**Altered profile of serum microRNAs in pancreatic cancer-associated new-onset diabetes mellitus**

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**Abstract**

**Background:** New-onset diabetes mellitus in pancreatic cancer has been recognized as a paraneoplastic phenomenon caused by the existence of the tumor. Circulating microRNAs (miRNAs) are emerging as non-invasive biomarkers for the detection of various cancers. In the present study, we hypothesized that a specific serum miRNA profile exists in pancreatic cancer-associated new-onset diabetes mellitus (PaC-DM).

**Methods:** Initial screening of differentially expressed miRNAs in pooled serum samples from 25 PaC-DM patients, 25 non-cancer new-onset type 2 diabetes mellitus (T2DM) patients, and 25 healthy controls was performed by TaqMan low-density arrays (TLDA). A stem–loop quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was conducted to confirm the relative concentrations of candidate miRNAs in 80 PaC-DM, 85 non-cancer new-onset T2DM patients, and 80 healthy controls.

**Results:** The TLDA identified 16 serum miRNAs that were significantly increased in PaC-DM compared with non-cancer T2DM and healthy controls. A combination of six serum miRNAs (miR-483-5p, miR-19a, miR-29a, miR-20a, miR-24, miR-25) was selected by qRT-PCR as a biomarker for PaC-DM. The area under the receiver operating characteristic curve (AUC) for the six-miRNA panel training and validation sets was 0.959 (95% confidence interval [CI] 0.890–1.028) and 0.902 (95% CI 0.844–0.955), respectively. The combination of these six miRNAs enabled the discrimination of PaC-DM from non-cancer new-onset T2DM with an AUC of 0.885 (95% CI 0.784–0.986) and 0.887 (95% CI 0.823–0.952) for the training and validation sets, respectively.

**Conclusion:** The six-serum miRNA panel may have potential as a biomarker for the accurate diagnosis and discrimination of PaC-DM from healthy controls and non-cancer new-onset T2DM.

**Keywords:** biomarker, diagnosis, new-onset diabetes mellitus, pancreatic cancer, serum microRNAs.

**Significant findings of the study:** Six serum miRNAs were identified that were increased significantly in PaC-DM compared with non-cancer T2DM and healthy controls. This six-miRNA panel had superior diagnostic capacity to detect PaC-DM and separate PaC-DM from non-cancer T2DM.

**What this study adds:** An altered serum miRNA expression profile was identified in PaC-DM and the potential of the six-miRNA panel as a novel clinical biomarker for the diagnosis and differentiation of PaC-DM was demonstrated.
**Introduction**

Type 2 diabetes mellitus (T2DM) is an adult-onset metabolic disorder associated with an increased risk of several kinds of malignant tumors, including hepatoma, breast cancer, and pancreatic cancer. Previous studies have reported the effects of long-standing diabetes on the development of pancreatic exocrine neoplasia. The potential mechanism reportedly involves overexpression of insulin-like growth factor (IGF)-1 and G-protein-coupled receptors, and the chronic hyperinsulinemia caused by T2DM or obesity enhances the development and growth of this pancreatic cancer. New onset T2DM (<36 months) has been considered as a relative disorder with early pancreatic cancer. In a cohort study including 933 controls and 512 pancreatic cancer patients, Pannala et al. found that diabetes mellitus associated with pancreatic cancer is often new in onset, and suggested that new-onset diabetes mellitus in patients with pancreatic cancer is likely induced by the tumor. Either hyperglycemia or diabetes, present in nearly 80% of pancreatic cancer patients, could exist during the asymptomatic phase of pancreatic cancer. Unlike T2DM, which is associated with weight gain and obesity, pancreatic cancer-induced diabetes is associated with weight loss. Diabetes and weight loss are paraneoplastic phenomena induced by pancreatic cancer. Pancreatic cancer cells have been reported to secrete some tumor-mediated factors, such as adrenomedullin, to induce insulin resistance and β-cell dysfunction in pancreatic cancer-associated diabetes. Given the distinctive outcomes and pathologic mechanisms underlying pancreatic cancer with new-onset diabetes mellitus (PaC-DM), a reliable, non-invasive biomarker for PaC-DM would facilitate improvements in the diagnosis of pancreatic cancer during the early stages within this specific population.

