

## Original Article

# Circulating serum microRNAs as diagnostic biomarkers for pseudomyxoma peritonei

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**Abstract:** For this study, microRNA (miR) expression profiles in 3 pooled pseudomyxoma peritonei (PMP) serum samples and 3 pooled healthy controls were analyzed with Aligent microarray chips. Three miRs (miR-423-5-p, miR-6728-5p, and miR-187-5-p) expressed statistically significant differences between the two groups and were chosen as candidates. qRT-PCR was then applied to validate expression of 3 candidate miRs in another 38 PMP and 43 healthy control samples, with miR-423-5p and miR-6728-5p confirmed as promising biomarkers for PMP patients. miR-423-5p was identified as an independent risk factor for diagnosis of PMP by logistic regression.

**Keywords:** Pseudomyxoma peritonei, microRNA, serum, qRT-PCR

## Introduction

Pseudomyxoma peritonei (PMP) is a rare and progressive disease in the peritoneum with an estimated incidence of about 2 out of every 10,000 laparotomies [1]. Five-year survival rate for PMP is only about 70% [2]. PMP originates most frequently from the appendix, but occasionally from ovaries, colorectum, gallbladder, stomach, urachus, lungs, and some other organs [3, 4]. Clinical symptoms are not typical for PMP patients and are closely related with disease progression and source. "Jelly belly" may present in some patients due to the production of mucinous. For other patients, abdominal distension and abdominal pain related to obstruction may be the main reasons for hospitalization. For female patients, pelvic pressure and ovarian masses may be the initial symptoms [5].

Due to atypical clinical manifestations, diagnosis of PMP can be difficult [6]. CT is the most widely used technology in diagnosis and preoperative planning of PMP [5]. High costs and ionizing radiation, however, have limited its application. Circulating tumor markers in the serum could reflect the extent of the disease. These have reportedly been used in screening and

early detection of cancer [6, 7]. CEA and CA19-9 are the two most frequently reported markers in PMP. They have been reported to correlate significantly with survival or described as predictors of PMP recurrence [8, 9]. Specificity of these two markers for disease has been poor. Serum CEA and CA19-9 could also be used to screen or predict recurrence in other diseases [10-12], implying that they are not suitable markers for screening or early detection of PMP. Therefore, discovery of new circulating markers for screening and early detection of PMP is an urgent matter.

MicroRNAs (miRs), short non-coding RNAs, could regulate gene expression and play important roles in various biological processes, such as cell development, differentiation, and proliferation [13]. It has been reported that more than 50% of miRs genes were located at cancer-associated genomic regions or fragile sites, implying that miRs may be involved in carcinogenesis as tumor suppressors or oncogenes [14]. Circulating miRs in serum could protect against degradation by RNase and be detected by qRT-PCR, holding great promise as novel noninvasive biomarkers for diagnosis and assessment of cancers [15, 16]. No relevant literature regarding expression of miRs in PMP

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**Table 1.** Characteristics of patients and controls in the study

Variables	Discovery stage (microarray)			Validation stage (qRT-PCR)		
	PMP (n=21)	Healthy control (n=21)	<i>p</i> value	PMP (n=38)	Healthy control (n=43)	<i>p</i> value
Age (year)			0.376			0.174
Mean ± SD	57±9.92	54.66±5.23		60.32±10.32	56.56±13.83	
Sex (%)			1.00			0.260
Male	57.14 (12)	57.14 (12)		55.26 (21)	67.44 (29)	
Female	42.86 (9)	42.86 (9)		44.74 (17)	32.56 (14)	
Source (%)						
Appendix	90.48 (19)			89.48 (34)		
Ovary	4.76 (1)			2.63 (1)		
Not clear	4.76 (1)			7.89 (3)		
Classification (%)						
Low grade	90.48 (19)			84.22 (32)		
High grade	4.76 (1)			7.89 (3)		
Not clear	4.76 (1)			7.89 (3)		

PMP = pseudomyxoma peritonei, qRT-PCR = quantitative reverse transcriptase polymerase chain reaction.

was available for retrieval. Therefore, the present study aimed to explore expression and the clinical significance of serum miRs in PMP.

