Abstract. It has been reported that the expression of angiomotin (AMOT) is upregulated in breast cancer. However, the regulatory mechanism remains unknown. In the present study, we aimed to ascertain whether the expression of AMOT is regulated by microRNAs (miRNAs) in breast cancer. In the present study, miR-205 was significantly downregulated in breast cancer samples and it was identified to directly target the 3'-untranslated region (3'-UTR) of AMOT in breast cancer MCF-7 cells by luciferase assay. miR-205 and small interfering RNA (siRNA)-mediated AMOT-knockdown experiments revealed that miR-205 significantly inhibited the proliferation and the invasion of MCF-7 cells through a decrease in the expression of AMOT, yet had no effect on apoptosis. Furthermore, we observed that the overexpression of AMOT partially reversed the inhibitory effect of miR-205 on the growth and the invasion of MCF-7 cells. The data indicated that miR-205 regulated the proliferation and the invasion of breast cancer cells through suppression of AMOT expression, at least partly. Therefore, the disordered decreased expression of miR-205 and the resulting AMOT upregulation contributes to breast carcinogenesis, and miR-205-AMOT represents a new potential therapeutic target for the treatment of breast carcinoma.

Introduction

Breast cancer is one of the most common types of cancer originating from breast tissues, and is the second most notorious cause of cancer-related deaths after lung cancer. Currently, the prognosis of breast cancer is encouraging in virtue of the progress in diagnosis and effective systemic therapy, including surgical operation, chemotherapy and radiotherapy. However, such as for other solid tumors, the death rate of breast cancer is more than 90% due to distant metastases (1). Numerous factors affect the occurrence and progression of breast cancer, such as inactivation of tumor-suppressor genes and activation of oncogenes by DNA hypermethylation or histone deacetylase (2-4). Previous studies indicate that the membrane-associated protein AMOT is one of the most important biochemical characteristics of breast cancer (5,6). An angiostatin binding protein AMOT was first discovered in 2001 (7) and plays an important role in the regulation of endothelial cell migration and tube formation (7,8). Research has demonstrated that the expression of AMOT is upregulated in breast cancer tissues (5). However, the mechanism involved in the upregulation of AMOT in breast cancer remains unclear.

MicroRNAs (miRNAs) are a class of endogenous non-coding small RNAs of ~18-25 nucleotides long that play an important role in post-transcriptional regulation and are found in all eukaryotic cells. miRNAs usually have imperfect binding to the 3'-untranslated regions (3'-UTRs) of target mRNAs, resulting in translational silencing or mRNA degradation (9,10), and only a handful of miRNAs are able to enhance the expression of mRNAs (11). miRNAs play important roles in multifarious cellular processes, such as apoptosis and proliferation (12), and are frequently upregulated or downregulated in human types of cancers (13), acting as either tumor suppressors or oncogenes (14). The expression of microRNA-205 (miR-205) was found to be downregulated (15) or upregulated (16) in tumor tissues when compared with matched adjacent normal tissues. Various studies have found that miR-205 is downregulated in breast cancer tissues and in human breast cancer cell lines (17-19). However, the exact role of miR-205 in breast cancer at present remains unclear.

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In order to lay the foundation for later study, we performed quantitative real-time PCR and western blot analysis of AMOT in human breast cancer tissues, and found that the expression of AMOT was significantly upregulated in tumor tissues when compared with that in matched adjacent normal tissues both at the protein and mRNA levels, consistent with Holmgren et al (5). The aim of the present study was to investigate the potential involvement of miR-205 in the regulation of membrane-associated protein AMOT expression on the mechanism of occurrence and development of breast cancer. In
the present study, we showed that overexpression of miR-205 significantly suppressed the proliferation and invasion of breast cancer cells. Furthermore, we identified that miR-205 directly targets AMOT by binding to its 3'-UTR, resulting in inhibition of AMOT translation. Moreover, overexpression of AMOT reversed the inhibitory effect of miR-205 on the growth and the invasion of MCF-7 cells, in part.

Materials and methods

Tissues and cell lines. Tumor tissues and matched adjacent normal tissues (20 breast cancer tissue samples and 20 normal adjacent tissue samples) were obtained from breast cancer patients at the Department of General Surgery, Sun Yat-Sen University Cancer Center, from 2010 to 2013. All of the patients had an accurate histological diagnosis according to the clinicopathological criteria of the International Union for Cancer Control (UICC). The median age of the patients was 49.6 years (range, 34-72 years). All of the malignant tissues were from stage II-III tumors, according to the International Federation of Gynecology and Obstetrics (FIGO) classification. All patients provided consent for the use of their specimens in research, and this use was approved by the Institutional Research Ethics Committee of Sun Yat-sen University Cancer Center.