Pancreatic cancer is one of the most lethal diseases, with a high rate of cancer related-deaths, and early diagnosis is recognized as the only method to cure this disease. Most clinical diagnoses for pancreatic cancer are made at an advanced stage because of the insidious onset of the disease and a lack of effective biomarkers. The most common clinical tumor marker for pancreatic cancer is carbohydrate antigen (CA) 19-9, with a median sensitivity of 79% and median specificity of 82%. However, CA 19-9 can be detected in patients with certain non-malignant diseases, such as chronic pancreatitis, cirrhosis, or other gastrointestinal tumors. Furthermore, CA 19-9 is a poor candidate for screening in the general population because of its limited diagnostic sensitivity in asymptomatic patients. Nonetheless, given the low incidence of pancreatic cancer, more accurate markers for diagnosis are required and screening for pancreatic cancer should be restricted to a more specific population with a potential risk for development of the tumor.

MicroRNAs (miRNAs) are endogenous single-stranded non-coding RNAs (~22 nucleotides in length) that control gene expression at the post-transcriptional level. As a result of imperfect base pairing with the 3’ untranslated region of the target mRNAs, miRNAs suppress protein translation by either impeding translation initiation or accelerating the degradation of mRNAs. Stable expression of miRNAs in serum, plasma, milk, and other types of bodily fluids has been demonstrated. In recent years, it has been shown that different combinations of serum miRNAs could be used to identify various human diseases, especially cancers, including prostate cancer, non-small cell lung cancer, renal cell carcinoma, hepatocellular carcinoma, gastric cancer, and pancreatic cancer.

In the present study, 25 PaC-DM, 25 non-cancer new-onset T2DM, and 25 healthy control sample pools were analyzed using reverse transcription–polymerase chain reaction (RT-PCR)-based TaqMan low-density array (TLDA). For confirmation, quantitative (q) RT-PCR was used to identify specific miRNA profiles in 80 serum samples from PaC-DM patients, which were compared with samples from 80 healthy controls and 85 non-cancer new-onset T2DM patients. The findings of the present study may suggest a new non-invasive diagnostic tool for PaC-DM and help discriminate PaC-DM from non-cancer T2DM.

**Methods**

**Study population**

In all, 80 PaC-DM patients (diabetes mellitus duration <3 years before the diagnosis of pancreatic cancer) hospitalized in Shanghai Ruijin Hospital and 85 non-cancer new-onset T2DM patients were enrolled in the study between April 2009 and October 2012. All patients who donated serum samples were diagnosed as having pancreatic ductal adenocarcinoma, confirmed by pathologic examination, and the diagnosis of T2DM was made in accordance with the criteria of the American Diabetes Association (ADA). The diagnosis and tumor stage of the pancreatic cancer were determined on the basis of surgical findings according to the World Health Organization’s classification system for pancreatic cancer. The absence of cancer in T2DM patients and healthy controls was confirmed by imaging examinations. Eighty subjects were recruited from the Healthy Physical Examination Center of Shanghai Ruijin Hospital as a parallel control...
group; this group underwent a health checkup that included a detailed medical history, physical and imaging examinations, and blood tests. Written informed consent was obtained from all subjects recruited to the study. This study was performed in accordance with the guidelines of Shanghai Ruijin Hospital and all experimental protocols were approved by the Ethic Committee of Shanghai Ruijin Hospital.

**Patient characteristics and collection of clinical data**

Basic information (age, gender, smoking status, alcohol consumption, and medical history) and clinicopathologic data were collected from PaC-DM, T2DM patients, and healthy controls from medical records and using a simple epidemiologic questionnaire. All clinicopathologic and histological information for pancreatic cancer and PaC-DM subjects was obtained from the medical and pathologic records in hospital. The characteristics of the patients and healthy controls enrolled in the training and validation sets are given in Table 1.

### TaqMan low-density array for serum miRNAs

Blood samples were collected from PaC-DM patients prior to surgery, as well as from non-cancer new-onset T2DM patients and healthy controls. The samples were then centrifuged (3000 g, 10 min, room temperature) and the supernatant collected and stored as described previously. For TLDA analysis, three serum pools were

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**Table 1** Patient characteristics and clinical features

