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Upregulation of serum miR-103 predicts unfavorable prognosis in patients with colorectal cancer

D.-S. WANG[1], B. ZHONG[2], M.-S. ZHANG[1], Y. GAO[1]

[1]Department of General Surgery, the Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, China
[2]Department of Hyperbaric Oxygen, the Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, China

Abstract. – OBJECTIVE: Circulating microRNAs (miRNAs) have been reported as biomarkers for the early detection of colorectal cancer (CRC). We aimed at evaluating the diagnostic and prognostic value of serum miR-103 in CRC patients.

PATIENTS AND METHODS: Quantitative reverse-transcription PCR (qRT-PCR) was applied to measure the miR-103 levels in blood samples of 96 patients and 60 controls.

RESULTS: Our results demonstrated that serum miR-103 was overexpressed in CRC subjects and the receiver operating characteristic (ROC) curve analysis showed that serum miR-103 could differentiate CRC cases from controls with relatively high accuracy. In addition, serum miR-103 level was more frequently detected in CRC patients with positive lymph node metastasis, distant metastasis and advanced tumor stage. Moreover, serum miR-103 levels in 23 postoperative blood samples were lower than paired preoperative plasma specimens, and serum miR-103 levels were re-elevated in seven patients at recurrence. Furthermore, serum miR-103 was significantly correlated with worse clinical factors, as well as poorer recurrence-free survival or overall survival. Finally, multivariate analysis confirmed that serum miR-103 was an independent prognostic marker for CRC.

CONCLUSIONS: Taken together, serum miR-103 might be a promising biomarker for diagnosis and prognosis of CRC.

Key Words:
Colorectal cancer, Serum miR-103, Prognosis, Diagnosis, Biomarker.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and the fourth leading cause of cancer death worldwide. In China, the mortality of CRC ranked fifth among tumor-related deaths, with an estimated 310,000 new cases and 149,000 people died from this disease in 2011[1,2]. Although surgical and medical treatments have substantially improved over the past decades, the overall 5-year survival rate remains dismal. Currently, most cases are diagnosed in the advanced stage because of the asymptomatic nature of CRC at earlier stage[3,4]. Hence, it is of critical importance to identify novel and reliable biomarkers for improving the prognosis of CRC patients. MicroRNAs (miRNAs) are a class of small, non-coding RNAs, which function as post-transcriptional regulators by binding to the 3′-untranslated region (3′-UTR) of target messenger RNA. Many miRNAs have been found to be aberrantly expressed in tumors and involve in the initiation and progression of various cancer types[5,6]. Additionally, miRNAs have been reported to be stably detected in serum or plasma, and used as biomarkers for early diagnosis of human cancers[7]. Previous studies have revealed that some circulating miRNAs could discriminate CRC patients from normal controls. For example, serum miR-200c overexpression was closely correlated with poor prognosis and showed great potential for CRC screening[8]. Similarly, serum miR-139-3p expression was greatly downregulated in CRC patients, and could well distinguish CRC patients from control subjects with high accuracy[9]. miR-103 has been reported to be dysregulated in several human cancers, such as CRC[10-13], malignant mesothelioma14, gastric cancer[15], hepatocellular carcinoma[16], triple-negative breast cancer[17,18]. However, the clinical significance of serum miR-103 for the diagnosis and prognosis of CRC patients is poorly understood. In this study, we aimed to detect the expression levels of serum miR-103 in

Corresponding Author: Bei Zhong, MD; e-mail: beizhong_ahqu@163.com

CRC patients and healthy controls, and evaluate its potential as indicator for CRC screening.

Patients and Methods

Study Population and Blood Samples

This study included pre-operative blood samples from 96 subjects diagnosed with CRC. Patients treated with chemotherapy or radiotherapy prior to any treatment were excluded. Moreover, twenty-three paired plasma specimens were collected from stage II-III CRC patients 2 months after curative resection. Blood samples were also obtained from 60 healthy volunteers and used as controls. Tumors were staged according to the classification guidelines of the American Joint Committee on Cancer (AJCC). All samples were centrifuged at 2000 x g for 10 min at room temperature, and the supernatant was stored in cryotubes at -80°C until further analysis. All the CRC patients received regular follow-up. Overall survival (OS) was defined as the time from surgery to the date of death or last follow-up. Recurrence-free survival (RFS) was measured as the time from surgery to the date of recurrence or death or last follow-up. This study was approved by the Ethics Committee of Affiliated Hospital of Qingdao University and written informed consent was obtained from each participant.

