Original Article
miR-26a suppresses the growth and metastasis via targeting matrix metalloproteinase 14 in pancreatic ductal adenocarcinoma

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Abstract: Pancreatic ductal adenocarcinoma (PDAC) is most lethal cancers in the world with one of the worst outcome. Increasing evidence suggest that aberrant expression of microRNAs (miRNAs) contribute to the development and progression of PDAC. The aim of our study was to explore the role of miR-26a in PDAC. Here, our data showed that miR-26a was significantly decreased in PDAC tissues and cell lines. Overexpression of miR-26a inhibited PDAC cell proliferation, migration and invasion in vitro. In addition, matrix metalloproteinase 14 (MMP14) was identified as a target of miR-26a in PDAC cells. Restoration of MMP14 remarkably attenuated the tumor suppressive effects of miR-26a on PDAC cells. Collectively, these data suggested that miR-26a could suppress PDAC growth and metastasis through targeting MMP14, suggesting miR-26a could act as a novel potential therapeutic target for PDAC treatment.

Keywords: Pancreatic ductal adenocarcinoma, miR-26a, matrix metalloproteinase 14, proliferation, migration, invasion

Introduction

Pancreatic cancer is one of the leading causes of cancer-related deaths worldwide [1]. Pancreatic ductal adenocarcinoma (PDAC) accounts for approximately 90% of all cancers, with an overall 5-year survival of 5% [2]. Pancreatic carcinogenesis is known to be a multistep process involving multiple genetic and epigenetic alterations [3]. Despite the great efforts and progressions in PDAC in recent decades, the molecular mechanism of PDAC remains unclear [4]. Thus, it is necessary to explore the molecular mechanisms of PDAC and provide novel agents for PDAC treatment.

MicroRNAs (miRNAs) are small non-coding RNAs which regulate a variety of cellular processes via binding to the 3'-untranslated region (3'-UTR) of target mRNAs, resulting in thedegradation of the mRNAs or inhibition of mRNAs translation [5]. Accumulating evidence revealed that miRNAs play essential roles in cancer biology, including proliferation, metastasis and angiogenesis [6, 7]. Dysregulation of miRNAs

was associated with PDAC progression. For example, He et al found that miR-371-5p was dramatically upregulated in PDAC and could act as a novel prognostic factor and therapeutic target for pancreatic cancer treatment [8]. Li et al showed that miR-184 was increased expression in PDAC and upregulated expression of miR-184 in PDAC might facilitate the proliferation and invasion ability of tumor cells [9]. Sun et al reported that miR-615-5p could function as a tumor suppressor in PDAC by targeting AKT2 [10]. However, the role of miR-26a in PDAC carcinogenesis remains unclear.

In this study, we found that the expression of miR-26a was significantly down-regulated in PDAC tissues and cell lines. Overexpression ofmiR-26a suppressed PDAC cells proliferation, migration and invasion. matrix metalloproteinase 14 (MMP14) was identified as a target of miR-26a in PDAC cells, and restoration of MMP14 remarkably reversed the tumor-suppressive effects of miR-26a. Therefore, our study revealed that miR-26a could act as a novel target for the treatment of PDAC.
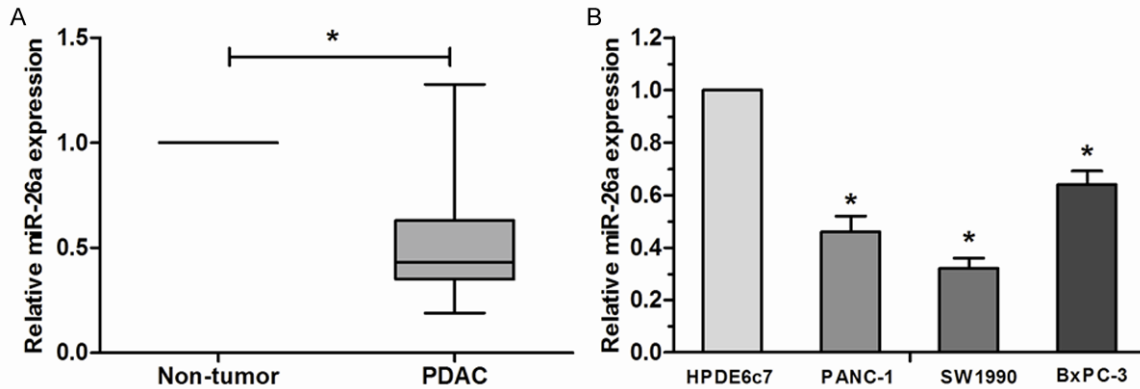
Figure 1. miR-26a is downregulated in PDAC tissues and cell lines. A. The expression of miR-26a in 18 paired PDAC tissues and adjacent non-tumor tissues was examined by using qRT-PCR. B. The expression of miR-26a in PDAC cell lines (PANC-1, SW1990 and BxPC-3) and human pancreatic normal cell line HPDE6c7. *P<0.05.