Despite low incidence of PMP, the Special Treatment Center of PMP provided sufficient patient samples for research. In the discovery stage, microarrays were initially applied to screen expression levels of miRs in 3 pooled PMP serum samples and 3 pooled healthy control samples. Three candidates expressing statistically significant differences between the two groups were evaluated in the validation stage. Finally, miR-423-5p and miR-6728-5p were confirmed as promising biomarkers for screening and early detection of PMP, while miR-423-5p was verified as a risk factor for diagnosis of PMP.

## Material and methods

### Study design

This study was designed in two different stages, with no sample overlapping between stages.

First, 21 serum samples from PMP and 21 from controls were randomly chosen and pooled as 3 PMP samples and 3 control samples (7 samples were pooled as 1 pooled sample). MiR arrays were then used to discover candidates expressing statistically significant differences between 3 PMP pools and 3 control pools. Those miRs were chosen as candidates for fur-

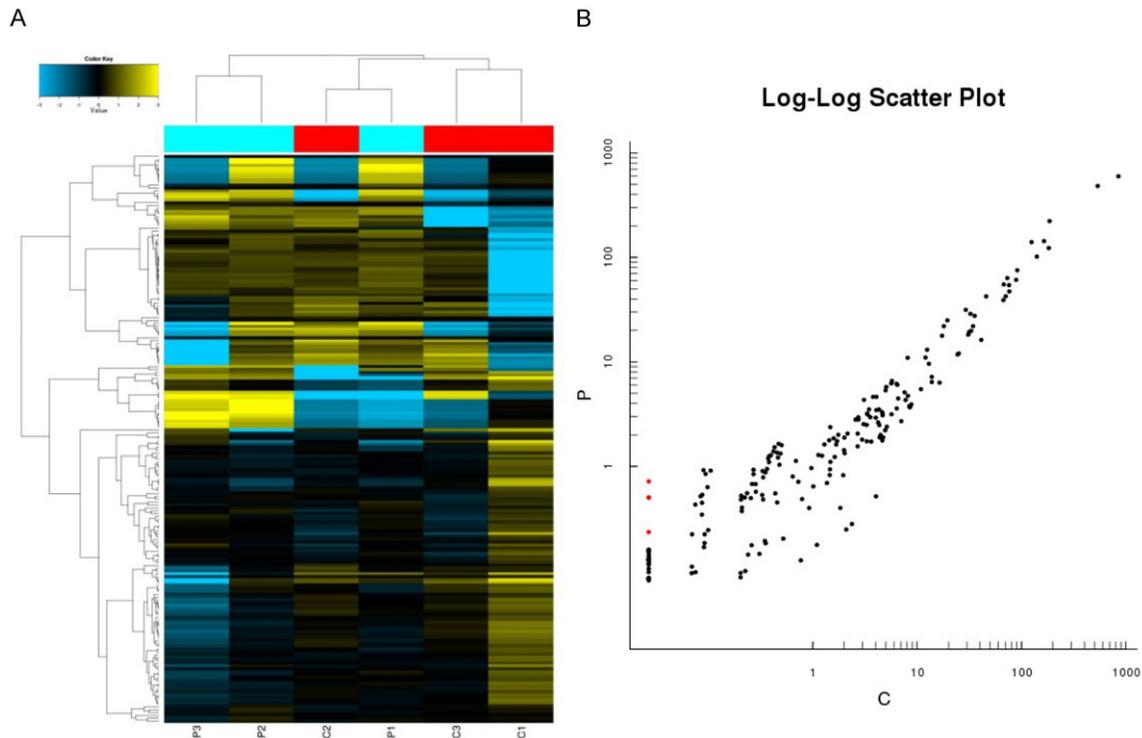
ther research, with *p* value < 0.05 and fold expression change > 2.0.

The three candidate miRs, discovered via microarray, were further validated with another cohort of 81 plasma samples with quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Logistic regression analysis was applied to identify risk factors for the disease.