Total RNA Extraction and qRT-PCR

Total RNA extraction from all serum samples was processed using the miRNeasy RNA isolation Kits (Qiagen Inc, Valencia, CA, USA). The reverse transcription reaction was performed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). PCR reaction for quantification of miR-103 was carried out in triplicate using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The relative serum miR-103 expression was normalized against the small nucleolar RNA RNU6b using the 2-ΔΔCt method.

Carbohydrate Antigen 19-9 (CA 19-9) and Carcinoembryonic antigen (CEA) Detection

CA 19-9 and CEA were usually used as biomarkers in CRC. In this study, we detected the levels of serum CA 19-9 and CEA in 96 cases with CRC by using electrochemiluminescence (ECL) on a Cobas e-602 analyzer (Roche Diagnostics, Mannheim, Germany).

Statistical Analysis

Statistical analyses were performed using MedCalc 7.2 (MedCalc, Mariakerke, East Flanders, Belgium) and Prism 5.01 (GraphPad Software Inc., San Diego, CA, USA). Mann-Whitney U-test was used to compare the differences in serum miR-103 levels between the two groups. Receiver operating characteristic (ROC) curves and area under the ROC curve (AUC) were established to analyze the diagnostic value of serum miR-103 for CRC. The association between serum miR-103 expression and clinical factors was evaluated by the Pearson x2-test. Survival curves were constructed using the Kaplan-Meier method, and differences were determined using the log-rank tests. Multivariate Cox proportional hazards model was used to assess risk ratios for prognostic analysis. *p*-value less than 0.05 was defined as a statistically significant difference.

Results

Up-regulation of miR-103 in CRC Blood Samples and ROC Curve Analysis

The relative miR-103 expression in plasma was detected in all the CRC patients and controls by qRT-PCR. As shown in Figure 1, miR-103 expression was dramatically elevated in CRC patients compared with healthy subjects ($p<0.01$). Next, ROC curve was plotted to assess the diagnostic value of serum miR-103. As shown in Figure 2, serum miR-103 could well distinguish CRC patients from normal controls with AUC value of 0.857. The diagnostic specificity and sensitivity of serum miR-103 were 80.0% and 88.5%, respectively.

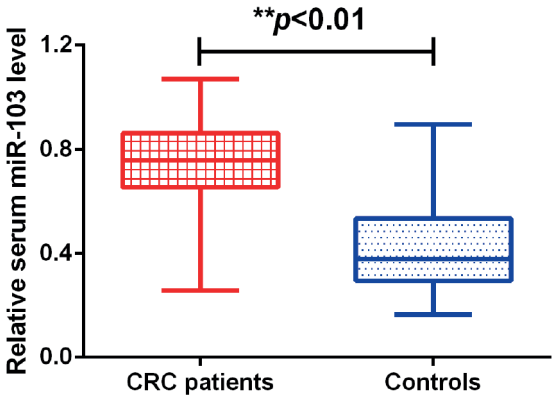

Figure 1. Serum miR-103 expression was significantly elevated in CRC patients compared to controls.

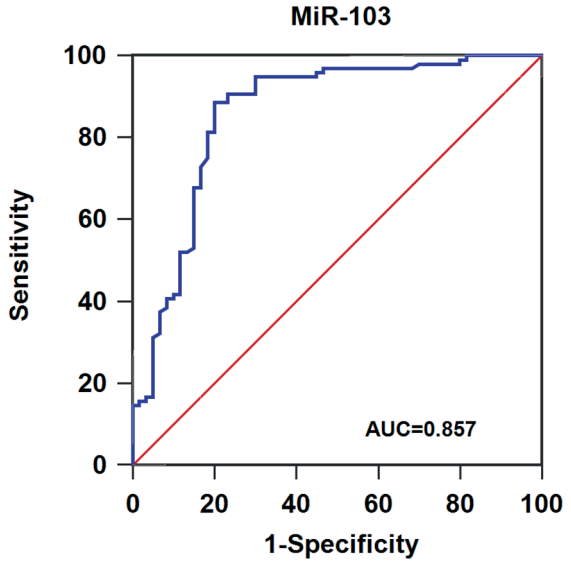

Figure 2. ROC curve was drawn for serum miR-103, and an AUC value of 0.857 was yielded.

Correlation of Serum miR-103 Expression with the Clinicopathological Features of CRC

All the CRC subjects were separated into high expression group (n=47) and low expression group (n=49) based on the median miR-103 level. As shown in Table I, the results revealed that high miR-103 expression was closely associated with distant metastasis (p=0.0209), lymph node metastasis (p=0.0395) and tumor stage (p=0.0021). In contrast, there was no significant correlation between miR-103 expression and patients' gender, age, tumor size, differentiation, CA 19-9 and CEA (all p>0.05).

