Combined serum CA19-9 and miR-27a-3p in peripheral blood mononuclear cells to diagnose pancreatic cancer

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**Accession number of repository for sequencing data:**

Our records have been approved and assigned GEO accession numbers as below.


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**Contributorship:**

Conception and design: Xiaolin Wang

Financial support: Xiaolin Wang

Administrative support: Xiaolin Wang

Provision of study materials or patients: Xiaolin Wang, Dayong Jin

Collection and assembly of data: Wansheng Wang, Lingxiao Liu, Guoping Li, Yi Chen, Changyu Li

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Abstract

MicroRNAs are potentially very useful biomarkers in the diagnosis of cancer. We sought to identify specific microRNAs in peripheral blood mononuclear cells (PBMCs) whose levels might facilitate diagnosis of pancreatic cancer (PC). We investigated PBMC microRNA expression in three independent cohorts (healthy, benign pancreatic/peripancreatic diseases [BPD], and PC), comprising a total of 352 participants. First, we used sequencing technology to identify differentially expressed microRNAs in PBMC of PC, BPD and healthy controls (n=20 in each group). Then the selected microRNAs were analyzed using the quantitative reverse-transcriptase polymerase chain reaction assays in the remaining 292 samples. The predictive value of the microRNAs was evaluated by logistic regression models and the receiver operating characteristic curve (AUC). We found that miR-27a-3p level in PBMCs could discriminate PC from BPD with a sensitivity of 82.2% and specificity of 76.7% (AUC=0.840; 95% CI, 0.787-0.885). Combination of PBMC miR-27a-3p and serum CA19-9 levels provided a higher diagnostic accuracy with a sensitivity of 85.3% and specificity of 81.6% (AUC=0.886; 95% CI, 0.837-0.923). The satisfactory diagnostic performance of the panel persisted regardless of disease status (AUCs for tumor-node-metastasis stages I, II, and III were 0.881, 0.884, and 0.893, respectively). PBMC miR-27a-3p level represents a potential marker for PC screening. A panel combining serum CA19-9 and PBMC miR-27a-3p level could have considerable clinical value in diagnosing
PC.
Introduction

Pancreatic cancer (PC) is a lethal malignancy with an overall 5-year survival rate of only approximately 5% (1). In 2008, PC was responsible for an estimated 268,800 deaths worldwide (2). The poor prognosis of this disease is partly due to late clinical presentation and the lack of effective early detection measures. As a result, only 15–20% of patients with PC are candidates for potentially curative treatments at the time of diagnosis (3,4). Therefore, it is important to identify new and more effective biomarkers for early detection of PC.

MicroRNAs are endogenous single-stranded RNA molecules that are 18–24 nucleotides in length (5). Mature microRNAs repress translation of mRNA into protein, and many microRNAs are highly conserved among species (6). Many studies have demonstrated that microRNAs play roles in the regulation of crucial biological processes, including cellular proliferation, development, differentiation, metabolism, apoptosis, and immunity (7-13).

Tumors are equipped with multiple mechanisms to evade early events in immunological surveillance by regulating their susceptibility to lysis (14). These mechanisms might involve modulation of microRNAs, which have significant impacts on the function of anti-tumor T cells (15). Based on this idea, it would be useful to study the role of microRNAs in peripheral blood mononuclear cells (PBMCs) in the context of diagnosis and prognosis of malignant tumors. Several studies have identified transcripts expressed differentially between
PBMCs from cancer patients and normal subjects, and some of these
eexpression changes appear to reflect specific immune responses of circulating
cells (16,17). However, few studies have been performed on PBMC
microRNAs, which could be of use as diagnostic biomarkers for PC.

Our study investigated PBMC microRNA expression profiles with
independent validation in a large cohort of 352 participants, with the purpose of
identifying microRNA markers for the diagnosis of PC. Healthy subjects,
patients with PC, and patients with benign pancreatic/peripancreatic diseases
(BPDs) were included in the cohort. The diagnostic performance of PBMC
microRNA levels was assessed and compared with the widely used marker
serum CA19-9.

