Abstract. MicroRNA (miRNA or miR)-506 is a novel miRNA related to the survival of breast cancer patients. However, the mechanism underlying miRNA-506 involvement in breast carcinogenesis remains unclear. In the present study, we found that miR-506 was downregulated in human breast malignant tissues and breast cancer cell lines by RT-qPCR analysis, and the expression level of miR-506 was decreased with the increasing of tumor stage. Subsequently, gain-of-function and loss-of-function experiments were performed in vitro, and the results from MTT assay, Transwell-Matrigel invasion assay and cell adhesion assay revealed that miR-506 suppresses cell proliferation, invasion and adhesion of breast cancer cells. Luciferase reporter assay revealed that IQ motif containing GTPase activating protein 1 (IQGAP1) is a direct target of miR-506. miR-506 represses the expression of IQGAP1 and its downstream extracellular signal regulated kinase (ERK) mitogen-activated protein kinase (MAPK) signaling pathways, as demonstrated by the RT-qPCR and western blot analysis. Furthermore, we found that IQGAP1 rescues the effect of miR-506 on cell proliferation, invasion, adhesion, and the activation of ERK MAPK signaling. In conclusion, the present study is the first to provide evidence that miR-506 acts as a tumor suppressor, at least partially, by directly downregulating IQGAP1 in breast cancer cells. The miR-506/IQGAP1/ERK pathway may be a novel therapeutic target in breast cancer.

Introduction

Breast cancer is the most common malignancy in women worldwide (1-3). Similar to many other solid tumors, distant metastasis are responsible for >90% of breast cancer-related mortality (4).

MicroRNAs (miRNAs or miRs) are a subclass of 19-25 nucleotides in length, non-coding RNAs that have received increasingly attention in recent years. miRNAs play important regulatory roles in a variety of biological processes, such as cellular proliferation, differentiation, apoptosis and motility (5). Growing evidence indicates that the alteration of miRNA expression in tumors is associated with tumor development and progression (6). miR-506 is a novel miRNA, and it has been demonstrated that the expression pattern of miR-506 is different in different types of malignant tumors, suggesting the role of miR-506 is complex in cancer progression (7-10). The meta-analysis revealed that miR-506 is related to the survival of breast cancer patients (11). However, the mechanism underlying miRNA-506 involvement in breast carcinogenesis remains unclear.

In the present study, we investigated the expression of miR-506 in different breast tissues and breast cancer cell lines. In addition, gain-of-function and loss-of-function experiments were performed in vitro to examine the role of miR-506 in breast cancer cell proliferation, invasion and adhesion. Furthermore, a novel target by which miR-506 exerts its effects on breast carcinogenesis was identified.

Materials and methods

Clinical breast tissues. The present study was approved by the Ethics Committee of the China-Japan Union Hospital of Jilin University. The surgical specimens, including 48 normal, 42 fibroadenoma and 48 malignant breast tissues were obtained from patients in the Breast Surgical Department of China-Japan Union Hospital of Jilin University. Among these 48 patients with malignant breast cancer, 8 patients had stage 0, 13 patients had stage I, 10 patients had stage II, 9 patients had stage III and the other 8 patient had stage IV, at the time of the diagnosis. None of these patients had received chemotherapy or radiation therapy treatment before surgery. Written informed consent was obtained from each patient prior to enrollment in this study.

Cell culture and cell transfection. Human breast cancer cell lines (T47D, MDA-MB-231, MCF7, SK-BR-3 and HCC1937), normal human epithelial mammary cell line MCF-10A and HEK293 cells were obtained from the American Type
Culture Collection (ATCC; Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS; Invitrogen). The cells were maintained at 37°C in 5% CO₂ and were passaged every 2-3 days. The miR-negative control (NC), miR-506 mimic, miR-506 inhibitor, IQGAP1-pcDNA3.1 and IQGAP1-shRNA were transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Luciferase assay. Luciferase constructs were made by ligating fragments containing the wild-type (WT) and mutant-type (MUT) 3' untranslatable region (UTR) of IQ motif containing GTPase activating protein 1 (IQGAP1) in pMIR-REPORT luciferase vector (Applied Biosystems, Foster City, CA, USA). The luciferase constructs were co-transfected with the miR-NC or the miR-506 mimic into the HEK293 cells. Firefly and Renilla luciferase activity were measured using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

MTT assay. Cells (5x10³) were suspended and cultured in the 96-well plates overnight. Following transfection, 10 µl MTT solution (0.5 mg/ml; Sigma, St. Louis, MO, USA) was added to each well, and the plates were incubated at 37°C for 4 h. The formazan granules were dissolved using DMSO (Sigma), and then the absorbance rates were measured at 570 nm using a microplate reader (Infinite M200; Tecan Group Ltd., Männedorf, Switzerland).

