Abstract. Epithelial-mesenchymal transition (EMT) plays a critical role in the process of cancer invasion and metastasis. The Wnt/β-catenin signaling pathway is known as a stimulatory factor, which may trigger EMT and metastasis of cancer cells. In addition, several microRNAs (miRNAs) have been proven to regulate the EMT process. Recent research revealed that miR-148a is downregulated in pancreatic cancer. However, the definite role of miR-148a in EMT and invasion of pancreatic cancer is still unknown. The present study attempted to demonstrate the underlying mechanism of miR-148a in the regulation of EMT and invasion of pancreatic cancer cells. Our data revealed that the expression of miR-148a was markedly downregulated in human pancreatic ductal adenocarcinoma (PDAC) cell lines and tissues. In addition, the downregulation of miR-148a was associated with poor prognosis and EMT phenotype. Furthermore, restoration of miR-148a expression inhibited the EMT process, as well as the migration and invasion of BxPC-3 pancreatic cancer cells. Wnt10b, a promoting molecule of the Wnt/β-catenin signaling pathway, was demonstrated by dual-luciferase reporter assay to be a direct target of miR-148a. Subsequently, we found that miR-148a negatively regulated the protein expression of β-catenin, cyclin D1 and MMP-9, which were important components of the Wnt/β-catenin signaling pathway. In conclusion, these findings revealed that miR-148a suppresses EMT and invasion of pancreatic cancer cells by targeting Wnt10b and inhibiting the Wnt/β-catenin signaling pathway, and thus, miR-148a may serve as a novel therapeutic target for pancreatic cancer.

Introduction

Pancreatic cancer is one of the most aggressive and lethal malignant tumors of the digestive system. The incidence rate of pancreatic cancer has been increasing over the past several years worldwide. In the USA, ~53,070 new cases of pancreatic cancer and 41,780 related deaths were estimated to occur in 2016, and it has been listed as the fourth leading cause of cancer-related deaths with a 5-year survival rate of ~8% (1). Surgical resection is still the most effective therapeutic method for pancreatic cancer. However, <20% of the patients with pancreatic cancer are clinically amenable to surgical resection at the time of diagnosis. The aggressive nature of pancreatic cancer and its propensity for invasion and metastasis are the major causes of unresectable tumors and poor prognosis. Therefore, further study on the related mechanisms underlying the invasion and metastasis of pancreatic cancer is essential to improve its prognosis.

A notable event in the process of cancer-related migration, invasion and metastasis is epithelial-mesenchymal transition (EMT), an essential biological process with marked morphological changes between the epithelial state and mesenchymal cell-like properties (2). EMT is characterized by the loss of cell-cell contact through the inhibition of epithelial markers, such as E-cadherin expression, and the acquisition of mesenchymal features, such as the upregulation of the mesenchymal markers N-cadherin, vimentin and fibronectin. Cancer cells are endowed with migratory and invasive properties that allow them to migrate to distant organs through the extracellular matrix during the EMT process. Some data have indicated that EMT plays a key role in the pathogenesis and progression of pancreatic cancer (3-6).

Recently, emerging evidence has revealed that microRNAs (miRNAs) play an important regulatory role in the EMT process as promotive or inhibitory factors. miRNAs are a class of 18-22 nucleotide-long small non-coding RNA molecules, which regulate the expression of target genes by binding to a complementary sequence predominantly in
their 3′-untranslated region (3′UTR) (7). miRNAs have been implicated in the regulation of genes which have an impact on cell differentiation, apoptosis and neoplastic transformation. miR-148a is a member of the miR-148/152 family and is located at chromosome 7p15.2. The expression of miR-148a is decreased in various types of tumors including gastrointestinal cancers (8,9), cholangiocarcinoma (10), hepatocellular carcinoma (11) and ovarian cancer (12), and miR-148a may be considered as a type of tumor-suppressor miRNA. In pancreatic cancer, miR-148a is also downregulated in cancer tissues and cancer cell lines (13-15). Previous studies have revealed that miR-148a has an inhibitory effect on the growth and apoptosis in pancreatic cancer by targeting DNMT1 (16), CCKBR, Bcl-2 (17) or CDC25B (18). However, the real role of miR-148a in EMT, migration and invasion of pancreatic cancer is still unknown.

