MicroRNA-148a inhibits breast cancer migration and invasion by directly targeting WNT-1

QIAN JIANG¹, MIAO HE¹, MENG-TAO MA¹, HUI-ZHE WU¹, ZHAO-JIN YU¹, SHU GUAN², LONG-YANG JIANG¹, YAN WANG¹, DA-DI ZHENG¹, FENG JIN² and MIN-JIE WEI¹

¹Department of Pharmacology, School of Pharmacy, China Medical University, Shenyang, Liaoning 110122, ²Department of Surgical Oncology, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning, P.R. China

Received September 19, 2015; Accepted October 30, 2015

DOI: 10.3892/or.2015.4502

MicroRNAs (miRNAs) are small endogenous non-coding RNAs that post-transcriptionally regulate gene expression through mRNA degradation or translational repression and monitor several biological processes (9). In general, individual miRNAs regulate multiple mRNAs and individual mRNAs can be targeted by multiple miRNAs (9). Several human miRNAs have been shown to regulate the metastasis of breast cancer cells (10,11). MicroRNA-148a (miR-148a), as a member of miR-148/152 family, plays an important role in the growth and development of normal tissues and is involved in the genesis and development of disease (12). The down-regulated expression of miR-148a has been found in human gastrointestinal (13) and pancreatic cancers (14,15), and other tumor types (16). Recent studies have shown that miR-148a is downregulated in breast cancer cells and tumors (17,18). However, the roles and mechanisms of miR-148a in breast cancer metastasis remain to be elucidated.
In the present study, we found downregulated expression of miR-148a in breast cancer tissues and cell lines. Furthermore, we demonstrated that miR-148a was able to inhibit the migration and invasion of breast cancer cells by transfecting miR-148a mimic in MCF-7 and MDA-MB-231 cells. Importantly, our results showed miR-148a directly inhibited the expression of WNT-1 and inactivated the Wnt/β-catenin pathway in breast cancer cells. These findings provide new insights into the molecular mechanisms of breast cancer metastasis and provide a therapeutic strategy for the treatment of breast cancer.

Materials and methods

Cell lines. Human embryonic kidney cell line 293T, breast cancer cell lines (MCF-7, MDA-MB-231, SKBR3, T47D, BT549 and MDA-MB-435S), and mammary epithelial cell (MCF-10A) were all purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C.

Cell transfection. The miR-148a mimic and negative control (NC) mimic were purchased from RiboBio (Guangzhou, China). MCF-7 and MDA-MB-231 cells were seeded on 6-well plates (3x10⁴/well), and cultured overnight. Cells were then transfected with 15 nM miR-100 mimic or miR-NC using Lipofectamine 2000 according to the manufacturer's instructions (Life Technologies, USA). After 48 h, the cells were used for western blotting and qRT-PCR analysis.

RNA isolation and qRT-PCR analysis. Total RNA and miRNAs from breast cancer cells were isolated using a miRNA isolation kit (BioTeke, China). qRT-PCR for miR-148a was performed using the TaqMan MicroRNA assay as described in our previous studies (19). For mRNA, 100 ng RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega, USA), followed by qPCR using SYBR Premix Ex Taq™ II kit (Takara, Japan) as described in our previous studies (19). The expression levels of miR-148a and WNT-1, TCF-4, LEF-1 mRNA were normalized to that of GAPDH. The fold-change for each miRNA and mRNA relative to the control was calculated using the 2⁻∆∆Ct method.

Western blot analysis. Cells were lysed and total proteins were extracted as previously described (19). Equal amounts of proteins (30-50 µg) were subjected to 10% SDS-PAGE separation, and then transferred to PVDF membranes. Membranes were incubated with primary antibodies against human WNT-1 (1:400; Boster), β-catenin (1:1,000; PeproTech), MMP-7 (1:500; Boster) or GAPDH (1:1,000) followed by incubation with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA). Protein bands were visualized by enhanced chemiluminescence (ECL; Amersham, Germany). The expression levels of proteins were quantitatively analyzed with FluorChem V2.0 software (Alpha Innotech Corp., USA).

