

MicroRNA-134 targets KRAS to suppress breast cancer cell proliferation, migration and invasion

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Abstract. The expression patterns and functions of microRNA-134 (miR-134) have been previously studied in numerous types of cancer. To the best of our knowledge, this is the first study of miR-134 in human breast cancer. In the present study, the expression patterns, biological functions and underlying molecular mechanisms of miR-134 in human breast cancer were investigated. Reverse transcription-quantitative polymerase chain reaction evaluated the expression of miR-134 in human breast cancer tissues, matched normal adjacent tissues, breast cancer cell lines and a normal mammary epithelial cell line. Following transfection with miR-134, an MTT assay, cell migration assay, cell invasion assay, western blot analysis and a luciferase assay were performed on the MCF-7 and MDA-MB-231 human breast cancer cell lines. The findings revealed that miR-134 expression levels were significantly downregulated in breast cancer cells. Statistical analysis demonstrated that low expression of miR-134 was significantly associated with lymph node metastasis, TNM stage and reduced cell differentiation. It was observed that miR-134 inhibited the growth, migration and invasion of breast cancer cells. Additionally, the present study indicated that miR-134 may directly target the Kirsten rat sarcoma viral oncogene homolog in breast cancer tissues. These results suggest that miR-134 may be used as a potential therapeutic biomarker in breast cancers.

Introduction

Breast cancer is the most common malignancy among women and the leading cause of cancer-associated mortality,

accounting for 14% of global cases (1,2). In the United States, there were ~234,190 recorded new cases and 40,730 mortalities due to breast cancer in 2015 (3). The etiology of breast cancer remains unclear, although genetic and epigenetic alterations are considered to contribute to tumorigenesis and progression (4). Despite advances in modern therapies for patients with breast cancer, including surgery, radiotherapy, hormonal therapy and various types of chemotherapeutic approaches using targeted and non-targeted drugs, numerous patients with breast cancer respond only transiently to conventional chemotherapy (5,6). A high proportion of these patients eventually exhibit tumor metastasis, which is a major contributor to cancer mortality (5,6). Therefore, it is necessary to explore the molecular mechanisms underlying the tumorigenesis and progression of breast cancer in order to develop more effective treatments.

Numerous previous studies have demonstrated that microRNAs (miRNAs) are involved in the tumorigenesis and progression of breast cancers (7-9). miRNAs are a novel group of non-protein-coding, single-stranded, short (generally 18-24 nucleotides in length) proteins, which regulate the translational or post-transcriptional levels of target mRNAs, through binding to the 3'-untranslated region (UTR) of those target mRNAs (10). These miRNAs are located in the introns of non-coding genes, the introns of protein-coding genes or the exons of non-coding genes (11). miRNAs have crucial functions in various physiological and pathological processes, including cell proliferation, survival, migration, invasion and the cell cycle (12). Previous studies have suggested that specific miRNAs may be downregulated or upregulated in certain types of tumors (13-15). Downregulated miRNAs may normally function as tumor suppressor genes, whereas upregulated miRNAs may normally function as oncogenes (16). Dysregulated miRNA expression has been observed in various types of human malignancies, including breast cancers (17). These previous studies indicated that specific dysregulated miRNAs may serve as useful biomarkers for breast cancer tumorigenesis, progression and clinical prognosis, as well as potential targets for breast cancer therapy (16,17).

The expression patterns and underlying mechanisms of miR-134 have been previously studied in various types of cancer. However, to the best of our knowledge, this is the first study of miR-134 in human breast cancer (18-20).

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In our current study, we examined miR-134 expression in breast cancer tissues and cell lines. The association between miR-134 expression and clinicopathological features was also analyzed. In addition, the effects of miR-134 on breast cancer cell proliferation, migration and invasion were evaluated. Furthermore, the molecular mechanism underlying the biological roles of miR-134 on breast cancer cells was explored. The results of the current study have potential therapeutic applications and may be used to develop current and novel treatments for breast cancers.