Transwell-Matrigel invasion assay. The Transwell inserts (Corning Incorporated, Corning, NY, USA) precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used to determine breast cancer cell invasion ability. Briefly, the cells were suspended in the FBS-free medium at the density of 5x10⁵ cells/ml and placed in the upper chambers. The lower chambers were filled with cell medium containing 8% FBS. Following incubation at 37°C for 24 h, the cells on the upper surface of the membrane were removed by a cotton swab, and the cells on the lower surface of the insert were fixed in 95% ethanol and stained with hematoxylin. The invaded cells were counted under a light microscope (Nikon, Tokyo, Japan).

Cell adhesion assay. For adhesion assay, the 96-well plates were precoated with fibronectin (Sigma), and then blocked with 1% bovine serum albumin (BSA; Sigma) for 2 h. The cells were suspended in the FBS-free medium at the density of 3x10⁵ cells/ml and seeded in the 96-well plates. Following incubation at 37°C for 2 h, the adhesive cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. The crystals were dissolved using SDS (Amresco LLC, Solon, OH, USA) and then the absorbance rates were measured at 570 nm using a microplate reader (Infinite M200; Tecan Group Ltd.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from the breast tissues and breast cancer cell lines was extracted using the TRIzol reagent (Invitrogen). For miR-506 amplification, mirVana RNA isolation kit (Ambion, Austin, TX, USA) was used for RNA isolation following the manufacturer's instructions. RNA samples were reverse transcribed to complementary DNAs using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). Subsequently, the cDNA was amplified by real-time PCR on an ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems) using the SYBR-Green PCR kit (Applied Biosystems). The conditions for PCR amplification were as follows: an initial 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec. The Ct value was calculated using the ΔΔCt method.

Western blot analysis. The total protein was extracted using the Total Protein Extraction kit (BioChain Institute Inc., Hayward, CA, USA). Protein (30 µg) was resolved on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) by electroblotting. The membranes were then incubated with 5% BSA (Sigma) at 4°C overnight. After washing in Tris-buffered saline with 0.1% Tween-20, the membranes were incubated with the primary antibodies, including rabbit polyclonal to IQGAP1 (1:800; cat. sc-10792; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal to B-Raf (1:400; cat. sc-9002; Santa Cruz Biotechnology), rabbit monoclonal to Erk1/2 (Thr202/Tyr204) (1:800; cat. 4695; Cell Signaling Technology, Beverly, MA, USA), rabbit monoclonal to phospho-Erk1/2 (Thr202/Tyr204) (1:500; cat. 4376; Cell Signalling Technology), and rabbit polyclonal to GAPDH (1:1,000; cat. sc-25778; Santa Cruz Biotechnology) at 37°C for 2 h, followed by the incubation of horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000; cat. sc-2004; Santa Cruz Biotechnology) at 37°C for 1 h. Chemiluminescence detection was carried out by using ECL Plus™ (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis. All data were expressed as the mean ± SD. Differences between 2 groups were assessed by the Student's t-test. Analysis was performed using SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL, USA). A P-value <0.05 was considered significant.

Results

Expression of miR-506 in human breast tissues and breast cancer cell lines. Expression of miR-506 in the normal, fibroadenoma and malignant breast tissues was analyzed using RT-qPCR. The results showed that miR-506 expression was not significantly different between the normal and fibroadenoma breast tissues. However, miR-506 expression was significantly reduced in the malignant breast tissues compared with the normal and fibroadenoma breast tissues (Fig. 1A). Furthermore, we found that miR-506 expression was decreased with the increasing of tumor stage (Fig. 1B).

