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Downregulation of miR-124 predicts poor prognosis in pancreatic ductal adenocarcinoma patients

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ABSTRACT

Background and aims: miRNA-124 (miR-124) expression was known to be downregulated in patients with pancreatic ductal adenocarcinoma (PDAC). Downregulation of miR-124 was significantly associated with poor prognosis in patients with PDAC. Recent studies have shown that circulating miRNAs could be the potential biomarkers for invasive diagnostic as well as prognostic purposes. The purpose of the current study was to characterise the serum miR-124 levels and assess the clinical significance of serum miR-124 in patients with PDAC.

Methods: Using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays, serum miR-124 levels were determined in serum from 126 patients with PDAC (53 resectable pancreatic cancer and 73 unresectable pancreatic cancer), 28 chronic pancreatitis patients and 47 healthy control individuals. The prognostic significance of miR-124 and other clinicopathological variables was determined using univariate and multivariate analyses.

Results: Serum miR-124 levels were significantly decreased in patients with PDAC. Serum levels of miR-124 distinguished PDAC from chronic pancreatitis ($P < 0.001$) and healthy control subjects ($P < 0.001$). Low serum levels of miR-124 were significantly associated with lymph node metastasis, tumour node metastasis (TNM) stage and shorter survival time after surgery. In multivariate analysis, serum miR-124 ($P = 0.001$, HR: 2.47, 95% CI: 1.25–4.05), high TNM stage ($P = 0.001$, HR = 3.24, 95% CI: 2.03–8.08) and lymph node metastasis ($P = 0.015$, HR = 1.66, 95% CI: 1.02–3.13) were significant predictors.

Conclusions: Serum miR-124 levels have utility as diagnostic biomarkers in patients with PDAC. These findings suggest, for the first time, that serum miR-124 levels may have prognostic impact in patients with PDAC.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a leading cause of cancer death and has the lowest survival rate for any solid cancer (~2%). Despite decades of effort, the five-year survival rate remains at only ~5%. There are no early detection tests and most patients with localised disease have no recognisable symptoms or signs. Therefore, most patients are not diagnosed until late in their disease after the cancer has metastasised to other organs.[1] However, as to the patients with curative resection of pancreatic cancer, the overall 5-year survival rate is 25%. This can be improved to 40% when the tumour is operated in its early stages. For example, when the tumour size is less than 2 cm and there are no lymph node metastases.[2,3] Therefore, early diagnosis of pancreatic cancer is of paramount importance in clinical practice. The early detection of pancreatic cancer is difficult because patients generally present at an advanced disease stage.

Serum is a preferred specimen for the early diagnosis of malignant tumours because samples are available readily by less invasive methods. The CA 19-9 serum marker is elevated in the majority of pancreatic cancer patients but does not achieve the performance required for either early detection or diagnosis, due to both false positive and false negative readings.[4] Other markers such as CEA and CA125 are being studied, but not specifically for the diagnosis of pancreatic cancer.[5,6]

MicroRNAs (miRNAs), which are small non-coding RNAs, regulate the translation of specific protein-coding genes. Since their discovery in 1993,[7] altered expressions of miRNAs have been associated with numerous diseases. In addition, tumour miRNAs are involved in tumourigenesis and the development of various cancers. In recent years, several studies have shown that miRNAs are detectable in plasma/serum, which clearly demonstrated that circulating miRNAs originate from cancerous tissues and are protected from endogenous

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RNase activity by unknown mechanisms.[8–10] miRNAs can be present in a remarkably stable form [10] and the expression level of serum miRNAs is reproducible and consistent among individuals.[9] Furthermore, secretory vesicles including miRNAs can function as intercellular transmitters.[11] miRNA expression profiles in pancreatic cancer cells are different from those in normal pancreatic cells and pancreatic cells in patients with chronic pancreatitis. Most miRNA expression profile analyses have demonstrated that miRNA expression is downregulated in pancreatic tumours, but upregulated in the normal pancreas and that the expression pattern is tissue specific.[12–15]

miR-124 is a brain-enriched miRNA that plays a crucial role in gastrulation and neural development.[16] miR-124 has been widely reported to regulate a plethora of target proteins involved in cell cycle, differentiation, cellular development and migration.[17] Using pyrosequencing, Wang and co-workers identified that miR-124 gene is highly methylated in pancreatic cancer tissues and its downregulation is significantly correlated with poor prognosis. Furthermore, they demonstrated that miR-124 inhibited pancreatic cancer cell proliferation by inhibiting Rac1 oncogene and MKK4-JNK-c-Jun pathway.[18]

