Circulating exosomal microRNAs as novel detection biomarkers in pancreatic cancer

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Abstract

Background

Circulating exosomal microRNAs are reflective of the characteristics of the tumor, are valuable biomarkers in different types of tumors, and play important roles in tumor progression and metastasis. The purpose of this study was to investigate the circulating exosomal microRNAs miRNA-21 and miRNA-210 as novel biomarkers for patients with pancreatic cancer (PC).

Methods

Serum exosomal microRNAs were extracted from the serum of PC and chronic pancreatitis (CP) patients using an RNA Isolation kit. To identify the exosomes in the serum, we used transmission electron micrographs for the crystalline structure, western blotting, and NanoSight for exosomal markers and nanoparticle characterization. The relative expression levels of exosomal microRNAs were quantified using quantitative PCR and compared between PC and CP patients.

Results

A total of 40 serum samples (30 PC and 10 CP) were collected. The expression levels of both exosomal miRNA-21 and miRNA-210 were obviously higher in PC patients compared with those in CP patients (both $P<0.001$). However, no significant difference in the relative serum levels of free miR-21 and miR-210 was observed between these two groups (both $P>0.05$). Exosomal miRNA-21 and miRNA-210 were related to tumor stage, as well as other factors. The diagnostic of exosomal miRNA-21 and miRNA-210 levels was 83% and 85%, respectively. Furthermore, when combining the expression of exosomal miRNA with serum CA19-9, the accuracy increased to 90%.

Conclusions

We herein identified that the serum exosomal miRNAs miRNA-21 and miRNA-210 may be of value as potential biomarkers and therapeutic targets for the diagnosis and treatment of PC.

Background

Pancreatic cancer (PC) is one of the most highly malignant cancers, with a mean 5-year survival rate of < 5% [1, 2]. According to a recent report, PC is responsible for ~ 227,000 deaths annually worldwide [3], due to its high recurrence rate, asymptomatic onset and < 25% resectable localized tumors. Unfortunately, early diagnostic and effective treatment strategies are lacking, and there is an urgent need to explore diagnostic tools with high sensitivity and specificity, repeatability.

Thus, it is urgent to explore the underlying molecular mechanisms and identify novel diagnostic biomarkers for PC. Identifying a minimally invasive/non-invasive and sensitive diagnostic method has recently become a research hotspot, and there has been some progression in the study of serum markers

[4, 5]. Further elucidating the related biological processes is crucial, as it may uncover novel potential biomarkers for early diagnosis of PC.

MicroRNAs (miRNAs) are 18–22 nucleotides in length, endogenous non-coding single-stranded RNAs that regulate gene expression by binding to the 3’- or 5’-untranslated regions post-transcriptionally or the open reading frames [6, 7]. mRNA or protein translation is degraded or suppressed by miRNAs through this interference process. Numerous studies have demonstrated that miRNAs are involved in a variety of malignant processes, such as PC, lung, breast and prostate cancer, among others [8–10]. In particular, the clinical applications of miRNAs have been investigated as biomarkers for early diagnosis and treatment of tumors, clinical response and histological classification. Due to the repeatability and ease of sample collection, circulating miRNAs are currently investigated as non-invasive biomarkers in early diagnosis of cancers. miRNAs in exosomes were first extracted and verified by Valadi et al. Exosomes are secreted by different types of cells and may be used for transporting proteins, mRNAs and miRNAs to target cells [11]. The study of miRNAs in exosomes is becoming a new focus of research. Moreover, it was found that exosomal miRNAs may play crucial roles in biological processes such as tumor progression, cell proliferation, invasion, metastasis, apoptosis and differentiation [12–14], and may be of value as novel serum biomarkers for the early diagnosis of cancers, such as PC.