Materials and methods

Ethics statement and clinical specimens. The Ethics Committee of the Chengdu Military General Hospital approved the present study. At initial diagnosis, written informed consent was obtained from all patients. A total of 85 pairs of breast cancer tissue and normal adjacent tissue (NAT) samples were obtained from patients (age range, 23-82 years) who had undergone breast surgery at the Chengdu Military General Hospital (Chengdu, China). In the current study, the patients involved had not received chemotherapy or radiotherapy prior to breast surgery. Clinicopathological data for these patients, including age, tumor diameter, lymph node metastasis, TNM stage and pathological differentiation, were also collected. The tissue samples were snap frozen in liquid nitrogen immediately following surgery and stored at -80°C until use in the present study.

Cell culture and transfection. The MCF-7 and MDA-MB-231 breast cancer cell lines, and the MCF-10A normal mammary epithelial cell line, were acquired from the American Type Culture Collection (Manassas, VA, USA). All cell lines were grown in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) or RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin at 37°C in an atmosphere containing 5% CO₂ and 100% humidity. miR-134 mimics, negative control (NC) and luciferase reporter plasmids were synthesized by GenePharma Co. Ltd. (Shanghai, China). Cells were seeded in a six-well plate at 40-50% confluence. Following overnight incubation, the cells were transfected with miR-134 mimics or NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 50 nmol/l.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the tissue samples and cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA concentration was determined with NanoDrop ND-1000 Spectrophotometer. Equal amounts of RNA were subjected to cDNA synthesis using a PrimeScript RT Regent kit (Takara, Bio, Inc., Otsu, Japan). RT-qPCR was subsequently performed using a SYBR green kit (Takara Bio, Inc.) with U6 as an internal control, according to the manufacturer's protocol. The reaction system contained 10 µl SYBR Green I mix, 2 µl cDNA, 2 µl forward primer, 2 µl reverse primer and 4 µl ddH₂O. The thermal cycling

conditions of the reaction were as follows: 95°C for 10 min; and 40 cycles of 95°C for 15 sec and 60°C for 1 min. U6 RNA was used as an internal control. Primers were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). All RT-qPCR was performed in ABI 7500 RT-qPCR detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). All assays were performed in triplicate.

MTT assay. An MTT assay was used to investigate the effect of miR-134 on breast cancer cell growth. Transfected cells (miR-134 and NC) were seeded into 96-well plates at a density of 3x10³ cells/well. The cells were incubated with 20 µl MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA). Following incubation for 4 h at 37°C, the formazan precipitates were dissolved in 200 µl dimethyl sulfoxide. The absorbance at 490 nm was evaluated using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments were repeated in triplicate.

Cell migration and invasion assay. To investigate the effect of miR-134 on cell migration and invasion, Transwell chambers with an 8-µm pore polycarbonate membrane (Costar; Corning Incorporated, Corning, NY, USA) were used. Diluted Matrigel (50 µl; 2 mg/ml; BD Biosciences, San Jose, CA, USA) was placed on the inner chamber membrane surface for the invasion assay. Transfected cells were collected, counted and resuspended in single cell suspension (FBS-free culture medium). Subsequently, 1x10⁵ cells were added to the upper chamber and 500 µl culture medium supplemented with 20% FBS, was added into the lower chamber as a chemoattractant. Following a 24 h incubation, any non-migrated cells were carefully removed from the top of the chamber using a cotton swab. Subsequently, the chambers were fixed with 100% methanol, stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Haimen, China) for 10 min and washed with PBS (Gibco; Thermo Fisher Scientific, Inc.) 3 times. The chambers were photographed and counted in five random fields under a light microscope with x200 magnification using Photoshop (Adobe, San Jose, CA, USA). All experiments were repeated in triplicate.

miR-134 target prediction. The target genes of miR-134 were predicted by using TargetScan (version 7.0; <http://www.targetscan.org/index.html>) (21).

