Identification of Circulating MiR-25 as a Potential Biomarker for Pancreatic Cancer Diagnosis

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Key Words
MiR-25 • Pancreatic cancer • Serum • Biomarker • Circulating microRNAs

Abstract

**Background:** The aim of this study was to identify novel microRNAs for potential use in the diagnosis of pancreatic cancer (PaC). **Methods:** A total of 1063 serum samples from 303 patients with PaC were collected, and the expression level of miR-25 was measured using quantitative real-time PCR (qRT-PCR). **Results:** We found that miR-25 had significant diagnostic value for the differential diagnosis of PaC in normal controls with an AUC (the area under the ROC curve) of 0.915 (95% CI: 0.893-0.937) that was significantly higher compared with an AUC of 0.725 for serum tumor marker carcinoembryonic antigen (CEA) and an AUC of 0.844 for CA19-9. **Conclusions:** These data suggest that serum miR-25 has strong potential as a novel biomarker for the early detection of PaC.

Introduction

Pancreatic cancer (PaC) is the fourth leading cause of cancer-related deaths with the poorest survival rate (less than 5%) among common malignancies [1, 2]. Early diagnosis of PaC is still difficult, and currently, no credible blood biomarkers exist to help identify patients with PaC at an early stage [3, 4]. Because of a lack of timely diagnosis, only approximately 20% of patients with PaC can undergo surgical treatment with curative intent, and the remaining cases have locally advanced or metastatic PaC at the time of diagnosis [3]. Therefore, it is
critical to identify specific biomarkers for the early diagnosis of PaC, especially for early-stage malignant tumors.

MicroRNAs (miRNAs) are single-stranded RNA molecules of approximately 22 nucleotides (nt) long comprising noncoding RNAs that function as repressors of gene expression, particularly as posttranscriptional repressors. MicroRNAs have been shown to play important roles in oncogenesis and tumor metastasis. Studies involving miRNAs have been expanded to various categories for malignant tumors, including PaC [5, 6], suggesting that miRNAs might be potential biomarkers for the diagnosis and prognosis of cancer. Diagnostic biomarkers from the blood could be valuable because it is difficult to obtain useful tissue biopsies from patients suspected of having PaC. Retrospective studies have shown that expression of specific miRNAs in the plasma or serum can distinguish patients with PaC from healthy controls [7-9].

miR-25 is one of the most studied and well-described miRNAs related to human disorders including cancer. It is transcribed from the minichromosome maintenance protein-7 (MCM7) gene and is 22 nt long. It is overexpressed in various human carcinomas including brain tumors, gastric adenocarcinoma, prostate carcinoma, and ovarian cancer [10, 11]. A previous study carried out by our group used a genome-wide miRNA expression profile in serum from 197 PaC patients and 158 cancer-free controls and indicated that miR-25 was overexpressed in PaC patients; therefore, it might be a potential biomarker for the diagnosis of PaC [9]. However, a group of miRNAs was screened in this study; however, it would be more convenient and less expensive if we could screen for one miRNA with the most potential. Furthermore, more clinical samples are needed to further characterize the role of miR-25 for the diagnosis of PaC. In the present study, PaC patients, controls with other diseases, and normal controls (cancer-free) were recruited to investigate whether miR-25 was potent enough to be a biomarker for the diagnosis of PaC.

 Patients and Methods

Patient selection

A total of 1063 serum samples from PaC patients or control participants admitted to three national hospitals were collected. The samples from PaC patients met the following criteria: a) PaC diagnosis confirmed by pathological examination; b) no acute infection; c) no jaundice; and d) alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels less than 2.5 times the upper limit of normal (ULN). This project was approved by the Clinical Research Ethics Committee of all three hospitals.