Exosomes originate from internal multivesicular bodies, and are membranous vesicles with a diameter of 30–120 nm [15, 16]. Exosomes have been identified in the serum, plasma, breast milk, and other human bodily fluids. Recent findings have reported that exosomal miRNAs contain miRNA, mRNA and cell-specific proteins, and have been used as a diagnostic biomarker in tumor cells, as they may reflect genetic information and molecular signatures of the various cells of origin [17–19]. Tumor cell-derived exosomes contain tumor-specific miRNAs, and their roles in malignant progression, tumor cell invasion and migration are emerging [20, 21]. However, the role of circulating exosomal miRNAs in PC has not been extensively investigated to date.

Recent studies have demonstrated that miRNA-21 and miRNA-210 are associated with a poor prognosis in PC; however, those results were based on tumor tissues and the small sample size limited the clinical significance. Moreover, there are fewer reports on the significance of circulating exosomal miRNAs as diagnostic markers of PC. The aim of the present study was to investigate the diagnostic relevance of two circulating serum exosomal miRNAs, namely miRNA-21 and miRNA-210, as novel serological biomarkers for PC.

Methods

Patients

A total of 40 patients at the Renmin Hospital of Wuhan University, China, were enrolled in the present study, and their clinicopathological characteristics were evaluated. Serum samples were obtained from all patients prior to specific treatment. Of those patients, 30 had PC and 10 had chronic pancreatitis (CP). The serum exosomal microRNAs in patients with PC were detected prior to surgical resection. The study

protocol was approved by the Ethics Committee of Renmin Hospital of Wuhan University. Informed consent was obtained from all patients.

Isolation and identification of exosomes from the serum of PC patients

Blood samples were collected from the patients and centrifuged at 500 x g for 5 min at 4 °C. The supernatants were preserved at -80˚C until use. Exosomes were isolated from serum samples using the Exosome extraction Kit (System Biosciences, Mountain View, CA, USA). Briefly, 500 µl serum was mixed with 120 µl ExoQuick solution and then incubated at 4 °C for 30 min. The mixed solution (ExoQuick/serum) was centrifuged at 12,000 x g for 2 min. Then, exosome pellets were obtained. Transmission electron microscopy (HT7800) was used to visualize and verify the exosomes that were extracted from the serum. The nanoparticle size distribution and concentration of vesicles were analyzed on the NanoSight LM10-HS instrument (NanoSight, Amesbury, UK) using NTA 3.0 software. Western blot analysis was performed using anti-CD63 (ARG57952, Arrigo Biolaboratories Corp, Taiwan, 1:500), anti-TSG101 (ab125011, Abcam, Cambridge, UK, 1:1000), and anti-CD81 (MA1-10290, Life Technologies Corporation, Carlsbad, CA, USA, 1:500) antibodies, which are protein markers and enriched in exosomes.
MicroRNA (miRNA) Extraction

miRNA extraction was performed using a miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The final volumes of RNA were standardized by dilution with 30 µL nuclease-free water. The RNA concentration was quantified using a Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Measurement Of MicroRNA Levels Using Reverse Transcription-quantitative PCR (qPCR)

To explore exosomal microRNAs, miRNA-21 and miRNA-210 were selected [22, 23]. The RNA was reverse-transcribed using the TaqMan microRNA Reverse Transcription Kit (Qiagen, Hilden, Germany). The mixture was incubated at 37 °C for 60 min and 94 °C for 5 min. qPCR was performed by using the TaqMan miRNA assay according to the manufacturer's protocol (Qiagen, Hilden, Germany) and Cq values were calculated. The miR-39 value was used as the internal control. The amplification conditions of the mixture were as follows: 95 °C for 15 min, followed by 45 cycles at 94 °C for 15 sec and at 55 °C for 30 sec. The relative miRNA expression values were normalized to Cel-miR-39 and calculated using the the $2^{-\Delta\Delta Cq}$ method.

Statistical analysis

The data were statistically analyzed using SPSS version 20.0 (IBM Corp.). The miRNA values are presented as mean ± standard deviation. The Student's t-test was used to analyze the differences between two groups. The diagnostic value of the candidate miRNAs was evaluated by calculating specificity and sensitivity. A P-value < 0.05 was considered to indicate a statistically significant difference.