<table>
<thead>
<tr>
<th></th>
<th>PaC-DM (n = 80)</th>
<th>T2DM (n = 85)</th>
<th>Control (n = 80)</th>
<th>P-value PaC-DM vs control</th>
<th>P-value PaC-DM vs T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>60.1 ± 10.7</td>
<td>57.4 ± 9.6</td>
<td>59.5 ± 13.5</td>
<td>0.584§</td>
<td>0.441§</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>42 (52.50%)</td>
<td>44 (51.76%)</td>
<td>43 (53.75%)</td>
<td>0.197¶</td>
<td>0.204¶</td>
</tr>
<tr>
<td>Female</td>
<td>38 (47.50%)</td>
<td>41 (48.24%)</td>
<td>37 (46.25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥24 kg/m²</td>
<td>0 (0%)</td>
<td>43 (50.59%)</td>
<td>27 (33.75%)</td>
<td>0.669¶</td>
<td>0.372¶</td>
</tr>
<tr>
<td>&lt;24 kg/m²</td>
<td>80 (100%)</td>
<td>42 (49.41%)</td>
<td>53 (66.25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ever/current</td>
<td>28 (31.25%)</td>
<td>32 (37.65%)</td>
<td>27 (33.75%)</td>
<td>0.198¶</td>
<td>0.215¶</td>
</tr>
<tr>
<td>Never</td>
<td>52 (68.75%)</td>
<td>53 (62.35%)</td>
<td>53 (66.25%)</td>
<td></td>
<td></td>
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<tr>
<td>Alcohol consumption</td>
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</tr>
<tr>
<td>Ever/current</td>
<td>27 (33.75%)</td>
<td>25 (29.41%)</td>
<td>23 (28.75%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>53 (66.25%)</td>
<td>60 (70.59%)</td>
<td>57 (71.25%)</td>
<td></td>
<td></td>
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<tr>
<td>TNM stage</td>
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</tr>
<tr>
<td>0</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>7 (8.75%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<td></td>
</tr>
<tr>
<td>IIA</td>
<td>13 (16.25%)</td>
<td>5 (6.11%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td>16 (20%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>19 (23.75%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>25 (31.25%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
<td></td>
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<tr>
<td>FBG†</td>
<td>6.96 ± 1.79</td>
<td>8.25 ± 3.64</td>
<td>5.35 ± 1.52</td>
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<td></td>
</tr>
<tr>
<td>≥6.1</td>
<td>40 (57.14%)</td>
<td>58 (68.24%)</td>
<td>5 (6.25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;6.1</td>
<td>30 (42.86%)</td>
<td>27 (31.76%)</td>
<td>75 (93.75%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum CA 19-9‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (U/L)</td>
<td>492 ± 1548</td>
<td>14.1 ± 10.5</td>
<td>8.56 ± 1.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>43 (55.71%)</td>
<td>7 (8.24%)</td>
<td>4 (5.00%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>37 (44.29%)</td>
<td>78 (91.76%)</td>
<td>76 (95.00%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD or as n (%).

*Body mass index (BMI) was calculated as weight divided by height squared; a BMI ≥24 kg/m² is regarded as overweight.
†Fasting blood glucose (FBG) ≥6.1 mmol/L is regarded as impaired.
‡Serum CA 19-9 levels >35 U/L are considered positive, ≤35 U/L are considered negative.
§Student’s t-test.
¶Two-sided χ² test.

PaC-DM, pancreatic cancer-associated new-onset diabetes mellitus; T2DM, type 2 diabetes mellitus.
formed for 25 PaC-DM patients, 25 T2DM patients, and 25 controls with 500 μL serum from each subject. Total RNA was extracted from each serum sample with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The 25 T2DM patients and 25 healthy controls were age- and sex-matched with the 25 PaC-DM patients (see Table S1).

The total RNA extracted from each serum sample pool was reverse transcribed, followed by cDNA pre-amplification using the TaqMan PreAmp Mastermix (Life Technologies, Carlsbad, CA, USA). The miRNA profiling of 754 different human miRNAs was then examined by TLDA on an ABI PRISM 7900HT Sequence Detection System (TaqMan Array Human MiRNA A 1 B Cards Set v3.0; Life Technologies).

Quantitative RT-PCR of serum miRNAs

For the qRT-PCR of individual serum samples, total RNA was extracted from 100 μL serum mixed with 300 μL denaturing solution using a one-step phenol–chloroform purification protocol as described previously. Then, total RNA from all individuals in the three groups was isolated and stored at −80°C until detection of candidate miRNAs.

