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Original Article

Expression of microRNA-200c in human pancreatic ductal adenocarcinoma and its prognostic significance

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Abstract: Introduction: MicroRNAs (miRNAs) have emerged as potential therapeutic candidates due to their ability to regulate multiple targets involved in tumor progression. MiRNA-200c (miR-200c) was previously shown to be correlated with aggressive clinicopathological features and poor prognosis in several cancers. The aim of this study was to analyze miR-200c expression in pancreatic ductal adenocarcinoma (PDAC) and to determine whether miR-200c has an independent prognostic value in PDAC. Methods: Quantitative real-time PCR (qRT-PCR) assay was performed to detect the expression of miR-200c in human PDAC cells and tissue samples. The association of miR-200c expression with clinicopathologic features was analyzed. Kaplan-Meier analyses were used to assess survival of patients. Univariate and multivariate analyses were performed using the Cox proportional hazards model to analyze the prognostic significance of miR-200c expression. Results: Our data showed that the relative level of miR-200c in PDAC cells was significantly lower than that in normal human pancreatic duct epithelial cell line. The expression of miR-200c in PDAC tissues was significantly lower than that in adjacent non-tumor tissues. By statistical analyses, low miR-200c expression was observed to be closely correlated with clinical stage, liver metastasis and lymphnode metastasis. Kaplan-Meier survival analysis revealed that patients with low miR-200c expression had a poor overall survival compared with the high miR-200c group ($P < 0.05$). Univariate and multivariate analyses showed that low miR-200c expression was an independent poor prognostic factor for PDAC patients. Conclusion: Our data demonstrated that reduced miR-200c in PDAC tissues was correlated with tumor progression, and miR-200c might be a potential molecular biomarker for predicting the prognosis of patients.

Keywords: microRNA-200c, overall survival, pancreatic cancer, pancreatic ductal adenocarcinoma

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in Western countries and the sixth in China, is characterized by aggressive invasion, early metastasis, and lack of specific symptoms [1, 2]. The 5-year survival of PDAC is only approximately 5%, and this figure has remained nearly unchanged over the past two decades, but the incidence of PDAC has been rising worldwide [3, 4]. Pancreatic carcinogenesis is known to be a multi-step process involving multiple genetic and epigenetic alterations [5]. The identification of biomarkers that accurately predict disease recurrence or response to chemotherapy would be of substantial aid in individual risk assessment and treatment selection and may even lead to novel therapies by becoming targets for molecular intervention in specific subsets of patients. Hence, a better understanding the molecular mechanisms involved in pancreatic carcinogenesis will be helpful for identification of new and effective biomarkers for early diagnosis and prognosis of PDAC.

MicroRNAs (miRNAs) comprise a class of 19~23 nucleotides of noncoding RNAs regulating gene expression by targeting translational cleavage or repression [6]. In recent years, a growing number of evidence had demonstrated that miRNA expression levels were directly associated with cancer cell formation, development and pathology [7-9]. With the growing knowledge of relation between miRNAs and cancer, it also has been shown that the sensitivity of cancer cells to anticancer drug was affected by miRNAs [10]. Among them, miR-200c has been

