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Original Article

Aberrant expression of microRNAs in serum may identify individuals with pancreatic cancer

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Abstract: Pancreatic cancer (PC) has the poorest survival rate among all types of human cancer due to the lack of sensitive and non-invasive diagnostic screen methods for PC screening. Our aim was to identify novel serum microRNA (miRNA) biomarkers for the early detection of PC. We used microarray to screen differential expression of miRNAs in two pooled serum samples (6 PC patients and 6 healthy controls). A panel of miRNAs (22 over-expression and 23 decreased) were deregulated in serum of PC patients in comparison to controls. The expressions of 8 selected miRNAs were further evaluated in sera from 49 PC patients and 27 controls using quantitative reverse transcription-polymerase chain reaction. The levels of serum miR-492 and miR-663a were significantly decreased in PC patients compared with controls ($P < 0.05$). ROC curve analysis showed that serum miR-492 and miR-663a yield an AUC of 0.787 with 75.5% sensitivity and 70.0% specificity and 0.870 with 85.7% sensitivity and 80.0% specificity, respectively, for discriminating between PC patients and healthy controls. In addition, the level of miR-663a was significantly and inversely associated with TNM stage ($P = 0.027$). These results suggested that serum miR-492 and miR-663a could have strong potential as novel non-invasive biomarkers for the early detection of PC.

Keywords: MicroRNA, pancreatic cancer, miR-492, miR-663a, serum

Introduction

Pancreatic cancer (PC), eighty-five percent of which arises from the epithelial lining of the exocrine pancreatic duct [1], is the fourth leading cause of cancer-related death worldwide [2]. Due to its asymptomatic onset, only ~20% of PC patients were considered eligible for surgery and only about a half undergoes successful resection when diagnosed [3]. Therefore, the annual incidence and mortality rate of PC are almost identical, which simultaneously leads to its lowest survival rate for any solid cancers [3-7]. So far, the only chance to acquire the relatively long-term survival in PC patients is to be diagnosed in the relatively earlier stage, when a potentially curative resection can be performed. Unfortunately, there are still no clinical screening algorithms used in pancreatic cancer, such as colonoscopy examining in colorectal cancer, mammographic screening in breast cancer and even low dose spiral computed tomography (LD-CT) used in lung cancer, which could increase the early detection and then reduce the mortality [8-12]. Although helical CT, Magnetic Resonance Imaging (MRI) and the endoscopic ultrasonography (EUS)-guided fine needle aspiration (FNA) are the most reliable imaging methods for the early detection of PC to date, the invasive and costly nature, as well as low accuracy tends to restrict their use. In addition, most PC patients have no indication for the disease and are not candidates for screening. Although numerous tumor-specific molecular alterations, such as CA19-9, K-ras, P53 and mucin, have shown the potential as biomarkers for PC [1, 12-14], no biomarker thus far have sufficiently high discriminating power for the diagnosis of PC. Therefore, there is a pressing need for the development of novel screening tools and new minimally invasive diagnostic biomarkers to complement and

Table 1. Demographic and clinical characteristics of the PCs and healthy individuals

		PCs (n = 49)	Controls (n = 27)
Age (year, Mean ± SD)		61.7 ± 10.7	60.8 ± 10.2
Gender	Male	27	15
	Female	22	12
Histological grades	high	11	
	moderate	17	
	low	21	
Stages	I	6	
	II	14	
	III	15	
	IV	14	

improve on current strategies for the early detection of PC.

MiRNAs are a class of 19-25 nt non-coding RNAs that negatively regulate the expression of more than 30% of human mRNAs containing respective target sequences [15]. Altered expressions of miRNAs have been found to be associated with many diseases including cancer. Furthermore, cancer-specific miRNA profiles have been found in various types of cancer, and thus could be considered as potential cancer biomarkers [16, 17]. Unlike messenger RNAs (mRNAs), which are vulnerable and easily degraded by ribonucleases, miRNAs have been found not only in tissues but also in serum, plasma and other body fluids in a stable form [18]. The alteration of circulating miRNAs may reflect physiological or pathological changes in cancer cells, and may also be used as crucial surrogate biomarkers [19, 20]. Additionally, blood is an easily accessible and rich body fluid. Therefore, developing minimally-invasive, relatively low-cost and easily repeatable techniques with respect to circulating miRNAs is becoming more and more attractive clinically and may offer new revenue as next generation of biomarkers.