Materials and Methods

Study Design and Patients

Blood samples from 352 patients who met the eligibility criteria
(Supplementary Table S1) were collected at Zhongshan Hospital between
January 2010 and January 2012. The samples were allocated to two
sequential phases (Fig. 1).

1. Discovery phase. Pre-operative PBMCs were collected from 20
patients with PC and 20 patients with BPD. As an additional control, PBMCs
were also collected from 20 healthy subjects. Patient characteristics are
presented in Supplementary Table S2. MicroRNA profiles were generated by
sequencing small RNAs from the three groups of samples. By comparing microRNA profiles between the PC and healthy groups, and between the PC and BPD groups, we established two differential microRNA expression patterns and subsequently compared them to each other. MicroRNAs that were significantly upregulated (fold change ≥2, normalized expression level of microRNA ≥100 in each sample) in both pairwise comparisons were chosen for further testing by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Subsequently, five of the microRNAs identified via sequencing were selected as candidates for further testing by qRT-PCR.

2. Validation phase. Five differentially expressed microRNAs identified via sequencing were first tested by qRT-PCR in an independent cohort of PBMC samples from 100 participants. Three microRNAs that were differentially expressed between the PC group and both control groups (healthy subjects and BPD patients) were further tested in an additional 192 participants.

In each study phase, blood samples were obtained from three categories of participants: healthy subjects, patients with BPD, and patients with PC. The investigation protocol was approved by local institutional review boards, and informed consent was obtained from all study participants. No patient received chemotherapy or radiotherapy before blood sampling.

**PBMC Preparation and RNA Isolation**

For preparation of PBMCs, peripheral blood (5 ml) was drawn into EDTA
tubes and transferred to the laboratory within 30 min for blood processing.

PBMCs were isolated using lymphocyte separation medium (Sigma-Aldrich, USA), following the manufacturer’s instructions.

For the PBMC samples, total RNA (covering all the small non-coding RNAs) was extracted using Trizol Reagent (Invitrogen, USA) according to the manufacturer’s instructions. The concentration was quantified using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA, USA). Each total RNA pellet was resuspended in 30 µl nuclease-free water and stored at -80°C.

**Sequencing and qRT-PCR**

First, to identify candidate microRNAs for use in diagnosing PC, we applied HiSeq 2000 technology (Illumina, CA, USA) to sequence small RNAs from 60 PBMC samples (see Supplementary Text S1 for details). For this sequencing procedure, we used pools of equal amounts (1 µg) of total RNA from 20 PC patients, 20 BPD patients, and 20 healthy subjects.

To validate the candidate microRNAs identified by sequencing, we performed qRT-PCR using Taqman microRNA assays (Applied Biosystems, Foster City, CA). The assays were first performed on 100 samples for five candidates (miR-27a-3p, miR-16-5p, miR-15b-5p, miR-26a-5p, and miR-342-3p) that met the aforementioned criteria for significant upregulation. The expression level of RNU6B snRNA was used as a stable endogenous control for purposes of normalization. All assays were carried out in triplicate.
ΔC_{T} was calculated by subtracting the average C_{T} value of RNU6B snRNA from the average C_{T} value of the microRNAs of interest. Relative expression levels were expressed as $2^{-\Delta C_{T}}$. Subsequently, the assays were performed on an additional 192 samples for three candidates (miR-27a-3p, miR-16-5p, and miR-15b-5p) that were significantly differentially expressed in the first validation.