Western blotting. A western blot analysis was used to quantify the changes in Kirsten rat sarcoma viral oncogene homolog (KRAS) protein expression levels. Cells transfected with miR-134 and NC were lysed with a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The protein concentration was determined by using the bicinchoninic acid assay (Thermo Fisher Scientific, Inc., Rockford, IL, USA). An equal amount of protein (20 µg) from each cell line was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and the proteins were subsequently transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk (Beyotime Institute of Biotechnology) in Tris-buffered saline. The membranes were then incubated with a mouse anti-human

monoclonal KRAS primary antibody at a 1:500 dilution (cat. no. ab157255; Abcam, Cambridge, MA, USA) overnight at 4°C. After washing with Tris-buffered saline with 0.5% Tween 20 (Beyotime Institute of Biotechnology) 3 times, the membranes were incubated with a goat anti-mouse horse-radish peroxidase-conjugated secondary antibody (1:1,000 dilution; cat. no. ab97023; Abcam) at room temperature for 1 h. The protein bands were visualized using an enhanced chemiluminescence solution (Pierce Biotechnology, Inc., Rockford, IL, USA). Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. The protein blots were quantified using AlphaEase FC software (Cell Biosciences, Inc., San Jose, CA, USA).

Luciferase assay. To determine whether KRAS was a direct target of miR-134, a luciferase assay was used. Cells were seeded in 12-well plates at 30-40% confluence, and then were transfected with miR-134 mimics or NC, following which the cells were co-transfected with a reporter plasmid containing the wild-type (Wt) and mutant 3'-UTR of KRAS; Lipofectamine® 2000 was used as a transfection reagent. Following a 48 h transfection, a luciferase assay was performed using a dual-luciferase reporter assay system (Promega Corporation, Madison, WI, USA). The firefly luciferase activity was normalized to the corresponding *Renilla* luciferase activity. All Luciferase assays were repeated in 3 independent experiments.

Statistical analysis. Data were presented as the mean \pm standard deviation. Data were compared with SPSS 17 software (SPSS, Inc., Chicago, IL, USA) using a Student's t-test. A 2-tailed value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-134 is downregulated in breast cancer tissue samples and cell lines. The expression levels of miR-134 in breast cancer tissue samples, matched NATs, breast cancer cell lines and a normal mammary epithelial cell line were quantified using RT-qPCR. As indicated in Fig. 1A, miR-134 expression levels were significantly downregulated in breast cancer tissue samples compared with matched NATs ($P = 0.001$). As indicated in Fig. 1B, downregulation of miR-134 was also observed in MCF-7 ($P = 0.010$) and MDA-MB-231 ($P = 0.005$) cells compared with the MCF-10A normal mammary epithelial cell line. The results indicate that miR-134 may have an important role in breast cancer.

The association between miR-134 expression levels and the clinicopathological features of patients with breast cancer. The present study examined whether the expression levels of miR-134 were associated with the clinicopathological features of breast cancer. Statistical analysis revealed that low expression levels of miR-134 were significantly associated with lymph node metastasis ($P = 0.021$), TNM stage ($P = 0.037$) and reduced cell differentiation ($P = 0.01$; Table I). However, there was no significant correlation between miR-134 expression levels and other clinicopathological factors, including patient age and tumor diameter.