Now rare reports focused on circulating miRNA associated with Chinese patients with PC have been found. In this study, we reported that the levels of serum miR-492 and miR-663a could have the potential as biomarkers for the early detection of PC by using genome-wide microarray-based expression profiling and relatively large scale validation using quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Materials and methods

Study population

A total of 49 patients with pathologically confirmed diagnosis of pancreatic cancer were recruited between 2011 and 2013. Furthermore, serum samples from 27 age- and sex-matched healthy individuals without cancer history were also recruited. In the first phase, serum pools from 6 PCs and 6 normal controls were used to generate circulating miRNAs profile through microarray analysis. In considering our aim of finding novel serum biomarkers, then in the second phase, 8 deregulated and relatively seldom reported miRNAs were selected for further validation analysis in the total 49 cases and 27 controls by qRT-PCR. The participants population characteristics with respect to age, sex, histological grades as well as stages of disease, were described in **Table 1**.

All blood samples were collected prior to any therapeutic procedures, including surgery. Ten milliliters of venous blood were collected from each participant at his/her first admission to the hospitals. To harvest cell-free serum, the blood was drawn into a sterile tube without anticoagulant. After leaving the tube in standing position for 30-40 min, samples were centrifuges at 20°C 1500 g for 10 min, and the supernatant serum was quickly removed and stored as multiple aliquots in fresh tubes immediately at -80°C until use. The pilot study was approved by Institutional Research Board (IRB) of Taizhou People's Hospital, and all written confirmed consents were obtained from the participants.

RNA isolation

Total RNA was extracted from 100 μL serum by using miRNeasy Kit (Qiagen, Germany) according to the protocol of the miRNeasy Serum/Plasma Handbook. The RNA concentration and purity were checked by OD260/OD280 (≥ 1.6) and OD260/OD230 (≥ 1.0), and the RNA yield and quality were accessed (RIN ≥ 5.0) using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For miRNA profiling, serums of 6 patients were pooled together along with 6 controls (normal subjects) into two

Table 2. miRNAs differentially expressed in PC tissues versus the normal controls by microarray

miRNAs	log2 (Ratio)	*P*-value (Differentially expressed)
Up		
miR-1238	1.292379	0.003578
miR-1260a	0.863487	0.00038
miR-1228-3p	1.244691	0.000399
miR-1228-5p	0.847836	0.013527
miR-1273f	0.710763	0.000247
miR-197-3p	0.596422	0.009921
miR-2392	1.322312	0.000046
miR-296-3p	0.693487	0.000812
miR-3656	1.379091	0.005216
miR-3665	0.96061	0.003321
miR-3622a-3p	0.827692	0.000068
miR-4290	0.771779	0.005266
miR-4463	0.985322	0.001412
miR-4507	1.03733	0.004281
miR-4656	1.378242	0.004189
miR-4685-3p	1.351687	0.000978
miR-4763-5p	1.446675	0.001509
miR-483-5p	0.636053	0.002465
miR-532-3p	0.704699	0.000315
miR-634	1.110041	0.008287
miR-762	1.375178	0.001302
miR-765	0.911852	0.002048
Down		
miR-1224-3p	-1.0475	0.000213
miR-1228-3p	-1.244691	0.000399
miR-1237	-1.143345	0.00002
miR-1280	-1.041495	0.001724
miR-1469	-0.772993	0.000634
miR-1470	-1.183234	0.000189
miR-3162-3p	-1.233538	0.000001
miR-3178	-1.283955	0.000445
miR-3179	-0.81245	0.000003
miR-3198	-0.709559	0.007617
miR-4258	-1.195613	0.000023
miR-4294	-0.743065	0.001106
miR-4443	-0.94938	0.000091
miR-4455	-0.844675	0.000018
miR-4497	-0.901158	0.001526
miR-466	-0.908588	0.000043
miR-492	-1.04923	0.000042
miR-574-3p	-1.044789	0.000029
miR-590-3p	-1.054462	0.010108
miR-595	-0.882519	0.001009
miR-637	-0.836802	0.000017
miR-638	-0.877963	0.000245
miR-663a	-0.731414	0.000033

separate groups to select miRNAs whose expression were differentially expressed in PC patients compared to the normal control. For normalization of sample-to-sample variation during RNA isolation and as internal control, same amounts of synthetic C. elegans miRNA-39 (cel-miR-39) were added into each serum sample for miRNA profiling and qRT-PCR analysis as described by Mitchell et al [18].