Homo sapiens breast cancer SKBR-3, MDA-MB-435S and MCF-7 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The breast cancer cells were maintained according to the vendor's instructions. In brief, SKBR-3 cells were cultured in McCoy's 5A medium (modified) (ATCC) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin). MDA-MB-435S cells were cultured in Leibovitz's L-15 medium (ATCC) with 10% FBS, 0.01 mg/ml bovine insulin, 0.01 mg/ml glutathione and 1% penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin). MCF-7 cells were cultured in Eagle's minimal essential medium (EMEM) (ATCC) with 10% FBS and 1% penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin). All cells were cultured and maintained in a humidified incubator under standard conditions.

Plasmid construction. AMOT 3'-UTR containing putative binding sites for miR-205 were amplified from the normal human genome DNA and cloned into downstream of the psi-CHECK2 vector (Promega, Madison, WI, USA), and named AMOT-3'-UTR-WT. AMOT mutant 3'-UTR recombinant plasmid was generated by the QuikChange Site-Directed Mutagenesis kit (Stratagene, USA), which generated a mutation of 7 bp from ATGAAGG to TACGGTC in the predicted binding sites for miR-205. The full length cDNA encoding human AMOT was amplified by PCR and the recombinant plasmid, pcDNA3.1/AMOT was constructed. All plasmids were confirmed by DNA sequencing.

miRNA mimics and siRNA transfection. The miR-205 mimics, and the scrambled sequence pre-miR negative control (NC) were purchased from a commercial manufacturer (RiboBio, China) and small interfering RNA (siRNA) targeting AMOT was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells (1x10^5/well) were seeded in 24-well plates and incubated for 24 h, and then the cells were transfected with miRNA mimics (50 nM) or miR-NC (50 nM) or siRNA (50 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in serum-free medium in accordance with the manufacturer's instructions.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of miRNA and mRNA. Total RNA and miRNA were extracted from the tissues or the cultured cells using TRIzol reagent (Invitrogen). The relative expression of miRNA-205 was quantitated using a SYBR PrimeScript miRNA RT-PCR kit (Takara, China) in accordance with the manufacturer's instructions, and the relative amount of miRNA was normalized to U6 using the comparative threshold cycle method. For quantitative analysis of mRNA expression, 2 μg of total RNA was used to synthesize complementary DNA (cDNA) using M-MLV reverse transcriptase (Promega, USA), and the corresponding cDNA was used for quantitative PCR using SYBR-Green Real-Time Master Mix (Toyobo, Japan), and a constitutive expression gene, GAPDH, was used as an internal control to verify the fluorescent real-time RT-PCR reaction. The sequence-specific primers were synthesized by Sangon (Shanghai, China) (Table I). qPCR was performed using the Applied Biosystems 7500 Sequence Detection system (ABI, USA).

Protein extraction and western blotting. Total protein was extracted with SDS lysis buffer (Beyotime, China) and the concentration of total proteins was determined with the BCA protein assay kit (Pierce, USA). Equal amounts of total proteins were separated on 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were subjected to overnight blocking in Tris-buffered saline (TBS) containing 5% non-fat dried milk and incubated with a primary antibody [AMOT, 1:1,000; and GAPDH, 1:3,000 (both from Abcam)] for 1 h at

<table>
<thead>
<tr>
<th>Name</th>
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<tr>
<td>miR-205-RT</td>
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<td>GCAATTCGGTGGAGCAGACTCC</td>
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<tr>
<td>U6-RT</td>
<td>CGCTTCAGAGTGGTTGCTCAT</td>
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<tr>
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<td>AMOT-F</td>
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<td>GAPDH-F</td>
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<tr>
<td>GAPDH-R</td>
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</tr>
<tr>
<td>F, forward primer; R, reverse primer; RT reverse transcription primer; AMOT, angiomotin.</td>
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37°C, and then the membranes were washed and subsequently treated with the secondary antibody (goat anti-rabbit IgG; Boster) at a 15,000 dilution for 1 h at room temperature and visualized by chemiluminescence.

Luciferase assays. For the reporter assay, the MCF-7 cells were cultured in 24-well plates one day before transfection. AMOT-3'-UTR-WT or -MUT vectors (100 ng) were co-transfected with 100 nM miR-205 mimics or negative control into MCF-7 cells using Lipofectamine 2000 reagent as previously described. After forty-eight hours of transfection, luciferase activities were measured using a Dual-Luciferase Reporter assay system (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized to Renilla luciferase activity. Three independent experiments were performed, and the data are presented as the mean ± SD.