Sample processing and RNA extraction

All serum samples were recovered and kept at −80°C until analysis. The total RNA was extracted from 100 μl of serum through phenol/chloroform purification followed by centrifugation in isopropyl alcohol. Because U6 and 5S rRNAs were degraded in the serum samples and because there is no current consensus on housekeeping miRNAs for a qRT-PCR analysis of serum miRNAs, the expression levels of miRNAs were directly normalized to the serum volume in this study. Briefly, 2 μl of total RNA was reverse-transcribed to cDNA using an AMV reverse transcriptase (Takara, Dalian, China) and a stem-loop RT primer (Applied Biosystems, Foster City, CA, USA).

miRNA quantification by qRT-PCR

Hydrolysis probes (Applied Biosystems, Foster City, CA, USA) were used for the qRT-PCR analysis; this system is highly specific for the target miRNA, but not for longer pre-processed precursors or other highly homologous miRNAs, which differ in sequence by as little as one nucleotide [12, 13]. qRT-PCR was performed using a TaqMan PCR kit on an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All reactions were run in triplicate. After the reaction, the C values were determined based on fixed threshold settings. The experimenters were blinded to the identities of the cases and controls, and samples from cases and controls were mixed on the qRT-PCR plates to avoid batch effects.
Statistical analysis

Expression levels of serum miRNAs were compared using Student’s t-test. Receiver operator characteristic (ROC) curves were established to evaluate the diagnostic value of serum miRNAs for differentiating cancer patients from controls. A p value of less than 0.05 and Z value of more than 1.96 were considered significant. All statistical analyses were performed with SPSS 18.0 software (SPSS Inc, Chicago, IL). The classified points can be divided into four categories: true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN).

\[
\text{Sensitivity} = \frac{TP}{TP + FN} \\
\text{Specificity} = \frac{TN}{FP + TN} \\
\text{Positive predictive value} = \frac{TP}{TP + FP} \\
\text{Negative predictive value} = \frac{TN}{TN + FN}
\]

Results

Patient characteristics

Clinical characteristics of the study participants are presented in Table 1. A total of 600 samples were collected from normal controls (cancer-free), 160 samples from patients with other diseases (including 40 with chronic pancreatitis, 20 with gastric cancer, 20 with lung cancer, 20 with esophageal cancer, 20 with colorectal cancer, 20 with liver cancer, and 20 with breast cancer), and 303 samples from patients with PaC. For patients with PaC, the PaC was confirmed by a pathological diagnosis. Because of restrictions in obtaining samples from the participants, there was a significant difference in age between normal controls and PaC patients, as well as controls with other diseases and PaC patients (both \( p \) <0.0001).

CA19-9 and CEA for the diagnosis of PaC

The expression levels of carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) in PaC patients and those with other diseases were quantitatively measured and statistically compared (Table 2). CA19-9 was measured in 288 PaC patients with different stages of cancer, and 72.9\% of them were positi-

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal control</th>
<th>Other disease Control</th>
<th>PaC</th>
</tr>
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<tbody>
<tr>
<td>No. of cases</td>
<td>600</td>
<td>160</td>
<td>303</td>
</tr>
<tr>
<td>Age (mean±SD)</td>
<td>49±16</td>
<td>56±13</td>
<td>62±10</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>359</td>
<td>102</td>
<td>188</td>
</tr>
<tr>
<td>Female</td>
<td>241</td>
<td>58</td>
<td>115</td>
</tr>
<tr>
<td>Stage(TNM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
<td>9 (2.97%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>39 (12.87%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>47 (15.51%)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>123 (40.59%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>85 (28.05%)</td>
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Table 2. Positive ratio for known serum biomarkers CA19-9 and CEA. *: The cut-off values for CA19-9 and CEA were 39 kU/L and 5 μg/L, respectively

<table>
<thead>
<tr>
<th>Classification</th>
<th>CA19-9</th>
<th>CEA</th>
</tr>
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<tbody>
<tr>
<td>Stage I</td>
<td>6/9</td>
<td>66.67</td>
</tr>
<tr>
<td>II</td>
<td>27/39</td>
<td>69.23</td>
</tr>
<tr>
<td>III</td>
<td>31/46</td>
<td>67.39</td>
</tr>
<tr>
<td>IV</td>
<td>88/119</td>
<td>73.95</td>
</tr>
<tr>
<td>PaC case in total</td>
<td>210/288</td>
<td>72.92</td>
</tr>
<tr>
<td>Other diseases case</td>
<td>32/117</td>
<td>27.35</td>
</tr>
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</table>
ve (above the 39 kU/l-cutoff level), whereas 27.4% of the patients with other diseases were also positive for CA19-9. However, the positive percentage of CEA was only 47.7% with a high false-positive rate of 24.0% in 281 PaC patients and 125 controls. These results demonstrated a false-positive rate of <50% and 24% for CA19-9 and CEA, respectively.