Materials and methods

Patient samples and cell lines

18 paired PDAC and adjacent non-tumor tissues were obtained from Department of Hepatobiliary and Pancreatic Surgery, Tianjin Nankai Hospital. The study was approved by the Ethics Committee of Tianjin Nankai Hospital. Written informed consents were obtained from each patient. All tissues were stored in liquid nitrogen before RNA isolation and Western blot. The human pancreatic cancer cells, including PANC-1, SW1990 and BxPC-3 were obtained from the American Type culture Collection (ATCC). Human pancreatic normal cells HPDE6c7 were purchased from Guangzhou Jennio Biotech company. All cells were maintained in DMEM with 10% FBS (GIBCO), and were cultured at 37°C with 5% CO_2.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissues or cell lines using Trizol reagent (Invitrogen). MicroRNA was isolated with the mirVana miRNA IsolationKit (Ambion). The quantitative real-time PCR (qRT-PCR) was performed using a SYBR Green mix (Takara) on ABI 7900 (ABI). U6 were used as control. Primers for U6and miR-26a were obtained from Gene Copoeia (Carlsbad). The primers for MMP14 were sense 5'-TCGGCCCAAAGCAGCTTC-3' and antisense 5'-CTTCATGGTGTCTGCATCAGC-3'. qRT-PCR was conducted at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

The relative expression levels of miR-26a and MMP14 were calculated using the $2^{-\Delta\Delta CT}$ method.

Plasmids

MiR-26a and control mimics (miR-NC) were obtained from Ribio Bio (Guangzhou, China). MMP14 overexpression plasmid, pcDNA3-MMP14 was purchased from Promega. The MMP14 3'-UTR fragment containing putative miR-26a binding sites was amplified from complementary DNA. The 3'-UTR was inserted into pGL3 vector (Invitrogen), and named pGL3-MMP14 3'-UTR (WT). Mutation of the miR-26a target sites in pGL3-MMP14 3'-UTR (Mut) was performed using a Strata gene mutation kit (Stratagene).

HEK293 cells were cultured in 24-well plates, and eachwell was co-transfected with 100-ng Wt or Mut, and 50-ng pRL-SV40 (Promega) and 100-ng miR-26a mimics using Lipofectamine 2000. Renilla luciferase pRL-SV40 was used as a control. Cells were collected 48 hafter transfection and the dual-luciferase activity was examined with a Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's instructions.