We detected the expression of miR-506 in five breast cancer cell lines (T47D, MDA-MB-231, MCF7, SK-BR-3 and HCC1937). A normal human epithelial mammary cell line MCF-10A was used as control. The results obtained from RT-qPCR analysis revealed that compared with the control, miR-506 expression was significantly decreased in the breast cancer cell lines. Among these breast cancer cell lines, miR-506 had the lowest expression in MDA-MB-231 cells, and highest expression in HCC1937 cells (Fig. 1C).
Expression of IQGAP1 in human breast tissues and breast cancer cell lines. IQGAP1 expression was also examined in human breast tissues and breast cancer cell lines. In contrast to miR-506 expression, IQGAP1 protein was significantly upregulated in the malignant breast tissues compared with the normal and fibroadenoma breast tissues (Fig. 2A). In addition, IQGAP1 protein was significantly increased in the breast cancer cell lines compared with the normal control MCF-10A cells (Fig. 2B).

Effect of miR-506 on cell proliferation, invasion and adhesion of breast cancer cells. To reveal the effect of miR-506 on breast cancer cell proliferation, invasion and adhesion, the miR-506 mimic was transfected into the MDA-MB-231 cells, which have low endogenous miR-506 expression, to overexpress miR-506. In addition, we knocked down the expression of miR-506 by transfection of the miR-506 inhibitor into the HCC1937 cells, which show high miR-506 expression. As demonstrated in Fig. 3A, the expression level of miR-506 was increased ~4.6-fold in MDA-MB-231 cells following transfection with the miR-506 mimic; however, miR-506 expression
was decreased ~85% in HCC1937 cells following transfection with the miR-506 inhibitor (Fig. 3B).

We found that compared with the control, MDA-MB-231 cells with ectopic expression of miR-506 showed a significant...
decrease in cell proliferation by MTT assay (Fig. 4A). The number of invaded cells was also decreased in MDA-MB-231 cells transfected with the miR-506 mimic in comparison to the cells transfected with the miR-NC (Fig. 4B). Furthermore, cell adhesion assay revealed that following transfection with the miR-506 mimic, the adhesive ability of the MDA-MB-231 cells was significantly reduced compared with that of the miR-NC (Fig. 4C).

On the contrary, inhibition of miR-506 in HCC1937 cells led to the induction of cell proliferation, increased number of invaded cells and adhesive ability (Fig. 5).

**Effect of miR-506 on IQGAP1, B-Raf and phosphorylated (pho)-extracellular signal regulated kinase (Erk) 1/2 expression.** To investigate whether miR-506 regulates IQGAP1 expression, the miR-506 mimic and the miR-506 inhibitor was transfected into the MDA-MB-231 and HCC1937 cells, respectively, and IQGAP1 expression was analyzed both at the mRNA level and protein level. It was shown that IQGAP1 expression was significantly decreased in the MDA-MB-231 cells transfected with the miR-506 mimic (Fig. 6A). Transfection of miR-506 inhibitor into the HCC1937 cells led to increased expression of IQGAP1 (Fig. 6B).

In addition, we found that the ERK MAPK pathway was suppressed in MDA-MB-231 cells by transfection of the miR-506 mimic, as evidenced by the decreased expression of B-Raf and pho-Erk1/2 (Fig. 7A). In HCC1937 cells, the expression of B-Raf and pho-Erk1/2 was significantly increased by the miR-506 inhibitor (Fig. 7B).

**mir-506 regulates IQGAP1 expression by directly targeting its 3’UTR.** miRanda algorithms were employed to search for putative gene targets of miR-506, and IQGAP1 was identified as a potential target. Subsequently, luciferase reporter assay was performed to determine whether IQGAP1 was a direct target of miR-506. IQGAP1 3’UTR (wt 3’UTR or mut 3’UTR) luciferase reporter vector was co-transfected with the miR-506 mimic or miR-NC into the HEK293 cells. As shown in Fig. 8, the luciferase activity of IQGAP1 wt 3’UTR was notably decreased in the miR-506 mimic group compared with the miR-NC group. However, the luciferase activity of IQGAP1 mut 3’UTR had no significant difference between the miR-506 mimic group and the miR-NC group.

**IQGAP1 attenuates the effect of miR-506 on cell proliferation, invasion and adhesion of breast cancer cells.** Since IQGAP1 was shown to be a direct target of miR-506, its principle role was further investigated by its overexpression or suppression in MDA-MB-231 and HCC1937 cells. As shown in Fig. 9A, the expression of IQGAP1 was significantly increased in MDA-MB-231 cells following transfection with the miR-506 mimic and IQGAP1-pcDNA3.1 compared with the cells only transfected with the miR-506 mimic. In addition, transfection of the IQGAP1 shRNA in HCC1937 cells led to significantly decreased expression of IQGAP1 (Fig. 9B).
We found that the inhibition of cell proliferation, invasion and adhesion as a consequence of miR-506 mimic transfection in MDA-MB-231 cells was attenuated by the overexpression of IQGAP1 (Fig. 10).