Apart from the diagnostic miRNA signatures identified in specimens from tumour tissues, studies have also shown the diagnostic and prognostic usefulness of miRNAs in circulation. In fact, except for miRNAs in the biopsies and tumour samples, a substantial number of cancer-related miRNAs can be also detected in blood including total blood, serum and plasma, as well as other body fluids such as saliva, urine, breast milk, seminal fluid and tears.[19,20] In the current study, serum miR-124 levels was detected in patients with PDAC, pancreatitis and healthy subjects by reverse transcription-quantitative polymerase chain reaction (qRT-PCR) assay. The correlation of miR-124 levels with clinicopathological factors and prognosis was also statistically analysed. We hypothesised that **s**erum miR-124 has utility as diagnostic biomarkers in patients with PDAC, and serum miR-124 levels may have prognostic impact in patients with PDAC.

Materials and methods

Serum samples

Written informed consent was obtained from donors and no data allowing identification of patients was provided. Serum samples were collected from an 'unselected' patient population and processed at Biobank in Yantaishang Hospital and Yuhuangding Hospital according to the standard procedures. Serum samples were from patients of histologically or cytologically confirmed PDAC and pancreatitis. Healthy subjects were from the centre of physical examination. All the samples were from Yuhuangding Hospital and Yantaishan Hospital (December 2005 and January 2010). No preoperative chemotherapy and/or radiotherapy case was included. Of these, 201 samples, including 126 pancreatic cancer patients (53 resectable pancreatic cancer and 73 unresectable pancreatic cancer), 28 chronic pancreatitis patients and 47 healthy control individuals. The mean age of the patient group was 60.4 y (range, 43–74 y) in resectable groups, 62.7 y (range, 48–72 y) in unresectable groups, 59.6 y (range, 39–74 y) in chronic pancreatitis groups and 61.4 y for the healthy control group (range, 42–76 y). The control subjects were healthy with no evidence of pancreatic, biliary or liver disease. The size of tumour was analysed by maximum diameter. The patients were staged according to the international tumour node metastasis (TNM) system by International Union against Cancer (UICC). Postoperative adjuvant chemotherapy with gemcitabine, S-1 and oral administration of tegafur was administered to 42, 9 and 2 patients, respectively. Intraoperative therapy was not performed on any patient. The survival period after surgical resection was used as survival time for statistical analysis. The median length of the survival time at the last follow up of the patients was 10.8 months (range, 0–147). Clinical outcome data were available in all 53 patients with PDAC, and overall survival was analysed. The resectable patients' characteristics with respect to age, sex, histopathology and TNM stage are described in Table 1. All samples were stored at –80 °C and sent frozen on dry ice. Each aliquot had been thawed no more than three times before use.

qRT-PCR

The total RNA, including microRNA, was extracted from the serums. Real-time RT-PCR method was used to assess the levels of miR-124 with Express SYBR® GreenER qPCRs supermix Universal kit (Invitrogen) on a Rotor-gene 6000 system (Qiagen, Valencia, CA, USA). U6 RNA was used as an endogenous reference for normalising the expression levels of miR-124. Initially, we calculated a $^{\Delta}Ct$ (target-reference), which is equal to the difference between threshold cycles for miR-124 (target) and those for U6 RNA (reference). The fold-change for serum miR-124 level was calculated with the $2^{\Delta\Delta}Ct$ method, in which $^{\Delta\Delta}Ct = {}^{\Delta}Ct$ (target reference in tumour samples) – $^{\Delta}Ct$ (target-reference in normal samples). The qRT-PCR primers for miR-124 were designed as follows: forward: miR-124 :5′-5′-TGGCAGGCTGCGACGT-3′ and 5′-CGGCTCTTTGTGTATCGTAC-3′. U6:5′-CTCGCTTCGGCAG CACA-3′ and 5′-AACGCTTCACGAATTTGCGT-3′. The relative expression levels of miR-124 in serum of patients with PDAC compared to healthy or pancreatitis controls were calculated using the method of $2^{-\Delta\Delta}Ct$. The reaction parameters were: 60 °C for 2 min, 95 °C for 10 min, followed by 36 cycles of 95 °C for 15 s and 55 °C for 1 min. PCR cycle threshold (*C*t) values were recorded for each

Table 1. Serum miR-124 levels and clinicopathological features of 53 patients with resectable PDAC.

