Int J Clin Exp Pathol 2017;10(12):11673-11678
www.ijcep.com /ISSN:1936-2625/IJCEP0047553

Original Article

Serum miR-133b is a potential novel prognostic biomarker for colorectal cancer

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Received December 28, 2016; Accepted January 25, 2017; Epub December 1, 2017; Published December 15, 2017

Abstract: Background: Deregulation of microRNA-133b (miR-133b) has been demonstrated to contribute to the initiation and development of various cancers including colorectal cancer (CRC). However, the correlation between serum miR-133b levels and the prognosis of CRC remains elusive. Methods: A total of 98 CRC patients, 30 patients with precancerous polyps and 40 healthy volunteers were enrolled in this study. Quantitative real-time PCR (qRT-PCR) was performed to measure the serum miR-133b expression level in all the participants. The chi-square test was carried out to compare the categorical variables. Furthermore, survival analysis and cox proportional hazards *regression model* were used to determine the prognostic significance of serum miR-133b in CRC. Results: Serum miR-133b level was progressively down-regulated from healthy controls, patients with precancerous polyps to CRC patients. CRC patients with higher clinical stage or lymph node metastasis had lower serum miR-133b levels. A positive correlation was found between decreased serum miR-133b levels and distant metastasis, lymph node metastasis and clinical stage. Additionally, CRC patients with high serum miR-203 levels had better 5 year overall survival and relapse free survival rates. Finally, low serum miR-133b level was demonstrated to be an independent risk factor for CRC. Conclusion: These findings suggest that miR-133b might serve as a novel potential prognostic biomarker for CRC.

Keywords: Colorectal cancer, microRNA-133b, serum, prognosis

Introduction

Colorectal cancer (CRC) is one of the most frequent lethal malignancies worldwide and the fourth leading cause of cancer-related death in China. Though the incidence and mortality rates of CRC in China are still considerably lower than those in developed countries, the number of CRC deaths is reported to be steadily increasing [1-4]. Unfortunately, most CRC patients present at advanced stages, leading to the poor clinical outcome of this deadly disease. Therefore, identifying biomarkers for surveillance, diagnosis and prediction of prognosis is important for individualized therapy of CRC.

MicroRNAs (miRNAs) are small size, highly conserved non-coding RNAs which regulate expression of multiple genes *via* interactions with the 3' untranslated regions (UTR) of target mRNAs, leading to target degradation or translation suppression [5]. Biologically, miRNAs play a central role in regulating cancer cell proliferation, migration and invasive capacity. Pathologically, aberrant miRNAs expression was observed in various types of tumors including CRC [6, 7]. For instance, Feng et al showed that miRNA-141 levels were downregulated in the clinical samples derived from CRC patients. In addition, miRNA-141 might exert its tumor suppressive function by downregulating mitogen-activated protein kinase 4 [8]. MiR-410 was overexpressed in CRC tissues and demonstrated to function as an oncomiR by decreasing FHL1 expression, which was a known tumor suppressor in many types of cancers [9].

Previous studies have shown that miR-133b acted as a tumor suppressor in many different types of cancers including CRC [10-12]. However, the correlation between serum miR-133b expression level and the prognosis of CRC *has* not *yet* been *elucidated*. In this study,

Table 1. Relation between serum miR-133b levels and clinical characteristics in CRC patients

Variables	No. of patients	miR-133b low	miR-133b high	*P*
Gender				0.300
Male	54	29	25	
Female	44	19	25	
Age (years)				0.193
<60	37	15	22	
≥60	61	33	28	
Differentiation				0.372
Well/moderate	81	38	43	
Poor	17	10	7	
Location				0.849
Colon	46	23	23	
Rectum	52	25	27	
T classification				0.527
T1/T2	21	9	12	
T3/T4	77	39	38	
Distant metastasis				0.040
M0	83	37	46	
M1	15	11	4	
Node stage				0.005
N0	57	21	36	
N1-N2	41	27	14	
AJCC stage				0.001
I/II	45	14	31	
III/IV	53	34	19	

we aimed to explore the potential clinical significance of serum miR-133b in CRC.

Materials and methods

CRC patients

In this study, 98 patients with CRC and 30 patients with precancerous polyps were recruited prior to any treatment. Forty volunteers between 34 to 68 years old were also enrolled as healthy controls. Clinical stage of tumors was assessed according to the American Joint Committee on Cancer (AJCC) Staging System. The clinical characteristics of CRC patients were summarized in **Table 1**. This study was performed with the approval of Qilu Hospital of Shandong University and written informed consent was received from all the patients.

Serum sample collection and storage

Pre-operative blood samples were taken from healthy controls, patients with precancerous polyps patients with CRC. A sample (approximately 5 ml) of fasting venous blood was withdrawn from each participant. Within 30 min after collection, all samples were processed and centrifuged at 1500 g for 20 min. Then the separated supernatant was divided into aliquots and stored in cryotubes at -80°C until further processing.

RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from 200 μL serum samples by using a miRVana PARIS Kit (Ambion, Austin, TX, USA) and quantified by *Eppendorf* Biophotometer according to the manufacturer's instructions. Reverse transcription was carried out with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Shanghai, China). Amplifications were performed using the Maxima SYBR Green qPCR Kit (Thermo Scientific, CA, USA) on a Roche LC480II lightcycler (Roche, Indianapolis, IN, USA). Each sample was analyzed in triplicate. The expression levels of miR-133b were normalized with respect to RNU6, and relative quantification values of miR-133b expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All statistical analyses were performed using SigmaPlot 13.0 (Systat, Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software Inc., San Diego, California, USA). The statistical difference of serum miR-133b expression levels among different groups was analyzed using Kruskal-Wallis test. The Chi-square test was performed to compare the categorical variables. Moreover, Kaplan-Meier survival analysis plus a log rank test were used to analyze the correlation between serum miR-133b expression levels and the status of the CRC patients with respect to overall survival (OS) or relapse free survival (RFS). Cox proportional hazards models were used for multivariate analyses of prognostic factors. Differences were considered statistically significant at $P<0.05$.

Results

Decreased expression of serum miR-133b in CRC patients

The expression level of serum miR-133b in CRC was determined by qRT-PCR. The results

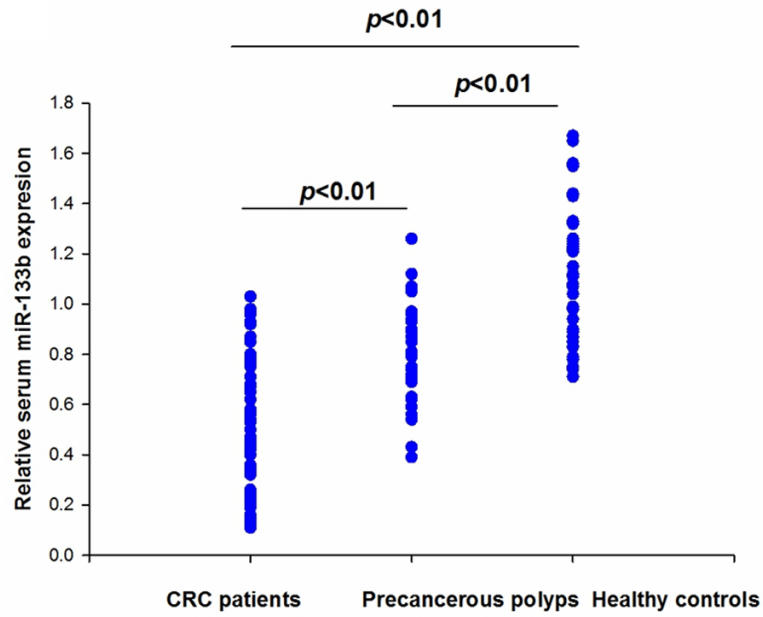

Figure 1. Serum miR-133b expression levels were progressively downregulated from healthy controls, patients with precancerous polyps to CRC patients.

showed that serum miR-133b expression level was highest in healthy controls, while progressively reduced from patients with precancerous polyps to CRC patients (P<0.01) (**Figure 1**).

CRC patients in the higher AJCC stage had a significantly lower serum miR-133b levels compared to those in the lower stage (P<0.01) (**Figure 2A**). In addition, the expression level of serum miR-133b was lower in CRC patients with lymph node metastasis compared to the patients without lymph node metastasis (P<0.01) (**Figure 2B**).

Association between serum miR-133b and clinical parameters of CRC patients

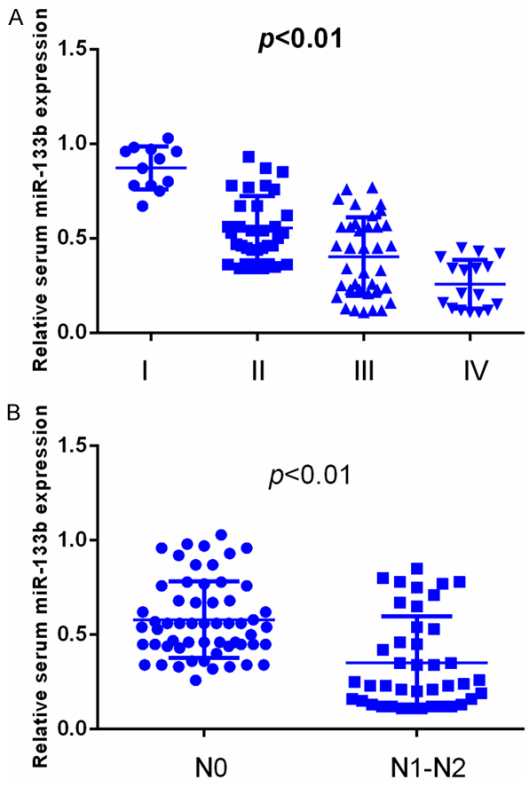

Figure 2. CRC patients with higher AJCC stage or lymph node metastasis had lower serum miR-133b levels.