A hydrolysis probe-based qRT-PCR was conducted to quantify the relative concentration of serum miRNAs using the ABI 7500 RT-PCR system (Applied Biosystems, Grand Island, NY, USA). All reactions were run in triplicate, as described previously. The combination of let-7d, let-7g, and let-7i (let-7d/g/i) was used as an endogenous reference gene for the normalization of serum miRNAs, as reported previously. The comparative threshold cycle (ΔCT) method was used to compare each treatment with the internal control, and values are expressed as 2−ΔΔCT.

Statistical analysis

Statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). The miRNA data are presented as the mean ± SEM. Student’s t-test was used to compare differences in serum miRNA concentrations among the PaC-DM, T2DM, and matched healthy control groups. For evaluation of the diagnostic value using the combination of the six miRNAs, data were divided into three groups: PaC-DM versus control, PaC-DM versus new-onset T2DM, and PaC-DM versus both new-onset T2DM and healthy control. Logistic regression was used, receiver operating characteristic (ROC) curves were constructed, and the area under the curve (AUC) was calculated in each group. Furthermore, odds ratio (OR) were determined in addition to logistic regression analysis to evaluate the effects of serum miRNAs on PaC-DM. Two-sided P < 0.05 was considered significant.

Results

TaqMan low-density array analysis and identification of candidate miRNAs

A multiphase case-control study was performed to identify miRNAs that were markedly increased in the serum of patients with PaC-DM (an overview of the strategy is shown in Fig. 1). Initially, TLDA was performed on the three serum pools from 25 PaC-DM, 25 T2DM, and 25 healthy controls to identify altered miRNA profiles. Of the 754 miRNAs scanned, more than 40 were observed to be differentially expressed across the patient and control samples (Fig. 2). The miRNA was considered upregulated if there was a 1.5-fold change in the concentration between the PaC-DM sample and either the T2DM or control samples. As indicated in Table S2, 16 miRNAs were considered elevated in the PaC-DM group. Based on the miRNA expression levels (Cq values <30 in PaC-DM), 12 of the upregulated miRNAs (miR-7, miR-212, miR-19a, miR-202, miR-29a, miR-195, miR-483-5p, miR-20a, miR-24, miR-205, miR-21, and miR-25) were then chosen as candidate miRNAs for further confirmation and validation by individual qRT-PCR.

Confirmation of increased serum miRNAs by qRT-PCR

To confirm and validate the serum miRNA signature panel for diagnosing PaC-DM, we subsequently established a training set (30 PaC-DM, 30 T2DM, and 30 controls) and a validation set (50 PaC-DM, 55 T2DM, and 50 controls), which were randomly derived from a total of 245 serum samples to refine the 12 candidate serum miRNAs by a hydrolysis probe-based qRT-PCR assay. As indicated in Table 1, there were no significant differences between patients with PaC-DM and T2DM, or between patients with PaC-DM and healthy controls in terms of age, sex, smoking status, and alcohol consumption. Fasting blood glucose levels in the PaC-DM and T2DM groups were higher than in the control group, revealing the dysfunctional glucose metabolism of the patients. Serum CA 19-9 is one of the most widely used tumor makers for cancer screening tests, particularly pancreatic cancer. In the present study, the positive predictive value (PPV) and negative predictive value (NPV) of serum CA 19-9 in all cases from the training and validation sets for pancreatic cancer diagnosis was 79.63% and 80.63%, respectively.

The 12 candidate miRNAs selected were confirmed by individual qRT-PCR assay in the training set. The inclusion criteria for significantly upregulated miRNAs, as
reported previously, were: (i) a mean 1.5-fold change; (ii) \( P < 0.01 \) for comparisons of cases and controls; (iii) a Cq value of 35; and (iv) a detection rate of 75% in either the cases or controls. Based on the qRT-PCR results of the training set, six miRNAs (miR-483-5p, miR-19a, miR-29a, miR-20a, miR-24, and miR-25) were significantly increased in PaC-DM cases compared with either T2DM cases or controls (\( P < 0.01 \); Table 2). The fold change in serum levels for each miRNA in PaC-DM was at least 1.5 greater than in the two non-cancer groups (Table 3). However, the expression of the remaining six miRNAs did not differ significantly between the PaC-DM and T2DM cases or controls (Table S3). In addition, we assessed the expression of the six selected miRNAs in 30 age- and sex-matched patients with pancreatic cancer but without diabetes (Table S4). As indicated in Table S5, the expression of the six selected miRNAs was increased between 1.6- and 2.9-fold in pancreatic cancer patients compared with the healthy controls.