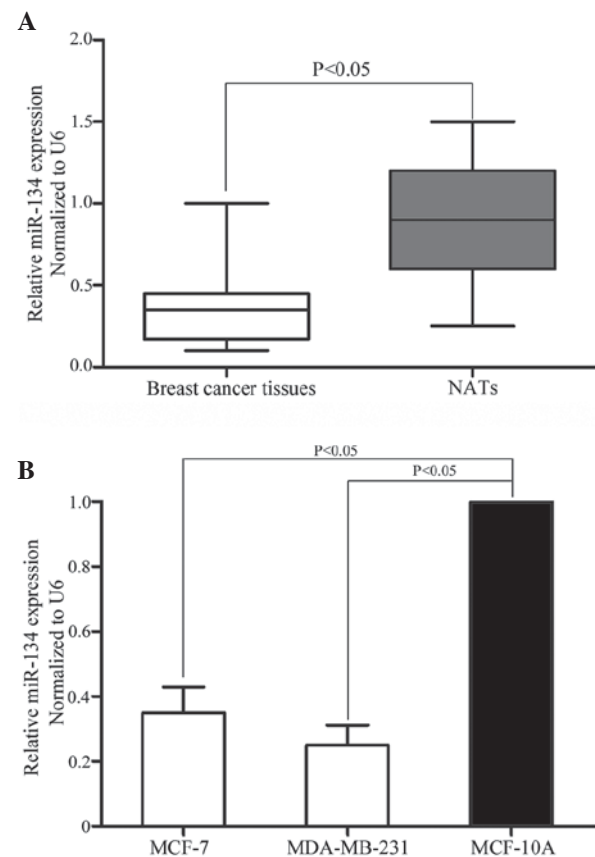


Figure 1. miR-134 expression in breast cancer tissue samples and cell lines. (A) miR-134 expression levels were significantly downregulated in breast cancer tissue samples compared with NATs. (B) miR-134 was significantly downregulated in MCF-7 and MDA-MB-231 breast cancer cell lines compared with the MCF-10A immortalized control cells. miR-134, microRNA-134; NAT, normal adjacent tissue.

miR-134 suppresses the proliferation of certain breast cancer cells. In order to assess the effect of miR-134 on breast cancer cell proliferation, miR-134 mimics were transfected into the MCF-7 and MDA-MB-231 cells. Following transfection (48 h incubation period), miR-134 was significantly upregulated in MCF-7 ($P = 0.001$) and MDA-MB-231 ($P = 0.005$) cells (Fig. 2A).

MTT assays were used to assess the effects of miR-134 on cell proliferation. It was observed that miR-134 significantly inhibited cell proliferation in MCF-7 ($P = 0.015$) and MDA-MB-231 ($P = 0.012$) cells (Fig. 2B), demonstrating that miR-134 may function as a tumor growth suppressor in human breast cancers.

miR-134 decreases the migration and invasion of breast cancer cells. Cell migration and invasion assays were performed to investigate the effects of miR-134 on cell motility. As indicated in Fig. 3, the migration and invasion of MCF-7 ($P = 0.024$ for migration; $P = 0.030$ for invasion) and MDA-MB-231 cells ($P = 0.032$ for migration; $P = 0.018$ for invasion), transfected with miR-134, was significantly decreased compared with the NC ($P < 0.05$). These results demonstrated that miR-134 may inhibit cell metastasis in breast cancer.

KRAS is a direct target gene of miR-134. To identify the targets of miR-134, TargetScan version 7.0 was used. As

Table I. Correlation between miR-134 expression and the clinicopathological features of patients with breast cancer.

Clinical feature	Case number	miR-134 expression		P-value
		Low	High	
Age				
<50 years	34	23	11	0.819
≥50 years	51	33	18	
Tumor diameter				
<2.5 cm	45	29	16	0.821
≥2.5 cm	40	27	13	
Lymph node metastasis				
Positive	30	35	10	0.021
Negative	55	21	19	
TNM stage				
I-II	47	26	21	0.037
III	38	30	8	
Pathological differentiation				
Moderately and highly differentiated	50	27	23	0.010
Poorly differentiated	35	29	6	

miR-134, microRNA-134.

indicated in Fig. 4A, KRAS was identified as a target of miR-134. Subsequently, western blot analysis was used to determine whether the protein expression levels of KRAS were downregulated following the transfection of MCF-7 and MDA-MB-231 cells with miR-134. As indicated in Fig. 4B, KRAS expression levels were significantly downregulated in MCF-7 ($P=0.015$) and MDA-MB-231 ($P=0.008$) cells following their transfection with miR-134 ($P<0.05$).

Luciferase assays were also performed to determine whether miR-134 directly targets KRAS. As indicated in Fig. 4C, miR-134 significantly inhibited the KRAS Wt, but not the KRAS mutant luciferase activity in MCF-7 ($P=0.028$) and MDA-MB-231 ($P=0.022$) cells. Principally, KRAS was observed to be a gene directly targeted by miR-134 *in vitro*.