MTT assay. The cell proliferation activities of the MCF-7 cells with miRNA mimics or siRNA duplexes or miR-205 and AMOT were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) assay at 0, 24, 48, 72 and 96 h. After a 24-h transfection, the MCF-7 cells were seeded into 96-well culture plates at 1x10^4/well in a final volume of 100 µl. At the indicated time points, 20 µl of MTT (5 mg/ml) was added into each well for a 4-h incubation at 37°C. Following removal of the culture medium, the remaining crystals were dissolved in 200 µl dimethylsulfoxide (DMSO) (Sigma), and the absorbance at 450 nm was measured. Three independent experiments were performed.

Statistical analysis. For quantitative data, all experiments were performed at least three times, and all samples were tested in triplicate. All data are expressed as the mean ± SD. Statistical significance between groups was determined using one-way analysis of variance (ANOVA) or an unpaired Student's t-test using SPSS 18.0 (SPSS, Inc., USA). A P-value of <0.05 was considered to indicate a statistically significant result.

Results

AMOT is upregulated in breast cancer tissues. We first detected the expression level of AMOT by real-time PCR and western blotting. As shown in Fig. 1, the expression of
AMOT was significantly upregulated in the tumor tissues when compared with that of the matched normal tissues both at the mRNA (Fig. 1A) and protein levels (Fig. 1B). The data revealed that AMOT is overexpressed in breast cancer tissues, suggesting that increased AMOT protein expression contributes to breast cancer development.

**AMOT 3'-UTR is a predicted target of miRNA-205.** In order to determine whether miR-205 regulates AMOT expression in breast cancer, miRNA target prediction databases were used for computational analyses, including TargetScan (www.targetscan.org), miRDB (http://mirdb.org/miRDB) and microRNA (www.microrna.org). miR-205 was found to have one predictive target site in the human AMOT 3'-UTR (Fig. 2A). The expression of mature miR-205 in the tumor and matched normal tissues was examined by real-time PCR. As shown in Fig. 2B, miR-205 was significantly down-regulated in the cancer tissues when compared with that in the matched normal tissues. The results showed that the expression of miR-205 and AMOT in the tumor tissues is inversely correlated. Therefore, miR-205 was selected to analyze the role of AMOT in breast cancer.

**AMOT is a direct target of miR-205 and is downregulated by miR-205.** To investigate whether or not miR-205 inhibits the expression of the endogenous AMOT protein, the expression level of miR-205 and AMOT in three breast cancer cell lines were examined by real-time PCR and western blotting, respectively. miR-205 was significantly expressed at a low level in the MCF-7 cells (Fig. 3A), and the expression level of AMOT was significantly higher in the MCF-7 cells (Fig. 3B). Thus, the MCF-7 cell line was chosen to be used in the present study. The dual-luciferase reporter vectors were constructed containing wild-type or mutant-type seed sequences in the 3'-UTR of AMOT (Fig. 3C). Overexpression of miR-205 markedly reduced the expression of AMOT at the protein level in the MCF-7 cells (Fig. 3D), yet did not affect the AMOT mRNA level (Fig. 3E). The MCF-7 cells were co-transfected with AMOT-3'UTR-WT and miR-205 mimics which resulted in a significantly reduced activity of the luciferase reporter gene, yet the luciferase activity was not significantly attenuated in the target region of the mutated AMOT-3'UTR-MUT construct (Fig. 3F). Based on the results, miR-205 directly targets the 3'UTR of AMOT and downregulates AMOT expression.
Overexpression of miR-205 suppresses the proliferation and invasion of MCF-7 cells in vitro. To evaluate the role of miR-205 in tumor cell proliferation and invasion, miR-205 was overexpressed in the MCF-7 cells by transfection with miR-205 mimics (Fig. 4A). Overexpression of miR-205 as well as transfection with AMOT-siRNA markedly reduced the expression level of the AMOT protein (Fig. 4B). As shown in Fig. 4C, the growth of miR-205- and AMOT-siRNA-transfected cells was significantly suppressed compared with the NC-transfected MCF-7 cells (P<0.05 at 72 and 96 h). The role of miR-205 in tumor cell invasion was detected using Transwell assay. miR-205- and AMOT-siRNA-transfected cells exhibited much less invasive ability when compared with the negative control cells (Fig. 4D and E). When MCF-7 cells were transfected with miR-205 inhibitors, cell proliferation and invasion were not affected (data not shown). These results indicate that the miR-205-mediated growth and inhibition of invasion occur in an AMOT-dependent manner in MCF-7 cells.

miR-205 does not affect breast cancer cell apoptosis. In order to evaluate the effect of miR-205 on cell apoptosis, the Annexin V-FITC/PI staining method was used to perform the apoptosis assays. The data demonstrated that overexpression of miR-205 as well as AMOT-siRNA transfection did not affect cell apoptosis in the MCF-7 breast cancer cell line when compared with the negative controls (Fig. 5).