Cell proliferation assay

Transfected cells were seeded into 96-well plates with a density of 4000 cells/well, and cultured for different time. 10 μL of MTT was added into each well, and incubated for 4 h. Then the supernatant was discarded, and 200 μL of DMSO was added to each well. Optical
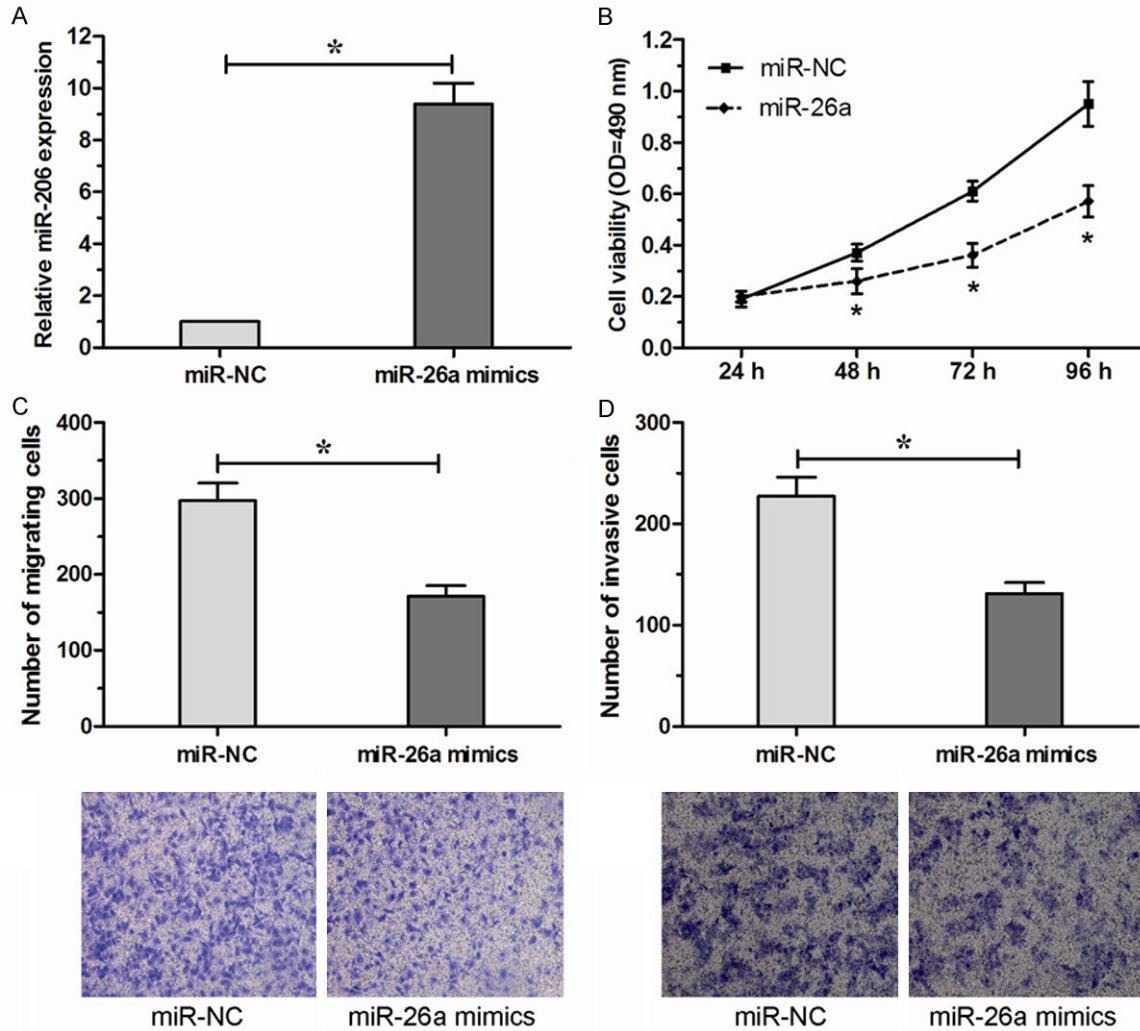
Figure 2. MiR-26a suppresses PDAC cell proliferation, migration and invasion. A. miR-26a mimics increased the expression level of miR-26a compared to control mimics (miR-NC) in SW1990 cells. B. MTT assay showed that over-expression of miR-26a suppressed the proliferation of SW1990 cells compared to the miR-NC group. C. Transwell migration assay showed that upregulation of miR-26a inhibited the migration ability of SW1990 cells compared to the miR-NC group. D. Transwell invasion assay showed that upregulation of miR-26a inhibited the invasion capacity of SW1990 cells compared to the miR-NC group. $^{*}P<0.05$.

density (OD) was detected at the wavelength of 490 nm. Data were derived from three independent experiments.

