Malignant potential in pancreatic neoplasm; new insights provided by circulating miR-223 in plasma

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Malignant potential in pancreatic neoplasm; new insights provided by circulating miR-223 in plasma

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Kyoto Prefectural University of Medicine, Division of Digestive Surgery, Department of Surgery, Kyoto, Japan

Background: Recent studies have identified that microRNAs are stably detectable in plasma/serum because of their binding to specific proteins or being packaged in secretory vesicles.

Methods: We tested miR-223 as a candidate of novel plasma biomarker in pancreatic cancer (PCa) and intraductal papillary mucinous neoplasm (IPMN).

Results: i) miR-223 expression was significantly higher in PCa tissues (p = 0.0069) than in normal tissues. ii) Plasma miR-223 levels were significantly higher in 71 PCa patients than 67 healthy volunteers (p < 0.0001). iii) Plasma miR-223 levels were significantly reduced in postoperative samples (p = 0.0297). iv) Plasma miR-223 levels tended to discriminate the malignant potential between benign IPMN and malignant IPMN (p = 0.0963), and the progressive extent of invasiveness between malignant IPMN and pancreatic invasive ductal carcinoma (PIDC) (p = 0.0004). Multivariate logistic regression analysis revealed that a low level of plasma miR-223 was an independent risk factor for PIDC (p = 0.0012, odds ratio 7.90 [95% CI: 2.06 – 41.2]). v) There was no significant correlation between plasma miR-223 levels and the number of any blood cell types in the peripheral blood.

Conclusion: Plasma miR-223 might be a clinically useful biomarker for screening PCa, and predicting malignant potential of IPMN and the invasiveness of PCa.

Keywords: biomarker, intraductal papillary mucinous neoplasm, liquid biopsy, microRNA, pancreatic cancer, pancreatic invasive ductal carcinoma, plasma, prognosis

criteria for putative malignancy. To date, however, there is no highly sensitive biomarker to predict the malignancy in patients with branch duct-type IPMN. Serum CA19-9 levels have been demonstrated to be useful to predict the malignancy and the development of invasive ductal carcinoma in patients with IPMN [9,10]. Serum CA19-9 levels, however, lack sufficient sensitivity and specificity. Hence, the development of novel molecular biomarkers for pancreatic neoplasm using less invasive technology is necessary.

MicroRNAs (miRNAs), which are small non-coding RNAs, regulate the translation of specific protein-coding genes. Since their discovery in 1993 [11], miRNAs have been intensively studied in cancer research. Altered expressions of miRNAs are related to several diseases, and contribute to the development of various cancers [12-15]. In recent years, several studies demonstrated that miRNAs are detectable in plasma/serum and present in a remarkably stable form [13,16-19]. Tumor-derived miRNAs are resistant to endogenous ribonuclease activity in plasma/serum, because they bind to some proteins, such as the Argonaute 2 protein and high-density lipoproteins [20,21], or are packaged by some kind of secretory vesicles, including apoptotic bodies and exosomes in plasma/serum [16,22-24]. The expression level of each miRNA in serum is consistent in all healthy individuals [16,17]. Furthermore, secretory particles, which contain specific miRNAs, can function as intercellular transmitters. For example, secreted miRNAs from donor cells can be transferred to and function in recipient cells [25-27].

Concerning plasma/serum miRNAs for diagnosing malignant potential of IPMN, several research groups, including our own, reported the potential utility of only a few miRNAs that are circulating in plasma/serum in clinical application [28-30]. However, these miRNAs are not all candidates for diagnosing malignant potential of IPMN, and more sensitive and promising candidates could be found in clinical settings. In this study, we tested plasma miR-223 for diagnosing PCa and predicting malignant potential in IPMN. miR-223 was previously reported to be highly expressed in PCa tissues [31,32] and to have a potential oncogenic function in various cancers to target the tumor suppressor genes such as EPB41L3 [33], FBWX7 [34], NFI-A [35] and SEPT6 [36]. Our results provide the evidence that the plasma level of miR-223 contributes to detecting PCa and predicting malignant potential in IPMN and the invasiveness of PCa.