**Statistical Analysis**

Differential expression of PBMC microRNAs, and differences in serum CA19-9 between PC and control groups (healthy and BPD) was analyzed using the Mann-Whitney test. The correlation between PBMC microRNA expression and serum CA19-9 in patients with PC and clinicopathological features was evaluated using the Mann-Whitney or Kruskal-Wallis test. A multivariate logistic regression model was used to select diagnostic microRNA markers based on the validation dataset of PBMC microRNA expression. PBMC miR-27a-3p and serum CA19-9 were analyzed using a logistic regression model to select significant predictors for PC based on the validation dataset of PC and BPD. Factors that are associated with the level of miR-27a-3p and CA19-9 (expressed as the natural logarithm of their relative expression values) were evaluated using a multiple linear regression model including PC, jaundice (cases with serum total bilirubin level >17.1 μmol/L) in the validating dataset (PC, healthy, and BPD groups). The area under the ROC curve (AUC) was used as an accuracy index to evaluate the diagnostic
performances of the individual markers and the panel. Methods used for sample-size estimation are presented in Supplementary Text S2. All P-values are two-sided, and P < 0.05 was considered statistically significant. MedCalc (version 10.4.7.0; MedCalc, Mariakerke, Belgium) software was used to perform the ROC and regression analysis. The predictive panel was validated using the open source package “pROC” for R and S-PLUS (18).

Results

Patient Characteristics

The characteristics of the study participants in the validation phase are presented in Table 1. Serum total bilirubin and CA19-9 of the study participants are also presented in Supplementary Table S3. A total of 352 participants were recruited, including 149 patients with PC, 80 healthy subjects, and 123 patients with BPD. The BPD group used for sequencing (n=20) included patients with chronic pancreatitis (n=11), serous cystadenoma (n=4), and pseudocyst (n=5). The BPD group used in the validation datasets (n=103) included patients with chronic pancreatitis (n=33), pseudocyst (n=15), autoimmune pancreatitis (n=4), serous cystadenoma (n=15), benign cyst (n=2), lymphoepithelial cyst (n=1), and biliary calculus disease (n=33). The PC group used for sequencing (n=20) comprised 20 patients with pancreatic ductal adenocarcinoma (PDAC). The PC group used in the validation datasets (n=129) included patients with PDAC (n=106), neuroendocrine carcinoma
(n=10), intraductal papillary mucinous carcinoma (n=6), solid pseudopapillary carcinoma (n=5), and acinar cell carcinoma (n=2).

**MicroRNA Screening**

First, HiSeq 2000 sequencing was performed to identify microRNAs that were significantly differentially expressed among the PC, healthy, and BPD groups. Supplementary Figure 1 illustrates the hierarchical clustering of the differentially expressed microRNAs in the three possible pairwise comparisons: PC vs. healthy, PC vs. BPD, and BPD vs. healthy. By comparing microRNA profiles between the PC and healthy groups and between the PC and BPD groups, we established two differential microRNA expression patterns that we then compared to each other. Five microRNAs, miR-27a-3p, miR-16-5p, miR-15b-5p, miR-26a-5p, and miR-342-3p, were significantly upregulated in the PC group relative to both the healthy and BPD groups (fold change ≥2, normalized expression level of microRNA ≥100 in each sample; Supplementary Table S4). We selected these five differentially expressed microRNAs as candidates for further testing via qRT-PCR.

**Independent Validation on PBMCs Samples**

The five microRNAs identified via sequencing were first tested by qRT-PCR in an independent cohort of PBMC samples from 100 participants (Supplementary Table S5). Three of the five microRNAs were significantly upregulated in the PC group (n=40) relative to both the healthy group (n=30) and the BPD group (n=30). Those three microRNAs were further tested in an
additional 192 participants. The combined set of 292 PBMC samples (including 129 PC patients, 103 BPD patients, and 60 healthy subjects) was used to evaluate the differential expression of the three microRNAs (Supplementary Tables S3). Significantly upregulated expression of miR-27a-3p, miR-16-5p, and miR-15b-5p was observed in patients with PC compared with subjects in both the healthy and BPD groups: average fold-change = 3.16, 2.30, and 2.40 for miR-27a-3p, miR-16-5p, and miR-15b-5p, respectively (Table 2; Fig. 2A-C). Thus we analyzed these three microRNAs using a multivariate logistic regression model. In this model, as shown in Table 2, only miR-27a-3p (P<0.001) effectively discriminated the PC group from the healthy and BPD groups. The corresponding AUCs were 0.857 (95% CI, 0.812–0.895; sensitivity = 82.2%; specificity = 79.1%).