Discussion

Previous studies have demonstrated that the aberrant expression of miRNAs is a characteristic of certain malignancies, including breast cancers (22-24). miR-134 was first identified as a brain-specific miRNA located at 14q32 (25); it was observed to be localized in the synapto-dendritic compartment of hippocampal neurons and involved in the regulation of the neuronal microstructure (26). Previous studies have indicated that miR-134 was involved in several physiological and pathological processes; Han *et al* (27) identified that miR-134 has a crucial role in the translation-dependent guidance of nerve growth cones. miR-134 was also identified as a potential plasma biomarker in the diagnosis of acute pulmonary embolism (18). In the present study, the results demonstrated that miR-134 is downregulated in breast cancer tissues and cell lines. The expression levels of miR-134 were significantly associated with lymph node metastasis, TNM stage and reduced cell

differentiation in patients with breast cancer. Furthermore, the upregulation of miR-134 expression inhibits breast cancer cell proliferation, migration and invasion. This study improved our current understanding of the expression pattern and function of miR-134 in breast cancer. It was also observed that the restoration of normal miR-134 expression may serve as a novel therapeutic approach for breast cancer treatment.

miR-134 has been previously demonstrated to function as a tumor suppressor in several types of tumors (18-20). In hepatocellular carcinoma, miR-134 suppressed cell metastasis by regulating the expression of integrin $\beta 1$ (19). In glioma, the downregulation of miR-134 expression is typical, and cell growth, migration and invasion was inhibited by the upregulation of miR-134; miR-134 overexpression also enhanced cell apoptosis in human glioma cells (20). Li *et al* (28) demonstrated that the expression levels of miR-134 were associated with the invasive potential and epithelial-mesenchymal transition (EMT) phenotype of non-small-cell lung cancer (NSCLC) cells. Functional assays determined that miR-134 inhibited EMT by targeting Forkhead Box M1 in NSCLC cells (28). Furthermore, miR-134 was demonstrated to be associated with drug resistance by targeting the multidrug resistance-associated protein 1/ATP binding cassette subfamily C member 1 in H69AR lung cancer cells (29). However, miR-134 has also been demonstrated to function as an oncogene (30). Liu *et al* (30) observed that miR-134 expression was upregulated in head and neck squamous cell carcinoma (HNSCC). miR-134 overexpression promoted the oncogenicity, carcinogenesis and metastasis of HNSCC cell lines (30). These contradictory studies suggested that the role of miR-134 in cancers may be tissue-type dependent.

Identification of the miR-134 target genes is important for understanding the role of miR-134 in breast cancer