MiRNA profiling

Small RNA was pre-enriched by Nanoseplook (Pall Corporation, USA) from 2.5 μg total RNA samples and labeled with miRNA ULSTM Labeling Kit (Kreatech Diagnostics, The Netherlands). Labeled miRNA targets were hybridized to the Human miRNA OneArray® v3 with OneArray® Hybridization System. After 16 hours hybridization at 37°C, non-specific binding targets were washed away by three different washing steps (Wash I 37°C 5 min; Wash II 37°C, 5 min 25°C 5 min; Wash III rinse 20 times), and the slides were dried by centrifugation and scanned by an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). The Cy5 fluorescent intensities of each probe were analyzed by GenePix 4.1 software (Molecular Devices, Sunnyvale, CA, USA). The raw intensity of each probe was processed by R program. Probes that passed the criteria were normalized by 75% median scaling normalization method. Normalized spot intensities were transformed to gene expression log2 ratios between the control and treatment groups. The spots with |log2 (Ratio)| ≥ 0.585 (fold change ≥ 1.5) and *P*-value < 0.05 are tested for further analysis.

Quantification of miRNAs by real-time RT-PCR

A SYBR Green-based quantitative RT-PCR assay was employed for miRNA quantification in extracted serum samples. In brief, 20 μL of total RNA sample containing miRNA was reversely transcribed to cDNA using miScript-II-RT-Kit (Qiagen, Germany) according to the manufacturer's instructions. Real-time PCR was then performed according to the miScript-PCR-System -Handbook in ABI Vii 7 PCR system (Applied Biosystems, NY, USA). For the 20 μL reaction, cDNA (1 μL) was combined with 5 μL of 2× Master Mix, 1 μL of 10× universal primer, 1 μL of 10× miSCRIPT primer, 2 μL of H2O and 1 μL of Template. The PCR was carried out as follows: initial denaturation at 95°C for 15 s, followed by 45 cycles of 94°C for 15

s, 55°C for 30 s and then 70°C for 35 s. All assays were repeated in triplicates. The expression levels of miRNAs were normalized to the spiked-in cel-miR-39, and were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The data analysis of gene expression profiling, including data filtering, normalization and statistical calculations, was processed by R version 2.12.1. Arrays (including technical replicates) of any compared sample set were normalized together after filtering probes according flag note from gpr files. The log2 (Ratio) were calculated by pair-wise combination and error weighted average. The significant differential expressed genes (DE genes) were selected according to log2 (Ratio) and *P*-value with criteria: |log2 (Ratio)| ≥ 0.585 (fold change ≥ 1.5) and *P*-value < 0.05. Probes were being filtered with flag note of gpr file, flag = 0, to select those were considered as detected for further analysis, Clustering and PCA.

The ANOVA test was used to compare the expression of serum miRNAs between the different groups. Receiver-operating-characteristic (ROC) curves and the area under the ROC curve (AUC) assessed the feasibility of using serum miRNAs as a diagnostic tool for PC. The Younden index determined the threshold for the serum miRNA concentrations. The correlation between clinicopathologic features and serum miRNA levels was determined by Mann-Whitney U test and Student's t-test. All tests were 2-tailed and a significance level of P < 0.05 (95% CI) was considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA).

Results

Demographic and clinical characterization of study population

A total of 49 PC patients and 27 healthy controls were included in this study. As shown in **Table 1**, all 49 patients enrolled in the study were both clinically and pathologically diagnosed for pancreatic cancer (PC). These patients were at various clinical stages including 6 with stage I, 14 with stage II, 15 with stage III and 14 with stage IV, as determined according to the International Union against Cancer (UICC) guidelines. The cases and controls were well-matched on age (mean age: 61.7 vs 60.8 (*P* = 0.229) and gender (*P* = 0.970).

miRNA profiling

At first, we made case-versus-control comparisons using 12 participants (i.e., 6 vs. 6). The differentially expressed miRNAs were identified in serum pools of 6 PCs and 6 controls using Human miRNA OneArray® v3 (Phalanx Biotech Group, Taiwan). Among 1711 miRNAs analyzed, 22 showed up-regulated while 23 demonstrated down-regulated changes in PC serum pools (**Table 2**). Eight deregulated miRNAs with extremely alteration and relatively seldom reported in previous studies involving 4 increased expression (miR-1238, miR-296-3p, miR-4290 and miR-483-5p) and 4 low expressed (miR-1280, miR-492, miR-595, miR-663a) were selected as the candidates for further qRT-PCR validation to explore the potent novel serum biomarkers for PC.