Figure 1 Diagram showing the research design strategy for identifying biomarkers of pancreatic cancer-associated new-onset diabetes mellitus (PaC-DM). T2DM, type 2 diabetes mellitus; TLDA, TaqMan low-density arrays; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

Table 2 Relative content of the six selected serum microRNAs in the three groups

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Training set</th>
<th>Validation set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls (n = 30)</td>
<td>T2DM (n = 30)</td>
</tr>
<tr>
<td>miR-483-5p</td>
<td>31.8 ± 5.9</td>
<td>57.6 ± 5.4</td>
</tr>
<tr>
<td>miR-19a</td>
<td>0.191 ± 0.025</td>
<td>0.188 ± 0.017</td>
</tr>
<tr>
<td>miR-29a</td>
<td>1.25 ± 0.14</td>
<td>1.56 ± 0.15</td>
</tr>
<tr>
<td>miR-20a</td>
<td>0.533 ± 0.042</td>
<td>0.603 ± 0.037</td>
</tr>
<tr>
<td>miR-24</td>
<td>0.476 ± 0.044</td>
<td>0.495 ± 0.055</td>
</tr>
<tr>
<td>miR-25</td>
<td>0.215 ± 0.040</td>
<td>0.159 ± 0.018</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM and normalized against let-7d/g/i.
PaC-DM, pancreatic cancer-associated new-onset diabetes mellitus; T2DM, type 2 diabetes mellitus.
We next evaluated changes in the serum levels of the six miRNAs in the validation set. As indicated in Tables 2 and 3, the expression of the six miRNAs was greater in the PaC-DM cases compared with the other two non-cancer groups (P < 0.01).

Diagnostic value of the six miRNAs for PaC-DM

Receiver operating characteristic curve analyses was used to determine the diagnostic value of the six validated miRNAs. The ROC curve analyses indicated that miR-483-5p, miR-19a, miR-29a, miR-20a, miR-24, and miR-25 could be used to discriminate patients with PaC-DM from healthy controls, with the AUCs of 0.766, 0.819, 0.789, 0.894, 0.847, and 0.875, respectively (Table S6). Using an AUC cut-off value of ≥0.750, the serum levels of the six miRNAs identified were able to distinguish patients with PaC-DM.

For further estimation of the diagnostic value of the six-miRNA panel, we calculated the risk score function for patient and control samples using a risk score formula, as described previously (see also Supplementary Methods). The frequency table and the AUC values were then applied to evaluate the diagnostic effect of the combined six-miRNA panel. The combination of these six miRNAs provided an increased AUC of 0.959 (95% CI 0.890–1.028) and 0.902 (95% CI 0.844–0.955) for distinguishing PaC-DM from healthy controls in the training and validation sets, respectively. The ROC curve revealed a high diagnostic accuracy, which was superior to most of the individual miRNAs (Fig. 3a,b; Table S6). Conversely, using a cut-off value of 35.0 U/mL CA 19-9 currently used in clinically, the ROC curve for CA 19-9 had a lower diagnostic accuracy (AUC 0.747; 95% CI 0.665–0.830) than the six-miRNA panel (Fig. S1).

Figure 2  Clustering analysis based on data from the TaqMan low-density arrays miRNA profile performed using pooled serum samples from pancreatic cancer-associated new-onset diabetes mellitus (PaC-DM) patients, patients with type 2 diabetes mellitus (T2DM), and healthy controls.

Table 3  Fold changes in the expression of the six selected serum microRNAs

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Training set</th>
<th>Validation set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PaC-DM vs control</td>
<td>PaC-DM vs T2DM</td>
</tr>
<tr>
<td>miR-483-5p</td>
<td>3.58</td>
<td>1.45 × 10⁻⁶</td>
</tr>
<tr>
<td>miR-19a</td>
<td>2.21</td>
<td>0.003</td>
</tr>
<tr>
<td>miR-29a</td>
<td>2.08</td>
<td>5.82 × 10⁻⁷</td>
</tr>
<tr>
<td>miR-20a</td>
<td>1.88</td>
<td>2.19 × 10⁻⁴</td>
</tr>
<tr>
<td>miR-24</td>
<td>1.65</td>
<td>0.003</td>
</tr>
<tr>
<td>miR-25</td>
<td>3.00</td>
<td>4.88 × 10⁻⁶</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM and normalized against let-7d/g/i.
PaC-DM, pancreatic cancer-associated new-onset diabetes mellitus; T2DM, type 2 diabetes mellitus.
Discrimination between PaC-DM and T2DM using the six miRNAs