Expression level of miR-25 in PaC patients

The serum miR-25 expression level was detected with qRT-PCR. Expression miRNA levels were expressed as copies/μl of serum. The cutoff value for miR-25 was 20,000 copies/μl. As shown in Figure 1, the median concentration of miR-25 was 8391.82, 10377.70, and 45659.24 copies/μl for normal controls, patients with other diseases, and PaC patients, respectively. These results revealed that miR-25 expression was significantly upregulated in PaC patients compared with that of normal controls and patients with other diseases (both \( p < 0.0001 \)) (Fig.1).

The distribution of miR-25 in PaC patients was also studied (Table 3). In the 303 PaC patients, 74 (24.42%), 150 (49.51%), and 79 (26.07%) of the cases were negative, weak positive, and strong positive, respectively, for miR-25. Overall, 229 (75.58%) of PaC patients were positive for miR-25; this percentage was comparable to that of CA19-9 (72.9%) but was significantly higher than that of CEA (47.7%) \( (p = 0.0001) \). The sensitivity was calculated as 75.58%, and the specificity was 93.03%. The positive predictive value was 81.21%, and the negative predictive value was 90.52%, indicating that miR-25 had a strong potential as a biomarker for the early diagnosis of PaC.

ROC curve of miR-25 in PaC patients

An ROC curve analysis illustrated the use of serum miR-25 in the differential diagnosis of PaC (Fig. 2). Significance was noted between CEA and CA19-9, but no overlap was noted among miR-25, CEA, and CA19-9 \( (p<0.05 \) and \( Z=1.96) \), as illustrated in Table 4. Serum miR-25 yielded an AUC of 0.915 (95% CI: 0.893-0.937). At a cutoff value of 20,000 copies/μl, the sensitivity and specificity of miR-25 were detected to be 75.58% and 93.03%, respectively. As shown

Table 3. Distribution of miR-25 in PaC patients (n=303). *Standards for classification: negative, \( \leq 20,000 \) copies/μl; weak positive, between 20,000 to 50,000 copies/μl; strong positive, \( \geq 50,000 \) copies/μl

| Classification* | No. of cases | proportion
<table>
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</thead>
<tbody>
<tr>
<td>Negative</td>
<td>74</td>
<td>24.42%</td>
</tr>
<tr>
<td>Weak positive</td>
<td>150</td>
<td>49.51%</td>
</tr>
<tr>
<td>Strong positive</td>
<td>79</td>
<td>26.07%</td>
</tr>
</tbody>
</table>

Table 4. Significant differences in the AUC between miR-25 and CEA/CA19-9

<table>
<thead>
<tr>
<th>Significant difference</th>
<th>miR-25 vs. CEA</th>
<th>miR-25 vs. CA19-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z</td>
<td>6.7304</td>
<td>3.5392</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.000001</td>
<td>0.000402</td>
</tr>
</tbody>
</table>
in Figure 2, the AUC was 0.725 (95% CI: 0.685-0.765) for CEA and 0.844 (95% CI: 0.811-0.876) for CA19-9 and was significantly lower as compared with an AUC of 0.915 (95% CI: 0.893-0.937) for serum miR-25.