Results

Patient Characteristics

The clinical characteristics of the patients are shown in Table I. The mean age was 62 years. The median diameter of the tumor was 4.2 cm in the PC group. The median level of serum CA19-9 was higher in the PC group compared with that in the CP group ($P < 0.05$), whereas no statistically significant differences were found in gender or age.

Identification Of Circulating Serum Exosomes

Exosomes were collected and observed using TEM (Fig. 1a), consistently with the characteristics of exosomes [24] (Fig. 1b). Abundant small vesicles were observed by a NanoSight in the range of 50–100 nm (Fig. 1c); the size and overall concentration were approximately 93 nm and 1.3×10^9 particles per mL, respectively (Figure. 1c). Known exosome-specific markers, such as CD63, CD81 and TSG101, were identified in the vesicles by western blotting; the vesicles obtained from pancreatic serum exosomes were compared with those of healthy controls (Fig. 1d).

Expression Level Of Serum Exosomal And Serum Circulating MiRNAs

The Cq value of Cel-miRNA-39 was used as the internal control. Compared with CP patients, the expression levels of serum exosomal miRNA-21 and miRNA-210 were obviously higher in PC patients (Fig. 2; $P < 0.05$). By contrast, there was no difference in the relative expression levels of serum circulating free miRNA-21 and miRNA-210 between the PC and CP groups (Fig. 2; $P > 0.05$).

Comparison of the Diagnostic Values of exosomal miRNAs, serum circulating miRNAs, and CA19-9 Quantitation

The diagnostic value of serum circulating free or exosomal miRNA-21and miRNA-210 was investigated. The accuracies of exosomal miRNA-21 (80%) and exosomal miRNA-210 (85%) were superior to those of serum free miRNA-21 (73%) and miRNA-210 (78%). When combining the results of exosomal miRNA-21 and exosomal miR-210, the accuracy, specificity and sensitivity were 90%, 80% and 93%, respectively. Taking the positive results of either exosomal miRNA-21 or exosomal miRNA-210 levels as the criterion for diagnosis of PC, the specificity of the combined test (with CA19-9) was 90%/90% and the sensitivity was 90%/90% (Table II).

Serum exosomal miRNA-21 and miRNA-210 levels are correlated with multiple prognostic factors of PC

The associations between the relative levels of these miRNAs and clinical characteristics were evaluated. According to high and low expression levels of miRNA-21 (≥ 0.09 vs. <0.09) and miRNA-210 (≥ 0.0016 vs. <0.0016), the patients were divided into two groups. In the present study, we found that the expression levels of miRNA-21 and miRNA-210 were associated with the TNM stage, and the levels of these miRNAs are closely correlated with advanced disease ($P < 0.05$) and other prognostic factors (Table III).

Discussion

Recently, the abnormal expression of serum exosomal miRNAs has emerged as a novel potential biomarker of tumor diagnosis and progression, due to their stability in the plasma/serum and their specific expression profiles, which are reflecting the gene information and biological properties of tumor cells [25, 26]. These serum exosomal microRNAs may be used as non-invasive, specific and sensitive biomarkers for the diagnosis and prognosis of tumors [27]. Alterations of circulating microRNAs have been investigated in patients with PC. However, studies on serum exosomal microRNAs in PC are lacking. In the present study, we investigated the levels of free and exosomal miRNA-21 and miRNA-210 in PC patients.

Numerous studies indicate that serum exosomal miRNAs are closely correlated with tumor-derived microRNAs, and that these miRNAs may be of value as diagnostic biomarkers for cancer [28, 29]. In the present study, compared with the CP group, the expression levels of serum exosomal miRNA-21 and miRNA-210 were obviously higher in the PC group. By contrast, the expression levels of serum free miRNA-21 and miRNA-210 did not differ statistically significantly between these two groups. Whole circulating peripheral blood contains other miRNAs in addition to exosomal miRNAs, which are easily degraded, probably because these miRNAs are not enclosed in exosomes [30, 31]. We hypothesized that exosomal rather than free serum miRNAs were preferable and may serve as a new biomarker for PC, as serum free miRNAs could not distinguish between PC and CP patients. In fact, serum exosomal miRNAs originating from tumor-derived miRNAs may be used as novel diagnostic biomarkers [32]. Furthermore, exosomal miRNAs in the serum or plasma and other bodily fluids may be more stable and valuable as biomarkers for PC detection compared with serum free miRNAs.