In the present study, we found that downregulated miR-148a was associated with poor prognosis and EMT phenotype in pancreatic ductal adenocarcinoma (PDAC). Then, we focused on two other important questions in this study: i) whether restoration of miR-148a expression could inhibit EMT, as well as the migration and invasion of pancreatic cancer cells; and ii) whether miR-148a negatively regulated the Wnt/β-catenin signaling pathway by targeting Wnt10b. The answers to these questions could provide new insights concerning the molecular mechanism of the development of pancreatic cancer, as well as a new strategy for the diagnosis and treatment of pancreatic cancer in the future.

Materials and methods

Cell lines and cell culture. The normal human pancreatic ductal epithelial cell line HPDE, and human PDAC cell lines BxPC-3, AsPC-1 and Mia PaCa-2 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone), penicillin (100 U/ml) and streptomycin (100 µg/ml) in an incubator with 95% air and 5% CO2. The cells were used when they were in the logarithmic growth phase.

Patients and tissue samples. Thirty-three paired PDAC and adjacent non-tumorous tissues (ANT) were obtained from PDAC patients who underwent surgery between January 2013 and December 2015 in our hospital. These patients included 19 males and 14 females with an average age of 54.7±8.4 years (range: 36-69 years). None of the patients had received local or systemic treatment before surgery and all patient tissues were pathologically confirmed as PDAC. The histological grade and TNM stage of cancers were assessed according to criteria set by the 8th UICC/AJCC TNM staging system of pancreatic cancer. The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University and all samples were obtained with informed consent.

Synthetic miRNA transfection. BxPC-3 cells were seeded into plate wells, incubated overnight, and then transfected with miR-148a mimic, miR-148a inhibitor and their respective negative controls (Ambion, USA). Lipofectamine™ 2000 (Invitrogen, USA) was used for cell transfection according to the manufacturer’s protocol. The cells were collected for RNA and protein extraction or for further assays after an additional 48-h incubation.

RNA isolation and quantitative real-time PCR. Total RNA was isolated from snap-frozen fresh samples and BxPC-3 cells using TRIzol (Invitrogen) and cDNA were obtained using M-MLV Reverse Transcriptase kit (Takara, Japan) according to the manufacturer’s protocol. Quantitative PCR was performed using the Applied Biosystems (ABI) 7500 Fast Real-Time PCR system with SYBR-Green PCR kit (Applied Biosystems, USA) to detect E-cadherin and vimentin mRNA expression. β-actin served as an internal control. For the detection of miR-148a expression, 100 ng of total RNA was reverse transcribed by stem-loop RT primers, followed by RT-PCR on the ABI 7500 Fast Real-Time PCR system with SYBR-Green PCR kit. Human U6 RNA served as an internal control. Cycle threshold (Ct) values were used to quantify the expression of each gene, and miRNA or mRNA levels were calculated according to the 2-ΔΔCt method. The primer sequences are shown in Table I.

Protein extraction and western blot analysis. Total protein was extracted with lysis buffer (50 mM Tris-HCl, pH 6.8; 150 mM NaCl; 0.5% sodium deoxycholate; 1% NP-40) and supernatants collected by centrifugation. Equal amounts of protein were denatured in SDS sample buffer at 100°C for 5 min, and separated in 10% polyacrylamide gels, and then transferred to cellulose nitrate (CN) membranes (Millipore, USA). The membranes were blocked with 5% non-fat milk in TBST (TBS containing 0.05% Tween-20) and incubated with primary antibodies overnight at 4°C. After washing the membranes with TBST, they were incubated with a secondary antibody. The protein expression was detected by Western Lightning®-ECL, enhanced chemiluminescence substrate (NEL100001EA; Perkin Elmer). The following primary antibodies were used: E-cadherin, vimentin, N-cadherin, β-catenin, matrix metalloproteinase-9 (MMP-9) and cyclin D1 (Cell Signaling Technology, USA), and β-tubulin (Santa Cruz Biotechnology, USA) was used as a loading control.