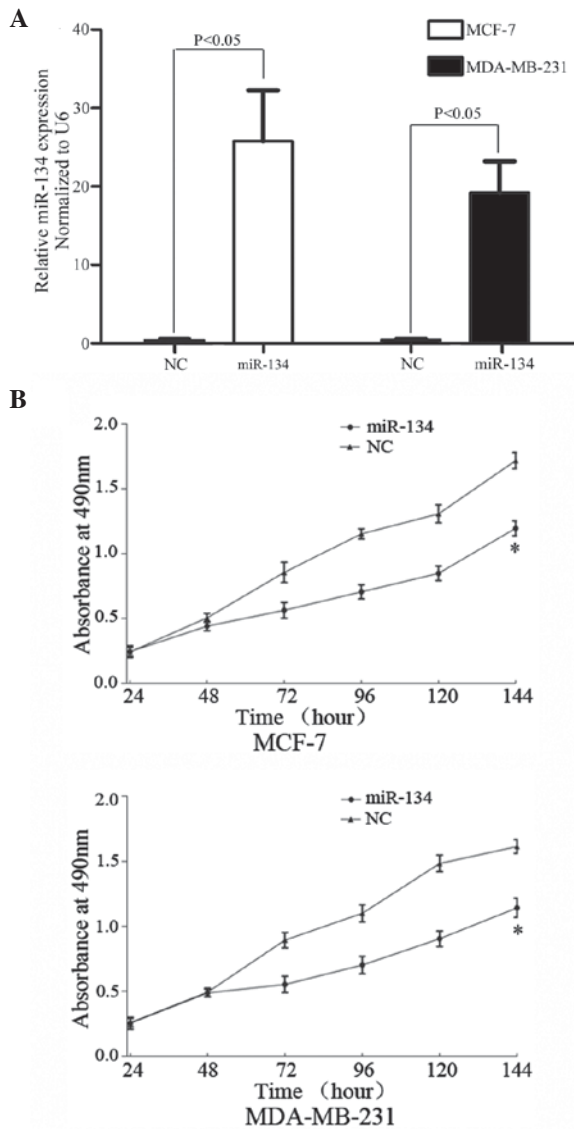


Figure 2. (A) Transfection of miR-134 mimics into MCF-7 and MDA-MB-231 cells significantly increased the expression levels of miR-134. (B) The MTT assay revealed that the upregulation of miR-134 significantly suppressed cell proliferation in MCF-7 and MDA-MB-231 cells. miR-134, microRNA-134; NC, negative control. * $P < 0.05$.

tumorigenesis and development. It is also essential for the development of novel targeted therapies for patients with breast cancer. In the present study, an important molecular link between miR-134 and KRAS was observed in breast cancer. Firstly, TargetScan predicted that KRAS mRNA was a direct target of miR-134. Secondly, western blotting revealed that miR-134 suppressed the expression of KRAS in breast cancer cells. Finally, a luciferase assay demonstrated that miR-134 directly targeted the KRAS 3'-UTR. These results suggested that miR-134 may have a tumor suppressor role in breast cancer tumorigenesis and development by targeting KRAS.

The rat sarcoma (RAS) genes encode a family of homologous 21 kDa GTP-binding proteins, including Harvey RAS, neuroblastoma RAS and KRAS (31). Among the RAS proteins, KRAS was first identified as the transforming factor in the Harvey and Kirsten strains of the rat/mouse sarcoma virus (32). KRAS primarily functions as a critical 'on-off'

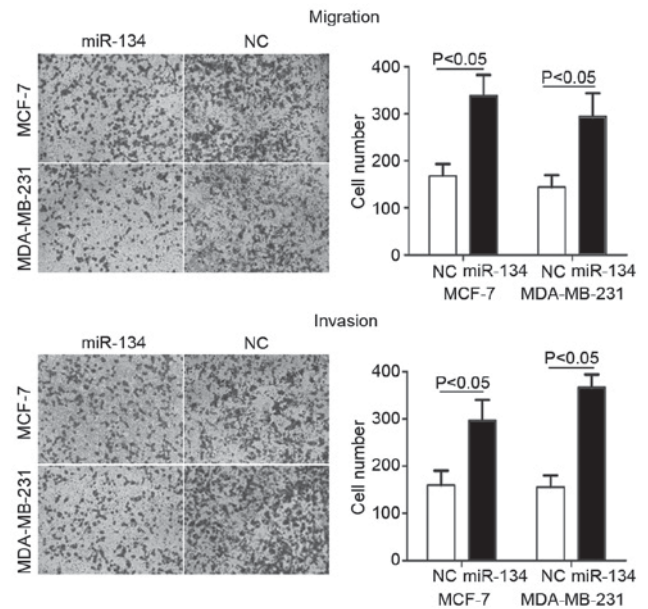


Figure 3. miR-134 suppressed the migration and invasion of MCF-7 and MDA-MB-231 cells. The migration and invasion assays were performed using transwell chambers. Ectopic miR-134 inhibited the migration and invasion of MCF-7 and MDA-MB-231 cells. miR-134, microRNA-134; NC, negative control.