Cell migration and invasion assays

Cell migration and invasion were determined by using transwell chambers (Millipore). 1×10^5 transfected cells were seeded into the top chamber and grown in DMEM, while DMEM containing 10% FBS was added to the bottom chamber. After 24 h of incubation, cells remaining on the top surface of membranes were removed, and the membranes were stained with 0.5% crystal violet, visualized using an inverted microscope (Olympus). While for invasion assay, the top chamber was precoated with Matrigel (BD).

Luciferase reporter assays

For luciferase activity assay, HEK-293 cells were co-transfected with miR-26a or control mimics and wild-type (Wt) or mutant (Mut) 3'-UTR of MMP14. 48 h after transfection, HEK-293 cells were harvested, and luciferase activity was assayed using Dual-Luciferase Reporter Assay System (Promega).
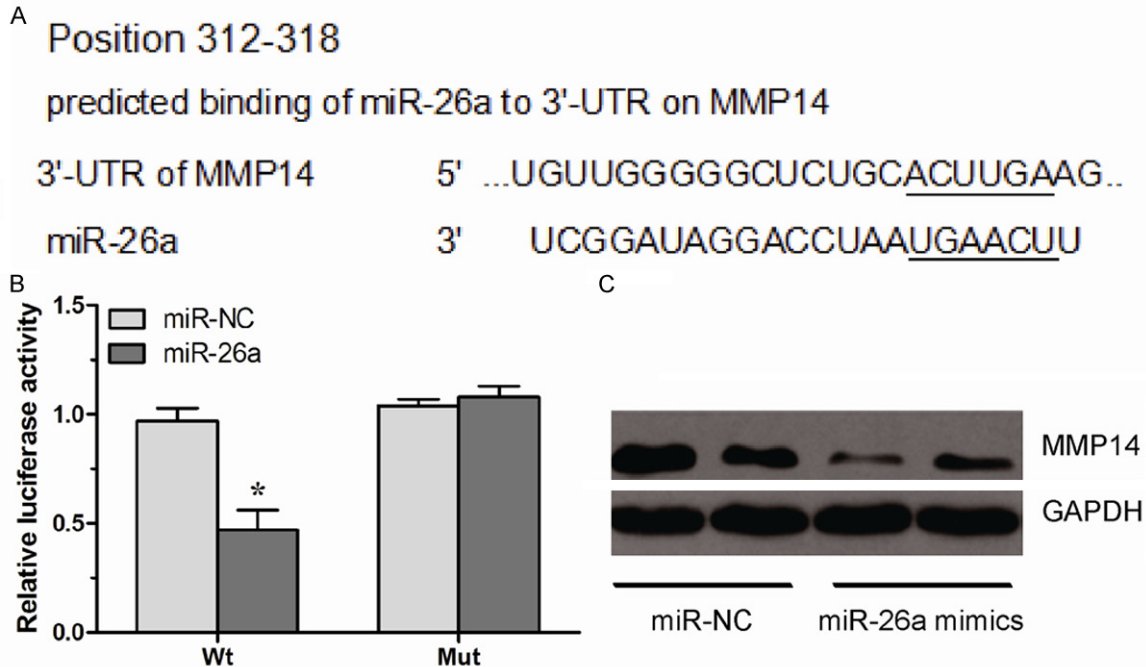
Figure 3. MMP14 is a target of miR-26a in PDAC cells. A. MMP14 was predicted as the targetgenes of miR-26a. B. HEK293 cells were co-transfected with miR-26a mimicsor the miR-NC with Wt or Mut MMP14 3'-UTR. Luciferase activity was assayed. C. SW1990 cells were transfected with miR-26a mimics or miR-NC. The protein level of MMP14 was analyzed by Western blot. *P<0.05.

