Original Article
miR-499a promotes PANC-1 cell proliferation by down-regulating PDCD4 expression in pancreatic ductal adenocarcinoma

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Abstract: Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive digestive system tumors, but study of the molecular mechanism of occurrence and development of PDAC is considerably limited. In order to better understand the potential pathogenesis, the differentially expressed miRNAs were screened in PDAC and adjacent tissues using miRNAs microarrays. We found that miR-499a was significantly up-regulated in PDAC tissues compared with adjacent tissues, and protein-protein interaction (PPI) and gene ontology (GO) analyses indicated programmed cell death protein 4 (PDCD4) is a key target gene of miR-499a, which is involved in the regulation of transcription, cellular biosynthetic process, RNA metabolic process, and other multiple biologic processes. Moreover, PDCD4 mRNA and protein expression were obviously down-regulated in PDAC tissues compared with adjacent tissues. In vitro, up-regulating of miR-499a could decrease PDCD4 expression and promote cell proliferation in PANC-1 cells transfected with miR-499a mimics. Similarly, promoting proliferation was also observed in PANC-1 cells transfected with PDCD4 siRNA. In conclusion, we first found miR-499a was significantly up-regulated in PDAC tissues, and we promoted PANC-1 cell proliferation by down-regulating PDCD4 expression.

Keywords: miR-499a, PANC-1, cell proliferation, PDCD4 expression, pancreatic ductal adenocarcinoma

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is one of the most malignant tumors, and its morbidity is comparable to its mortality [1]. Rapid progression, early metastasis, and low sensitivity to chemotherapy or radiotherapy, all lead to a poor prognosis for patients with PDAC. Although progress in treatment and diagnosis has been made, outcome remains unsatisfactory [2]. Therefore, it is necessary to better understand the molecular mechanism of pancreatic cancer.

MicroRNAs (miRNAs) play an important role in the occurrence and development of tumors [3] and may be diagnostic and prognostic markers. In pancreatic cancer, some novel miRNAs had been discovered, some of which may relate to tumorigenesis and development [4]. For example, microRNA-224 can increase the ability of proliferation and migration in pancreatic cancer cells [5]. Besides, some miRNAs (miR-1301, miR-598, miR-1180, miR-155, miR-496, miR-203, miR-193b, miR-135b) are considered as independent predictors for survival in patients with PDAC [6]. However, almost all of them have not been applied to clinical practice. Hence, we still need to explore more novel and meaningful miRNAs. miRNAs were identified that are differentially expressed between normal and PDAC tissues by miRNA microarrays. Next, an integrated bioinformatics analysis was performed to explore the key target genes of miRNAs and the potential molecular mechanism. Finally, the effect of differentially expressed miRNAs on cell proliferation ability was evaluated in vitro.

Material and methods
Sample collection
Twenty paired fresh PDAC and adjacent tissues were collected during operation from February 2015 to May 2018 in Tianjin Nankai Hospital (Tianjin, China), and were at -80°C. These pa-
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target genes of miR-499a were input this database, a protein-protein interaction network was constructed as previous report (3016-8505). In String database, GO analysis of all genes in this network was performed by its analysis function. The biological process (BP) list is downloaded and shown.

**Human protein atlas database**

All immunohistochemical images of PDCD4 in PDAC (n=11) and adjacent tissues (n=3) were downloaded from Human Protein Atlas (HPA) database [9], the integral optical density (IOD) of these image was calculated by Image-Pro Plus software (Media Cybernetics; version 6.0), and the IOD value represents the relative expression level of PDCD4.

**Cell culture and transfection**

Human pancreatic cancer (PANC-1) cells were purchased from American Type Culture Collection (ATCC), and grown in DMEM medium supplemented with 10% fetal bovine serum (Invitrogen), and maintained at 37°C, 5% CO$_2$ in a humidified incubator. miR-499a mimics, siRNA targeting PDCD4, and corresponding negative control (NC) sequences were designed and synthesized by Sangon Biotech (Shanghai, China). Lipofectamine 2000 reagent (Invitrogen) was used to transfect miR-499a mimics, siRNA targeting PDCD4, and corresponding negative control (NC) sequences into PANC-1 cells respectively, according to the manufacturer’s protocol.