miRNA-21 is upregulated in different types of tumors and targets tumor-suppressive mRNAs. It was demonstrated that overexpression of serum ex-miRNA-21 was diagnostic of pancreatic ductal adenocarcinoma [33]. By contrast, miRNA-210 acts as an oncogene, and has been shown to be upregulated in various types of tumors compared with adjacent normal tissues, contributing to the progression and development of various cancers, including lung cancer and PC via different signaling pathways [34, 35]. The possible clinical application of miRNA-210 in diagnosing and detecting cancers was previously investigated [36]. Moreover, the serum levels of miRNA-210 were previously found to be induced under hypoxic conditions and linked to adverse prognosis in some cancers [37]; as hypoxic environment is common in PC [38, 39], it may be worth investigating the potential diagnostic value of serum exosomal miRNAs for patients with PC. The analysis of exosomal miRNA-21 and miRNA-210 levels among circulating serum miRNAs may represent a feasible strategy for PC diagnosis. It may be inferred that exosomal miRNAs in the serum directly reflect the properties of tumor cells, as well as various diseases, as the quantity and content of exosomes may be reflective of the pathophysiological state of the cells [40].

In the present study, we compared the potential diagnostic value of serum exosomal miRNAs with the serum levels of CA19-9. Exosomal miRNA-21 and miRNA-210 levels may be a new diagnostic or therapeutic target for early PC, while CA19-9 levels remained within the normal range. The sensitivity of the serum test was increased to 90% when combining positive results for exosomal miRNA-21 and

miRNA-210 levels with serum CA19-9 levels, while the specificity remained 90%. Serum exosomes are a valuable tool, and different methods may be used to explore their components, which may lead to more accurate, sensitive, cost-effective, and high-throughput diagnoses. The present study demonstrated that the serum exosomal miRNA-21 and miRNA-210 levels were closely related to disease state and other prognostic factors, and they may be used for early diagnosis and prediction of prognosis non-invasively. These investigations reveal that these serum exosomal miRNAs are potential valuable biomarkers for the diagnosis, screening and prognosis of PC.

The limitations of the present study included the insufficient sample size, which may affect the diagnostic value of exosomal miRNAs. In addition, the expression level of serum exosomal miRNAs was not compared between normal controls and PC tissues. The mechanisms regulating transfer of exosomal miRNAs from tumor cells into the blood is rarely reported, while the association between serum and tissue levels of exosomal miRNAs should be a focus of future research. Furthermore, exosomal miRNAs in the plasma, pancreatic juice, bile, urine, saliva, breast milk and other bodily fluids have not yet been investigated as diagnostic biomarkers, and will be examined in future studies. In addition, the level of change of circulating exosomal miRNAs was not examined before and after treatment in PC patients. Finally, the effect of the degree of circulating exosomal miRNA expression on disease progression, tumor cell metastasis and overall survival cannot be concluded from our results due to the small number of PC patients and the short follow-up period, and must be addressed in the future.

Conclusions

The present study demonstrated that serum exosomal miRNA-21 and miRNA-210 levels distinguished effectively between patients with PC and those with CP. Furthermore, the combination of exosomal miRNAs with CA19-9 was more specific and sensitive compared with serum tests alone. These findings indicate that circulating exosomal miRNAs miRNA-21 and miRNA-210 may represent novel biomarkers and therapeutic targets for PC. Moreover, the quantitation of exosomal miRNAs may be used as a clinical examination for further confirmation of diagnosis. Further investigation of a large number of cases is needed to determine the potential diagnostic, prognostic and clinical value of miRNA-21 and miRNA-210 in PC patients.

Abbreviations

PC: pancreatic cancer; CP: chronic pancreatitis; CA19-9: Carbohydrate antigen199.