Furthermore, serum miR-103 expression was more frequently detectable in patients with lymph node metastasis (n=51) than in those without (n=45, p<0.05, Figure 3A). Moreover, a significant increase in serum miR-103 expression was observed in patients (n=32) with distant metastasis compared to those without (n=64, p<0.05, Figure 3B). In addition, miR-103 levels were greatly upregulated in the serum of advanced stage (III-IV) patients (n=63) compared with those in early stage (I-II) cases (n=33, p<0.05, Figure 3C).

Correlation Between Serum miR-103 Expression and Treatment Response

We found the serum miR-103 levels in 23 pre-operative serum were significantly higher than paired post-operative serum (p<0.01, Figure 4). More interestingly, serum miR-103 levels were re-elevated in 7 cases that developed recurrence during follow-up.

Correlation between Serum miR-103 Expression and Prognosis in CRC Patients

The Kaplan-Meier method plus log-rank test revealed that CRC patients in high miR-103 expression group displayed significantly shorter OS (p=0.014, Figure 5A) and RFS (p=0.008, Figure 5B). Multivariate analysis revealed that lymph node metastasis (RR=1.54, 95% CI=0.95-2.13, p=0.029), distant metastasis (RR=1.88, 95% CI=1.16-2.71, p=0.020), tumor stage (RR=3.73, 95% CI=1.51-6.14, p=0.003) and serum miR-103 (RR=2.36, 95% CI=1.32-3.50, p=0.014) were strongly associated with RFS. Also, multivariate analysis showed that lymph node metastasis (RR=1.32, 95% CI=0.87-1.85, p=0.034), distant metastasis (RR=1.67, 95% CI=1.09-2.37, p=0.025), tumor stage (RR=3.55, 95% CI=1.47-

Table I. Correlation of serum miR-103 expression with clinicopathological characteristics of 96 CRC cases.

Clinicopathological factors	Cases (n=96)	High miR-103 (n=47)	Low miR-103 (n=49)	p
Gender				0.5601
Male	58	27	31	
Female	38	20	18	
Age				0.0674
< 60	40	24	16	
≥ 60	56	23	33	
Lymph node metastasis				0.0395*
Positive	51	30	21	
Negative	45	17	28	
Tumor size (cm)				0.2916
< 4	42	18	24	
≥ 4	54	29	25	
Distant metastasis				0.0209*
Positive	32	21	11	
Negative	64	26	38	
Differentiation				0.1467
Well/Moderate	52	29	23	
Poor	44	18	26	
Tumor stage				0.0021*
I-II	33	9	24	
III-IV	63	38	25	
CA 19-9				0.2270
< 35 U/mL	39	22	17	
≥ 39 U/mL	57	25	32	
CEA				0.1502
< 5 ng/mL	46	19	27	
≥ 5 ng/mL	50	28	22	

*Statistically significant (p<0.05).