Validation of the differential expressed miRNAs

The expressions of 8 candidate miRNAs were measured in individual serum samples of 49 PCs and 27 controls including which were used for array test. Among them, only 2 miRNAs (miR-492 and miR-663a) demonstrated significantly decreased expression in PC group with more than 2-fold change compared with control group (P < 0.05) (**Figure 1**) by using qRT-PCR, and these altered miRNAs were thus chosen for further analysis.

Diagnostic value of miR-492 and miR-663a for pancreatic cancer

To further evaluate the discriminating effect of serum miRNAs on PC, ROC curves were established for each of the 2 miRNAs using 49 PCs and 27 controls in the validation phase (**Figure 2**). ROC curve analysis results revealed that miR-492 and miR-663a yielded the largest areas under the ROC curves (AUCs) and indicated their potential diagnostic value with the AUCs for miR-492 and miR-663a were 0.787 (95% CI: 0.689-0.885) and 0.870 (95% CI: 0.793-0.948), respectively. For each miRNA,

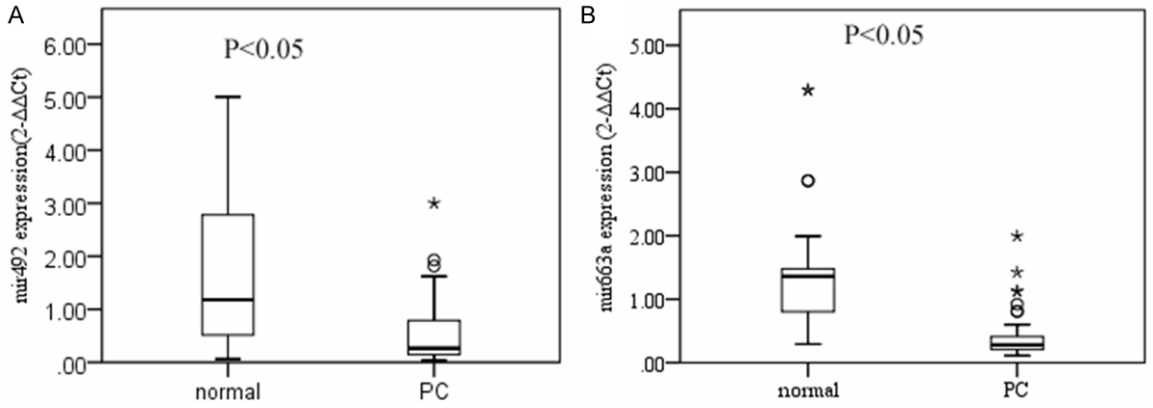

Figure 1. Serum levels of miR-492 (A) and miR-663a (B) in 49 PCs and 27control subjects in the second-stage validation. The relative levels of miRNAs (Y-axis) were normalized to the spiked-in cel-miR-39. Data indicated significantly decreased expression of miR-492 and miR-663a in PC group with more than 2-fold change compared with the normal control group.

the threshold for miR-492 at which the Youden's index (sensitivity + specificity-1) for PC diagnosis was largest at the ROC curve, produced optimal sensitivity and specificity were 75.5% and 70.0% in separating PC from normal controls. While for miR-663a, the sensitivity and the specificity were 85.7% and 80.0%, respectively. Simultaneous analysis of miR-492 and miR-663a showed a similar AUC of 0.869 (95% CI: 0.791-0.947) as miR-663a, with 85.7% sensitivity and 80.0% specificity, indicating a poor additive effect of the 2 miRNAs (**Figure 2**).

Association between serum miRNA expression and clinical characteristics

Furthermore, we sought to investigate the correlation between serum expression levels of miR-492 and miR-663a and clinical parameters of PCs in this pilot study. As shown in **Figure 3**, no correlations were found between the two miRNAs and age, gender as well as the histological differentiation grades. However, serum miR-663a demonstrated significantly positive correlation with clinical stage of PC ($P = 0.027$). Further analysis results showed that miR-663a expressed higher level in stage I than in relatively advanced stages (II-IV) but no difference among the cases with II, III and IV stage were found.

