Reduced expression of serum miR-204 predicts poor prognosis of gastric cancer

X. Chen[1,2], X.S. Liu[1,3], H.Y. Liu[4], Y.Y. Lu[1,3] and Y. Li[5]

[1]Qingdao University Medical College, Qingdao, Shandong, China
[2]Department of Gastroenterology, Yantai Municipal Laiyang Central Hospital, Yantai, Shandong, China
[3]Department of Gastroenterology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China
[4]Department of Infection Diseases, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China
[5]Department of Gastroenterology, Qingdao Municipal Hospital, Qingdao, Shandong, China

Corresponding author: X.S. Liu
E-mail: xishuangliuqdu@163.com

ABSTRACT. Deregulation of microRNAs (miRNAs) is implicated in the initiation and progression of gastric cancer (GC). Previous studies have demonstrated that miR-204 was downregulated in GC tissues. However, its expression profile in serum samples and its potential for clinical value remain unknown. Real-time PCR was performed to evaluate the expression level of serum miR-204 in patients with GC. The association between serum miR-204 expression level and the clinical outcome of GC was then investigated. Our results showed that the expression of miR-204 in serum samples from GC patients was significantly lower than that in the healthy controls (P < 0.01). Serum miR-204 expression level of GC patients was significantly upregulated after receiving surgical resection (P < 0.01). In addition, serum miR-204 was associated with lymph node metastasis (P = 0.016), tumor

differentiation (P = 0.001), and TNM stage (P = 0.005). GC patients with low serum miR-204 expression had shorter overall survival than those with high serum miR-204 expression (P = 0.004). Multivariate analysis revealed that serum miR-204 expression level was an independent risk factor for this malignant disease (HR = 3.629, 95%CI = 2.828-8.146, P = 0.015). In conclusion, our findings indicate that serum miR-204 may be employed as a novel biomarker for monitoring the treatment response and predicting the prognosis of GC.

Key words: Gastric cancer; miR-204; Serum; Prognosis

INTRODUCTION

Gastric cancer (GC) is a heterogeneous, multi-factorial disease and the second most frequently diagnosed cancer (Jemal et al., 2006; Rahman et al., 2014). Despite a sharp decline in incidence and advances in surgical techniques, GC remains the second most common cause of cancer (Han et al., 2003). One of the major reasons for the poor clinical outcome of GC is that most patients are at an advanced clinical stage at the time of diagnosis (Dicken et al., 2005). Unfortunately, biomarkers with high sensitivity and specificity for GC are still lacking. Thus, identifying novel biomarkers for early detection and prediction of the prognosis of GC is important.

MicroRNAs (miRNAs) are a class of small, highly conservative, non-coding RNAs that mediate gene expression through complimentary binding with the 3'-untranslated regions (UTRs) of target genes (He and Hannon, 2004). Abundant studies have shown that miRNAs regulate a variety of cellular processes, such as cell proliferation, migration, and differentiation (Bueno et al., 2008). Aberrant expression of miRNAs is closely correlated with many pathological processes, including human cancer (Dalmay and Edwards, 2006; Nana-Sinkam and Croce, 2011). Zhang et al. (2015b) showed that miR-153 was downregulated in GC tissues and that low expression of miR-153 was correlated with poor prognostic features. In addition, forced expression of miR-153 suppressed the migration and invasion capacity of GC cell lines and vice versa, indicating that miR-153 functions as a tumor suppressor in GC. Xu et al. (2015) reported that miR-374a was overexpressed in human GC cell lines and tissues. Inhibition of miR-374a was able to suppress GC cell proliferation, migration, and invasion *in vitro* and retarded tumor growth *in vivo*, suggesting that miR-374a may promote the tumorigenesis of GC.

Dysregulation of miR-204 is a common feature in a number of cancers, such as breast cancer, acute myeloid leukemia, and prostate cancer (Butrym et al., 2015; Wang et al., 2015; Todorova et al., 2016). Although miR-204 has been found to be downregulated in GC tissues (Zhang et al., 2015a), its expression profile in serum samples and its potential for clinical use are unknown. Therefore, the purpose of the current study was to investigate the clinical significance of serum miR-204 for GC.