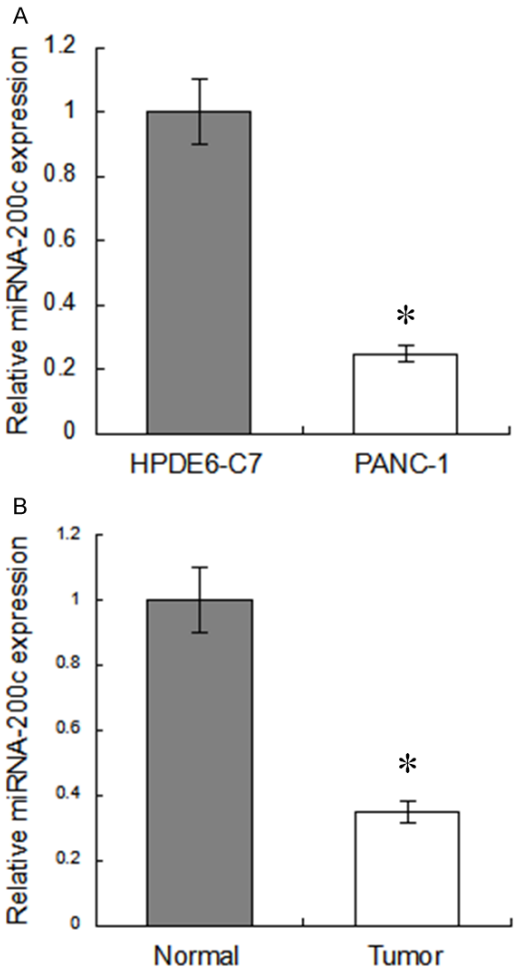

Figure 1. qRT-PCR analysis of miR-200c expression in PDAC cell lines and tissue samples. A. The expression level of miR-200c in a normal human pancreatic duct epithelial cell line (HPDE6-C7) and PDAC cell line (PANC-1). B. The relative level of miR-200c expression in PDAC tissues and non-tumor tissues. Results are expressed as mean ± SD for three replicate determination $*P < 0.05$.

shown to be expressed in various carcinoma tissues and its over- or down-expression played essential role in tumor formation or cancer cell apoptosis. However, the relationship between expression of miR-200c and the prognosis of patients with PDAC remains unclear.

In our study, qRT-PCR assay was performed to detect the expression of miR-200c in PDAC and adjacent non-tumor tissues. Furthermore, the correlations of miR-200c expression with clinicopathologic features of PDAC patients were statistically analyzed. Finally, we determined the potential role of miR-200c in PDAC prognostic prediction. Our data indicated that miR-200c was significantly downregulated in PDAC tissues and could be served as a potential molecular biomarker for the prediction of poor prognosis.

Materials and methods

Patients and specimens

A total of 75 primary PDAC and paired adjacent non-tumor tissues (located > 2 cm from the tumors) were collected from patients who undergone pancreatic surgical resection with informed consent at the Henan Provincial People's Hospital, China, from 2008 to 2013. Both tumor and non-tumor tissues were confirmed by two experienced pathologists. None of these patients received neoadjuvant or adjuvant treatment before operation. Median follow-up time of surviving patients was 10.8 months. The study was approved by the Medical Ethical Committee of Zhengzhou University. Informed consent had been obtained from all of the patients for use of the clinical specimens.

Cell lines and cultures

A normal human pancreatic duct epithelial cell line (HPDE6-C7) and PDAC cell line (PANC-1) were purchased from the Cell Bank of Chinese Academy of Sciences. Cells were cultured in RPMI 1640 and DMEM medium, respectively, with 10% fetal bovine serum, at 37°C in a humidified incubator with 5% CO_2.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with the Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Complementary DNA was reverse-transcribed using a reverse transcription kit (Invitrogen, USA). Briefly, cDNA were synthesized using a miR200c specific primer in reverse transcription system. The reaction condition was as follows: 16°C 30 min, 42°C 42 min, 85°C 5 min. Quantitative detection of RT products was performed using specific sense and antisense primers of miRNA-200c and Sybergreen I dye. The PCR reaction condition was as follows: 95°C for 5 min, 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s, 40 cycles, to obtain fluorescence intensity. U6 was used as an internal control. The sequence of specific

Table 1. Association between miR-200c expression and clinicopathologic features of PDAC patients

Clinicopathological feature	Total	miRNA-200c expression		*P* value
		Low	High	
Age (years)				0.525
< 60	32	14	18	
≥ 60	43	22	21	
Gender				0.121
Male	51	20	31	
Female	24	14	10	
Tumor size (cm)				0.385
< 2	8	5	3	
≥ 2	67	31	36	
Differentiation				0.453
Well	6	2	4	
Moderate + Poor	69	34	35	
Clinical stage				0.000
I + II	28	5	23	
III + IV	47	30	17	
T classification				0.589
T1 + T2	42	19	23	
T3 + T4	33	17	16	
Lymphnode metastasis				0.000
Absent	40	10	30	
Present	35	25	10	
Liver metastasis				0.003
Absent	62	24	38	
Present	13	11	2	

primer for miRNA-200c was 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATGCACTGGATACGACTCCATC-3'; the sequence of sense primer of miRNA-200c was 5'-GGTAATACTGCCGGGTAAT-3'; the sequence of antisense primer of miRNA-200c was 5'-CAGTGCGTGTCGTGGAGT-3'. The Ct value was analyzed using the RFQ-PCR (Applied Biosystems Viia7, USA) analysis program. Relative mRNA expression levels were determined by the $2^{-\Delta\Delta Ct}$ method in comparison with control cells.