The expression level of miR-27a-3p in PBMCs was comparable between strata by sex, age (≤60 or >60 years of age), levels of fasting blood glucose (≤17.1 umol) and CA19-9 (≤37 or >37 U/mL), or TNM stage. However, the level of miR-27a-3p expression in PC and BPD patients seem to be associated with elevated levels of total bilirubin (>17.1 umol/L) with the median (range) level of 85.48 (5.39-873.10) in patients with elevated serum total bilirubin versus 73.77 (3.05-337.79) in their counterparts (P=0.013, Mann-Whitney test). Furthermore, we also noticed a higher level of total bilirubin [13.1(3.5-385.5)] in patients with elevated level of CA19-9 versus those with normal level [11.1(3.5-298.9)]
Multiple linear regression analysis revealed that both PC (partial regression coefficient = 1.15, \( P<0.001 \)) and jaundice (partial regression coefficient = 0.59, \( P<0.001 \)) were predictors for PBMC miR-27a-3p expression in the validating dataset (comprising PC, healthy, and BPD groups).

We next assessed the performance of miR-27a-3p in differentiating the PC group from the BPD and healthy groups. This analysis demonstrated that miR-27a-3p was moderately accurate at discriminating PC from the BPD group (AUC = 0.840; 95% CI, 0.787–0.885; sensitivity = 82.2%; specificity = 76.7%) and the healthy group (AUC = 0.886; 95% CI, 0.831–0.927; sensitivity = 82.9%; specificity = 83.3%).

**Evaluating the diagnostic performance of serum CA19-9**

Discrimination between PC patients and healthy subjects does not reflect the putative performance of the diagnostic test in a clinical setting. In this context, BPD patients represent a more suitable control. Therefore, we evaluated the diagnostic accuracy of serum CA19-9 in differentiating between the PC and BPD groups. For the purposes of this evaluation, we used the dataset from 232 patients from the validation phase (129 PC and 103 BPD) (Fig. 2D). The resulting AUC was 0.788 (95% CI, 0.730–0.839; sensitivity = 72.9%; specificity = 75.7%).

Multiple linear regression analysis revealed that both PC (partial regression coefficient = 2.10; \( P<0.001 \)) and jaundice (partial regression...
coefficient = 1.11; P<0.001) were predictors for serum CA19-9 in the validating dataset (PC, healthy, and BPD groups).

Establishing the Predictive Panel in a Clinical Setting

There were significant differences in the distributions of gender, total bilirubin, and fasting blood glucose between PC and BPD groups (Pearson χ² test, p<0.05) in a clinical setting. Thus the five variables (including gender, total bilirubin, fasting blood glucose, miR-27a-3p and CA19-9) were analyzed in the validation phase by a multivariate logistic regression model. Only miR-27a-3p and CA19-9 were sufficiently effective in the model to discriminate PC from BPD group:

\[ \text{logit}(p = \text{PC}) = -2.4597 + 0.0239 \times \text{miR-27a-3p} + 0.0064 \times \text{CA19-9} \]

The diagnostic performance of the established panel was then evaluated using ROC analysis. The corresponding AUC was 0.886 (95% CI, 0.837–0.923; sensitivity= 85.3%; specificity=81.6%). The panel consisting of PBMC miR-27a-3p level and serum CA19-9 was significantly more effective than either individual marker alone at discriminating PC from BPD (vs. PBMC miR-27a-3p level: P=0.005; vs. serum CA19-9 level: P<0.001; Table 3). We validated the final predictive panel using 2000 stratified bootstrap replicates (18), and the resulting 95% CI of resampling AUCs was 0.840–0.928.

Next, we evaluated the diagnostic performance of the predictive panel at different TNM stages. The corresponding AUCs for patients of TNM stages I, II, and III were 0.881, 0.884, and 0.893, respectively.
**Discussion**

Lack of effective early detection measures is one of the most important factors contributing to the poor prognoses of PC patients. Serum CA19-9 has served for many years as a serum marker for PC diagnosis and screening. However, the major limitation of CA19-9 as a diagnosis marker for pancreatic cancer is lack of specificity because it is also elevated in BPD(3,4). Extensive efforts to identify a better serum or plasma marker have met with limited success.