Groups	Number	Serum miR-124 levels	*P*-value
Age (y)			0.369
≤60	19	0.24 ± 0.07	
>60	34	0.25 ± 0.08	
Sex			0.546
Female	17	0.26 ± 0.05	
Male	36	0.24 ± 0.06	
Tumour size, cm			0.148
≤3	15	0.26 ± 0.08	
>3	38	0.24 ± 0.08	
Lymph node metastasis			0.004
Yes	28	0.13 ± 0.04	
No	25	0.39 ± 0.07	
Neural invasion			0.184
Yes	38	0.23 ± 0.05	
No	15	0.27 ± 0.09	
Tumour differentiation			0.374
Well	41	0.26 ± 0.06	
Poor/moderate	12	0.25 ± 0.06	
T stage			0.145
T1+T2	19	0.27 ± 0.05	
T3	34	0.21 ± 0.04	
TNM stage			0.001
I+IIA	25	0.32 ± 0.06	
IIB+IIIA	28	0.12 ± 0.04	

target gene and for normalisation controls and were averaged across three independent runs. Primers for miR-124 and U6 were custom-ordered from Songan.com (Shanghai, China). In addition, each measurement was performed in triplicate.

Statistical analysis

All data are presented as mean ± SD. Differences in serum miR-124 levels and clinicopathologic variables were analysed using the student *t* test. Age was dichotomized at the median value. Overall survival (OS) was displayed using Kaplan-Meier survival curves with 95% confidence intervals (CIs) and the differences between subgroups were compared using the log-rank test. All risk factors identified by univariate analysis were adopted in multivariate Cox proportional hazard analysis. OS was defined as the time between surgery and death from any cause. Survival curves were calculated using the method of Kaplan-Meier and compared using the log-rank test. Factors shown to be of prognostic significance in the univariate models were evaluated using a multivariate Cox regression model. A *P* value less than 0.05 was considered statistically significant. All statistical manipulations were performed using the SPSS.22 software program (SPSS Inc., Chicago, IL, USA).

Results

We assessed the utility of serum miR-124 levels as diagnostic biomarkers by comparing serum levels of miR-124 in patients with PDAC to serum levels of miR-124 in healthy control subjects and chronic pancreatitis patients. Serum miR-124 levels were 0.83 ± 0.07 (miR-124/U6) in healthy control subjects, 0.91 ± 0.08 in patients with chronic pancreatitis, and 0.14 ± 0.05 in patients with PDAC. There was a statistically significant difference between serum miR-124 levels in patients with PDAC and healthy control subjects and chronic pancreatitis patients ($P < 0.001$). Serum miR-124 levels were significantly decreased in patients with PDAC relative to patients with chronic pancreatitis and healthy control subjects. Although serum miR-124 levels was slightly increased in patients with chronic pancreatitis, there was no statistically significant compared to healthy control subjects ($P = 0.074$).

In order to improve patient outcomes, it is desirable that biomarkers have the ability to diagnose PDAC at an earlier (resectable) stage and later (unresectable) stage. In our 126 patients of PDAC, there was 53 resectable (stage I + II + IIIA) and 73 unresectable (stage IIIB + IV) patients. Of the 53 resectable cases examined, the serum miR-124 levels were 0.25 ± 0.07, which was significant higher than the unresectable patients (0.08 ± 0.02) ($P = 0.001$).

Overall diagnostic accuracy is represented by the AUC of the ROC curve. The relative sensitivity and specificity are also presented. The AUC values for discriminating between healthy people and patients with cancer or chronic pancreatitis are 0.98(0.79–1.0) and 0.61(0.47–0.76), respectively. In addition, discrimination between cancer and chronic pancreatitis was achieved 0.67(0.58–0.83), the discrimination between resectable and unresectable patients was achieved 0.84(0.72–0.96) (Figure 1).

Serum miR-124 levels in different groups of 53 resectable PDAC patients were compared. As shown in Table 1, the preoperative serum miR-124 levels correlated well with Lymph node metastasis ($P = 0.004$) and TNM stage ($P = 0.001$), but not with neural invasion ($P = 0.184$), tumour differentiation ($P = 0.347$), T stage ($P = 0.145$), tumour size ($P = 0.148$), age ($P = 0.369$) and gender ($P = 0.546$) (Table 1). These results suggest that decreased