Statistical analysis

All statistical analyses were performed by using the SPSS 18.0 statistical software package. The data were presented as the mean ± SD. The association between miR-200c expression level and clinicopathologic factors of the patients was analyzed using the chi-square test. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. The significance of different variables with respect to survival was analyzed using the multivariate Cox proportional hazards model. Differences were considered statistically significant when $P < 0.05$.

Results

Expression of miRNA-200c in human PDAC cells and tissue samples

To determine the expression of miR-200c expression in PDAC, qRT-PCR assay was performed to firstly detect the expression level of miR-200c in normal human pancreatic duct epithelial cell line (HPDE6-C7) and PDAC cell line (PANC-1). According to the results of qRT-PCR, the relative level of miR-200c expression in PDAC cell line was significantly lower than that in normal human pancreatic duct epithelial cell line (**Figure 1A**; $P < 0.05$). Next we detected the expression of miR-200c in 75 cases of PDAC tissues and adjacent non-tumor tissues, and results indicated that the mean level of miR-200c expression in PDAC tissues was significantly lower than that in adjacent non-tumor tissues (**Figure 1B**; $P < 0.05$).

Correlations of miRNA-200c expression with clinicopathologic features of PDAC patients

To further investigate the correlations of miR-200c expression with clinicopathologic features of PDAC patients. The relative expression of miR-200c was divided into two groups based on the mean value (0.33): High miR-200c expression group had miR-200c expression levels more than mean value and low miR-200c expression group had miR-200c expression levels less than mean value. Then, the correlations of miR-200c expression with clinicopathologic features of patients were statistically analyzed. As shown in **Table 1**, low miR-200c expression was observed to be closely correlated with advanced clinical stage, higher incidence of lymph node metastasis and liver metastasis (**Table 1**; $P < 0.05$). However, there were no significant correlations between miR-200c expression and other clinicopathologic factors including age, gender, tumor

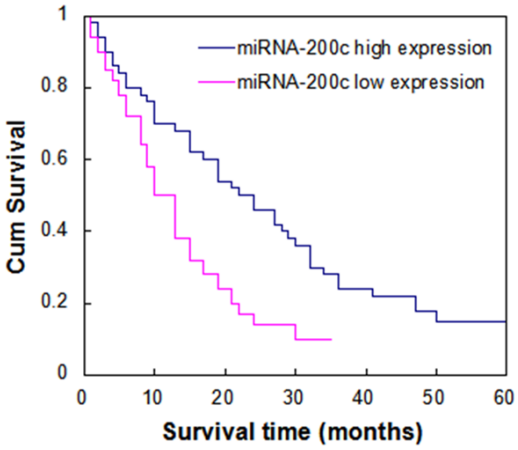

Figure 2. miR-200c expression is correlated with overall survival in patients with PDAC. Kaplan-Meier curve for overall survival in patient tissues with low versus high miR-200c expression. Corresponding P values analyzed by log-rank test.

size, differentiation and T classification (**Table 1**; $P > 0.05$).

Correlations of miRNA-200c expression with overall survival of PDAC patients

To further assess the correlation of miR-200c expression with survival of PDAC patients, Kaplan-Meier analyses were performed. As shown in **Figure 2**, the 5-year overall survival of low miR-200c expression group was significantly shorter than that of high miR-200c expression group (**Figure 2**; $P < 0.05$). Our results demonstrated that downregulation of miR-200c might be correlated with poor survival of PDAC patients.

Univariate and multivariate analyses of prognostic variables in PDAC patients

To further determine the prognostic significance of miR-200c expression for PDAC patients, survival data were obtained for each patient and univariate and multivariate analyses were performed (**Table 2**). Univariate Cox regression analysis showed that clinicopathological variables including clinical stage, lymph node metastasis, liver metastasis, and miR-200c expression were significantly associated with overall survival (**Table 2**; $P < 0.05$). Also, to evaluate whether low miR-200c expression (low vs. high) might be as an independent predictor for overall survival of PDAC patients, multivariate Cox regression analyses were performed. Along clinical stage, lymph node metastasis, and liver metastasis (**Table 2**; $P < 0.05$), miR-200c expression was an independent molecular biomarker for predicting of the poor overall survival of PDAC patients (RR: 2.314, 95% CI: 1.731-6.378, $P = 0.008$).

Discussion

There are currently no means for the reliable diagnosis of early stages of pancreatic cancer (PDAC) and the curative treatment of late stages. Consequently, the vast majority of patients (80%) display an advanced disease that results in a low resection rate leading to a dismal overall median survival of less than 6 months [1]. Thus, there is an urgent need to discover diagnostic as well as prognostic molecular markers together with reliable therapeutics to improve pancreatic cancer management.