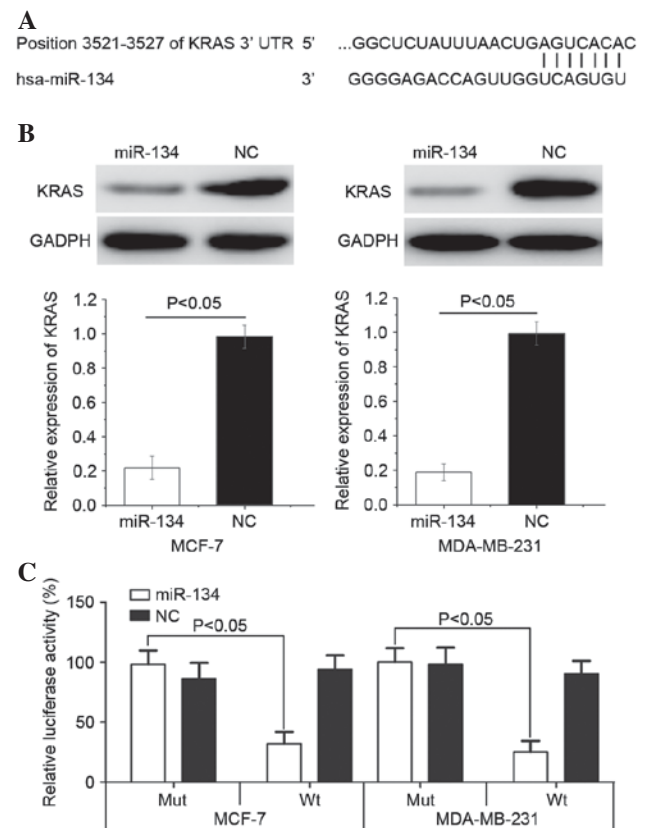


Figure 4. KRAS is directly targeted by miR-134. (A) TargetScan determined that KRAS mRNA contained an miR-134 seed match at position 3521-3527 of the KRAS 3'-UTR. (B) The western blot assay revealed that KRAS expression levels were significantly downregulated in MCF-7 and MDA-MB-231 cells following transfection with miR-134. (C) miR-134 significantly inhibited the KRAS Wt, but not the KRAS Mut, luciferase activity in MCF-7 and MDA-MB-231 cells. Wt, wild type; Mut, mutant, KRAS, Kirsten rat sarcoma viral oncogene homolog; miR-134, microRNA-134; UTR, untranslated region; MCF-7 cells, Michigan cancer foundation-7 cells; NC, negative control.

switch in cell signaling networks that relay extracellular signals to the nucleus and connect multiple upstream signals to various types of downstream signaling pathways (33). These signaling pathways are involved in cell differentiation, proliferation, survival rate, migration, invasion, cytoskeletal changes and the cell cycle (34,35). KRAS is a member of the epidermal growth factor receptor (EGFR)/RAS/mitogen activated protein kinases (MAPK) cell signaling pathway and has previously been demonstrated to be associated with physiological and pathological processes (36).

In breast cancer, KRAS/MAPK signaling has an important role in transfer growth signaling from the extracellular environment (37,38). The activation of the KRAS/MAPK signaling pathway induces numerous responses in breast cancer cells, resulting in the regulation of cell proliferation, differentiation, migration and invasion (39). In breast cancer tumorigenesis and progression, numerous facets may induce the upregulation of KRAS/MAPK signaling; this has been demonstrated to be regulated in breast cancer tissues by increased expression levels of EGFR, human epidermal growth factor receptor 2/erythroblastic leukemia viral oncogene homolog 2 and insulin-like growth factor receptor (40-42). Previous studies identified that KRAS was regulated by numerous miRNAs in breast cancer. Johnson *et al* revealed that the lethal-7 miRNA family regulates KRAS and cytoplasmic-myelocytomatosis (43). Kent *et al* (44) also demonstrated that miR-134 and miR-145 enhanced RAS signaling by downregulating KRAS and the RAS-responsive element-binding protein. Through overexpressing miR-134 in breast cancer cell lines, the present study demonstrated that miR-134 decreases cell proliferation, migration and invasion by downregulating KRAS expression levels. Therefore, miR-134 may act as a regulator of the KRAS oncogene, which may have certain clinical applications.

To the best of our knowledge, this study is the first to demonstrate that miR-134 is downregulated in breast cancer and is significantly associated with lymph node metastasis, TNM stage and reduced cell differentiation. It was observed that miR-134 inhibits cell proliferation, migration and invasion in breast cancer. The identification of the candidate target genes of miR-134 may provide an insight into the potential mechanisms underlying the function of miR-134 in breast cancer. miR-134 may contribute to breast cancer tumorigenesis and development through the downregulation of KRAS, indicating that miR-134 may function as a tumor suppressor in breast cancer.

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