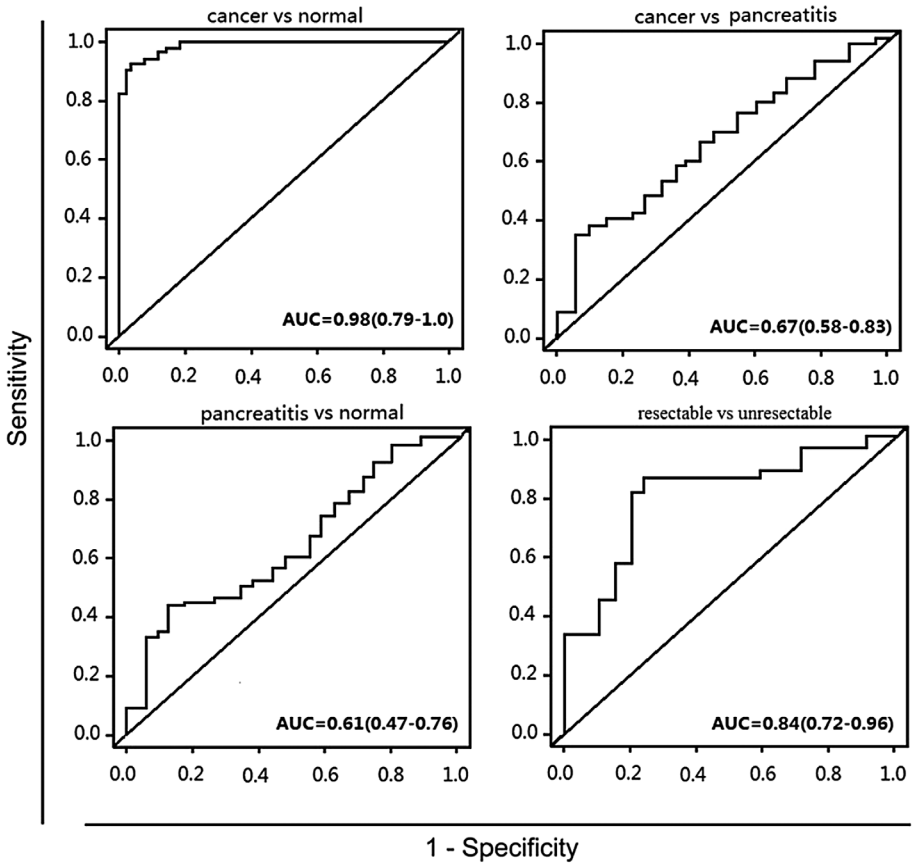

Figure 1. ROC curves calculated on the serum miR-124 measurements. Receiver operating characteristic (ROC) curves are a widely accepted indicator of diagnostic utility. Measure of accuracy is the corresponding area under the ROC curve, denoted as AUC. It ranges in value from 0.5 (chance) to 1.0 (perfect discrimination).

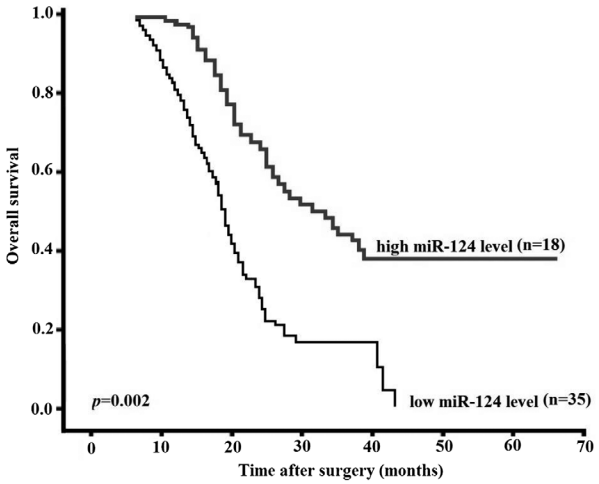

Figure 2. Kaplan-Meier survival curves for OS according to miR-124 levels in patients with resectable PDAC.

miR-124 expression may be correlated with poor prognosis in patients with PDAC.

Among the 53 patients examined, 38 died (cancer-related death) during the follow up period. Fifteen patients were alive at the last follow up and the longest survivor was alive at 147 months after surgery. The overall one, three, and five year survival rates of patients with surgical resection were 52.8, 17 and 11.3%, respectively.

The 53 patients with resectable PDAC were divided into high and low serum miR-124 level groups (18 and 35 patients, respectively) using the median miR-124 value (0.25 ± 0.07) in 53 patients with resectable PDAC as the cut-off point.

The one, three and five year survival rates of the low serum miR-124 group were 32.4, 7.3 and 0.0%, respectively, whereas those of the high serum miR-124 expression group were 65.7, 19 and 11.4%, respectively. The survival of patients with high serum miR-124 expression was significantly better than those with low serum miR-124 expression ($P = 0.002$; log rank test; Figure 2).

Univariate analyses indicated that decreased serum miR-124 ($P = 0.002$) was statistically significant risk predictors for poor OS in 53 patients with resectable PDAC (Table 2). In addition, high TNM stage ($P = 0.0001$) and lymph node metastasis ($P = 0.001$) was also statistically significant risk predictors for poor OS in 53 patients with resectable PDAC (Table 2).