To investigate the correlation of serum miR-133b expression level with the clinicopathological features of CRC patients, we divided the CRC patients into miR-133b-high (n=50) and miR-133b-low (n=48) expression groups according to the median expression level of serum miR-133b. As shown in **Table 1**, serum miR-133b expression level was dramatically correlated with several clinical parameters including distant metastasis (P=0.005), node stage (P=0.040) and TNM stage (P=0.001). No significant associations were found between serum miR-133b expression level with gender (P=0.300), age (P=0.193) differentiation (P=0.372), location (P=0.849) and T classification (P=0.527).

Decreased miR-133b expression predicts poor clinical outcome of CRC patients

Kaplan-Meier curves were constructed to evaluate 5 year OS and RFS of CRC patients. We found that the CRC patients in high serum miR-133b group had better 5 year OS (P=0.018) and RFS (P=0.006) than those in low serum miR-133b group (**Figures 3**, **4**), indicating that low serum miR-133b expression level was closely correlated with unfavorable prognosis of CRC.

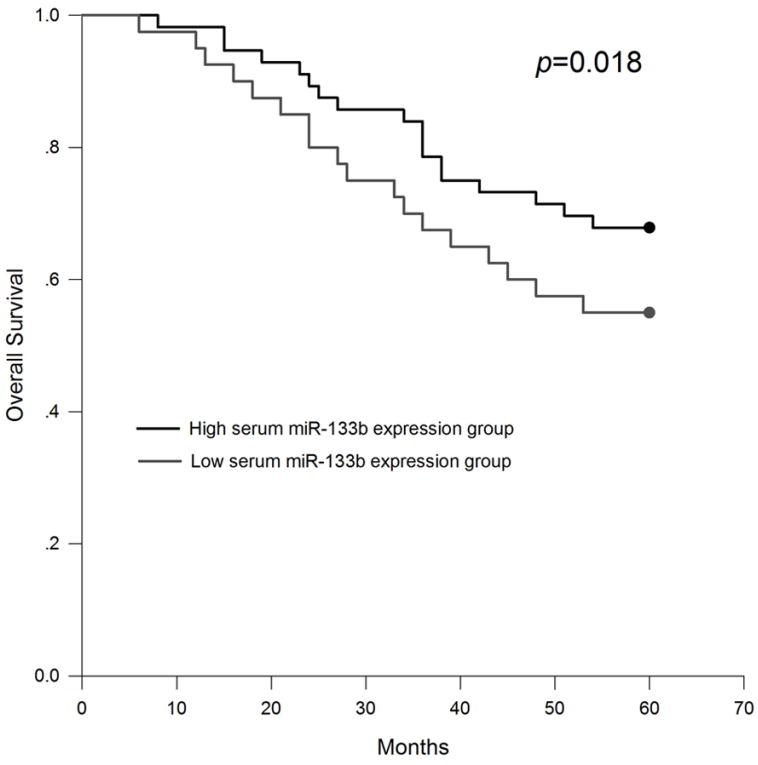

Figure 3. Kaplan-Meier curve of OS of CRC patients stratified by the serum miR-133b level.