**CCK-8 assay**

The proliferation ability of PANC-1 cells was assessed using cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Japan). PANC-1 cells transfected with miR-499a mimics, siRNA targeting PDCD4, and NC sequences were respectively plated in a 96-well plate (5×10$^4$ cells/well), and incubated at 37°C, 5% CO$_2$ in a humidified incubator. At 12, 24, and 36 hours after transfection, 10 µl CCK-8 reagent was added into each well, then incubated 1 hour at 37°C, 5% CO$_2$ in incubator. The absorbance of each group was detected at 450 nm by a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).
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Statistical methods
IBM SPSS 20.0 statistics software (IBM Corp, Armonk, NY) was used to analyze all data. The miR-499a, and PDCD4 mRNA level between the PDAC and adjacent tissues were compared with paired Student’s t-test. Independent t-test was used to analyze the IOD value of PDCD4 in PDAC and adjacent tissues. ANOVA post-hoc analysis was used to compare the cell proliferation ability under different conditions. P<0.05 was considered significant.

Results
miR-499a is up-regulated in PDAC tissues.

Expression profile microarray analysis indicated that 17 miRNAs were up-regulated and 4 miRNAs were down-regulated in PDAC tissues (Table 1; P<0.01). The heat map of differentially expressed miRNAs is shown in Figure 1A, and miR-499a was significantly up-regulated in PDAC tissues compared to adjacent tissues (log₂FC=3.61, P<0.01; FC=fold change).

Table 1. miRNAs differentially expressed between PDAC and normal tissue

<table>
<thead>
<tr>
<th>No.</th>
<th>miRNAs</th>
<th>log₂FC</th>
<th>Average expression level</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hsa-miR-499a-5p</td>
<td>3.61</td>
<td>8.95</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>hsa-miR-551a</td>
<td>3.46</td>
<td>8.92</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>hsa-miR-124-3p</td>
<td>3.71</td>
<td>8.93</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>hsa-miR-569</td>
<td>3.68</td>
<td>8.87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5</td>
<td>hsa-miR-5100</td>
<td>2.56</td>
<td>7.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6</td>
<td>hsa-miR-4535</td>
<td>2.93</td>
<td>7.70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>7</td>
<td>hsa-miR-24-1-5p</td>
<td>2.88</td>
<td>7.70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>8</td>
<td>hsa-miR-581</td>
<td>3.67</td>
<td>8.82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>9</td>
<td>hsa-miR-1234</td>
<td>-2.02</td>
<td>8.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10</td>
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<td>2.01</td>
<td>11.21</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>11</td>
<td>hsa-miR-217</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>12</td>
<td>hsa-miR-1178</td>
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<td>8.67</td>
<td>&lt;0.001</td>
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<tr>
<td>13</td>
<td>hsa-miR-649</td>
<td>3.61</td>
<td>8.93</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>14</td>
<td>hsa-miR-548u</td>
<td>-2.46</td>
<td>8.80</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>15</td>
<td>hsa-miR-151a-5p/hsa-miR-151b</td>
<td>2.97</td>
<td>7.75</td>
<td>&lt;0.001</td>
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<tr>
<td>16</td>
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<td>7.62</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>17</td>
<td>hsa-miR-5091</td>
<td>2.87</td>
<td>7.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>18</td>
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<tr>
<td>19</td>
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<td>2.02</td>
<td>8.80</td>
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<tr>
<td>20</td>
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<tr>
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<td>hsa-miR-1827</td>
<td>2.16</td>
<td>8.77</td>
<td>&lt;0.001</td>
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</tbody>
</table>

Moreover, qRT-PCR results further verified the relative miR-499a level was strongly up-regulated in PDAC tissues (n=10) compared with adjacent tissues (n=10) (Figure 1B, P<0.01).

PDCD4 is a key target gene of miR-499a and down-regulated in PDAC tissues.

In order to further explore the potential pathogenesis of miR-499a in PDAC occurrence and development, verified target genes were searched by TargetScan database, and 297 verified target genes were found. GO analysis found that they mainly involved in multiple biologic processes, such as regulation of transcription, cellular biosynthetic process, and RNA metabolic process (Figure 2A). Protein-protein interaction network of these target genes was constructed by String database, and results suggested that PDCD4 was a key target gene (Figure 2B), and it also is involved in regulation of transcription and cellular biosynthetic process. Therefore, PDCD4 aroused our interest.