### Patients and samples

This research was approved by the Ethics Committee of Aerospace Center Hospital. Written informed consent was obtained from all study participants. Blood samples from 59 PMP patients and 64 healthy controls were collected from November 2013 to July 2017. PMP patients were pathologically diagnosed for the first time and classified according to World Health Organization Classification of Tumors of the Digestive System [17]. They received no relevant treatment beforehand. Characteristics of these participants are shown in **Table 1**. There were no significant differences in distribution of age and sex between PMP and healthy control groups for both microarray and qRT-PCR (all *P* > 0.05). Sources and classification of PMP involved in both discovery and validation stages are also presented clearly in **Table 1**. Whole blood samples were collected and then centrifuged at 3000 g for 10 minutes, within 2 hours. Serum samples were then stored in Eppendorf tubes at -80°C until use.

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**Figure 1.** A. Hierarchical clustering of serum miR expression profiles on the microarrays. Hierarchical clustering was performed with 3 pooled PMP samples and 3 pooled healthy control samples. Clustering of 3 PMP pools placed 2/3 PMP pools in one group and 2/3 healthy pools in another group. Colored bars indicate the range of normalized log<sub>2</sub>-based signals. B. Expression difference about normalized log<sub>2</sub>-based signals of miRNAs was shown with scatter plot and 3 red spots represent 3 miRNAs upregulated in PMP pools. PMP: pseudomyxoma peritonei, P = PMP pools, C = control pools, miR = microRNA.

## MiR expression profile

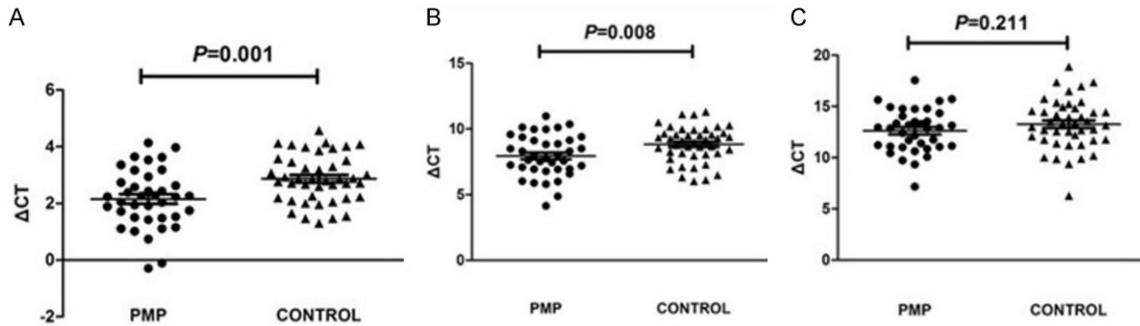
As described before, 3 pooled PMP samples and 3 pooled control samples were collected to examine miR expression profiles. MiR expression profiles were carried out with Agilent Human miR Microarray 21.0 (CapitalBio, China), according to manufacturer instructions. This array contained 2,549 human miRNAs. RNA was extracted using Qiagen miRNeasy Mini kit (Qiagen, Germany) and then fluorescein labeled for hybridization on miR microarray chips. Chips were then scanned using Agilent Microarray Scanner (CapitalBio, China). Images were analyzed using Agilent Feature Extraction software (CapitalBio, China). Filtered results were subject to cluster software (Cluster 3.0).

## RNA extraction and quantitative reverse transcriptase polymerase chain reaction

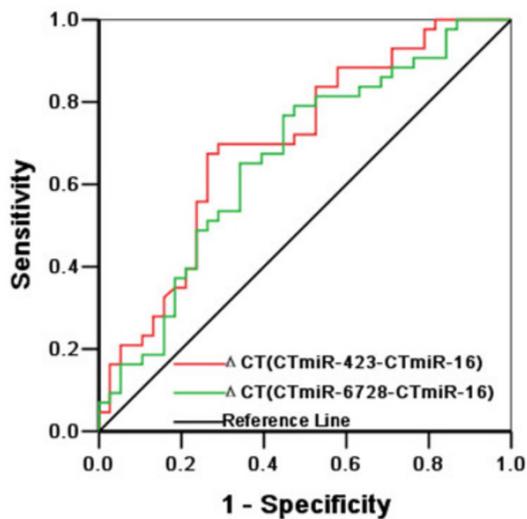
Thirty-eight PMP samples and 43 control samples were enrolled in the validation stage.