As detailed in Table 2, all six miRNAs were found to be consistently upregulated in the PaC-DM compared with T2DM groups in both the training and validation sets. The relative content of individual miRNAs in PaC-DM patients was approximately twofold higher than in T2DM cases for almost all miRNAs ($P < 0.05$; Table 3). Notably, the six miRNAs identified (miR-483-5p, miR-19a, miR-29a, miR-20a, miR-24, and miR-25) also distinguished patients with PaC-DM from patients with non-cancer new-onset T2DM, with AUC values of 0.788, 0.863, 0.823, 0.819, 0.737, and 0.831, respectively (Table S6). Using the risk scoring procedure on the ROC curves, the combined six-miRNA panel provided an improved AUC of 0.885 (95% CI 0.784–0.986) and 0.887 (95% CI 0.823–0.952) for the separation of PaC-DM from T2DM in the training and validation sets, respectively (Fig. 3c,d). In addition, we examined the diagnostic value of the six miRNAs in cases of pancreatic cancer without diabetes compared with healthy controls in the training set (Fig. S2). Although the combination of the six miRNAs exhibited diagnostic capacity for pancreatic cancer without diabetes with an AUC of 0.841 (95% CI 0.771–0.923), the AUC values were lower than either for
Serum miRNA profile in PaC-DM

PaC-DM versus healthy controls or PaC-DM versus T2DM. Collectively, these results suggest the six miRNAs selected may represent a potential diagnostic tool for PaC-DM and be suitable for separating PaC-DM from non-cancer T2DM.

As expected, the ROC curve of the six-miRNA panel (miR-483-5p, miR-19a, miR-29a, miR-20a, miR-24, and miR-25) exhibited high diagnostic accuracy for distinguishing PaC-DM from both T2DM and healthy controls, with AUC values of 0.762, 0.831, 0.826, 0.761, 0.792 and 0.863, respectively (Table S7). When the six-miRNA panel used to separate PaC-DM cases from both T2DM and healthy controls, the AUCs were 0.917 (95% CI, 0.836–0.998) and 0.895 (95% CI, 0.845–0.944) in the training and validation sets, respectively (Fig. 3e,f; Table 3), which indicates the potential of these six miRNAs as a biomarker for PaC-DM diagnosis in the general population.

Logistic regression analysis of the six miRNAs

To further weigh the relevance of the selected six miRNAs to PaC-DM, forward stepwise binary logistic regression analysis was performed using PaC-DM status as the dependent variable and controlling for other variables. When cut-off values for miR-483-5p, miR-19a, miR-29a, miR-20a, miR-24, and miR-25 were 99.792, 0.353, 2.659, 0.880, 0.767, and 0.455, respectively, the following significant ORs were obtained for PaC-DM cases: miR-483-5p, OR 2.209 (95% CI 1.089–7.056; \(P = 0.001\)); miR-19a, OR 2.219 (95% CI 1.220–6.381; \(P = 0.002\)); miR-29a, OR 1.768 (95% CI 1.156–9.531; \(P = 0.022\)); miR-20a, OR 2.615 (95% CI 1.002–5.546; \(P < 0.001\)); miR-24, OR 1.996 (95% CI 1.204–7.631; \(P = 0.009\)); and miR-25, OR 2.605 (95% CI 1.102–6.250; \(P < 0.001\)). Furthermore, the combination six-miRNA panel had the highest OR for PaC-DM (2.903; 95% CI 1.056–8.145; \(P = 0.004\)) when the cut-off value for the panel was 2.103. These results suggest that the altered expression of the six miRNAs in combination is highly relevant to the existence of PaC-DM.

Discussion

Pancreatic cancer has a dismal prognosis, with a 5-year survival rate of <5%. Multiple factors contribute to the malignancy of pancreatic cancer, such as delayed manifestation of clinical symptoms, rapid deterioration after diagnosis, metastasis, and resistance to chemotherapy. In addition, the lack of sensitive and specific tools for detection and early diagnosis markedly contributes to the poor prognosis of this disease. Indeed, a considerable proportion of PaC-DM patients have no symptoms of pancreatic cancer except impaired glucose tolerance.\(^{29,30}\) The association between diabetes mellitus and pancreatic cancer has been of interest since the 1950s.\(^{31}\) In the population-based study of Chari et al.\(^{32}\) on 2122 patients age >50 years with new-onset diabetes, 0.85% of patients were diagnosed with pancreatic cancer within 3 years of the onset of diabetes (eightfold higher risk than expected for the population). Similar results were reported in another population-based study among veterans in the San Francisco area.\(^{33}\) Although the causal relationship between diabetes and cancer remains to be fully clarified, the potential risk of pancreatic cancer in association with new-onset diabetes (<3 years) is widely accepted.\(^{1,34,35}\) Thus, a novel approach to effectively distinguish PaC-DM patients, particularly from non-cancer new-onset T2DM, is required.