2. Materials and methods

2.1 Patients and samples

The study was approved by the Institutional Review Board of both Kyoto Prefectural University of Medicine and Kyoto Second Red Cross Hospital, and each subject provided signed informed consent. Between January 2010 and April 2013, a total of 71 plasma samples of PCa patients with curative pan-creatctectomy (R0) and 68 samples of healthy volunteers were collected (Table 1). In this cohort, none of the patients received preoperative chemotherapy and/or radiotherapy. Forty-six validation samples were from Kyoto Prefectural University of Medicine (1st cohort), and 25 validation samples were from Kyoto Second Red Cross Hospital (2nd cohort). Sixty-six samples from healthy volunteers consisted of 45 validation samples from Kyoto Prefectural University of Medicine (1st cohort), and 22 validation samples from Kyoto Second Red Cross Hospital (2nd cohort). These healthy volunteers included medical personnel and patients with benign disease. From the patients who underwent surgery, a total of six PCa specimens were collected as well as six normal tissue specimens from adjacent normal pancreatic tissues which were resected as a combined pancreatic resection for gastric cancer, in order to exclude any influence of atypical or precancerous status of pancreatic tissue on the analysis. Tumor stages were assessed according to the Union for International Cancer Control classification [37].

Peripheral blood (7 ml) was obtained from each patient at the time of diagnosis or before surgery, and from the healthy volunteers. The blood was transferred into sodium heparin tubes (BD Vacutainer, Franklin Lakes, NJ, USA) and immediately subjected to the three-spin protocol (1500 r.p.m. for 30 min, 3000 r.p.m. for 5 min, and 4500 r.p.m. for 5 min) to prevent contamination by cellular nucleic acids. Plasma was collected and then stored at -80°C until further processing. The resected specimens were fixed in formalin and embedded in paraffin for pathological diagnosis. Histological evaluation was performed for tissues adjacent to specimens, according to the criteria of the World Health Organization. In all cases, two pathologists agreed with pathological observations and confirmed the diagnosis.

2.2 RNA extraction

Total RNA was extracted from 400 µl of plasma using the mirVana PARIS Kit (Ambion, Austin, TX, USA) and finally eluted into 100 µl of preheated (95°C) Elution Solution according to the manufacturer’s protocol. The reason why the volume of 400 µl of plasma was used as the common denominator in each microarray analysis is that there was no definite internal control in plasma miRNA analyses as shown in our previous studies [28,29,38-43]. Total RNA was also extracted from four 15-µm thick slices of the formalin-fixed paraffin-embedded tissue (total 60 µm in thickness) using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion) and then eluted into 60 µl of Elution Solution according to the manufacturer’s protocol.

2.3 Quantification of miRNA by qRT-PCR

The amounts of miRNAs were quantified by qRT-PCR using the human TaqMan MicroRNA Assay Kit (Applied Biosytems, Foster City, CA, USA). The reverse transcription reaction was carried out with a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) in 5 µl of solution, containing 1.67 µl of extracted RNA, 0.05 µl of 100 mM dNTPs, 0.33 µl of Multiscribe Reverse Transcriptase
Circulating miR-223 as a novel clinical biomarker in pancreatic neoplasm

Table 1. Association between plasma miR-223 level and clinicopathological characteristics in patients with pancreatic cancer.