Dual luciferase reporter assay. 293T cells (1.2x10⁴) in 24-well plates were co-transfected with 15 nM of miR-148a mimic or miR-NC and 10 ng of luciferase reporter plasmids containing either wild-type or mutant WNT-1-3'UTR using Lipofectamine 2000. Forty-eight hours after transfection, luciferase reporter assays were performed using the Dual Luciferase Reporter Assay kit (Promega), according to the manufacturer's protocol.

Transwell migration and invasion assays. The migration and invasion of cells were analyzed using 24-well Boyden chambers with 8-µm pore size polyethylene membranes (Corning, USA). For the invasion assay, the Transwell membranes were precoated with Matrigel (BD Biosciences, USA). For both assays, cells were seeded in starvation medium on the top chamber, and the bottom chamber was filled with 0.5 ml culture medium containing 10% FBS. After 24 h incubation, the cells that migrated or invaded to the lower chamber were fixed with 4% paraformaldehyde and stained with crystal violet solution. The cells were counted under a light microscope (magnification, x200; five random fields/well), and were analyzed using ImageJ software.

Human samples. Human breast cancer and adjacent normal tissues for qRT-PCR analysis were obtained from 69 breast cancer patients and for in situ hybridization and immunohistochemistry were obtained from 55 breast cancer patients, who underwent surgery at the First Affiliated Hospital of China Medical University between 2011 and 2012. Written informed consent was obtained from all patients. The study was approved by the Institutional Review Board of China Medical University Research Ethics Committee. This research was conducted in accordance with the Declaration of Helsinki.

Immunohistochemistry. Immunohistochemistry was performed as previously described (20). Briefly, 4-µm sections obtained from paraffin-embedded tumor tissues from breast cancer patients were incubated with primary antibody against WNT-1 (1:200; Boster). Images from each section were evaluated under a Nikon Eclipse 80i microscope (at a magnification of x200; Nikon, Japan). Five random fields without overlaps

Table I. Primer sequences used for the qRT-PCR analysis.

<table>
<thead>
<tr>
<th>Application</th>
<th>Oligonucleotides</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-148a</td>
<td>F</td>
<td>GGCAGTCTCAGTGC ACTACAG</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GTGCAGGTTCGGAGGT</td>
</tr>
<tr>
<td>U6</td>
<td>F</td>
<td>CTCGCTTTCGGAGCA</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AACGCTTCAAGA ATTGAGTC</td>
</tr>
<tr>
<td>WNT-1</td>
<td>F</td>
<td>TGCAGGCAACACC GCGTACTGAC</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAGGATGCAAGG AGGTTGATCG</td>
</tr>
<tr>
<td>TCF-4</td>
<td>F</td>
<td>GCAATGGGCAACTTGAC</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAGACCAAGCTCCTGACTTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F</td>
<td>AGGCCACATCGCTCAGACAC</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GCCCAATACGACC AAATCC</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
from each section were counted. The intensity score was defined as: for no staining (0), weak (1), moderate (2) or strong (3) staining. The percentage score was defined as 0 for <5% staining, 1 for 5-25% staining, 2 for 26-50% staining, 3 for 51-75% staining, and 4 for >75% staining. The intensity scores were multiplied with the percentage score to obtain the final scores.

**In situ hybridization.** In situ hybridization was performed using Enhanced SensitiveISH Detection KitⅡ as specified by the manufacturer (MK1030; Boster, China). Briefly, the slides were hybridized with 8 µg/ml probe complementary to miR-148a LNA-modified and DIG-labeled (Shanghai Sangon Biological Engineering Technology And Service Co., Ltd., China). After incubation with anti-DIG-HRP Fab fragments conjugated to horseradish peroxidase, the slides were detected by incubating with 3,3′-diaminobenzidine (DAB) and nuclei were counterstained with hematoxylin. Quantification of the staining intensity of miR-148a was performed through image analysis the same manner as immunohistochemistry.

**Statistical analysis.** Analyses were performed using SPSS 17.0. A two-tailed Student's t-test was used to evaluate the statistical significance of the differences between two groups. One-way analysis of variance (ANOVA) was used to compare the differences among three or more groups. The Pearson's rank correlation analysis was applied to assess the association between the expression of miR-148a and WNT-1. Probability values <0.05 were considered to indicate a statistically significant result.