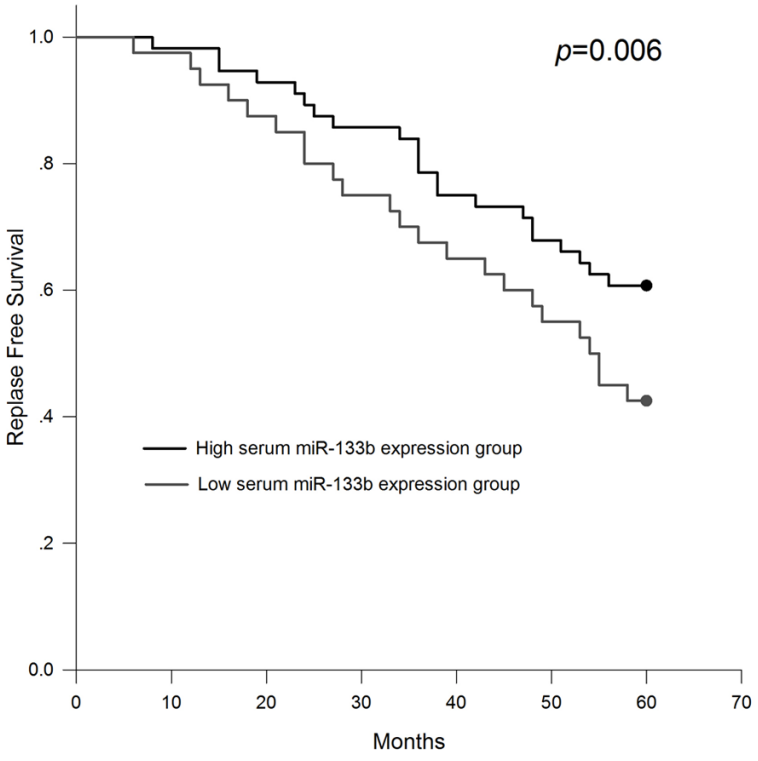

Figure 4. Kaplan-Meier curve of RFS of CRC patients stratified by the serum miR-133b level.

In addition, multivariate analysis identified node stage (OS: HR=2.79, 95% CI=1.36-4.22, P=0.027; RFS: HR=3.14, 95% CI=1.41-4.79, *P*=0.014), AJCC stage (OS: HR=4.57, 95% CI=1.89-7.05, P=0.006; RFS: HR=4.82, 95% CI=1.75-7.84, *P*=0.003) and serum miR-133b level (OS: HR=3.13, 95% CI=1.43-5.23, P=0.017; RFS: HR=3.59, 95% CI=1.55-5.98, P=0.009) as independent risk factors for both OS and RFS (**Table 2**).

Discussion

CRC is a major cause of *morbidity and mortality* throughout the world and remains to be a significant public health issue [1, 13, 14]. Therefore, revealing the hidden mechanisms is very important to offer efficient therapy for CRC patients.

In the current study, we found that serum miR-133b expression was greatly down-regulated in patients with CRC. Also, loss of serum miR-133b expression was associated with poor clinical parameters and shorter OS/RFS in CRC, suggesting that miR-133b acted as a tumor suppressor in CRC and enforced miR-133b expression might inhibit the development of this malignancy. Consistent with our findings, miR-133b was found to be remarkably underexpressed in CRC tissues compared to adjacent normal tissues. In addition, miR-133b suppressed the proliferation of CRC cells by directly inhibiting TATA box-binding protein-like protein 1 [10]. Similarly, miR-133b levels were especially lower in metastatic CRC tissues. Increased miR-133b expression inhibited invasion

Table 2. Multivariate analysis of the impact of clinical parameters on overall survival and relapse free survival in CRC patients

Parameters	Overall survival			Relapse free survival		
	HR	95% CI	*P*	HR	95% CI	*P*
Distant metastasis	1.82	0.91-2.76	0.081	1.65	0.89-2.91	0.157
Node stage	2.79	1.36-4.22	0.027	3.14	1.41-4.79	0.014
AJCC stage	4.57	1.89-7.05	0.006	4.82	1.75-7.84	0.003
Serum miR-133b	3.13	1.43-5.23	0.017	3.59	1.55-5.98	0.009

and stimulated apoptosis of CRC cells by down-regulating CXC chemokine receptor-4 [15].

Besides CRC, down-regulation of miR-133b has also been reported in various human malignancies. In non-small cell lung cancer and esophageal squamous cell carcinoma, it was demonstrated that suppression of miR-133b significantly promoted the migration and invasion of cancer cells by directly regulating fascin1 [11, 12]. Moreover, it was reported that ectopic expression of miR-133b inhibited cell proliferation, migration, invasion and progression in gastric cancer *via* regulating different downstream targets such as polypyrimidine tract-binding protein 1 [16], specificity protein 1 [17] and fibroblast growth factor receptor 1 [18]. Chang et al showed that miR-133b suppressed glioblastoma cell migration and invasion by regulating matrix metalloproteinase 14 in glioblastoma [19].

The role of miR-133b in tumorigenesis seems to be very complicated. It has been suggested to play an oncogenic role in cancers. Qin et al showed that enhanced miR-133b expression promoted cervical carcinoma cell proliferation *in vitro* as well as tumor growth and metastasis *in vivo*. Mammalian sterile 20-like kinase 2, cell division control protein 42 homolog and ras homolog gene family member A were identified as its downstream targets [20]. Hence, the controversial features of miR-133b in various cancers reflected that the biological function miR-133b was cell type dependent and closely related with the tumor microenvironment. Further exploration of the underlying mechanisms is required.

In conclusion, this study shows that serum miR-133b expression is markedly decreased in CRC patients and in turn contributes to aggressive progression of this deadly disease. These findings suggest that serum miR-133b might serve as a potential prognostic biomarker for CRC patients.

Acknowledgements

This work was supported by the National Natural Science Fund from the National Natural Science Foundation of China (grant nos. 81672427).

Disclosure of conflict of interest

None.

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