Cell migration and invasion assays. Cell migration and invasion of BxPC-3 cells were assayed using a Transwell chamber (Corning, NY, USA). Transwell filters coated with Matrigel (BD Biosciences, USA) were used for the tumor cell invasion assay, while Transwell filters without Matrigel were used for the migration assay. BxPC-3 cells (5x105) in serum-free medium were plated in the upper chamber, and 10% FBS was added to the bottom chamber. After 24 h of growth at 37°C, the cells that remained in the upper chamber were carefully wiped off using cotton wool. The cells that invaded or migrated to the lower surface of the membrane and the bottom chambers were fixed and stained with a dye solution containing 0.1% crystal violet and 20% methanol and then counted under a microscope.

Dual-luciferase reporter assay. The 3′UTR mRNA sequence of the Wnt10b gene containing the miR-148a binding site was amplified by PCR and cloned into the pmirGLO vector (wt-Wnt10b-3′UTR), and mut-Wnt10b-3′UTR vector was also synthesized with point mutation in the seed sequence. Then,
the BxPC-3 cells were cultured in 24-well plates and co-transfection of the wt-Wnt10b-3'UTR or mut-Wnt10b-3'UTR vector with miR-148a mimic or miR-NC using Lipofectamine™ 2000 (Invitrogen) was performed. The luciferase activity was detected using the dual-luciferase reporter assay kit (Promega, USA) after 48 h of transfection. The experiments were performed independently in triplicate.

RNA interference. To silence Wnt10b expression, Wnt10b-siRNA (Ambion, USA) with a scrambled siRNA (SCR) as a control was transfected into BxPC-3 cells. Lipofectamine™ 2000 was used for cell transfection according to the manufacturer's procedure. After 48 h of transfection, cell lysates were collected for further assays.

Statistical analysis. The results were analyzed using SPSS 17.0 software and expressed as the means ± standard deviation (SD) derived from three experiments independently, unless specifically needed. Comparative data between the groups were assessed by a two-tailed Student's t-test. Comparison of categorical variables between the groups was assessed by the Chi-square test or Fisher's exact test. The association between miR-148a and Wnt10b expression was calculated by Pearson's test. A P-value <0.05 was considered as statistically significant.

Results

Low expression of miR-148a is associated with poor prognosis and EMT phenotype of PDAC. The expression of miR-148a in BxPC-3, AsPC-1 and Mia PaCa-2 cell lines was markedly lower than that of the HPDE cell line (Fig. 1A). Moreover, the expression of miR-148a was also significantly decreased in PDAC specimens compared with that in the ANT specimens (Fig. 1B). Furthermore, miR-148a expression was revealed to be significantly lower in patients with advanced tumor stage and lymphatic metastasis, after the 33 cases were divided into low (n=17) and high expression (n=16) relying on the cut-off value of the expression of miR-148a (Table II). Compared with the ANT specimens, the expression of the E-cadherin protein was significantly lower in the PDAC specimens (Fig. 1C). The opposite was true for vimentin expression (Fig. 1D). In addition, the level of Wnt10b was significantly higher in the PDAC samples (Fig. 1E). Furthermore, the expression of miR-148a was negatively correlated with Wnt10b expression (R=-0.2635, P<0.01) (Fig. 1F). Collectively, these data revealed the existence of an inverse correlation between miR-148a and EMT phenotype, and low expression of miR-148a was associated with poor prognosis in pancreatic cancer.