Declarations

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to designing and drafting this manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

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Tables

Table 1 Characteristics of patients with pancreatic adenocarcinoma and chronic pancreatitis

	PC (N = 30)	CP (N = 10)	p-Value
Age, years [median (range)]	62 (30–80)	50.5 (34–80)	0.17
Sex (male/female)	18/12	8/2	0.64
Location (head/body/tail)	12/8/10	-	
Stagea (0/IA/IB/IIA/IIB/III/IV)	1/0/1/4/14/2/8	-	
T factor (cis/1/2/3/4)	1/1/4/20/4	-	
CA19-9, U/mL (median (range))	104.3 (0.5-1500)	15.00 (4.0-100.5)	0.01

PC pancreatic carcinoma, CP chronic pancreatitis, CA19-9 carbohydrate antigen 19-9

Table 2 Diagnostic value of Ex-miR or serum Fr -miRNA21 and -miRNA210 level for pancreatic cancer.

Ex-miR Exosomes microRNA, Fr -miRNA Free microRNA

	TP	FN	FP	TN	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)
Ex-miR-21	24	6	1	9	80	90	83	96	60
Ex-miR-210	25	5	1	9	83	90	85	96	64
Ex-miR-21/210	28	2	2	8	93	80	90	93	80
Free -miR21	22	8	3	7	73	70	73	88	47
Free -miR210	23	7	2	8	76	80	78	92	53
Ex-miR-21/ CA19-9	27	3	1	9	90	90	90	96	75
Ex-miR-210/ CA19-9	27	3	1	9	90	90	90	96	75

TP true positive, FN false negative, FP false positive, TN true negative, PPV positive predictive value, NPV negative predictive value, Ex-miR exosomal microRNA, Fr-miR free microRNA

	Serum exosomal miRNA-21 level			Serum exosomal miRNA-210 level		
	Low group (miRNA-21 <0.09) (n=12)	High group (miRNA-21 ≥0.09) (n=18)	P value	Low group (miRNA-21 <0.09) (n=16)	High group (miRNA-21 ≥0.09) (n=14)	P value
Age, years (<60/ ≥60)	5/7	7/11	0.06	5/11	6/8	0.007
male/female	8/4	12/6	1	12/4	11/3	0.311
Treatment history, no/yes	10/2	15/3	0.046	9/7	8/6	0.026
Location (head/body/tail)	5/3/4	5/7/6	0.01	4/6/6	3/5/6	0.004
Tumor variables						
Size of tumor<5cm/ ≥5cm	7/5	10/8	0.038	10/6	8/6	0.016
T stage, 1-2/3-4	4/8	7/11	0.011	16/0	12/2	0.006
N stage, 0/1	9/3	17/1	0.03	8/8	7/7	0.02
M stage, 0/1	10/2	18/0	0.25	14/2	11/3	0.201
CA19-9, U/ml (<37/≥37)	7/5	10/8	0.016	7/9	10/4	0.018
CRP, mg/L (<10/ ≥10)	4/8	10/8	0.02	9/5	10/4	0.013

Table 3. Correlations between expression levels of both serum exosomal miRNA-21 and exosomal miRNA-210 level and clinical characteristics (n=30)