The expression level of PDCD4 was assessed by IHC data from HPA database. We found that the IOD value of PDCD4 was obviously lower in PDAC tissues compared with normal tissues (Figure 3A and 3B; P<0.05). Furthermore, in clinical samples, qRT-PCR analysis confirmed that the PDCD4 mRNA level was also decreased in PDAC tissues (Figure 3C; P<0.05). Above results suggested that PDCD4 expression level was lower in PDAC tissues than adjacent tissues.

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miR-499a is associated with cell proliferation ability in multiple tumors [10]. In our study, miR-499a mimics were transfected into PANC-1 cells, and this increased the ability of proliferation after transfection 24 hours (Figure 4A). In
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In vitro, we also further verified that up-regulated miR-499a could decrease the expression level of PDCD4 (Figure 4B). Next, si-RNA targeting PDCD4 was transfected into PANC-1 cells, and down-regulated PDCD4 also promoted PANC-1 cell proliferation (Figure 4C). These results suggested that miR499 promoted the proliferation of PANC-1 cells by down-regulating PDCD4 expression.

Discussion

In this study, we first found that miR-499a was obviously up-regulated in PDAC tissues compared to normal tissues. Its target genes are mainly involved in regulation of transcription, and cellular biosynthetic process. Among these target genes, PDCD4 is a key target gene of miR-499a and is down-regulated in PDAC tissues. In vitro, we found that up-regulation of miR-499a promoted cell proliferation and decreased the expression of PDCD4 in PANC-1 cells. Furthermore, we also observed that down-regulation of PDCD4 could increase the ability of proliferation in PANC-1 cells. The above results suggested that miR-499a could promote PANC-1 cell proliferation by down-regulating PDCD4 expression.

A previous study confirmed that multiple miRNAs are involved in the occurrence and development of pancreatic cancer, such as miR-196b [11], miR-148a [12], and miR-365 [13]. Although a large number of abnormally expressed miRNAs were identified in PDAC, these miRNAs for clinical application are very limited. We first found that miR-499a was significantly up-regulated in PDAC tissues. However, the function of miR-499a is always controversial. miR-499a was considered as a carcinogenic miRNA in some studies; for example, miR-499 was highly expressed in squamous cell carcinoma tissues displaying a loss of PDCD4, which may be an important step in tonsillar carcinogenesis [14]. In contrast, another study found that miRNA-499a decelerated glioma cell proliferation while accelerating apoptosis through the suppression of Notch1 and the mitogen-activated protein kinase (MAPK) signaling pathway [15].

In order to further analyze the function and key target genes of miR-499a, PPI and GO analyses were performed in this study. GO analysis found that target genes of miR-499a mainly involved in regulation of transcription, cellular biosynthetic processes, RNA metabolic processes, and multiple biologic processes. Moreover, PPI analysis found that PDCD4 was a core gene in this interaction network. PDCD4, a novel tumor suppressor gene, inhibits tumor progression at transcriptional and translational levels and regulates multiple signal transduction pathways [16]. PDCD4 suppresses proliferation, migration, and invasion of endometrial
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Hence, the effect of miR-499a on proliferation ability of PANC-1 cells was assessed in vitro. We found that the proliferation ability of PANC-1 cells was increased compared with negative control groups after transfecting miR-499a mimics. Meanwhile, the PDCD4 mRNA level was decreased in PANC-1 cells transfected with miR-499a mimics. To further verify whether miR-499a promotes cell proliferation by down-regulating PDCD4 expression, si-RNA targeting PDCD4 was transfected into PANC-1 cells, then we observed that down-regulation of PDCD4 also promoted PANC-1 cell prolif-
miR-499a promotes PANC-1 cell proliferation by down-regulating PDCD4 expression. The above results suggested that miR-499a promotes PANC-1 cell proliferation by down-regulating PDCD4 expression in pancreatic cancer. However, this study still has some limits. For example, it is necessary to verify the expression level of miR-499a and association with prognosis in a large sample. Also, the role of promoting proliferation of miR-499a needs to be verified by animal experiments.

Briefly, our study first found that miR-499a was obviously up-regulated in PDAC tissues, and miR-499a overexpression could promote PANC-1 cell proliferation by down-regulation of PDCD4 expression. This work not only sheds light on differentially expressed miRNAs but also is meaningful for potential PDAC therapy.

Disclosure of conflict of interest
None.

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References
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