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>Plasma miR-223 concentration (atto M)</th>
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<tbody>
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<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Total</td>
<td>71</td>
<td>193.6</td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
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<tr>
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<td>202.4</td>
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<td>Female</td>
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<td>181.5</td>
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<tr>
<td>Age (year)</td>
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<td></td>
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<tr>
<td>&lt; 65</td>
<td>24</td>
<td>198.2</td>
</tr>
<tr>
<td>≥ 65</td>
<td>47</td>
<td>191.2</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant IPMN*</td>
<td>17</td>
<td>381.3</td>
</tr>
<tr>
<td>PIDC from IPMN§</td>
<td>11</td>
<td>98.5</td>
</tr>
<tr>
<td>PIDC §</td>
<td>43</td>
<td>143.7</td>
</tr>
<tr>
<td>Location</td>
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<td></td>
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<tr>
<td>Pancreatic head</td>
<td>51</td>
<td>225.9</td>
</tr>
<tr>
<td>Pancreatic body or tail</td>
<td>20</td>
<td>111.3</td>
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<tr>
<td>Size of tumor (mm)</td>
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<td></td>
</tr>
<tr>
<td>&lt; 3.0</td>
<td>27</td>
<td>169.0</td>
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<tr>
<td>≥ 3.0</td>
<td>44</td>
<td>208.6</td>
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<tr>
<td>T-stage (TNM)</td>
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</tr>
<tr>
<td>Tis/T1/T2</td>
<td>23</td>
<td>315.6</td>
</tr>
<tr>
<td>T3/T4</td>
<td>48</td>
<td>135.1</td>
</tr>
<tr>
<td>N-stage (TNM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>49</td>
<td>214.8</td>
</tr>
<tr>
<td>N1</td>
<td>22</td>
<td>146.3</td>
</tr>
</tbody>
</table>

Significant values are in bold.

*Mann-Whitney U-test.

IPMN*: Intraductal papillary mucinous neoplasm.

PIDC*: Pancreatic invasive ductal carcinoma.

3. Results

3.1 Study design to develop a novel biomarker of plasma miRNA

The study design is summarized in Figure 1. This study was divided into several parts: i) confirmation of higher miR-223 levels in primary PCa tissue than normal pancreatic tissues; ii) large-scale evaluation of plasma miR-223 concentrations using quantitative RT-PCR by comparing results from 71 patients with PCa and 67 volunteers; iii) evaluation of whether plasma miR-223 levels could be used to monitor tumor dynamics; iv) evaluation of whether plasma miR-223 levels could sensitively reflect the malignant potential of pancreatic neoplasm and v) evaluation of whether there was the correlation between plasma miR-223 levels and blood cells of peripheral blood.

3.2 Confirmation of higher miR-223 levels in primary PCa tissues than normal pancreatic tissues

To confirm previously reported high miR-223 expression levels in primary PCa tissues [31,32], we first examined the expression level of miR-223 in primary PCa tissues. We

(50 U/µl), 0.5 µl of 10× Reverse Transcription Buffer, 0.06 µl of RNase inhibitor (20 U/µl), 1 µl of gene-specific primer (has-miR-223, Assay ID: 002295 and RNU6B, Assay ID: 001093) and 1.39 µl of nuclease-free water. To synthesize cDNA, reaction mixtures were incubated at 16°C for 30 min, at 42°C for 30 min and at 85°C for 5 min, and then were held at 4°C. Next, 0.67 µl of cDNA was amplified using 5 µl of TaqMan 2 × Universal PCR Master Mix with no AmpErase UNG (Applied Biosystems), 0.5 µl of gene-specific primers/probe and 3.83 µl of nuclease-free water in a final volume of 10 µl. Quantitative PCR was run on a StepOne-Plus PCR system (Applied Biosystems), and reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold (Ct) values were calculated with StepOne Software v2.0 (Applied Biosystems).

The amounts of miRNAs in plasma were calculated on a standard curve constructed with the use of synthetic miRNAs, the mirVana miRNA Reference Panel (Ambion). Standard reference miRNAs were amplified for each reaction. The expression of miRNAs from tissue samples and cultured cells was normalized using the 2^ΔCt method relative to U6 small nuclear RNA (RNU6B). ΔCt was calculated by subtracting the Ct values of cel-miR-39 or RNU6B from those of the miRNAs of interest. Δ∆Ct was then calculated by subtracting the mean of ΔCt of plasma of healthy volunteer or normal pancreatic tissue from the ΔCt of PCa tissues. The change in gene expression was calculated with the equation 2^ΔΔCt