Restoration of miR-148a expression inhibits EMT as well as invasion and migration in vitro. As shown in Fig. 2A, compared to the control groups, there was a significantly higher miR-148a expression in the BxPC-3 cells of the experimental group which was transfected with miR-148a mimic. Marked morphological changes were also observed in the experimental group, such as a cobblestone-like morphology typical of epithelial cells which replaced the fibroblastic, spindle-like morphology (Fig. 2B). In addition, miR-148a mimic upregulated the expression of E-cadherin, and suppressed the expression of vimentin and N-cadherin (Fig. 2C). Moreover, the migration and invasion abilities were significantly decreased in cells transfected with miR-148a mimic (Fig. 2D). These data revealed that restoration of miR-148a expression inhibited EMT as well as the invasion and migration of BxPC-3 cells in vitro.

Table I. Real-time PCR primers for the target gene and primers for Wnt10b 3'UTR in the luciferase reporter assay.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>miR-148a</td>
<td>F: GGCAGCAAAAGTTCTGAGACAC</td>
</tr>
<tr>
<td></td>
<td>R: GTGCAGGGTCGGAGGTATTC</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>F: GACAAACAAGCCCGAATT</td>
</tr>
<tr>
<td></td>
<td>R: GGAAACTCTCTCGGTCCA</td>
</tr>
<tr>
<td>Vimentin</td>
<td>F: ATTCACCTTTTGCTTCAAGG</td>
</tr>
<tr>
<td></td>
<td>R: CTTCAGAGAGAGGAGCGCGA</td>
</tr>
<tr>
<td>Wnt10b</td>
<td>F: TGGAGAAATGCGCTCTGAC</td>
</tr>
<tr>
<td></td>
<td>R: AGAGTGACCTTGGAAGGAAATC</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: TGGATCAGCAAGCAGAGTA</td>
</tr>
<tr>
<td></td>
<td>R: TCAGCCACATTGTGAACCTTT</td>
</tr>
<tr>
<td>U6</td>
<td>F: CTCGGCTCCGGCACACACA</td>
</tr>
<tr>
<td></td>
<td>R: AACGTCACCGAAATGTCGT</td>
</tr>
<tr>
<td>Wt 3'UTR of Wnt10b</td>
<td>F: CTAGCTACGCCGGCGTCAGTTGGAAGTAAATGCAAATGCACCTGTG</td>
</tr>
<tr>
<td></td>
<td>R: TGGACACAGTGCACTTACTCTTAGTCATACGCGCCGCTAG</td>
</tr>
<tr>
<td>Mt 3'UTR of Wnt10b</td>
<td>F: CTAGCTACGCCGGCGTCAGTTGGAAGTAAATGCAAATGAGTCTG</td>
</tr>
<tr>
<td></td>
<td>R: TGGACACAGTACTCTTTTTCATCTTAGTCATACGCGCCGCTAG</td>
</tr>
</tbody>
</table>

F, forward; R, reverse; 3'UTR, 3'-untranslated region.
miR-148a directly targets Wnt10b 3' UTR and inhibits its expression. The aforementioned data revealed that there was a significantly inverse correlation between Wnt10b and miR-148a in PDAC tissues (Fig. 1F). Meanwhile, we found that Wnt10b was a potential target of miR-148a using bioinformatic prediction tools TargetScan 6.2 and miRBase (Fig. 3A). Therefore, a dual luciferase reporter assay was performed to ascertain the direct binding of miR-148a to Wnt10b mRNA 3'UTR. The results revealed that miR-148a mimic significantly decreased the luciferase activity of the reporter with wild-type Wnt10b-3'UTR, but unaffected the activity of the mutant type vector, which suggests that the 3'UTR of Wnt10b was a direct target site for the miR-148a silencing of Wnt10b (Fig. 3B).