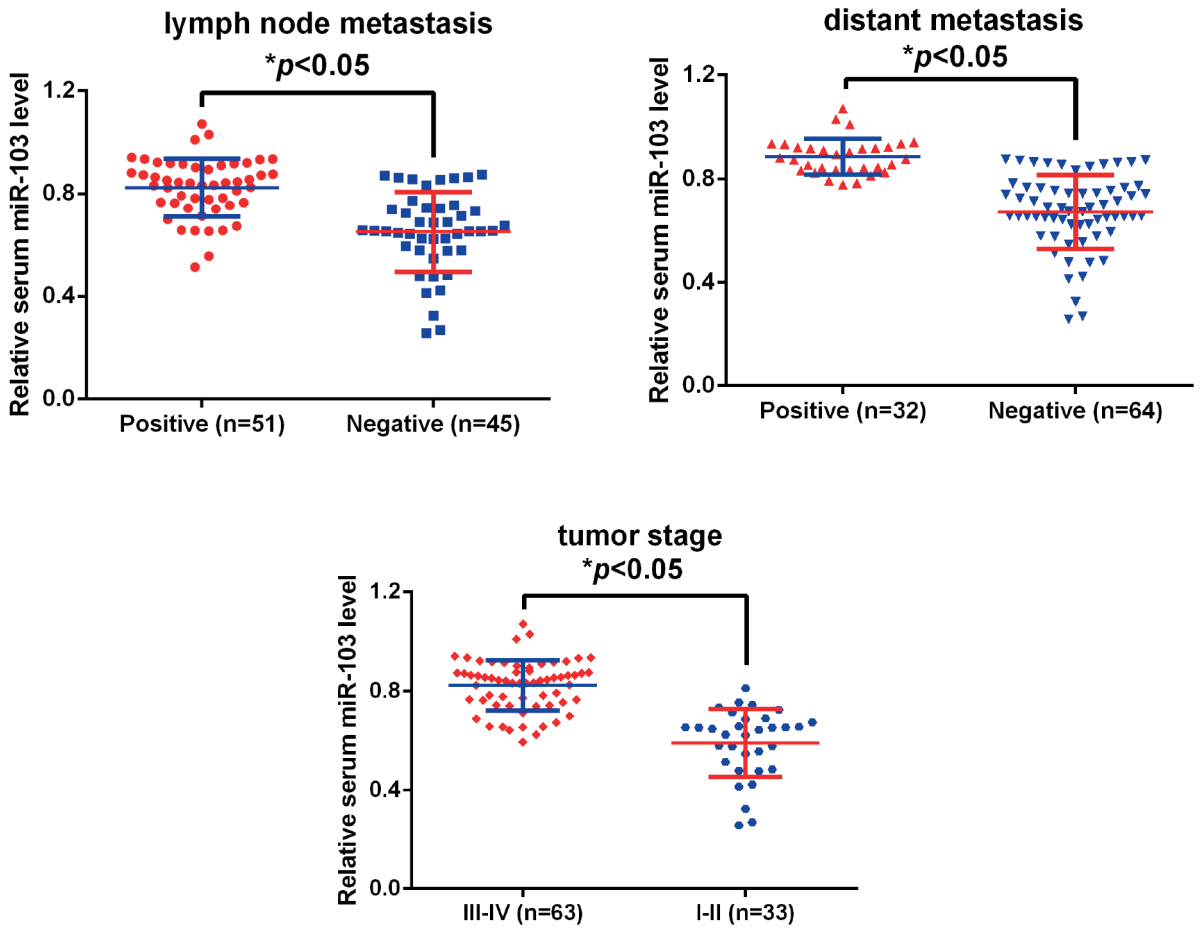

Figure 3. ***A,*** Serum miR-103 levels in patients with positive lymph node metastasis were greatly upregulated. ***B***, Serum miR-103 levels in patients with positive distant metastasis were greatly upregulated. ***C,*** Serum miR-103 levels in III/IV stage patients were significantly higher than those in I/II stage patients.

Table II. Multivariate analysis of the impact of parameters on recurrence free survival and overall survival in CRC patients.

	Multivariate analysis		
Parameters	**RR**	**95% CI**	***p***
Recurrence free survival			
Lymph node metastasis	1.54	0.95-2.13	0.029
Distant metastasis	1.88	1.16-2.71	0.020
Tumor stage	3.73	1.51-6.14	0.003
Serum miR-103	2.36	1.32-3.50	0.014
Overall survival			
Lymph node metastasis	1.32	0.87-1.85	0.034
Distant metastasis	1.67	1.09-2.37	0.025
Tumor stage	3.55	1.47-5.79	0.007
Serum miR-103	2.14	1.23-3.22	0.016

RR, risk ratio; CI, confidence interval; *Statistically significant ($p<0.05$). *Statistically significant ($p<0.05$).

5.79, $p=0.007$) and serum miR-103 (RR=2.14, 95% CI=1.23-3.22, $p=0.016$) were independent prognostic markers for predicting poorer OS in CRC cases (Table II).

Discussion

In the present study, we demonstrated that serum miR-103 overexpression was observed in CRC patients and strongly associated with aggressive clinicopathological features. Ectopic miR-103 expression was more frequently occurred in CRC patients with lymph node metastasis, distant metastasis and advanced tumor stage. Moreover, miR-103 levels in 23 postoperative blood samples were greatly lower than those in preoperative specimens, and serum miR-103 expression were

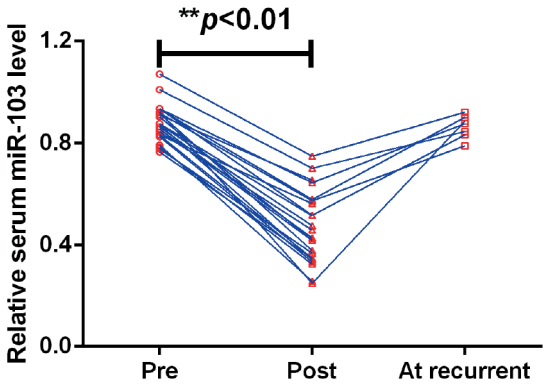

Figure 4. A significant decrease in serum miR-103 levels was noted in the post-operative serum, and serum miR-103 levels were re-elevated in seven patients at recurrence.