MATERIAL AND METHODS

Study population

This study was approved by the Research Ethics Committee of the Affiliated Hospital of Qingdao University, and written informed consent was obtained from all participants,

including 115 patients with GC and 40 healthy volunteers. GC patients were recruited from the Department of Gastroenterology, The Affiliated Hospital of Qingdao University; they were diagnosed with GC based on histopathological evaluation. No previous local or systemic treatment had been conducted on these GC patients before the first-time serum sample collection. Clinical data for the GC patients are summarized in Table 1.

Clinical samples and real-time PCR

Blood samples were collected from patients with GC or healthy donors. Serum was separated by centrifugation at 1200 g at 4°C for 15 min and then frozen at -80°C prior to total RNA isolation. The miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA, USA) was used to isolate RNA from serum samples according to the manufacturer instructions. Briefly, 20 μL total RNA sample was converted into cDNA using miScript-II-RT-Kit (Qiagen, Germany). Real-time PCR was then performed with the Applied Biosystems Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were performed in triplicate, and miRNA relative expression was calculated using a $2^{-\Delta\Delta Ct}$ method. RNU6 was used as an endogenous control.

Statistical analysis

Expression levels of serum miR-204 were compared between patients with GC and healthy volunteers using the Mann-Whitney U-test. The chi-square test was used to evaluate the association between serum miR-204 expression and clinical features of GC. An overall survival (OS) curve was plotted using the Kaplan-Meier method, and the association between serum miR-204 expression and OS in GC patients was analyzed using a log rank test. A Cox proportional hazard model was employed for multivariate analysis. All statistical analyses were performed using the SPSS version 22.0 for Windows software (SPSS, Inc., Chicago, IL, USA). Data are reported as means ± SD, and differences were considered statistically significant when $P < 0.05$.

RESULTS

Expression level of serum miR-204 in GC

The results of the real-time PCR showed that the expression level of miR-204 in the serum samples from GC patients was significantly lower than that in the healthy controls ($P < 0.01$; Figure 1). Moreover, the serum miR-204 expression level in GC patients was significantly upregulated after receiving treatments ($P < 0.01$; Figure 2), indicating that serum miR-204 might be employed to monitor treatment response.
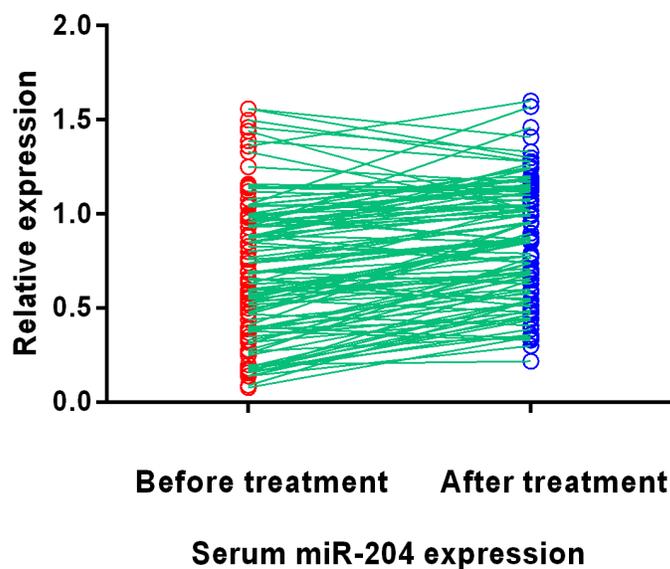
Association between serum miR-204 expression and clinicopathological parameters of GC

The median fold-change in serum miR-204 was used as a cutoff value to divide all 115 GC patients into two groups: the low-expression group (N = 61) and the high-expression group (N = 54). Serum miR-204 expression was associated with lymph node metastasis (P =

0.016), tumor differentiation (P = 0.001), and tumor, node and metastasis (TNM) stage (P = 0.005). It was not correlated, however, with gender (P = 0.755), age (P = 0.861), tumor size (P = 0.207), or distant metastasis (P = 0.095) (Table 1).
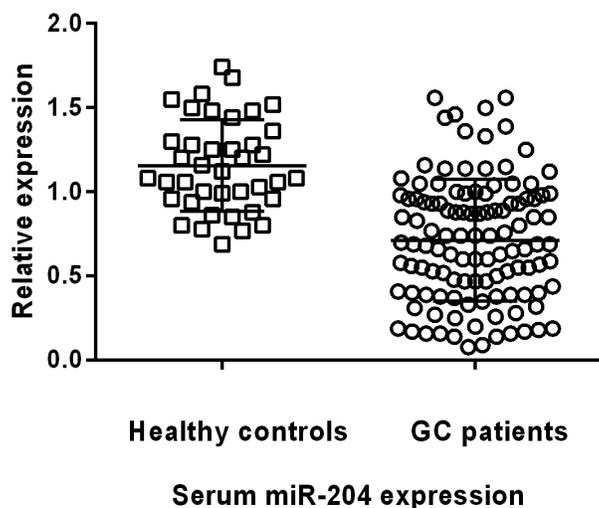
Figure 1. Expression level of serum miR-204 in gastric cancer (GC). The expression level of serum miR-204 was significantly downregulated in GC patients compared with that in healthy controls (P < 0.01).