Immune system evasion in cancer patients is partially due to functional repression of immune cells (14,15) and is initiated as early as the pre-malignant disease stage of PC (19). Several studies have shown that microRNAs play a crucial role in modulating adaptive or innate immune responses (20-23). Sasaki et al. (15) demonstrated that in the tumor microenvironment, which is skewed toward type-2 T cells, miR-17–92 expression in T cells is down-regulated; consequently, the persistence of tumor-specific T cells and the efficacy of tumor control are decreased. Furthermore, CD4+ T cells derived from miR-17–92 transgenic mice exhibit an enhanced type-1 phenotype associated with increased interferon-γ production and very late antigen (VLA)-4 expression. Baine et al. (17) reported the first in-depth comparison between global gene expression profiles of PBMCs from PC patients and healthy controls. Those authors identified a gene predictor set
that could potentially be further developed for use in diagnostic algorithms for PC. These discoveries regarding aberrant expression of both microRNAs in immune cells and mRNAs in PBMCs of cancer patients led us to evaluate the utility of PBMC microRNA levels in the diagnosis of PC.

For diagnosis of PC, the median sensitivity of serum CA19-9 is 79% (70–90%), and the median specificity is 82% (68–91%) (24), consistent with the diagnostic performance in our study. By contrast, a recent retrospective study showed that CA19-9 has poor clinical utility as a tumor marker for PC, with an AUC of only approximately 0.7 (25). This inconsistency might be related to case selection. Furthermore, elevation in serum CA19-9 is associated with hyperbilirubinemia irrespective of the presence of benign or malignant disease, compromising its diagnostic specificity (26). Our study also confirmed that hyperbilirubinemia is a predictor for elevation of serum CA19-9. Therefore, caution is warranted when interpreting the results in jaundiced patients. Marrelli et al. (27) demonstrated that serum CA19-9 decreases to normal levels when patients with benign pancreaticobiliary diseases are stented, but remains elevated in cases of malignancy. As with serum CA19-9, elevation of PBMC miR-27a-3p level was also related to hyperbilirubinemia in our study, potentially mitigating its diagnostic accuracy. However, owing to the complementary effect between the two biomarkers, the combination of serum CA19-9 and miR-27a-3p levels yielded higher accuracy in differentiating PC from BPD in a clinical setting, especially for patients at early stages (TNM
stage I). In addition, whereas serum CA19-9 has been reported (24) to correlate with tumor stage, we did not observe such a correlation in this study. This discrepancy could be attributed to the diversity of histological types of PC included in our cohorts.

Our study revealed that PBMC miR-27a-3p level was moderately accurate at differentiating PC from the control groups. In addition, its diagnostic performance was independent of disease staging; hence, it could serve as a potential diagnostic marker for PC at early stages. Previous reports of differential expression of miR-27a, both identified and verified, have primarily pertained to cancer cells (28–30). Liu et al. (28) showed that miR-27a is upregulated in human gastric adenocarcinoma, and that suppression of miR-27a inhibits gastric cancer cell growth. Their results supported the idea that miR-27a functions as an oncogene. Mertens-Talcott et al. (29) found that miR-27a exerts oncogenic activity by regulating ZBTB10, which results in overexpression of Sp proteins and Sp-dependent genes that are important for cell survival and angiogenesis. In the MCF-7 breast-cancer cell line, miR-27a indirectly regulates expression of estrogen receptor α, thereby influencing hormone responsiveness (30). Our study, however, is to our knowledge the first to report the importance of the miR-27a-3p expression profile in PBMCs from PC patients. Further functional study is needed to confirm the role of miR-27a-3p in PBMCs. In this study, although the expression levels of miR-16-5p and miR-15b-5p in the PC group were significantly higher than
those in the control groups, multivariate logistic regression analysis showed that these two microRNAs were not significant predictors for PC.