Protein extraction and Western blotting

Following the transfection, the cells were harvested and lysed. The protein concentration in the each lysate was determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology). Equivalent quantities of protein were separated by 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk and then incubated overnight with the appropriate primary antibody. They were next washed and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. Bound secondary antibody was visualized using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 for Windows. All of the experiments were conducted at least in triplicate, and the results were expressed as mean ± standard deviation (SD). The differences between groups were analyzed using Student's t test. P< 0.05 was considered a statistically significant difference.

Results

miR-26a was decreased in PDAC tissues and cell lines

We examined the expression of miR-26a in 18 paired PDAC tissues and adjacent non-tumor tissue samples by qRT-PCR. We found that miR-26a was significantly decreased in PDAC tissues compared with adjacent non-tumor tissues (**Figure 1A**; P<0.05). Furthermore, miR-26a was also downregulated in three PDAC cell lines (PANC-1, SW1990 and BxPC-3) compared with human pancreatic normal cell line HPDE6c7 (**Figure 1B**; P<0.05). This finding indicated that miR-26a might be involved in PDAC tumorigenesis.

miR-26a inhibited PDAC cell growth and motility

We further explored the role of miR-26a in the regulation of PDAC cell growth and motility. SW1990 cells were transfected with the miR-26a mimics and miR-NC, and miR-26a expression was examined by qRT-PCR. The expression of miR-26a was significantly up-regulated in miR-26a mimic-transfected cells compared
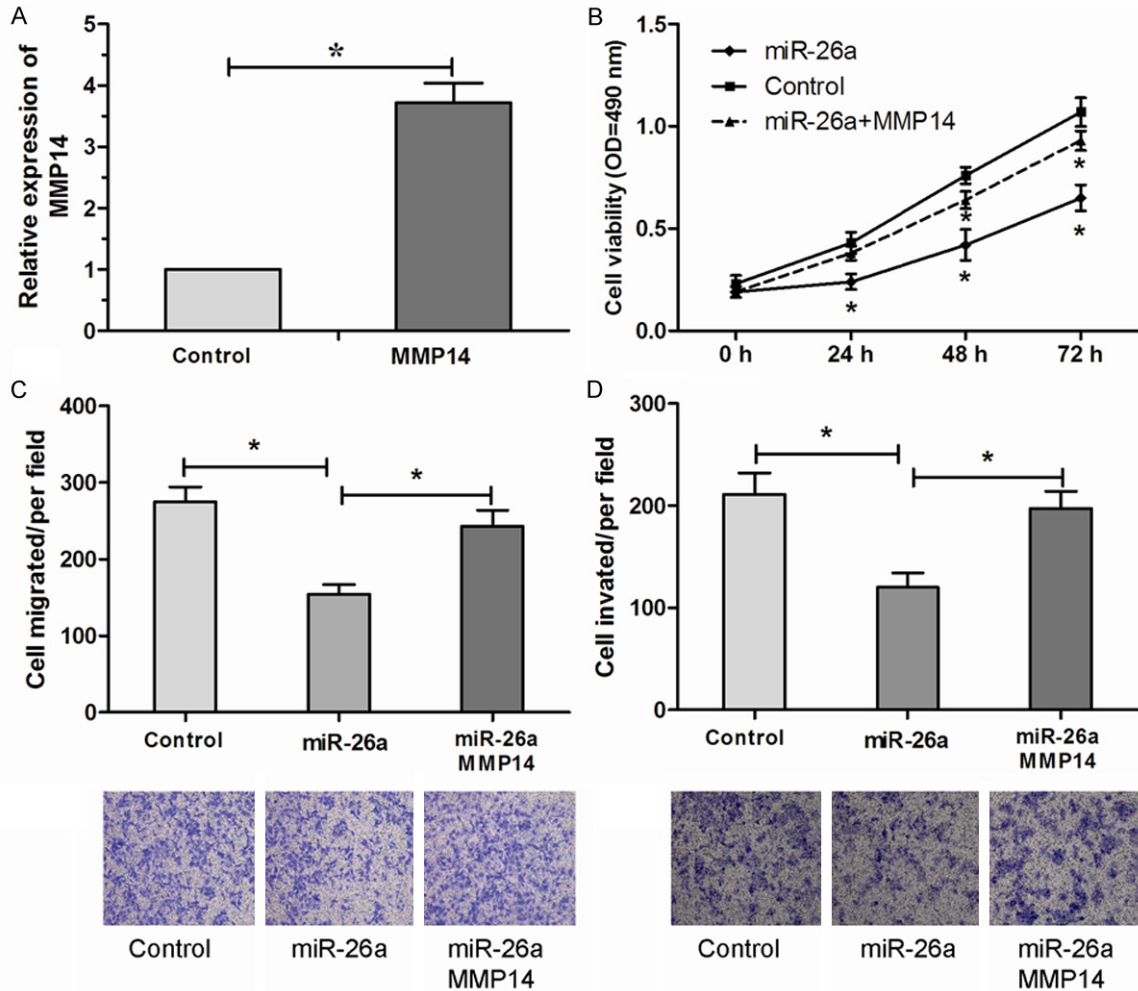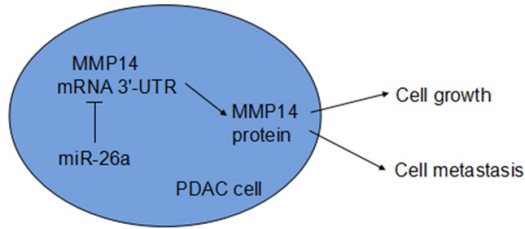