Figure 2. Serum miR-204 was upregulated in gastric cancer (GC) patients after therapy. Serum miR-204 expression level in GC patients was significantly increased after receiving treatments (P < 0.01).

Table 1. Correlations between serum miR-204 expression and clinicopathological features in GC patients.

Variables	N	Serum miR-204 expression		P
		Low	High	
Gender				
Male	72	39	33	0.755
Female	43	22	21	
Age (years)				
<60	48	25	23	0.861
≥60	67	36	31	
Tumor size (cm)				
<5	40	18	22	0.207
≥5	75	43	32	
Lymph node metastasis				
No	63	27	36	0.016
Yes	52	34	18	
Distant metastasis				
No	71	19	25	0.095
Yes	44	42	29	
Tumor differentiation				
Well + Moderate	56	21	35	0.001
Poor	59	40	19	
TNM				
I-II	46	17	29	0.005
III-IV	69	44	25	

Reduced serum miR-204 expression was associated with poor prognosis in GC patients

Our survival analysis showed that GC patients with low serum miR-204 expression exhibited shorter overall survival than those with high serum miR-204 expression. The 5-year OS rate in the low-expression group was 34.43%, compared with 57.41% in the high-expression group (P = 0.004; Figure 3).

Multivariate analysis revealed that tumor differentiation (hazard ratio (HR) = 4.206, 95% confidence interval (CI) = 3.058-9.633, P = 0.008), TNM stage (HR = 3.482, 95%CI = 2.674-7.410, P = 0.021), and serum miR-204 expression (HR = 3.629, 95%CI = 2.828-8.146, P = 0.015) were independent risk factors for GC (Table 2).
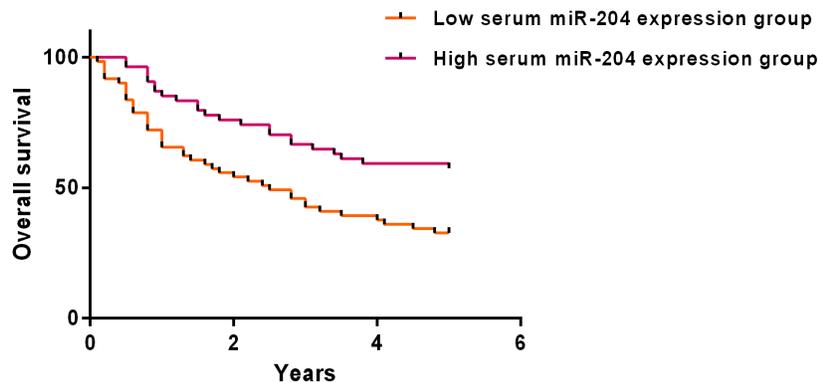
Figure 3. Association between serum miR-204 and overall survival in GC patients. The GC patients in the low serum miR-204 expression group had a significantly poorer overall survival than those in the high serum miR-204 expression group (P = 0.004).

Variables	Overal survival	
	HR (95%CI)	P
Lymph node metastasis	1.806 (0.981-2.893)	0.053
Tumor differentiation	4.206 (3.058-9.633)	0.008
TNM stage	3.482 (2.674-7.410)	0.021
Serum miR-204 expression	3.629 (2.828-8.146)	0.015

Table 2. Multivariate analysis of overall survival in GC.