re-elevated in 7 cases at recurrence. Also, serum miR-103 showed good diagnostic performance in discriminating CRC patients from controls. In addition, enhanced miR-103 expression was highly correlated with shorter RFS/OS in CRC. Finally, the Cox regression analysis confirmed that serum miR-103 was a risk factor for RFS/OS in CRC. The results indicated that serum miR-103 might play an oncogenetic role in CRC. In line with our findings, miR-103 was reported to involve in the initiation and progression of CRC by previous studies. For instance, serum miR-103 expression was markedly elevated in CRC patients in comparison with non-cancer subjects. Moreover, miR-103 overexpression was strongly correlated with poor clinical parameters[10]. Zheng et al[11] showed that up-regulation of miR-103 was closely associated with aggressive clinical variables and shorter overall survival. In addition, loss of miR-103 inhibited cancer cell proliferation, invasion and migration in vitro by targeting LATS2. Geng et al[12] found that ectopic miR-103 expression significantly promoted carcinogenesis both *in vitro* and *in vivo*. DICER and PTEN were identified to be the downstream targets of miR-103. Chen et al[13] demonstrated that miR-103 and miR-107 overexpression was correlated with lymph node, distant metastasis, as well as unfavorable prognosis in CRC patients by negatively regulating DAPK and KLF4. These findings suggested that miR-103 acted as an oncogenic miRNA in CRC. MiR-103 seems to have controversial roles in human cancers. On the one hand, the oncogenic role of miR-103 was reported in various cancer types. In gastric cancer, Zheng et al[14] found that miR-103 was highly expressed both in cancer cell lines and tissues. Enforced miR-103 expression was remarkably correlated with clinical outcome, as well as poor survival. In addition, miR-103 inhibition greatly suppressed cancer cell proliferation, migration, invasion and stimulated apoptosis in vitro and in vivo through directly regulating KLF4. In hepatocellular carcinoma, loss of miR-103 strongly repressed cancer cell proliferation and induced cell apoptosis in cancerous tissues and cell lines through negatively regulating AKAP12[15]. In triple-negative breast cancer (TNBC), Xiong et al[16] showed that miR-103 expression was up-regulated both in cancer tissues and cell lines, and positively associated with poor clinical outcome. Also, Kleivi et al[17] demonstrated that TNBC patients with high miR-103 expression experienced more frequent tumor recurrence and shorter overall survival. On the other hand, miR-103 played as a tumor suppressor. In prostate cancer, Fu et al[18] provided *in vitro* evidence to show that upregulation of miR-103 significantly inhibited cancer cell proliferation and migration by degrading PDCD10. Additionally, decreased miR-103 expression was strongly correlated with poor clinical characteristics. Weber et al[19] showed that miR-103 expression was markedly reduced in the peripheral blood of patients with malignant mesothelioma. Furthermore, ROC analysis revealed that serum miR-103 had a good performance to discriminate mesothelioma patients from normal controls.

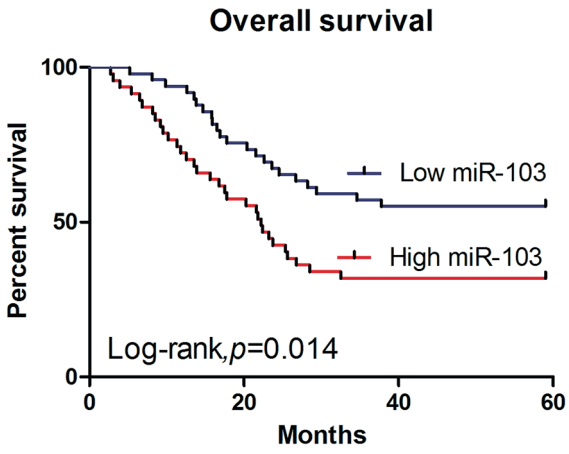

Figure 5. ***A***, Patients with high miR-103 expression had worse overall survival. ***B***, Patients with high miR-103 expression had worse recurrence free survival.

Conclusions

We found that serum miR-103 was overexpressed in CRC subjects and positively associated with dismal clinical outcome. Similarly, CRC patients with high serum miR-103 had shorter survival. Thus, we provided compelling evidence for the potential usefulness of serum miR-103 as a noninvasive screening and prognostic marker in CRC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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