To further investigate the interaction between miR-148a and Wnt10b, western blot analysis was conducted to assess the effect of miR-148a on Wnt10b expression. We found that Wnt10b protein expression was significantly downregulated in the miR-148a mimic-treated BxPC-3 cells (Fig. 3C), whereas it was upregulated in the miR-148a inhibitor-treated cells (Fig. 3D). It was evident from these data that Wnt10b was a functional target of miR-148a in BxPC-3 cells.

miR-148a suppresses the Wnt/β-catenin signaling pathway by targeting Wnt10b. Our data provided evidence that miR-148a functioned as a tumor suppressor and inhibitor of EMT in BxPC-3 cells. The ectopic overexpression of miR-148a suppressed the aggressiveness of the BxPC-3 cells by directly targeting Wnt10b, one of the major ligands of the Wnt/β-catenin signaling pathway (19,20). To further investigate whether miR-148a could inhibit the activation of the Wnt/β-catenin signaling pathway by targeting Wnt10b, BxPC-3 cells were transfected with miR-148a mimic, miR-148a inhibitor and their respective negative controls. Then, the protein expression...
levels of downstream targets of the Wnt/β-catenin signaling pathway, such as β-catenin, cyclin D1 and MMP-9, were detected by western blot analysis. These data revealed that miR-148a overexpression significantly decreased the protein expression of β-catenin, cyclin D1 and MMP-9 (Fig. 4A). However, miR-148a knockdown obviously increased β-catenin, cyclin D1 and MMP-9 protein expression (Fig. 4B).

To determine whether miR-148a could exert its tumor-suppressive function through Wnt10b, BxPC-3 cells were stably transfected with Wnt10b-siRNA. Wnt10b-siRNA
combined with miR-148a mimic and Wnt10b-siRNA combined with miR-148a inhibitor, respectively. Then, the protein levels of the Wnt/β-catenin signaling pathway molecules (β-catenin, cyclin D1 and MMP-9) and EMT markers (E-cadherin, vimentin and N-cadherin) were assessed by western blot analysis. The results revealed that the expression of these related proteins were significantly decreased after knockdown of Wnt10b. Meanwhile, the inhibitory efficiency of Wnt10b-siRNA was partly offset by the miR-148a inhibitor, and reinforced by the miR-148a mimic (Fig. 4C). To further identify whether miR-148a suppresses the invasion and migration abilities of BxPC-3 cells through the dysregulation of Wnt10b, the cell migration and invasion abilities were also assessed. The results revealed that the invasion and migration abilities of the BxPC-3 cells were significantly weakened after Wnt10b-siRNA transfection, and that miR-148a inhibitor partly offset this effect while miR-148a mimic strengthened this efficiency (Fig. 4D). Collectively, these findings demonstrated that miR-148a inhibited EMT and the invasion of pancreatic cancer cells by targeting Wnt10b and inhibiting the Wnt/β-catenin signaling pathway.

Discussion

EMT is a multistep biological process whereby epithelial cells change in plasticity by the transient dedifferentiation into a mesenchymal phenotype. Besides fundamental roles in embryonic development and differentiation of normal tissues, EMT also plays critical roles in the development and dissemination of malignant tumors, including pancreatic cancer. Recently, emerging data revealed that miRNAs play a key role in the regulation of the EMT process in many malignant tumors. Furthermore, some miRNAs, such as miR-15a, miR-652, and miR-200a, have been considered as regulators of EMT in pancreatic cancer (21-23). In the present
study, we demonstrated that miR-148a, acting as a tumor suppressor, inhibited the EMT process and suppressed the invasion and migration of pancreatic cancer cells. In addition, we also revealed that miR-148a exerted its effects, at least in part, by targeting Wnt10b, which could activate the Wnt/β-catenin signaling pathway.