DISCUSSION

The detection of biomarkers in serum samples provides several advantages over tissue biopsy. First, it is less invasive and more convenient. Serum biomarkers also may help clinicians classify GC patients into different subgroups before surgery resection and then provide reasonable treatment options. Lastly, biomarkers allow for the monitoring of treatment responses of patients in real-time, which may significantly improve treatment outcomes. In this study, our data showed that serum miR-204 expression was reduced in GC patients compared with healthy volunteers. In addition, miR-204 was significantly upregulated after the GC patients received surgical treatments. Overexpression of serum miR-204 was associated with advanced clinical stage and poor OS for GC. Moreover, serum miR-204 was an independent risk factor for this malignant disease.

Our findings indicate that miR-204 may function as a tumor suppressor in the development of GC, which is consistent with previous studies documenting the role of miR-204 in GC. The expression level of miR-204 has been reported to be downregulated in GC tissues and cell lines by different research groups (Zhang et al., 2013, 2015a). Zhang et al. (2015a) showed that miR-204-5p suppression could promote the proliferative capacity of GC cell lines, whereas forced expression of miR-204 inhibited this capacity. In addition, USP47 and RAB22A were identified as direct functional targets of miR-204-5p in GC (Zhang et al., 2015a). An inverse relationship was found between miR-204 expression and SIRT-1 expression in GC tissues, and overexpression of miR-204 was found to reduce GC cell invasion, anoikis resistance, and epithelial-mesenchymal transition by targeting SIRT-1 at the post-transcriptional level. Therefore, miR-204 is believed to play a crucial role in regulating the metastasis of GC (Zhang et al., 2013). Ezrin expression is also upregulated in GC tissues and cell lines, and overexpression of Ezrin has been correlated with poor prognosis of GC. As Ezrin is critical to Ras activation and is predicted to be a target of miR-204, this indicates that miR-204 downregulation may be a novel mechanism for aberrant Ras activation in gastric tumorigenesis (Lam et al., 2011). Further, miR-204 may target Bcl-2 expression and increase the responsiveness of GC cells to 5-fluorouracil and oxaliplatin treatment, indicating that miR-204 might be a therapeutic target for improving the prognosis of GC (Sacconi et al., 2012). Wang et al. (2014) compared differentially expressed miRNAs between *Helicobacter pylori*-related gastritis and gastric intestinal metaplasia lesions. MiR-204 was downregulated in GC precancerous lesions and may be closely correlated with the progression of GC. The expression of miR-204 has been found to be enhanced in *H. pylori*-negative GC compared with *H. pylori*-positive GC, suggesting that distinct GC pathogenesis mechanisms may exist in *H. pylori*-positive and *H. pylori*-negative GC and that miR-204 expression may be correlated with *H. pylori* infection (Chang et al., 2015).

As miRNAs may regulate many downstream target genes, it is common for a specific miRNA to act as an oncogene in one type of cancer while functioning as a tumor

suppressor in another. However, to the best of our knowledge, miR-204 has been shown to inhibit tumorigenesis in all types of cancers investigated thus far. The expression level of miR-204-5p was significantly downregulated in glioma tissues compared with normal brain tissues. Moreover, restoration of miR-204-5p expression resulted in suppression of tumor cell behaviors and improvement in overall host survival, suggesting that targeting miR-204-5p may be a practical and effective strategy for treating gliomas (Xia et al., 2015). Yin et al. (2014) revealed that miR-204-5p was frequently downregulated in colorectal cancer tissues and that low miR-204-5p expression was associated with poor clinical outcome. In addition, ectopic miR-204-5p expression not only inhibited the proliferation, migration, and invasion capacity of colorectal cancer cells, but also enhanced tumor sensitivity to chemotherapy by suppressing RAB22A expression. Similarly, Qiu et al. (2013) reported that the relative expression of miR-204 was reduced in human intrahepatic cholangiocarcinoma (ICC) as well as ICC cell lines and that reduced miR-204 expression was correlated with lymph node metastasis. Overexpression of miR-204 inhibited ICC cell migration, invasion, and the epithelial-mesenchymal transition process by targeting Slug directly. Although miR-204 plays a tumor suppressive role in many different types of cancers, its downstream targeted genes may be distinct in each type. Further investigations are needed to confirm the clinical significance of miR-204 in GC and elucidate its role in the carcinogenesis of GC.

CONCLUSIONS

In conclusion, we revealed that serum miR-204 was downregulated in patients with GC. A low level of serum miR-204 was associated with tumor progression and poor clinical outcome of GC. These findings indicate that serum miR-204 may be a good candidate for the prognosis prediction in GC patients.

Conflicts of interest

The authors declare no conflict of interest.

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