Discussion

Accumulating evidences have demonstrated the specificity of miRNAs for different types of cancers, all of which implied this small molecule might be a potential marker for the diagnosis and prognosis of cancers [16, 17]. However, the diagnostic potential and applicability in routine practice of tissue-based miRNAs are more limited because tissue specimens are difficult to obtain, particularly for advanced PC patients. Fortunately, circulating miRNAs were found to be stably existed in various body fluids, including serum and plasma at present [21-23]. As far as we known, tumor-derived circulating miRNAs firstly described by Mitchell et al opened up an extremely wide field for clinical application [18, 24-26].

Recently, published reports about circulating miRNAs in PC were limited. Morimura et al. demonstrated that plasma concentration of miR-18a was significantly higher in PC patients than that in controls, implied that circulating miR-18a might provide new complementary tumor biomarkers for PC [27]. Wang et al. also found a panel of four miRNAs, miR-21, miR-210, miR-155, and miR-196a over-expressed in PCs, which showed the feasibility of developing plasma miRNA profiling as a sensitive and specific blood-based biomarker assay for pancreatic cancer that had the potential of translation to the clinic with additional improvements in the future [28]. Ali et al., in the other research, reported that plasma level of miR-21 was significantly higher, whereas plasma levels of let-7 family (especially let-7d) and miR-146a was significantly lower in PC [29]. However, no relatively coincidence results were acquired till now. In the present study, we firstly identified the serum miRNA profiling of PC by using microar-