Discussion

miRNAs have been proven to serve as potential diagnostic biomarkers and prognostic factors in different types of cancers [14-18]. Meanwhile, miRNAs play a vital role in the pathogenesis, progression, and metastasis of PaC [19-24]. Serum miRNAs have been suggested as a biomarker for the diagnosis of PaC. The differential expression levels of miRNAs have been associated with the survival of PaC patients [25, 26]. In the search of novel biomarkers for PaC, Wang et al. described a combined analysis of the expression levels of four plasma miRNAs including miR-21, miR-210, miR-155, and miR-196a that have been known to be overexpressed in PaC tissues and were used as biomarkers to diagnose and differentiate patients with pancreatic adenocarcinoma from healthy controls with a relatively high sensitivity of 64% and specificity of 89% [7]. Circulating miRNAs combined with the serum marker CA19-9 has been used in the clinical diagnosis of PaC [8]. Ho et al. statistically compared the expression levels of miR-210 in plasma samples from patients diagnosed with PaC and age-matched healthy controls [27]. Dai et al. [28] selected a panel of six serum miRNAs including miR-483-5p, miR-19a, miR-29a, miR-20a, miR-24, and miR-25, and a qRT-PCR analysis demonstrated that the six-serum miRNA panel acted as potential biomarkers for the accurate diagnosis and discrimination of PaC from healthy controls and non-cancer T2DM.

CA19-9, which is significantly upregulated in approximately 80% of patients diagnosed with PaC, has been regarded as a useful biomarker for the diagnosis of PaC [3]. Moreover, CA19-9 has been used to estimate the therapeutic efficacy and clinical prognosis of patients with PaC [29]. CEA is commonly used as a serum biomarker in colorectal and PaC as part of preoperative staging and postoperative follow-up for clinical response in patients selected for treatment [30]. Therefore, the serum levels of CA19-9 and CEA were quantitatively measured and statistically compared with the expression of miR-25 in this investigation.

In our previous investigation [9], researchers from our study group screened multiple miRNAs in 197 patients diagnosed with PaC and 158 cancer-free controls and demonstrated that miR-25 is one of the most significantly upregulated miRNAs in PaC patients. In this large sample size study, a total of 1063 participants including 303 patients with PaC were recruited that further confirm that miR-25 serves as a biomarker for the diagnosis of PaC with a relatively high sensitivity and specificity. In addition to the cancer-free normal controls, 160 patients with other diseases such as chronic pancreatitis, gastric cancer, lung cancer, esophagus cancer, colorectal cancer, liver cancer, and breast cancer were enrolled in equal numbers and were used to investigate whether miR-25 was specific to PaC or only elevated in cancer patients. The results unambiguously showed that miR-25 could be used to not only distinguish PaC from healthy individuals, but also differentially diagnose PaC from alternative diseases.

The ROC curve analysis also confirmed that serum miR-25 levels could help differentiate patients with PaC from healthy controls; help determine a PaC diagnosis, with an AUC of 0.915 (95% CI: 0.893-0.937); and were significantly higher, as compared with an AUC of 0.725 (95% CI: 0.685-0.765) for CEA and 0.844 (95% CI: 0.811-0.876) for CA19-9. In the current study, the sensitivity of miR-25 for the diagnosis of PaC was comparable to that of CA19-9 (73%) and significantly higher than CEA (48%). The false-positive rate for miR-25 was as low as 8% that was considerably lower than CA19-9 (27%) and CEA (24%). These results suggested that miR-25 is a better biomarker for the diagnosis of PaC with a higher sensitivity and specificity than well-known serum biomarkers.

Healthy individuals of differing ages were recruited for this investigation. An older age was consistently correlated with the incidence of PaC. Previous studies have demonstrated
no association between age and miRNA expression in both study cases and healthy controls [5]. However, other investigations have suggested that serum miRNA expression is likely to be affected by aging [31]. In this study, it was more specific and accurate to evaluate the role of miR-25 in normal controls of differing ages.

Conclusion

In this large-sample investigation, 303 patients with PaC and 760 controls were recruited from three national hospital centers to help guarantee the accuracy and objectivity of the study findings. The findings in this investigation have demonstrated that miR-25 could be considered a novel biomarker for the diagnosis of PaC with sensitivity and accuracy comparable to those of CA19-9 but higher than those of CEA.

Acknowledgements

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Disclosure Statement

None declared.

References