In the present study, the six-miRNA panel (miR-483-5p, miR-19a, miR-29a, miR-20a, miR-24, and miR-25) was systematically determined by means of TLDA analysis of sera from PaC-DM patients, non-cancer new-onset T2DM patients, and healthy controls, followed by individual qRT-PCR validation. The ROC curves and logistic regression analyses revealed a superior diagnostic value of the six-miRNA panel for PaC-DM.

Diabetes mellitus has been described as a human disorder associated with miRNA.\(^{36}\) The expression of miR-375, which is abundant in islet cells, is modulated by pancreatic and duodenal homeobox 1 (Pdx-1) and neurogenic differentiation 1 (NeuroD1), two transcription factors critical for endocrine pancreas development.\(^{37}\) In insulin-secreting cells, miR-375 negatively regulates glucose-stimulated insulin secretion,\(^{38}\) which may be attributed to the control of insulin gene expression by this miRNA.\(^{39}\) Other studies have shown that miR-124a mediates the expression of several components (e.g. synaptosomal-associated protein 25 [SNAP25], Ras-related protein Rab3A, synapsin-1A, forkhead box protein A2 [Foxa2], Ca\(^{2+}\)) that can directly or indirectly regulate insulin secretion and exocytosis.\(^{40,41}\) The emerging roles of a panel of miRNAs, namely miR-375, miR-7, miR-195, miR-126, miR-9, miR-96, and miR-34a, in the process of pancreatic development and insulin secretion, and miR-7, miR-139, miR-145, and miR-1 in insulin growth factor-1 receptor expression, have been reported.\(^{42}\) In the present study, the TLDA data revealed altered expression of serum miRNAs, including miR-7, miR-195, miR-29a, and miR-21, which is consistent with the results from previous studies. Interestingly, there was dysregulated serum miRNA expression (miR-181a, miR-205, miR-374, and miR-520c-3p) in non-cancer new-onset T2DM compared with the PaC-DM and healthy controls (data not shown), suggesting a unique pathogenic mechanism underlying the dysfunctional glucose metabolism.
metabolism caused by pancreatic cancer compared with common T2DM. It is possible that the function of β-cells in the pancreas may be affected by tumor-secreted products such as secreted miRNAs in exosomes or microvesicles. However, the role of the miRNAs identified herein in the pathogenesis of PaC-DM is not clear and requires further investigation; however, our findings provide some clues as to the potential mechanism underlying PaC-DM.

New-onset T2DM has been proposed to be one of the early symptoms of pancreatic cancer. It has been shown that the risk of pancreatic cancer in people with new-onset T2DM is eightfold higher than in the general population. In a clinical retrospective study from Shanghai Ruijin Hospital on 1123 patients with pancreatic cancer, new-onset T2DM was consistently associated with neural invasion, poor tumor differentiation, and shorter post-surgical survival, providing clinical evidence of the relationship between the two disorders in a Chinese population. The relationships among pancreatic cancer, diabetes mellitus, and miRNAs may be uncovered by multiple assays that demonstrated that the antidiabetic metformin effectively reduces the prevalence of pancreatic cancer in patients with diabetes. The mechanism underlying this phenomenon may be explained, in part, by the inhibition of pancreatic cancer stem cell (CSC) by metformin, which has been found to hamper the expression of the CSC markers CD44, epithelial cell adhesion molecule (EpCAM), enhancer of zeste homolog 2 (EZH2), Notch-1, and octamer-binding transcription factor 4 (Oct4). Metformin treatment subsequently led to an increase in the expression of certain miRNAs (including let-7a/b, miR-26a, miR-101, and miR-200b/c) that are frequently downregulated in tissues from pancreatic cancer. In addition, the expression of diabetes-associated pancreatic cancer pathway genes such as Insulin receptor substrate-1 (IRS), Foxa2, and AKT has been shown to be mediated by miR-128a, miR-21, and miR-29a/b/c, selected by qRT-PCR. Collectively, these studies strongly indicate that a non-invasive method to screen for early pancreatic cancer in the new-onset T2DM population is feasible and cost-effective.