2.4 Statistical analysis

The Mann–Whitney U-test was used to compare unpaired data of continuous variables and the Kruskal–Wallis H-test was also used to compare more than two groups. The Chi-square test or Fisher’s exact probability test was used for categorical variables. Multivariate stepwise logistic regression analysis was performed to identify the independent risk factors associated with the invasiveness of PCa. The Wilcoxon test was used to compare the paired plasma samples obtained before and 1 month after pancreatectomy. Spearman’s correlation analysis was used to evaluate the correlation between plasma miR-223 levels and continuous variables. Receiver-operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to assess the feasibility of using plasma miRNA as a diagnostic tool for PCa and a prediction of the invasiveness of PCa. The Youden index was used to determine the cutoff value for the plasma miRNAs levels [46]. Survival curves were estimated using the Kaplan–Meier method, and statistical differences were examined using the log-rank test. A p-value < 0.05 was considered statistically significant.
used qRT-PCR to determine the expression of miR-223 in six PCa tissues and six normal pancreatic tissues. The miR-223 expression level was significantly higher in PCa tissues than in normal pancreatic tissues (p = 0.0069) (Figure 2A).

### 3.3 Large-scale analysis of the plasma level of miR-223 in PCa patients

We next validated our observations in large-scale settings. Before analyzing a large number of samples by qRT-PCR, the linearity of qRT-PCR was confirmed by various concentrations of 1 – 0.0001 fmol of each synthetic miRNA, namely miR-223 ($R^2 = 0.8198$) and cel-miR-39 ($R^2 = 0.9956$), between the logarithm of the amount of input miRNA and Ct values (Supplementary Figure S1). Plasma miR-223 was detectable in all samples from 71 PCa patients (mean; 193.5 atto mol/l, range; 8.9 – 1005.9) and 67 healthy volunteers (mean; 38.2 atto mol/l, range; 0.7 – 105.9). A waterfall plot demonstrated that the plasma level of miR-223 was significantly higher in the PCa patients than in the healthy volunteers (p < 0.0001) (Figure 2B). As a spike-in control for RNA samples [16], a synthetic RNA oligonucleotide, cel-miR-39 (Qiagen, Valencia, CA, USA), was also used for TaqMan qRT-PCR assays (Applied Biosystems).
normalized the data across samples using the $2^{-\Delta\Delta CT}$ method relative to cel-miR-39. These data showed almost same statistical result in comparison with the absolute quantification method (Supplementary Figure S2A and B).

Furthermore, to detect any cutoff points that could differentiate cancer patients from healthy volunteers, we utilized the AUC with the Youden index (Figure 2C) \cite{46}, and calculated that the value for the AUC was 0.8340. The optimal cutoff point was indicated at 85.0 atto M using plasma miR-223 with a sensitivity of 62.0%, a specificity of 94.1% and an accuracy of 77.7%. Our results provide evidence that the plasma level of miR-223 can be used to distinguish PCa patients from healthy volunteers.

3.4 Correlation between the plasma miR-223 levels and clinicopathological factors in PCa patients

We analyzed whether there was correlation between the plasma miR-223 levels and clinicopathological factors in PCa patients. In all 71 PCa patients, tumor location in pancreas body or tail ($p = 0.0003$), advanced T-stage ($p = 0.0029$), lymph node involvement ($p = 0.0489$) and invasive histological type ($p < 0.0001$) were significantly correlated with a low level of plasma miR-223 (Table 1). In concordance with these results, a low level of plasma miR-223 tended to be associated with a worse cause-specific survival rate of PCa patients with curative pancreatectomy ($p = 0.1478$). (E) The plasma level of miR-223 was significantly lower in PCa patients after surgery than before surgery ($p = 0.0297$).

**Figure 2.** The expression level of miR-223 in PCa tissues and plasma of PCa patients. (A) miR-223 expression was significantly higher in PCa tissues ($p = 0.0069$) than in normal tissues. (B) For a large-scale analysis, total RNA extracted from plasmas of 71 PCa patients and 67 age-matched healthy volunteers were used to analyze the expression level of miR-223 using qRT-PCR. Plasma miR-223 levels were significantly higher in PCa patients than in healthy volunteers ($p < 0.0001$). (C) Analysis of receiver-operating characteristic (ROC) curve to detect PCa patients. ROC analysis showed the greatest AUC of 0.8340 for miR-223. (D) Particularly in the 1st cohort, a low level of plasma miR-223 tended to be associated with a worse cause-specific survival rate of PCa patients with curative pancreatectomy ($p = 0.1478$). (E) The plasma level of miR-223 was significantly lower in PCa patients after surgery than before surgery ($p = 0.0297$).