Initially, RNA was isolated from 200  $\mu$ L serum using miRNeasy Mini Kit (Qiagen, Germany), according to manufacturer protocol. Total RNA was eluted with 14  $\mu$ L of RNase-free water and stored at  $-80^{\circ}\text{C}$  for further use. Quantitation of candidate miRNAs was carried out by qRT-PCR using TaqMan MicroRNA Assays kit (Applied Biosystems, USA), according to the manufacturer's instructions. Next, 5  $\mu$ L of total RNA from each sample was reverse transcribed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, USA). qRT-PCR was carried out in final volumes of 20  $\mu$ L using ABI 7500 and software (Applied Biosystems, USA). The thermal cycling program used for quantification was as follows: 10 minutes of incubation at  $95^{\circ}\text{C}$ , followed by 40 cycles at  $95^{\circ}\text{C}$  for 15 seconds and  $60^{\circ}\text{C}$  for 60 seconds. miR-16 was used as internal control. qRT-PCR was performed in duplicate, including no-template controls. Relative expression of miR was calculated from the following equation: miRNA expression levels were calculated using the  $2^{-\Delta\text{Ct}}$  method,

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**Figure 2.** Expression levels of the 3 miRNAs in the serum of 38 PMP patients and 43 healthy controls. qRT-PCR was applied to detect expression of miR423-5p (A), miR-6728-5p (B), and miR-187-5p (C). PMP = pseudomyxoma peritonei, CONTROL = healthy control, miR = microRNA, qRT-PCR = quantitative reverse transcriptase polymerase chain reaction.



**Figure 3.** ROC curve analysis for PMP diagnostic capability of miR-423-5p and miR-6728-5p. miR = microRNA, ROC = receiver-operating characteristic.

$\Delta Ct = (Ct_{miRNA}) \text{ mean} - (Ct_{miR-16}) \text{ mean}$ , where Ct is the threshold cycle for a sample.

### Statistical analysis

Continuous data are presented as mean  $\pm$  standard deviation (SD). Categorical data are presented as counts and proportions. Chi-square test was used to calculate *p*-values for sex. Comparisons between groups were examined using unpaired Student's *t*-tests. Receiver operating characteristic (ROC) curve analysis was applied to assess the diagnostic accuracy of miRs. Area under the ROC curve (AUC) was used to evaluate the diagnostic value of candidate miRs. Logistic regression was performed

to identify risk factors of PMP.  $P < 0.05$  is considered statistically significant. SPSS (13.0) was used to perform all statistical analyses.

### Results

#### MicroRNA expression profiles

In the initial discovery stage, expression of 2,549 miRs was measured in 3 PMP pools and 3 healthy control pools with Aligent microarray. **Figure 1A** shows the hierarchical clustering of serum microRNA expression profiles on the microarrays and clustering of 3 PMP pools, placing 2/3 PMP pools in one group and 2/3 healthy pools in another group. Three differentially expressed miRs were identified by the platform as candidates (3 red spot in scatter plot as shown in **Figure 1B**). These include miR-187-5p ( $P=0.003$ , upregulated in PMP pools with fold change 18.78), miR-423-5p ( $P=0.009$ , upregulated in PMP pools with fold change 8.75), and miR-6728-5p ( $P=0.002$ , upregulated in PMP pools with fold change 26.71).

#### Evaluation of candidate miRs

Expression of the three candidate miRs was further evaluated in another 81, with 38 patient samples and 43 control individuals. As presented in **Figure 2**, high expression levels of miR-143-5p (A,  $P=0.001$ , with fold change 0.77) and miR-6728-5p (B,  $P=0.008$ , with fold change 1.11) in PMP patients were observed, with statistically significant differences. No statistical differences were observed concerning expression of miR-187-5p between the two groups (C),  $P=0.211$ .

Next, ROC was performed to evaluate the diagnostic value of miR-423-5p and miR-6728-5p. AUC of miR-423-5p was 0.701 ( $P=0.002$ ), while sensitivity and specificity were 67.4% and 73.7%, respectively. For miR-6728-5p, AUC was 0.659 ( $P=0.011$ ), while sensitivity and specificity were 76.7% and 55.3%, respectively (**Figure 3**).