The recent discovery of microRNAs (miRNAs or miRs) has revealed a novel mechanism of gene regulation and provided new avenues for cancer research. MiRNAs are small, non-coding RNA molecules, which regulate the gene expression at post-transcription level [11, 12]. MiRNAs are involved in the regulation of various biological processes including proliferation, apoptosis, differentiation and development [13]. Cimmino et al reported that miR-15a and miR-16-1 negatively regulate the antiapoptotic B cell lymphoma 2 gene (*Bcl2*) causing CLL cells to undergo apoptosis [14]. Li Ma unraveled a mechanism in which miR-10b positively regulates cell migration and invasion in a non-metastatic breast cancer cell line in a multi-step process that ultimately leads to activation of RHOC, a pro-metastatic gene [15]. The Slack group introduced a novel strategy to efficiently inhibit miR-155, an oncomiR in a murine model of lymphoma, by using a peptide nucleic acid antagomiR attached to a pH-induced transmembrane structure (pHLIP) [16]. These data demonstrated the potential oncogenic or tumor suppressor role of miRNAs. However, the relationship between miRNAs and cancer patient prognosis remains largely unknown.

Here we reported miR-200c, which was previously shown to function as a tumor suppressor and to repress epithelial mesenchymal transition (EMT) and tumor metastasis. For instance, increased miR-200c expression leads

Table 2. Univariate analyses of different prognostic factors in PDAC patients

Clinicopathological feature	Univariate analysis			Multivariate analysis		
	Risk ratio	95% CI	*P*	Risk ratio	95% CI	*P*
Age (years)	1.176	0.353-1.894	0.267			
≥ 60 vs. < 60						
Gender	1.289	0.543-2.118	0.325			
Male vs. Female						
Tumor size (cm)	1.867	0.581-2.945	0.241			
≥ 2 cm vs. < 2 cm						
Differentiation	1.516	0.283-2.906	0.581			
Moderate + Poor vs. Well						
T classification	2.537	0.683-4.351	0.207			
T3 + T4 vs. T1 + T2						
Clinical stage	2.417	1.328-6.139	0.009	2.105	1.327-5.14	0.004
III + IV vs. I + II						
N classification	3.469	1.374-6.318	0.016	2.438	1.368-7.017	0.009
Present vs. Absent						
Liver metastasis	3.336	0.604-5.872	0.014	2.283	0.625-4.673	0.011
Present vs. Absent						
miRNA-200c	2.539	1.549-7.327	0.015	2.314	1.731-6.378	0.008
Low vs. High						

to a reversal of EMT in bladder cancer [17]. MiR-200c can also inhibit cancer stem cell self-renewal and attenuate differentiation [18]. MiR-200c were confirmed to be downregulated in human breast cancer stem cells as well as in normal human and murine mammary stem/progenitor cells. Moreover, miR-200c has a modulatory function in cell division and apoptosis [19]. However, the association between miR-200c expression and the clinicopathologic features and patient prognosis in PDAC is unknown. In this study, we aimed to explore the association between miR-200c expression and cancer prognosis and clinical pathology.

We found that the relative level of miR-200c in PDAC cell lines was significantly lower than that in normal human pancreatic duct epithelial cell line. Also, we compared the expression of miR-200c in PDAC tissues and adjacent non-tumor tissues, and showed that the expression of miR-200c in PDAC tissues was also significantly lower than that in adjacent non-tumor tissues. Then, we investigated the clinicopathologic significance of miR-200c expression in PDAC. Statistical analyses demonstrated that low miR-200c expression was significant correlated with advanced clinical stage, higher incidence of lymph node metastasis and liver metastasis. These data implied that downregulation of miR-200 might play critical roles in PDAC progression and development. Furthermore, we found miR-200c expression was observed to be significantly associated with overall survival of PDAC patients. Kaplan-Meier analysis of overall survival showed that patients with low miR-200c expression tended to have a significantly shorter overall survival than those with high miR-200c expression. Cox proportional hazards model proved that low miR-200c was an independent prognostic marker for predicting the poor prognosis of PDAC patients. Thus, miR-200c expression could be used as a molecular prognostic factor to identify patients who are more likely to have higher risk of death.

Taken together, this study showed that downregulation miR-200c was associated with tumor progression and poor prognosis in PDAC and was identified as an independent poor prognostic factor for PDAC patients.

Disclosure of conflict of interest

None.

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