Figure 4. Restoration of MMP14 reverse the effects of miR-26a. (A) SW1990 cells were transfected with MMP14 overexpression plasmid, and the expression of MMP14 was examined by qRT-PCR. (B-D) SW1990 cells were co-transfected with miR-26a mimics and MMP14 overexpression plasmidor the control vector. MTT (B), migration (C), and invasion (D) assays were performed. *P<0.05.

with the miR-NC transfected cells (**Figure 2A**; P<0.05). MTT assay was used to test PDAC cell proliferation ability. We found that miR-26a significantly inhibited SW1990 cell proliferation ability (**Figure 2B**; P<0.05). We further investigated whether miR-26a could also inhibit migration and invasion of PDAC cells. Transwell migration assay showed that overexpression of miR-26a dramatically suppressed tumor cell migration ability in SW1990 cell (**Figure 2C**; P<0.05). Similarly, transwell invasion assay demonstrated that miR-26a markedly decreased the invasive capacity of SW1990 cells (**Figure 2D**; P<0.05). These data suggested that miR-26a could inhibit cell growth and metastasis of PDAC cells.

MMP14 was a target of miR-26a in PDAC cells

To identify the target of miR-26a in PDAC, Targetscan software (http://www.targetscan.org/) was used. Our results showed that the 3'-UTR of human MMP14 contains putative target sites for miR-26a (**Figure 3A**). To confirm the target sites, the 3'-UTR and mutated 3'-UTR of human MMP14 were amplified and inserted into the vector. Luciferase activity assay indicated that miR-26a mimics significantly inhibited the Wt but not the Mut 3'-UTR luciferase activity in HEK-293 cells (**Figure 3B**; P<0.05). In addition, up-regulated expression of miR-26 significantly suppressed MMP14 protein level (**Figure 3C**; P<0.05). These data

Figure 5. Schematic miR-26a suppress PDAC cell growth and metastasisvia targeting MMP14.

revealed that MMP14 was a target of miR-26a in PDAC cells.