Finally, logistic regression was applied to verify risk factors for diagnosis of PMP on the validation cohort (81 samples), with two significant miRs (miR-423-5p and miR-6728-5p). After adjusting for age and sex, miR-423-5p ( $\Delta Ct$  ( $Ct_{miR-423-5p}$  mean- $Ct_{miR-16}$  mean)) was confirmed as an independent risk factor for PMP ( $P=0.002$ ). Odds ratio was 0.430 (95% CI=0.250-0.739,  $P=0.002$ ), suggesting that higher expression levels of miR-423-5p (lower  $\Delta Ct$  ( $Ct_{miR-423-5p}$  mean- $Ct_{miR-16}$  mean) indicate higher likelihood of developing PMP.

### Discussion

PMP is a rare disease, 2-3 times more common in women than in men [5]. In the 4th edition of WHO Classification of Tumors of the Digestive System, PMP is classified as low grade or high grade based on histological criteria. This classification is correlated significantly with outcomes. A review of 274 cases of PMP revealed an overall 5-year survival of 63% for low-grade mucinous adenocarcinoma, compared with 23% for high-grade mucinous adenocarcinoma [18]. Atypical manifestations of PMP make it difficult to detect this disease exactly. Biomarkers with high sensitivity and specificity would be helpful.

To the best of our knowledge, this is the first comprehensive study regarding expression and the clinical significance of serum levels of miRs in PMP. This study screened expression of 2,549 miRs in 3 pooled PMP patient samples and 3 pooled healthy controls with Aligent microarrays. Three miRs (miR-187-5p, miR-423-5p, and miR-6728-5p) expressing statistically significant differences between the two groups with a fold change > 2.0 were chosen as candidates. All three miRs were upregulated in PMP pools and fold changes were 18.78, 8.75, and 26.71 for miR-187-5p, miR-423-5p, and miR-6728-5p, respectively. They were then validated with qRT-PCR in 38 PMP samples and 43

healthy controls, with miR-16 chosen as an internal control. Results showed that expression levels of miR-423-5p and miR-6728-5p were statistically different between the two groups. No statistical differences were observed in miR-187-5p, suggesting that miR-423-5p and miR-6728-5p may be promising biomarkers for screening or early detection of PMP. For miR-423-5p,  $\Delta\Delta Ct$  was  $2.06\pm 1.09$  in the PMP group and  $2.83\pm 0.88$  in the control,  $P=0.001$ . For miR-6728-5p,  $\Delta\Delta Ct$  was  $7.57\pm 2.27$  in PMP group and  $8.67\pm 1.73$  in control,  $P=0.008$ . Diagnostic value of miR-423-5p and miR-6728-5p was evaluated by ROC. AUC were 0.701 and 0.659, respectively. Sensitivity and specificity were 67.4% and 73.7% for miR-423-5p. For miR-6728-5p, they were 76.7% and 55.3%, respectively. miR-423-5p was then confirmed as a risk factor for diagnosis of PMP on the validation cohort (81 samples) with logistic regression. Odds ratio was 0.430 (95% CI: 0.250-0.739,  $P=0.002$ ), implying that higher expression levels of miR-423-5p (lower  $\Delta Ct$  ( $Ct_{miR-423-5p}$  mean- $Ct_{miR-16}$  mean) indicate greater likelihood of developing PMP.

miR-6728-5p was discovered by Erik Ladewig [19]. No literature about its expression in any disease is currently available. Therefore, this study on miR-6728-5p in PMP is perhaps the first one, although the mechanisms remain unclear. miR-423-5p has been a research hotspot. It has been reported to be a promising biomarker for colorectal carcinoma [20, 21], bladder cancer [22], heart failure [23, 24], and some other diseases, but mechanisms remain unclear. MiRs are highly conserved noncoding RNAs that negatively regulate gene expression post-transcriptionally by binding to the 3'-untranslated region of target mRNAs, resulting in either mRNA degradation or translational repression [25]. Target genes of miR-6728-5p and miR-423-5p will be targets of research in the future.

In conclusion, this study was a multistep investigation to identify serum miRs in PMP detection. miR-423-5p and miR-6728-5p were confirmed as promising biomarkers, while miR-423-5p was confirmed as a risk factor for diagnosis of PMP. Further studies focusing on larger sample validation and mechanisms of miR-423-5p and miR-6728-5p are required.

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## Disclosure of conflict of interest

None.

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