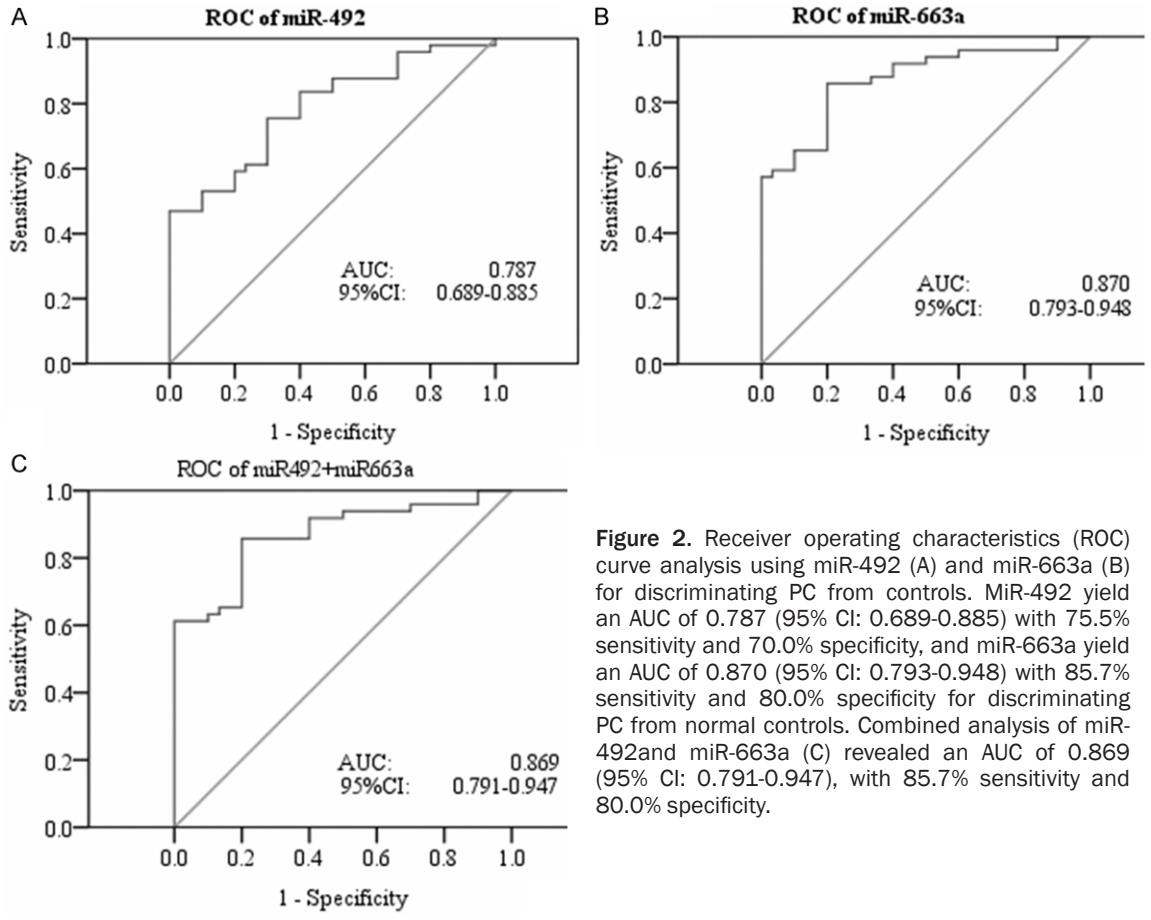

Figure 2. Receiver operating characteristics (ROC) curve analysis using miR-492 (A) and miR-663a (B) for discriminating PC from controls. MiR-492 yield an AUC of 0.787 (95% CI: 0.689-0.885) with 75.5% sensitivity and 70.0% specificity, and miR-663a yield an AUC of 0.870 (95% CI: 0.793-0.948) with 85.7% sensitivity and 80.0% specificity for discriminating PC from normal controls. Combined analysis of miR-492and miR-663a (C) revealed an AUC of 0.869 (95% CI: 0.791-0.947), with 85.7% sensitivity and 80.0% specificity.

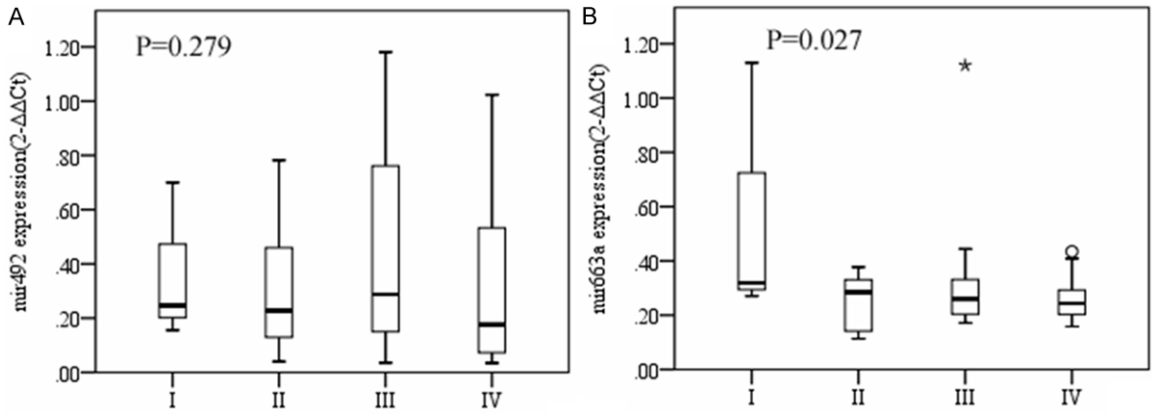

Figure 3. Correlation of serum levels of miR-492 and miR-663a and patients' different TNM stages. Box plots of serum levels of miR-492 (A) (P = 0.279) and miR-663a (B) (P = 0.027) in PC patients (n = 49) with different TNM stage.

ray analysis, and found 22 over-expressed and 23 down-regulated miRNAs. To further explored these novel markers and evaluated the possibility for clinical application in the diagnosis of PC, eight dysregulated miRNAs including four up-regulated (miR-1238, miR-296-3p, miR-4290 and miR-483-5p) and four down-regulated miRNAs (miR-1280, miR-492, miR-595 and miR-663a) were selected to evaluate the diagnostic potential in sera from 49 PC patients and 27 controls. ROC curve analysis manifested that the levels of serum miR-492 and miR-663a could contribute to the discrimination of PC with 75.5% sensitivity and 70.0% specificity

for miR-492 (AUC = 0.787) and 85.7% sensitivity and 80.0% specificity for miR-663a (AUC = 0.870), which demonstrated that miR-663a was more prominent than miR-492 as a PC biomarker. In addition, further combined miR-492 and miR-663a failed to improve the diagnostic efficiency of miR-663a indicated that there was no need to combination detection of these two molecules in early diagnosis of PC.