Restoration of MMP14 abolished the effects of miR-26a

We further explored whether restoration of MMP14 could reverse the tumor-suppressive effects of miR-26a in PDAC progression. The efficiency of pcDNA3-MMP14 transfection was determined by qRT-PCR (**Figure 4A**; P<0.05). MTT assay, transwell migration assay and transwell invasion assay showed that supplement of MMP14 by pcDNA3-MMP14 significantly attenuated the tumor suppressive effects of miR-26a in PDAC cells (**Figure 4B-D**; P<0.05). These data demonstrated that miR-26a could act as a tumor suppressor by targeting MMP14 in PDAC development (**Figure 5**).

Discussion

PDAC is characterized as a multi-cause and a prolonged multi-stage process, which involves a number of alterations in gene expression and physiological structure [11]. A large number of evidence showed that miRNAs were significant prognostic factors and also potential therapeutic targets for PDAC [12]. In the present study, our data revealed a tumor-suppressive role of miR-26a in PDAC progression. We showed that miR-26a was downregulated in PDAC tissues and cell lines. Overexpression of miR-26a suppressed proliferation, migration and invasion of PDAC cells. Furthermore, MMP14 was identified as a target of miR-26a in PDAC cells. Moreover, restoration of MMP14 substantially reversed the tumor-suppressive effects ofmiR-26a in PDAC cells. These findings demonstrated that miR-26a may act as a tumor suppressor in PDAC via targeting MMP14.

MicroRNA-26a (miR-26a), located in chromosome 3p22, a region with high frequency of loss of heterozygosity (LOH) in cancer, has

been proved to be a tumor suppressor gene [13]. Aberrant expression of miR-26a was thought to contribute to the malignant phenotype of several tumors. For example, Taheriazam et al suggested that downregulation of miR-26a contributed to aggressive progression of human osteosarcoma [14]. Zhang et al indicated that pathologically decreased miR-26a antagonized apoptosis and facilitated carcinogenesis by targeting MTDH and EZH2 in breast cancer [15]. Ma et al reported that miR-26a could suppress epithelial-mesenchymal transition in human hepatocellular carcinoma by repressing enhancer of zeste homolog 2 [16]. Lin et al showed that miR-26a could inhibit proliferation and motility in bladder cancer by targeting HMGA1 [17]. Thus, our study further elucidated the tumor-suppressive role of miR-26a in PDAC. Forced expression ofmiR-26a suppressed PDAC cells proliferation, migration and invasion by targeting MMP14.

Metalloproteinases 14 (MMP14) is a membrane-type metalloproteinase with collagenase activity and has been implicated to play a role in many biological processes in normal and tumor tissues [18]. The expression of MMP14 has been found to be elevated in a variety of cancers, and MMP14 plays critical roles in the development of many cancers, such as gliomas, nasopharyngeal carcinoma and breast cancer [19-21]. MMP14 has been found to be regulated by several miRNAs. For example, Jia et al showed that miR-34a Inhibited migration and invasion of tongue squamous cell carcinoma via targeting MMP14 [22]. Xu indicated that miR-133a suppressed cell proliferation, migration and invasion in human lung cancer by targeting MMP14 [23]. Zuo et al demonstrated that miR-22 inhibited tumor growth and metastasis in gastric cancer by targeting MMP14 [24]. In the study, our data showed that MMP14 was a target of miR-26a and supplement of MMP14 remarkably attenuated the tumor suppressive effects of miR-26a in PDAC cells.

In summary, our data showed that miR-26a could suppress tumor growth and metastasis of PDAC cells by targeting MMP14, highlighting the therapeutic potential of miR-26a in pancreatic cancer.

Disclosure of conflict of interest

None.

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