miR-148a has been investigated in many types of cancer, and its aberrant expression plays an important role in tumorigenesis and metastasis. Sakamoto et al (8) revealed that downregulation of miR-148a was significantly correlated with an advanced clinical stage, lymph node metastasis, and poor clinical outcome in gastric cancer (GC). Meanwhile, miR-148a played an important role in GC invasion by regulating MMP7 expression. Zhang et al (24) found that miR-148a was significantly decreased in hepatocellular carcinoma tissues, especially in those with portal vein tumor thrombus. Downregulation of miR-148a has also been noted in a variety of tumors, such as ovarian, colorectal, bladder and breast cancer (10,25-27). Moreover, some studies have suggested that EMT is regulated by miR-148a. Wang et al (28) demonstrated that miR-148a suppressed GC metastasis and EMT by targeting SMAD2. Li et al (29) revealed that miR-148a acted as an EMT suppressor in non-small cell lung cancer cells, at least in part through the modulation of ROCK1. However, the role and functional target of miR-148a in the EMT of pancreatic cancer is still unknown.

Using miRNA microarrays, Bloomston et al (13) revealed that miR-148a was significantly downregulated in pancreatic cancer tissues, compared with chronic pancreatitis or normal pancreas tissues. Similarly, miR-148a expression was significantly downregulated in various types of pancreatic cancer cell lines (18,30). Similar to those previous studies, our study revealed that miR-148a expression was significantly decreased in PDAC tissues, especially in patients with advanced tumor stage and lymphatic metastasis. Furthermore, we also found that epithelial marker E-cadherin was significantly inhibited whereas mesenchymal marker vimentin was significantly upregulated in PDAC specimens. In addition, restoration of miR-148a expression suppressed the invasion and migration of BxPC-3 cells in vitro, suggesting the existence of an inverse correlation between miR-148a and EMT in pancreatic cancer.

The mechanisms of regulation of EMT are very complex, involving many cellular signaling pathways like Notch, Wnt, TGF-β/Smad, Hedgehog and PI3K/Akt signaling. The Wnt/β-catenin signaling pathway is a type of canonical Wnt pathway. Aberrant Wnt/β-catenin signaling involves the stabilization of β-catenin and activation of downstream target genes such as c-Myc, MMP-9 and cyclin D1, which are essential oncogenes for the development of pancreatic cancer. Wnt10b is a member of the Wnt ligand gene family that encodes secreted signaling proteins, which activate the ancient and highly conserved Wnt signaling cascade, specifically canonical Wnt/β-catenin signaling (31,32). It has been demonstrated that miR-148a is inversely correlated with Wnt10b in oral and endometrial cancer (32,33). In addition, some studies also revealed that Wnt10b promoted the invasion and migration of cancer cells (34,35). In the present study, it was confirmed that Wnt10b was a direct target of miR-148a in pancreatic cancer BxPC-3 cells. We also demonstrated that the expression of three important downstream target genes of Wnt/β-catenin signaling, namely β-catenin, cyclin D1 and MMP-9 were significantly decreased after miR-148a mimic transfection. While the expression of these target genes was obviously upregulated after miR-148a inhibitor transfection. In addition, silencing of Wnt10b decreased the expression of the downstream genes of the Wnt/β-catenin signaling pathway and EMT markers, and inhibited the migration and invasion of BxPC-3 cells. Furthermore, the inhibitory effect of Wnt10b-siRNA could be rescued by miR-148a inhibitor, whereas miR-148a mimic had a synergistic effect with Wnt10b-siRNA. Collectively, these results revealed that miR-148a suppresses EMT and invasion of pancreatic cancer cells by targeting Wnt10b and inhibiting the Wnt/β-catenin signaling pathway.

In summary, our present study demonstrated that the downregulated expression of miR-148a was associated with the aggressive phenotype and EMT of pancreatic cancer. The effects of miR-148a on EMT and invasion in pancreatic cancer BxPC-3 cells observed in this study may be partially due to its regulation of the Wnt/β-catenin signaling pathway by targeting Wnt10b. All of these results indicate that miR-148a may be applied as a potential therapeutic target in pancreatic cancer.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81660401), the Natural Science Foundation of Jiangxi Province (grant no. 2016BAB205242) and the Scientific Research Foundation of the Education Office Jiangxi Province (grant no. GJJ14018).

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