PCa: Pancreatic cancer; ROC: Receiver-operating characteristic.
To validate whether the plasma miR-223 level reflects tumor dynamics during the treatment of PCa patients, we evaluated the plasma level of miR-223 in paired samples that were collected before and almost 1 month after surgery from 10 PCa patients who underwent curative pancreatectomy, and we observed that plasma miR-223 levels were significantly reduced in postoperative plasma samples \( p = 0.0297 \) (Figure 2E). Plasma miR-223 level of almost all healthy volunteers presented below 100 atto M (Figure 2B and Supplementary Figure S2). Therefore, PCa patients with preoperative plasma miR-223 level below 100 atto M could not present any change of plasma miR-223 level after surgery. PCa patients

**Figure 3. Plasma levels of miR-223 according to histological types of resected pancreatic tumor.** (A) Each plasma level of miR-223 in healthy volunteers, patients with benign IPMN, patients with malignant IPMN, patients with PIDC from IPMN and patients with PIDC. (B) Plasma miR-223 levels tended to be higher in malignant IPMN patients than in benign IPMN patients \( p = 0.0963 \). (C) There was no correlation between the tumor size and the plasma level of miR-223 by Spearman’s correlation analysis. (D) Plasma miR-223 levels were significantly lower in patients with pancreatic invasive ductal carcinoma (PIDC) than in those with malignant IPMN \( p = 0.0004 \). (E) A representation of the data using a ROC plot showed a strong separation between the malignant IPMN and PIDC, with an AUC of 0.7849. An optimal cutoff point was indicated at 125 atto M with a sensitivity of 82.4%, specificity of 62.9% and accuracy of 66.2% (continued).

IPMN: Intraductal papillary mucinous neoplasm; PIDC: Pancreatic invasive ductal carcinoma.
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Figure 3. Plasma levels of miR-223 according to histological types of resected pancreatic tumor. (A) Each plasma level of miR-223 in healthy volunteers, patients with benign IPMN, patients with malignant IPMN, patients with PIDC from IPMN and patients with PIDC. (B) Plasma miR-223 levels tended to be higher in malignant IPMN patients than in benign IPMN patients (p = 0.0963). (C) There was no correlation between the tumor size and the plasma level of miR-223 by Spearman’s correlation analysis. (D) Plasma miR-223 levels were significantly lower in patients with pancreatic invasive ductal carcinoma (PIDC) than in those with malignant IPMN (p = 0.0004). (E) A representation of the data using a ROC plot showed a strong separation between the malignant IPMN and PIDC, with an AUC of 0.7849. An optimal cutoff point was indicated at 125 atto M with a sensitivity of 82.4%, specificity of 62.9% and accuracy of 66.2%.

IPMN: Intraductal papillary mucinous neoplasm; PIDC: Pancreatic invasive ductal carcinoma.
with preoperative plasma miR-223 level > 100 atto mol/l presented the decrease of plasma miR-223 level after surgery.

3.6 Evaluation of whether plasma miR-223 level could sensitively predict the malignant potential of pancreatic tumor

Figure 3A shows each level of plasma miR-223 in healthy volunteers, patients with benign IPMN, patients with malignant IPMN, patients with PIDC from IPMN and patients with PIDC. Each plasma miR-223 level of pancreatic tumor was higher than that of healthy volunteers. However, plasma miR-223 levels tended to be extremely higher in patients with malignant IPMN than in patients with benign IPMN (p = 0.0988) (Figure 3B). Plasma level of miR-223 could discriminate the malignant potential between benign IPMN and malignant IPMN. Moreover, there was no correlation between the tumor size of IPMN and plasma level of miR-223 by Spearman’s correlation analysis (Figure 3C). These results indicated that plasma level of miR-223 facilitate to predict malignant potential of IPMN, independent of tumor size. Plasma level of miR-223 was significantly lower in patients with PIDC than in patients with malignant IPMN (p = 0.0004) (Figure 3D). Plasma miR-223 could significantly discriminate the progressive extent of invasiveness between malignant IPMN and PIDC. These results were also proved in two independent cohorts, respectively (Supplementary Figure S3).