**Results**

Expression of miR-148a is downregulated in breast cancer tissues and cell lines. We measured miR-148a expression in 69 pairs of human breast cancer tissues and adjacent normal breast tissues by qRT-PCR to observe the clinical relevance of miR-148a in human breast cancer patients. The findings showed that the expression of miR-148a in human breast cancer tissues was significantly lower than in the adjacent normal breast tissues (Fig. 1A; P<0.05). In addition, we found that miR-148a expression was decreased at least 2-fold compared with adjacent normal breast tissues in 15.9% (11/69) of human breast cancer cases (Fig. 1B). Furthermore, the low expression of miR-148a was shown to be closely correlated with lymph node metastasis in 69 cases of human breast cancer tissues by Mann-Whitney U test (P<0.05; Fig. 1C). We also found that the expression of miR-148a was significantly downregulated in SKBR3, MCF-7, T47D, BT549, MDA-MB-231, and MDA-MB-435S cells.
MDA-MB-231 and MDA-MB-435S breast cancer cells compared with human mammary epithelial MCF-10A cells by qRT-PCR analysis (Fig. 1D). Overall, these results suggested that the expression of miR-148a was downregulated in breast cancer tissues and established cell lines, and the low expression of miR-148a may be relevant to metastasis of breast cancer.

**Ectopic miR-148a expression inhibits the migration and invasion of breast cancer cells.** To observe whether miR-148a can inhibit the migration and invasion of breast cancer cells, we first transfected MCF-7 and MDA-MB-231 breast cancer cells with miR-148a mimic for 48 h, and then detected the expression levels of miR-148a using qRT-PCR analysis. It is noteworthy that the expression of miR-148a was increased by ~280- and 300-fold, respectively, in MCF-7 and MDA-MB-231 cells transfected with the miR-148a mimic relative to those transfected with NC (P<0.01; Fig. 2A). We measured the changes of migration and invasive abilities of MCF-7 and MDA-MB-231 cells transfected with the miR-148a mimic by Transwell migration and invasion assays. The results showed that the overexpression of miR-148a suppressed the migration ability of MCF-7 and MDA-MB-231 cells to 40 and 45% of the control (P<0.05; Fig. 2B), and decreased the invasion abilities of MCF-7 and MDA-MB-231 cells to 50 and 45% of the control (P<0.05; Fig. 2C). The data suggested that miR-148a inhibited breast cancer cell migration and invasion.

**WNT-1 is a direct target of miR-148a.** To ascertain the possible mechanisms of miR-148a suppressing the migration and invasion of breast cancer cells, we predicted the putative targets...
We focused on the genes related to Wnt/β-catenin signaling pathway involved in the tumor metastasis. We found that WNT-1, one of the major ligands of Wnt/β-catenin signaling pathway, was one of the targets of miR-148a (Fig. 3A). To further test whether WNT-1 was a direct target of miR-148a, we constructed a luciferase reporter plasmid containing WNT-1 3'-untranslated region (3'-uTR) harboring a conserved miR-148a binding site (pGL3-WNT-1-3'uTR) and a plasmid containing WNT-1-3' uTR with miR-148a target sequences mutated (pGL3-WNT-1-3' uTR mu). The pGL3-WNT-1-3'uTR or pGL3-WNT-1-3'uTR mu was cotransfected with the miR-148a mimic or NC into 293T cells. The reporter assay showed that miR-148a mimic significantly decreased the luciferase activity by ~50% in 293T cells co-transfected with the pGL3-WNT-1-3'uTR. However, the luciferase activity in the cells co-transfected with the pGL3-WNT-1-3'uTR mu was not significantly reduced (P<0.05; Fig. 3B). These findings suggested that WNT-1 was a direct target of miR-148a.