As expected, IQGAP1 suppression also reversed the effect of miR-506 inhibitor on cell proliferation, invasion and adhesion in HCC1937 cells (Fig. 11).

**IQGAP1 attenuates the effect of miR-506 on B-Raf and pho-Erk1/2 expression.** In addition, we found that the inhibitory effect of miR-506 mimic on the expression of B-Raf and pho-Erk1/2 in MDA-MB-231 cells could be reversed in the presence of IQGAP1 overexpression (Fig. 12A). However, the increased expression of B-Raf and pho-Erk1/2 by the transfection of miR-506 inhibitor was abolished by IQGAP1-shRNA (Fig. 12B).

**Discussion**

In the present study, we demonstrated downregulation of miR-506 in both the breast malignant tissues and human breast cancer cell lines. In addition, expression level of miR-506 was decreased with the increasing of tumor stage. These results indicate that miR-506 is clearly involved in the development of human breast cancer. Subsequently, in vitro experiments were performed to investigate the role of miR-506 on breast cancer cell proliferation, invasion and adhesion.

miR-506 was cloned relatively recently, and it acts as a tumor suppressive miRNA in various cancers and malignantly transformed cells (7-9,12,13). Downregulation of miR-506 has been identified in various tumors, and overexpression...
of miR-506 shows inhibitory effect on the development and progression of hepatocellular carcinoma, cervical cancer and ovarian cancer (7,9,13). However, Streicher et al (10) reported that miR-506 was upregulated and acts as an oncogene in melanomas. Taken together, these findings suggested that the function of miR-506 appears to be cell type-specific. In the present study, we clearly demonstrated the anti-oncogenic role of miR-506 in breast cancer cells. Both gain-of-function and loss-of-function experiments revealed that miR-506 suppresses cell proliferation, invasion and adhesion of breast cancer cells. These findings are consistent with the reports by Arora et al (8).

To date, there has been no report on the molecular mechanisms underlying the role of miR-506 in breast cancer. In this study, we first identified IQGAP1 as a direct target of miR-506. IQGAP1 is a scaffold protein which has ubiquitous expression (14,15). It contains multiple protein-interacting domains, including one calponin homology domain, one Ras-GAP-related domain, a polyproline binding domain and four calmodulin-binding motifs. IQGAP1 interacts with components of the cytoskeleton, the intercellular adhesion complex, and several signaling molecules, thus, having roles in many different aspects of cell physiology (16).
IQGAP1 has attracted attention because IQGAP1 plays important roles in the control of cell adhesion, polarization and migration (17). Overexpression of IQGAP1 has been shown in glioblastoma, hepatocellular carcinoma, lung and pancreatic cancer (18-22). IQGAP1 expression is also associated with the development of breast cancer (23,24). In the present study, we demonstrated that IQGAP1 expression is regulated by miR-506. Furthermore, IQGAP1 could attenuate the effect of miR-506 on breast cancer cell proliferation, invasion and adhesion.

The ERK mitogen-activated protein kinase (MAPK) pathway signaling is downstream of IQGAP1 (25), and this pathway was found to be repressed by miR-506 in the present study. There is a direct interaction between IQGAP1 and B-Raf, which modulates the activation of B-Raf (26). Raf was able to phosphorylate and activate the dual specificity protein kinases MEK1 and MEK2, thus leading to the phosphorylation of Erk1 and Erk2 (27). In addition, it has been demonstrated that IQGAP1 contributes to the regulation of epidermal growth factor (EGF)-stimulated ERK activity (28,29). The Ras/Raf/MEK/ERK cascade is suggested to play vital roles in several fundamental cellular activities (30). In the present study, we first proved that miR-506 represses the activation of B-Raf and Erk1/2, at least partially, by downregulation of IQGAP1.

Collectively, these findings revealed that miR-506 expression is downregulated in breast cancer and associated with the tumor stage. miR-506 inhibits breast cancer cell proliferation, invasion and adhesion, as well as the ERK MAPK pathway, at least partially, by directly downregulating IQGAP1. The miR-506/IQGAP1/ERK pathway may be a novel therapeutic target in breast cancer.

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References