In order to detect any cutoff points that could differentiate PIDC patients from malignant IPMN patients, we utilized the AUC with the Youden index [46] (Figure 3E), and calculated that the value for the AUC was 0.7849. The optimal cutoff point was indicated at 125.0 atto M using plasma miR-223 with a sensitivity of 82.4%, a specificity of 62.9% and an accuracy of 66.2% (Figure 3E). Multivariate logistic regression analyses for the progressive extent of invasiveness using this cutoff value and preoperative clinical factors revealed that a low level of plasma miR-223 was an independent risk factor for PIDC (p = 0.0018, odds ratio 7.90 [95% CI: 2.06 – 41.2]) (Table 2).

3.7 Evaluation of the correlation between plasma miR-223 levels and blood cells of peripheral blood

Recent reports indicated that some circulating miRNAs may be derived from peripheral blood cells [47]. In this study, no significant correlation was observed between the plasma level of miR-223 and the number of any types of peripheral blood cells in 47 consecutive PCa patients of the 1st cohort (Figure 4). Furthermore, we investigated the effect of plasma platelet on the plasma levels of miR-223 in six consecutive PCa patients, using previously described similar method with differential centrifugation and 0.22 µm filtration, in order to exclude the platelet (Figure 5A) [47]. As a result, there was a significant correlation of the plasma level of miR-223 between standard plasma and filtered plasma by Spearman’s analysis (p = 0.0006, R² = 0.95) (Figure 5B). Also in these six PCa cases, there was no correlation between platelet counts and plasma miR-223 levels (Figure 5C). These data strongly indicated that the plasma level of miR-223 was

### Table 2. Multivariate logistic regression analysis using preoperative clinical factors for the progressive extent of invasiveness in patients with pancreatic cancer.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Malignant IPMN (n = 17)</th>
<th>All PIDC (n = 54)</th>
<th>Univariate* p-value</th>
<th>OR 95% CI</th>
<th>Multivariate‡ p-value</th>
</tr>
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<td>Sex</td>
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</tr>
<tr>
<td>Male</td>
<td>11 (65%)</td>
<td>30 (56%)</td>
<td>0.3532</td>
<td>1.44</td>
<td>0.39 – 5.53</td>
</tr>
<tr>
<td>Female</td>
<td>6 (35%)</td>
<td>24 (44%)</td>
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<tr>
<td>Age</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>≥ 65</td>
<td>10 (59%)</td>
<td>37 (69%)</td>
<td>0.3244</td>
<td>1.46</td>
<td>0.40 – 5.19</td>
</tr>
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<td>&lt; 65</td>
<td>7 (41%)</td>
<td>17 (31%)</td>
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<td></td>
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<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pbt§</td>
<td>3 (18%)</td>
<td>16 (30%)</td>
<td>0.2606</td>
<td>1.21</td>
<td>0.22 – 7.34</td>
</tr>
<tr>
<td>Ph†</td>
<td>14 (82%)</td>
<td>38 (70%)</td>
<td></td>
<td></td>
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<tr>
<td>Tumor size</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>≥ 30 mm</td>
<td>11 (65%)</td>
<td>33 (61%)</td>
<td>0.5129</td>
<td>1.25</td>
<td>0.34 – 4.60</td>
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<tr>
<td>&lt; 30 mm</td>
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<td>21 (39%)</td>
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<td>Plasma miR-223</td>
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<td></td>
<td></td>
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<tr>
<td>≥ 125 atto M</td>
<td>14 (82%)</td>
<td>20 (37%)</td>
<td><strong>0.0012</strong></td>
<td>7.90</td>
<td>2.06 – 41.2</td>
</tr>
<tr>
<td>&lt; 125 atto M</td>
<td>3 (18%)</td>
<td>34 (63%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Significant values are in bold.

*Univariate analysis was assessed using Chi-square test and Fisher’s exact probability test.

‡Multivariable logistic regression was used.

§Pancreatic body or tail.

†Pancreatic head.

OR: Odds ratio; IPMN: Intraductal papillary mucinous neoplasm; PIDC: Pancreatic invasive ductal carcinoma.
not affected by the plasma platelet in preoperative PCa patients.