Next, we found that the overexpression of miR-148a significantly decreased the protein expression levels of β-catenin in MCF-7 and MDA-MB-231 cells, compared with NC-transfected cells (Fig 4A; P<0.05). In addition, the results also showed that the ectopic miR-148a expression obviously decreased the mRNA expression levels of T cell factor-4 (TCF-4), one of the important transcription factors of Wnt/β-catenin pathway, in MCF-7 and MDA-MB-231 cells, compared with NC-transfected cells (Fig 4B; P<0.05). Taken together, the findings suggested that miR-148a could suppress the migration and invasion of breast cancer cells by targeting WNT-1 and inhibiting the activation of Wnt/β-catenin signaling pathway.

miR-148a expression is negatively correlated with the expression of WNT-1 in human breast cancer tissues. To further evaluate the relevance of the endogenous expression of miR-148a and WNT-1, we measured the expression of miR-148a using in situ hybridization and the expression
of WNT-1 protein by immunohistochemistry in 55 pairs of human breast cancer tissues and adjacent normal tissues with tissue microarray (TMA). As shown in Fig. 5A and B, the expression of WNT-1 was significantly higher in human breast cancer tissues compared with the adjacent normal tissues (P<0.0001). Pearson rank correlation analysis showed that the expression of miR-148a was inversely related to the expression of WNT-1 protein in breast cancer tissues (Fig. 5C; P<0.01).

Discussion

Wnt/β-catenin signaling pathway influences embryonic development, cell polarity and adhesion, apoptosis and tumorigenesis (21,22). It is known that Wnt/β-catenin pathway is upregulated in breast cancer (6) and other types of tumors (8). WNT-1 was the original Wnt identified as an oncogene in mouse mammary tumors (23). Wong et al reported that there was a higher positive expression rate in human breast tumors (24). In our study, we also found that the WNT-1 was obviously upregulated in human breast cancer tissues when compared with the adjacent normal tissues. Wnt/β-catenin pathway has been shown to be involved in the tumor development and metastasis (5). Targeting the Wnt/β-catenin pathway would be very important to inhibit the metastasis of breast cancer.

miRNAs function as regulators of many oncobiological processes, such as tumorigenesis and metastasis (9). It has been demonstrated that many miRNAs can target and inhibit the main factors of WNT/β-catenin pathway and regulate the biological function of cancer cells. Wen et al reported that miR-126 suppressed papillary thyroid carcinoma cell proliferation and migration by directly repressing the expression of LRP6, a major regulator of the Wnt/β-catenin signaling cascade (25). miR-577 was found to directly target the Wnt/β-catenin pathway components LRP6 and β-catenin, and inhibit glioblastoma multiforme growth (26). Subramanian et al found that miR-29b decreased the transactivation of β-catenin target genes in human colorectal cancer cells (27).

In the present study, we found that miR-148a could inhibit the migration and invasion of breast cancer cells by directly targeting WNT-1 and inhibiting the activation of Wnt/β-catenin pathway. Furthermore, we also demonstrated that the expression of miR-148a was inversely related to the expression of WNT-1 in breast cancer tissues. Similarly, Yan et al also reported that WNT-1 was a target gene of
miR-148a in hepatocellular carcinoma cells (28). In addition, Joshi et al found that miR-148a reduced lung tumorigenesis in vitro and in vivo through the downmodulation of matrix metalloproteinase 15 (MMP15) and Rho-associated kinase 1 (ROCK1) (29). miR-148a was also demonstrated as a prognostic oncomiR to target mitogen-inducible gene 6 (MIG6) and BIM, and regulate EGFR and apoptosis in glioblastoma (30). Obviously, miR-148a plays different roles either as an oncomiR or as an antimiR in the tumor cells of different types by directly targeting different target genes.

In conclusion, our studies suggest that miR-148a can inhibit the migration and invasion of breast cancer cells by directly targeting WNT-1 and downregulating the Wnt/β-catenin signaling pathway. This will provide a new strategy for treating metastasis of breast cancer. However, the complex regulatory network of miR-148a in regulating the migration and invasion of breast cancer should be further explored.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (grant no. 81373427), the Program for Liaoning Innovative Research Team in University, LNIRT, China (grant no. LT2014016), the Liaoning Provincial Science and Technology Program, China (grant no. 2014021085), the Program for Liaoning Excellent Talents in University, China (grant no. LJQ2014084), and the S&T Projects in Shenyang, China (grant no. F14-232-6-05).

References