In multivariate analysis, significant predictors were serum miR-124 ($P = 0.001$, HR: 2.47, 95% CI: 1.25–4.05), high TNM stage ($P = 0.001$, HR = 3.24, 95% CI: 2.03–8.08) and lymph node metastasis ($P = 0.015$, HR = 1.66, 95% CI: 1.02–3.13) (Table 2). Serum miR-124 and TNM stage were significant poor risk factor affecting the outcome of the patients.

Table 2. Uni- and multivariable analyses of the effect of serum miR-124 level on survival.

	Univariable analysis			Multivariable analysis		
Factors (*n*)	Hazards	95%CI	*P*-value	Hazards	95%CI	*P*-value
miR-124 expression						
High/Low (18/35)	2.87	1.126–4.53	0.002	2.47	1.25–4.05	0.001
Age(Y)						
⩽60 vs. >60 (19/34)	1.36	0.74–1.68	0.42	–	–	–
Gender						
Male vs. Female (36/17)	1.15	0.67–1.48	0.56	–	–	–
Tumour size (mm)						
⩽30 vs. >30 (15/38)	2.25	1.08–3.28	0.094	2.32	1.17–3.42	0.107
Lymph node metastasis						
Yes vs. No (28/25)	5.32	3.86–10.4	0.001	1.66	1.02–3.13	0.015
TNM stage						
I+ IIA vs. +IIB+ IIIA(25/28)	3.24	2.03–8.08	0.0001	2.96	1.24–4.14	0.001
Neural invasion						
Yes vs. No(38/15)	1.56	1.15–3.06	0.254	1.63	1.23–3.48	0.273
T stage						
T1+T2 vs. T3(19/34)	1.56	1.04–5.29	0.012	2.18	1.56–3.24	0.138
Tumor differentiation						
Well/Moderate vs. poor(41/12)	1.56	1.23–2.83	0.36	1.48	1.26–2.94	0.384

Discussion

It is often difficult to diagnose pancreatic cancer because of limited access and the need for invasive diagnostic procedures; thus, substantial research effort has been applied to the identification of diagnostic and prognostic biomarkers in circulating samples.[21] The detection of miRNAs in samples acquired through minimally or non-invasive procedures, such as serum, plasma and saliva, can have a positive impact on the clinical management of these patients.

Wang et al. demonstrated that detecting miRNAs (miR-21, -210, -155 and -196a) in serum of patients with PDAC could clearly distinguish patients with and without cancer (sensitivity of 64% and specificity of 89%).[22] Using serum samples, miRNAs could be used to correctly discriminate patients with PDAC from controls, and also shown to predict prognosis.[23] A recent study also revealed that the expression of two miRNAs (miR-21 and miR-34a) in serum samples could individually discriminate PDAC and healthy controls.

In the present study, we evaluated serum miR-124 levels in patients with PDAC. Serum miR-124 levels were significantly decreased in patients with PDAC than the patients with pancreatitis and healthy controls. Serum miR-124 levels could correctly distinguish patients with and without the PDAC. Serum levels of miR-124 were also shown to predict prognosis: patients with unresectable PDAC (stages IIIB and IV) had significantly lower miR-124 levels, whereas patients with high miR-124 expression levels were in the early stages of the disease (stages I and II) (resectable PDAC).

The relationship between serum miR-124 and certain clinicopathologic parameters was evaluated in 53 patients of sectable PDAC. Reduced serum miR-124 levels in patients with PDAC were correlated with TNM stage and lymph node metastasis. Reduced serum miR-124 levels were also correlated with microvascular invasion, although no significant statistics was shown (data not shown). Importantly, miR-124 could be used to reliably predict survival: patients with a low level of miR-124 expression showed short-term survival, whereas patients with a high level of miR-124 expression showed long-term survival.

Univariate analysis showed that reduced miR-124 levels were significantly associated with the overall survival rate in patients with PDAC. Multivariate analysis indicated that serum miR-124 levels were an independent risk factor for poor prognosis in patients with PDAC. Collectively, these results suggest that serum miR-124 levels might be used as a novel prognostic marker in patients with PDAC. This study demonstrated that reduced serum miR-124 expression is associated with poor survival in PDAC patients. miR-124 could be used to correctly discriminate PDAC patients from normal controls. miR-124 expression may serve as an important prognostic marker and may represent a potential molecular target for the treatment of PDAC. This study represents an advance in biomedical science because it shows that miR-124 was first found to be low expressed in serum of patients with PDAC and may be as an important prognostic marker.

Disclosure statement

No potential conflict of interest was reported by the authors.

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