Our study has two unique advantages: First, we screened PBMC microRNAs using HiSeq 2000 sequencing technology, which enabled us to identify potential diagnostic markers with high accuracy. Moreover, our control group included not only healthy subjects, but also patients with BPD; as noted above, in a clinical setting, BPD patients represents a more suitable control group than healthy subjects. Our results indicate that our diagnostic panel could effectively differentiate PC from BPD.

Although our results are promising, there are several limitations in this study. First, we used pools of equal amounts of total RNA from 20 PC patients, 20 BPD patients, and 20 healthy subjects in the sequencing procedure, compromising the ability to identify differentially expressed microRNAs in PBMC. Moreover, PC group in this study included different types of pancreatic cancer. The potential marker miR-27a-3p we identified needs to be validated in a homogenous patient population with PDAC, which accounts for the vast majority of PC. In addition, the sample size is still small in present study, and we only performed internal validation of the algorithm using the open source package "pROC" for R and S-PLUS. Thus the panel identified in our study needs to be further validated using a large independent cohort.

In summary, our study demonstrates that the combination of serum CA19-9 and PBMC miR-27a-3p level can differentiate PC from BPD with a
higher degree of accuracy. Thus, this panel could have considerable clinical value for the diagnosis of PC.

REFERENCES


Table 1  Characteristics of study participants in the validation datasets

<table>
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<tr>
<th>Characteristic</th>
<th>PC (n=129)</th>
<th>BPD (n=103)</th>
<th>Healthy (n=60)</th>
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<tr>
<td><strong>Age</strong></td>
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<tr>
<td>&lt; 60 years</td>
<td>63 (48.8%)</td>
<td>59 (57.3%)</td>
<td>29 (48.3%)</td>
</tr>
<tr>
<td>≥60 years</td>
<td>66 (51.2%)</td>
<td>44 (42.7%)</td>
<td>31 (51.7%)</td>
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<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>78 (60.5%)</td>
<td>48 (46.6%)</td>
<td>31 (51.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>51 (39.5%)</td>
<td>55 (53.4%)</td>
<td>29 (48.3%)</td>
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<td><strong>TNM stage</strong></td>
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<tr>
<td>I A</td>
<td>10 (7.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I B</td>
<td>23 (17.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II A</td>
<td>23 (17.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II B</td>
<td>31 (24.0%)</td>
<td></td>
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<tr>
<td>III</td>
<td>32 (24.8%)</td>
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<tr>
<td>IV</td>
<td>10 (7.8%)</td>
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<tr>
<td><strong>CA19-9</strong></td>
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<tr>
<td>&lt; 37U/mL</td>
<td>32 (24.8%)</td>
<td>73 (70.9%)</td>
<td>60 (100%)</td>
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<tr>
<td>≥37U/mL</td>
<td>97 (75.2%)</td>
<td>30 (29.1%)</td>
<td>0 (0%)</td>
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<tr>
<td><strong>Total bilirubin</strong></td>
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<tr>
<td>&lt; 17.1 umol/L</td>
<td>92 (71.3%)</td>
<td>58 (56.3%)</td>
<td>60 (100%)</td>
</tr>
<tr>
<td>≥17.1 umol/L</td>
<td>37 (28.7%)</td>
<td>45 (43.7%)</td>
<td>0 (0%)</td>
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<td><strong>Fasting blood glucose</strong></td>
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<tr>
<td>&lt; 6.1 mmol/L</td>
<td>76 (58.9%)</td>
<td>83 (80.6%)</td>
<td>60 (100%)</td>
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<tr>
<td>≥6.1 mmol/L</td>
<td>53 (41.1%)</td>
<td>20 (19.4%)</td>
<td>0 (0%)</td>
</tr>
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</table>

Abbreviations: PC, pancreatic cancer; BPD, benign pancreatic/peripancreatic
diseases; TNM tumor-node-metastasis staging system (the International Union Against Cancer (UICC) tumor-node-metastasis classification in 2002).