Abbreviations: CA19-9: carbohydrate antigen 19-9; CRP, C-reactive protein

Figures

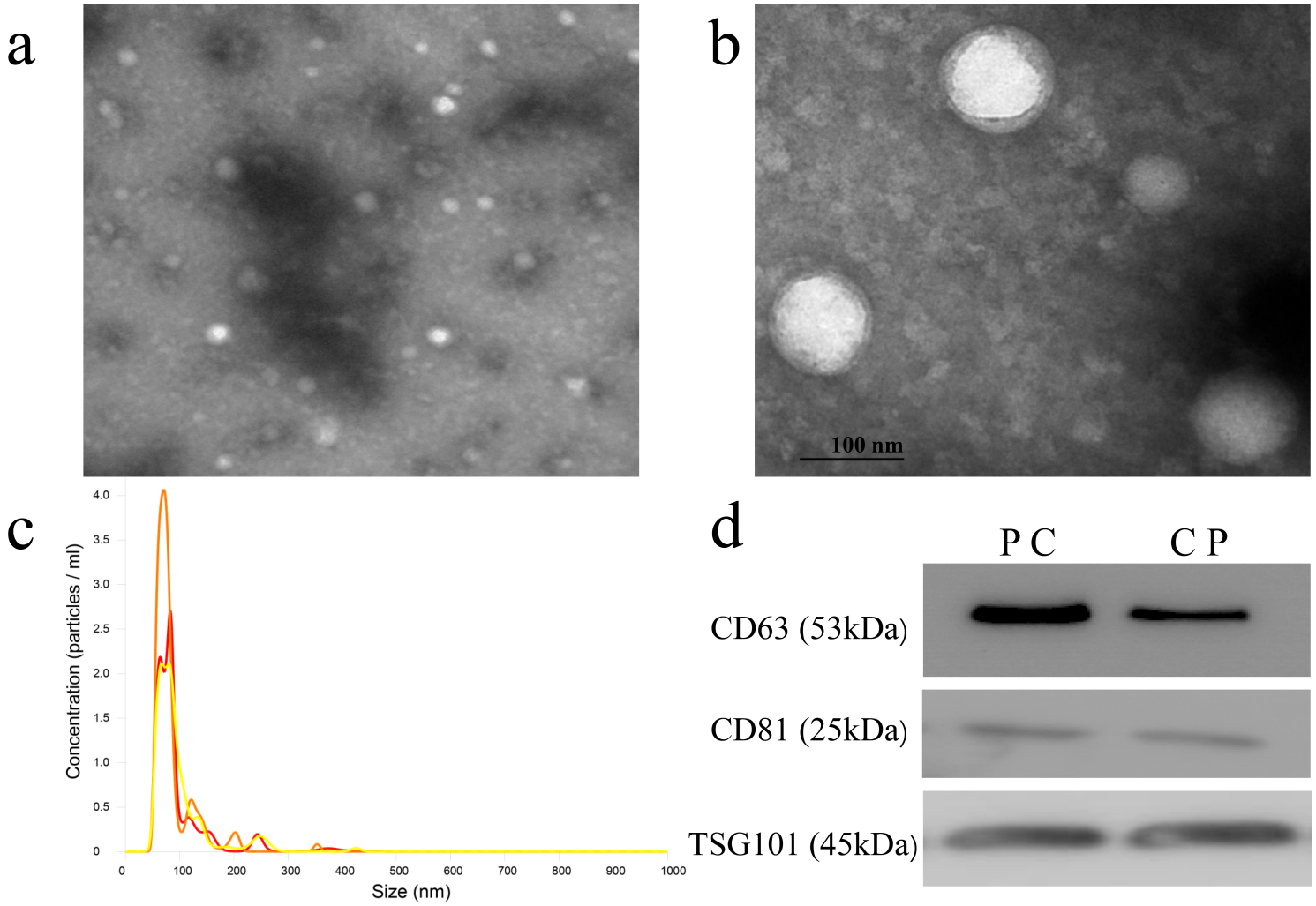

Figure 1

Exosomes are seen as small vesicles in the serum imaged using transmission electron microscopy with the negative stain method (a, b). The particle size distribution and concentration of exosomes isolated from serum were 93 nm and 1.3x109 particles/mL using a NanoSight (c). Exosomes extracted from serum were analyzed by western blotting using anti-CD63, -CD81 and -TSG101 antibodies. CD63, CD81 and TGS101 were present in the exosomes. PC pancreatic cancer, CP chronic pancreatitis.