As potent small molecule biomarkers, the differential expression of miRNAs in tissues or body fluids, including serum, plasma, and bile from pancreatic cancer, has been studied in the recent years. Elevated expression of miR-196a, miR-183, and miR-21 was found to predict poor survival of pancreatic cancer; miR-196a and miR-217 appear to be valuable in distinguishing normal pancreas from chronic pancreatitis and cancerous tissues; and miRNA-21, miR-10a, and miR-1246 mediate chemoresistance of multiple drugs and stemness in pancreatic cancer. Recently, miR-483-3p was observed to be significantly higher in the plasma of patients with pancreatic cancer and useful for discriminating pancreatic cancer from intraductal papillary mucinous neoplasm. In a previous study, a set of seven serum miRNAs (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, and miR-191) selected by qRT-PCR effectively discerned pancreatic cancer cases at different disease stages from controls (healthy controls and patients with chronic pancreatitis). Similarly, in the present study, the expression of miR-20a, miR-24, and miR-25 was upregulated in pancreatic cancer regardless of the presence of diabetes. These findings imply important roles for the three miRNAs in the development of pancreatic cancer, which may be independent of the new-onset diabetes associated with the tumor. Notably, miR-19a and miR-20a of the miRNAs identified in the present study belong to the first known oncomiRs (miR-17-92 cluster), which are overexpressed and critical to the onset and progression of solid tumors or hematological malignancies. In addition, miR-29a, one of the six miRNAs identified in the present study, has been shown to regulate proliferation, metastasis, and chemoresistance in pancreatic cancer cells. Some of these individual miRNAs have been reported to be significantly elevated in the serum or plasma of patients with other types of cancers. For example, miR-29a expression is high in colorectal cancer and is associated with TNM staging. Plasma miR-25 levels are considered as a novel biomarker not only for diagnosis, but also for monitoring of hepatocellular carcinoma and esophageal squamous cell carcinoma. In the present study, the ROC curves and logistic regression analyses indicated the advantage of the combination of the six miRNAs for the diagnosis of PaC-DM with the highest AUC and OR values compared with each individual miRNA. Together, the results suggest that a combination of several tumorigenesis-relevant miRNAs may be more suitable for the detection of PaC-DM and contribute to its differential diagnosis from other diseases.

In conclusion, we identified the first serum miRNA-based biomarker for the accurate discrimination of PaC-DM from non-cancer T2DM and the healthy population, and have provided a mechanistic insight into the pathogenesis and progression of PaC-DM.

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Disclosure
No potential conflicts of interest are declared.

References

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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Supplementary methods:** Risk score analysis.
**Figure S1** Diagnostic value of CA 19-9 for pancreatic cancer. (a) Receiver operating characteristic curve analysis for CA 19-9 and (b) distribution of CA 19-9 expression in 80 pancreatic cancer-associated new-onset diabetes mellitus.
**Figure S2** Receiver operating characteristic curve analysis of the six-miRNA panel to differentiate pancreatic cancer from healthy controls in the training set.
**Table S1** Patient characteristics and clinical features in the TaqMan Verso running title-density arrays.
**Table S2** Upregulated miRNAs in pooled serum samples from pancreatic cancer-associated new-onset diabetes mellitus and type 2 diabetes mellitus compared with healthy controls determined by TaqMan low-density arrays.
**Table S3** Relative content of some serum miRNAs determined by quantitative reverse transcription–polymerase chain reaction in the training set.
**Table S4** Characteristics and clinical features of patients with pancreatic cancer.
**Table S5** Relative content and fold changes of the six selected miRNAs in pancreatic cancer determined by quantitative reverse transcription–polymerase chain reaction in the training set.
**Table S6** Receiver operating characteristic curves and the corresponding areas under the curve of the six selected miRNAs for pancreatic cancer-associated new-onset diabetes mellitus, type 2 diabetes mellitus, and control groups in the validation set.
**Table S7** Receiver operating characteristic curves and the corresponding areas under the curve of the six selected miRNAs for discrimination of pancreatic cancer-associated new-onset diabetes mellitus from both type 2 diabetes mellitus and the control group in the validation set.