AMOT ameliorates the inhibitory effect of miR-205 on cell proliferation and invasion. For further study, AMOT was overexpressed in miR-205-overexpressing MCF-7 cells as shown by
ZHANG and FAN: AMOT IS A DIRECT TARGET OF miR-205

2168

western blotting (Fig. 6A). Moreover, overexpression of AMOT in the MCF-7-overexpressing miR-205 cells significantly decreased the inhibitory effect of miR-205 overexpression on breast cancer cell proliferation and invasion (Fig. 6B-D), but did not affect cell apoptosis (Fig. 6E and F).

Discussion

MicroRNAs (miRNAs) are endogenous, non-coding small RNAs that target the 3′-untranslated regions (3′-UTR) of certain messenger RNAs (mRNAs) to negatively regulate gene expression, including mRNA degradation and translation inhibition (9). According to statistics, more than 30% of all human genes as well as cellular processes are regulated or controlled by miRNAs (20,21). More and more evidence has shown the crucial impact of miRNAs on the occurrence and development of human types of cancers (22-24). Dysregulation of miRNAs plays important roles in cancer cell growth (25), invasion (26) and apoptosis (27).

Although miR-205 was identified many years ago, its biological function has only recently been investigated. miR-205 acts as a tumor suppressor and is involved in many physiological and pathological processes, such as hepatocellular carcinoma (28), oxidative and endoplasmic reticulum (ER) stress (29) and endometrial cancer (30). A previous study indicated that the expression of miR-205 is frequently reduced in various types of cancer (31,32), including breast cancer (17), which suggests that miR-205 plays an important role in the tumorigenesis and tumor progression of breast cancer. However, the function of miR-205 in breast cancer is poorly understood.

In the present study, we showed that miR-205 was significantly downregulated in breast tumor tissues, indicating a potential role of miR-205 in breast cancer. In order to understand whether miR-205 downregulation bears a biological role, the role of miR-205 in breast cancer cell growth, invasion and apoptosis was analyzed. Overexpression of miR-205 significantly inhibited cell proliferation and reduced the migration...
and invasion of breast cancer cells, and downregulated the expression of angiomotin (AMOT). However, overexpression of miR-205 did not markedly affect cell apoptosis. Overexpression of AMOT, a target gene of miR-205, partially ameliorated the inhibitory effect on breast cancer cell proliferation and invasion that was caused by miR-205 overexpression. These results suggest that miR-205 suppressed breast cancer cell proliferation and invasion, which strongly argues for the existence of a close correlation between miR-205 and breast cancer occurrence and development.

AMOT is a membrane-associated protein that is involved in controlling cell migration (33). Furthermore, it binds to the endothelial cell surface of angiogenic tissues (6). p80-AMOT is an isoform of AMOT, which enhances cell migration and stabilizes tubes in vitro (34). A previous study showed that the ERK1/2 pathway was activated through the RalGTPase indirectly by AMOT (35). In addition, the expression of AMOT was upregulated in breast cancer (5), and expression of AMOT enhanced ERK1/2-dependent proliferation of MCF-7 cells (36). AMOT also plays an important role in altering tumor vessel permeability and hampering the growth of tumors (37). In the present study, we identified that AMOT is a direct target of miR-205 in breast cancer through western blotting and luciferase assays. The results showed that overexpression of miR-205 as well as AMOT knockdown by AMOT-siRNA markedly reduced the expression of AMOT, and exhibited significant suppression of proliferation and invasion. In addition, overexpression of AMOT ameliorated the inhibitory effect of miR-205 overexpression on breast cancer cell growth and invasion in part. All of the results indicate that downregulation of miR-205 promotes the proliferation and invasive capacity of breast cancer through the AMOT-mediated signal pathway.

In conclusion, we demonstrated that miR-205 is downregulated in breast cancer tissues, and targets AMOT. Overexpression of miR-205 downregulated the expression of AMOT and inhibited the proliferative and the invasive capacities of breast cancer cells. Collectively, the present study may lead to new diagnostic and therapeutic approaches for breast cancer, and enhances our understanding of the post-transcriptional regulation of AMOT.

Acknowledgements

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References