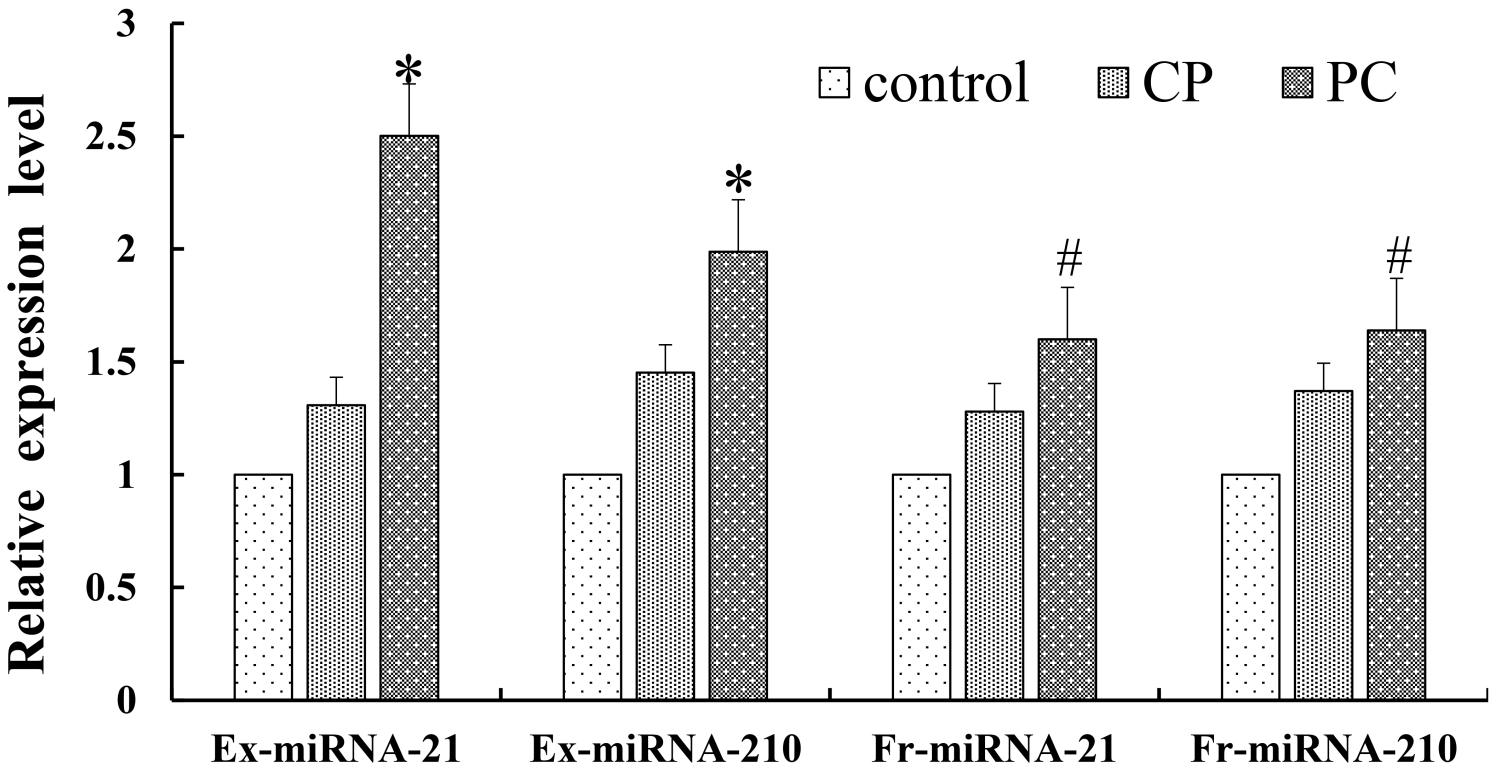

Figure 2

Expression level of miRNA-21 and miRNA-210 in the serum of patients with PC and CP. Compared with CP patients, the expression levels of miRNA-21 and miRNA-210 in exosomes purified from serum (ex-miR-21 and miR-210) were obviously higher in PC patients (*P<0.05). The relative expression of free miRNA-21 and free miRNA-210 in the serum was not significantly different between PC and CP patients (#P>0.05). CP chronic pancreatitis, PC pancreatic carcinoma, Ex-miRNA exosomal microRNA, Fr-miRNA free microRNA.

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