Furthermore, we also investigated the associations between these two serum miRNAs and clinical parameters of PC patients. A lower serum miR-663a expression was found in advanced PC patients with TNM stages II, III and IV than those with TNM stage I. At the same time, no significant difference between the levels of miR-492 and miR-663a and the histological grades, age as well as gender. Although the mechanism behinding this phenomenon was unclear, down-regulated levels of serum miR-663a might have some value for indicating the progression and treatment selection of PC.

MiR-492 and miR-663a were two miRNAs of less concern previously in human diseases involving the malignancies. Information on their relationships with carcinogenesis remains limited. In cultured cells, miR-492 could be up-regulated by the tumor suppressor p53 [30]. Moreover, a microarray profiling of stage II colon cancer revealed that miR-492 was down-regulated in cancers with recurrence of metastases in the liver and/or lungs [31]. Thus, it seems that the expression of miR-492 inversely correlates with metastases formation. In addition, Francesca Patella et al discovered that miR-492 inhibited in vivo angiogenesis promoted by tumor cells engrafted in a zebrafish model [32]. Recently, Schultz et al. also found that miR-492 was dysregulated in PC tissues [33]. All of these findings plus our results made miR-492 a promising tool to be tested in tumors as anti-angiogenic drug and suggested a role of miR-492 in modulating the secretion of pro-angiogenic factors. Little information was even available in the literature on the role of miR-663a. Langenberger et al. mentioned in their paper that miR-663a, a Dicer-independent miRNAs, was an important tumor suppressor [34]. Cheng Long showed that miR-663a, likely play important roles in the regulation of MAPK pathways which is consistently enriched in the chordoma tissue [35]. Our novel findings in this study suggested that serum miR-492 and miR-663a could act as suitable diagnostic markers for PC, which might bring the light on the potent pivotal role in PC cancer of these 2 miRNAs in the future.

However, although there was a partial overlap among their signatures in several studies which found altered miRNAs during the initiate and progress of pancreatic carcinoma, the majority of the miRNAs, particularly the circulating miRNAs, were different in PC patients [27-29]. For instance, the deregulated expression of miR-492 and miR-663a in our study was seldom found in previous reports. The reason for this difference is presently unclear. Intrinsic genetic heterogeneity between groups of patients of these patients coming from various studies might contribute to this discrepancy while differences in the analyte could also have an impact on the results of studies. In addition, the release of the miRNAs by the tumor cells into the circulation being affected by multiple parameters might attribute to the inconsistency. Therefore, the potential role of our signature of miRNAs in the pathogenesis of PC deserves further experimental attention and more large samples validated studies.

Previous studies and present research have actually demonstrated that aberrant expressions of circulating miRNAs have been found in many types of cancer. However, the origins of these extracellular circulating miRNAs still remain essentially unknown and conflict. Some researchers have detected the extracellular miRNAs in exosomes isolated from peripheral blood and culture media of several cell lines [36, 37]. On the contrary, Turchinovich and Wang [38, 39] reported in two individual studies that extracellular miRNAs were predominantly exosomes/microvesicles free and were associated with Ago proteins or nucleophosmin (NPM1). Although exploring the mechanism of origin of circulating miRNAs in serum was out of range of this present study, the origin and the function of extracellular miRNA in certain cancers deserve to further investigate in our future study which may shed light on the extracellular miRNAs' role in the pathogenesis in pancreatic cancer.

Our findings demonstrated the potential utility of circulating miRNAs as cancer-specific biomarkers; however, it is important to note that there were several limitations in this study.

Firstly, the sample size of cancers evaluated in this study was relatively small. Secondly, the panel of miRNAs selected for evaluation might be biased toward our search for pancreatic cancer-specific markers. Moreover, as in mRNA profiling studies, miRNA signatures identified by different groups could vary from one another. Large prospective cohorts and cross validation are needed to consolidate the important findings. Therefore, multi-center, large, independent, well-characterized, family and population-based case-control and additional validation studies are warranted.

In conclusion, serum miR-492 and miR-663a levels in PC were significantly decreased, suggesting the possibility that these two miRNAs are novel biomarkers for the clinic diagnosis of PC. With the accessibility of large sample sets, the range of technologies available, the prospect for circulating miRNAs as minimally invasive biomarkers for pancreatic cancer is excellent, although there are some challenges that the researchers have to conquer before these small non-coding RNAs can be fully understood and utilized.

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Disclosure of conflict of interest

None.

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