4. Discussion

PCa must be detected at an early stage with curative intent to improve survival rates because the only chance for cure of PCa is surgical resection with macroscopic tumor clearance. Moreover, if possible, patients with benign pancreatic disease must be diagnosed more accurately to avoid surgical impairments which decrease their quality of life and the operation-related death, because curative pancreatectomy for PCa has been one of the most aggressive and life-threatening surgeries among digestive cancers. To date, however, there has been no highly sensitive molecular biomarker to diagnose malignancy in pancreatic neoplasm such as IPMN. This prompted us to find more clinically useful miRNAs, which might facilitate better decision-making for pancreatic neoplasm.

In this study, we clearly demonstrated that miR-223, which is highly expressed in PCa tissues and reported to have an oncogenic function in various cancers, could be a novel molecular biomarker of plasma to detect PCa and predict the malignant potential of IPMN and the progressive extent of invasiveness in PCa. Specifically, plasma level of miR-223 was significantly higher in PCa patients than in healthy volunteers and tended to be higher in patients with malignant IPMN than in patients with benign IPMN (Figure 3B). However, contrary to our exceptions, plasma miR-223 level of patients with PIDC was significantly lower than that of patients with malignant IPMN (Figure 3D). Indeed, a low level of plasma miR-223 contributes to poor prognosis (Figure 2D) and was significantly associated with advanced T-stage, lymph node involvement and invasive histological type (Table 1). From the present results, therefore, miR-223 may function as an oncogenic factor in early stage of pancreatic carcinogenesis, and not mainly function as an invasive-promoting factor in tumor development of PCa. Detail molecular mechanisms through miR-223 may be highly complex and is currently unclear; nonetheless, these tendencies of plasma miR-223 levels were clearly demonstrated by two independent cohorts, respectively (Supplementary Figure S3).

Concerning the plasma level change of miR-223 from preoperative state to postoperative state in PCa patients, the
decrease of plasma miR-223 level in PCa patients after curative pancreatectomy indicate the tumor volume decrease. In this study, the mean plasma miR-223 levels of PCa patients and healthy volunteers were 193.5 atto mol/l (range; 8.9 – 1005.9) and 38.2 atto mol/l (range; 0.7 – 105.9), respectively. Therefore, we could clearly monitor that plasma miR-223 level in PCa patients was reduced to the level in healthy volunteers, although the difference of plasma miR-223 level between preoperative PCa patients and healthy volunteers was smaller than that between preoperative patients with malignant IPMN and healthy volunteers.

Recently, Pritchard et al. suggested a caution in a cancer biomarker study of blood-based miRNAs because some blood-based miRNAs might have been derived from peripheral blood cells [47]. Therefore, we also evaluated the correlation between plasma miR-223 levels and peripheral blood cells in consecutive PCa patients. As a result, no association was observed between plasma miR-223 levels and the number of any peripheral blood cells (Figure 4). These results might exclude the possibility that the secretion of miRNAs from blood cells or hemolysis affected the miR-223 level in clinical settings.

Furthermore, Pritchard et al. also reported that plasma level of miR-223 was specifically correlated with myeloid blood cell counts such as platelets and white blood cells [47]. Therefore, we examined the reason of the discrepancy between our group
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Taken together, we identified a plasma miR-223, as a novel biomarker, which could be a promising liquid biomarker for screening and monitoring PCa and evaluating the malignant potential of IPMN and the invasiveness of PCa. This is the first report to demonstrate the utility of plasma miR-223 level for decision-making in the treatments of IPMN and PCa. miR-223 is reported to be highly expressed in various cancer tissues and have an oncogenic function in various cancers. Although there may be the limitation to distinguish PCa and other types of cancer, plasma miR-223 might also be a useful biomarker for screening, predicting malignant potential and the invasiveness of other cancers. Moreover, we believe that more sensitive plasma miRNAs could be identified as biomarkers for evaluating malignant potential of pancreatic neoplasm using high-throughput technologies such as next-generation sequencing or digital PCR-based approaches. These strategies are currently under evaluation and we will report in the near future.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties.
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Supplementary material available online

Supplementary Figures S1–S3