it is not notable that the deregulation of miRNAs has been associated with tumor progression in certain human cancers. Numerous miRNAs have been observed in breast cancer development (23-27), by functioning alone or in combination. Therefore, investigating miRNAs that are aberrantly expressed in breast cancer may aid the understanding of the mechanisms that cause breast cancer carcinogenesis and progression. The present study demonstrated that miR-520e was markedly upregulated in breast cancer tissues compared with paired adjacent normal tissues. Furthermore, the ectopic expression of miR-520e promoted breast cancer cell proliferation, migration and invasion and inhibited cell apoptosis. The current study suggests that miR-520e may act as a novel oncomir in breast cancer and may be a potential therapeutic target.

Evading cell apoptosis is crucial for the malignant transformation of a tumor and tumor progression. It aids cells to avoid immune surveillance and survive in challenging tumor environments, including those that are low in nutrition and hypoxic (28). The present study demonstrated that miR-520e could suppress the apoptosis of breast cancer cells. Therefore, the upregulation of miR-520e may facilitate the survival and development of breast cancer.

Tumor metastasis is responsible for the rapid recurrence and poor prognosis of patients; therefore, the identification of novel anti-metastasis therapeutics is imperative (29). Previous studies using clinical specimens and *in vitro* and *in vivo* models have identified a limited number of miRNAs that promote metastasis, including miR-200, miR-10b and miR-21 (30-33). The present study revealed that miR-520e is associated with metastasis; upregulation of miR-520e may facilitate the metastasis of breast cancer cells and therefore the progression of breast cancer.

In summary, the present study investigated the potential role of miR-520e in breast cancer development. The findings of the current study suggest that upregulation of miR-520e may favor malignant transformation and tumor progression of breast cancer cells, and indicate that miR-520e possesses a potential application in anti-cancer therapy.

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