Table 2  MicroRNA profile and diagnostic performance in the validating dataset of PC, Healthy group and BPD

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>PC Versus Healthy group and BPD</th>
<th>Univariate</th>
<th>Multivariate</th>
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<tr>
<td></td>
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<td>P*</td>
<td>Average Fold Change</td>
</tr>
<tr>
<td>miR-27a-3p</td>
<td>&lt; 0.001</td>
<td>3.16</td>
<td>0.857</td>
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<tr>
<td>miR-16-5p</td>
<td>&lt; 0.001</td>
<td>2.30</td>
<td>0.773</td>
</tr>
<tr>
<td>miR-15b-5p</td>
<td>&lt; 0.001</td>
<td>2.40</td>
<td>0.782</td>
</tr>
</tbody>
</table>

NOTE. Abbreviations: AUC, area under the receiver operating characteristic curve; PC, pancreatic cancer; BPD, benign pancreatic/peripancreatic diseases.

* Mann–Whitney test; P < 0.05, significant predictor for PC.
### Table 3  Diagnostic performance of PBMCs miR-27a-3p and serum CA 9-9 in the validating dataset of PC and BPD group

<table>
<thead>
<tr>
<th>Variable</th>
<th>PC Versus BPD</th>
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<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>AUC (95% CI)</td>
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<tr>
<td>miR-27a-3p</td>
<td>82.2%</td>
<td>76.7%</td>
<td>0.840 (0.787-0.885)</td>
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<tr>
<td>CA 19-9</td>
<td>72.9%</td>
<td>75.7%</td>
<td>0.788 (0.730-0.839)</td>
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</tr>
<tr>
<td>combination*</td>
<td>85.3%</td>
<td>81.6%</td>
<td>0.886 (0.837-0.923)</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** *Combination of PBMC miR-27a-3p and serum CA19-9.

† logit(p=PC) = - 2.4597 + 0.0239×miR-27a-3p + 0.0064×CA19-9.

**Abbreviations:** AUC, area under the receiver operating characteristic curve; PC, pancreatic cancer; BPD, benign pancreatic/peripancreatic diseases.

**Fig 1.** Study design. PC, pancreatic cancer; BPD, benign pancreatic...
/peripancreatic diseases; ROC, receiver operating characteristics; RT-PCR, reverse transcriptase polymerase chain reaction.

**Fig 2.** MicroRNA validation by quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. Box plots of expression levels of (A) miR-27a-3p, (B) miR-16-5p, (C) miR-15b-5p and (D) CA19-9 in healthy subjects (H) (n=60) and patients with pancreatic cancer (PC) (n=129) and benign pancreatic /peripancreatic diseases (BPD) (n=103). Expression levels of the microRNAs (log\(_{10}\) scale at y-axis) are normalised to RNU6B. The lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the interval between the 10th and 90th percentiles. Filled circles indicate data points outside the 10th and 90th percentiles. Statistically significant differences were determined using Mann–Whitney tests.
Discovery Phase (n=60)
- P C n=20
- BPD n=20
- Healthy n=20

Validation Phase (n=292)
- First validation (n=100)
  - P C n=40
  - BPD n=30
  - Healthy n=30
- Further validation (n=192)
  - P C n=89
  - BPD n=73
  - Healthy n=30

Hiseq2000 sequencing

Screening

Candidate microRNAs (n=5)

Quantitative RT-PCR

Significant microRNAs (n=3)

Logistic Regression (combined 292 samples including 129 PC, 103 BPD and 60 healthy subjects)

Diagnostic Performance (ROC analysis)

Effective predictor Mi-27a-3p

CA19-9

Logistic Regression on the dataset of PC and BPD group (232 samples) in the clinical setting

Diagnostic Performance (ROC analysis)
Fig 2

miR-27a-3p expression (normalized)

BPD

H

PC

P < 0.001

P < 0.001

P = 0.04
CA19-9

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<tr>
<th></th>
<th>BPD</th>
<th>H</th>
<th>PC</th>
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- P < 0.001
- P < 0.001
- P < 0.001
Combined serum CA19-9 and miR-27a-3p in peripheral blood mononuclear cells to diagnose pancreatic cancer

Wansheng Wang, lingxiao liu, Guoping Li, et al.

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