Original Paper

Diagnostic and Prognostic Value of Serum MicroRNA-206 in Patients with Gastric Cancer

Cheng-Gong Hou^a Xiang-Yu Luo^b Gang Li^c

^aDepartment of Laboratory Medicine, Zhumadian Central Hospital, Zhumadian, ^bDepartment of Laboratory Medicine, Yuzhou Maternal and child health care hospital, Xuchang, ^cDepartment of General Medicine, Zhumadian Central Hospital, Zhumadian China

Key Words

MiRNA-206 • Biomarker • Gastric cancer • Diagnosis • Prognosis

Abstract

Aims: Recent studies have demonstrated that microRNAs (miRNAs) can serve as useful biomarkers for human cancers. The aim of this study was to evaluate the expression level of serum miRNA-206 in patients with gastric cancer (GC) and investigate its diagnostic and prognostic value. **Methods:** Quantitative real-time PCR was performed to evaluate serum miRNA-206 levels in 150 GC patients and 150 healthy volunteers. The association between miRNA-206 expression and clinicopathological factors as well as patient's survival was analyzed. Receiver operator curve (ROC) analysis was carried out to assess the potential value of serum miRNA-206 for GC diagnosis. **Results:** Serum miRNA-206 was down-regulated in GC patients compared with healthy controls ($P < 0.001$). Decreased serum miRNA-206 expression was significantly associated with deep local invasion, positive lymph node metastasis, and advanced clinical stage. Serum miRNA-206 expression was found to be significantly up-regulated in paired post-operative samples and reduced in patients with GC recurrence. ROC curve analysis showed that serum miRNA-206 was a useful marker for GC diagnosis, and could discriminate between recurred and non-recurred patients. Multivariate Cox regression analysis confirmed low serum miRNA-206 expression as an independent unfavorable prognostic factor for both DFS and OS of GC patients. **Conclusions:** These results suggest that serum miRNA-206 might not only serve as a novel diagnostic biomarker for GC, but also predict cancer recurrence and patient's prognosis.

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Introduction

Gastric cancer (GC) is the fourth most common malignancy and the second leading cause of cancer-related deaths worldwide, with approximately 1 million new cases and 0.7 million deaths per year [1]. Due to vague initial symptoms, the majority of GC patients are diagnosed at an advanced stage [2]. Despite recent improvements in multimodal therapy

Cheng-Gong Hou, MD,

Department of Laboratory Medicine, Zhumadian Central Hospital, Zhumadian 463000, Henan Province, (China)
Tel. +86-0374-8222990, E-Mail manuhouc@163.com

including surgery, chemotherapy, radiotherapy, and targeted therapy, the prognosis for patients with advanced GC remains dismal [3]. Currently, gastroscopic biopsy is the gold standard for diagnosis of GC [4]. However, the approach is considered invasive and the results are affected by the operator's experience. On the other hand, conventional GC-associated serum markers, such as carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), carbohydrate antibody 12-5 (CA12-5), and carbohydrate antibody 72-4 (CA72-4), lack sufficient sensitivity and specificity to facilitate early detection. Therefore, it is urgent to elucidate the regulatory network underlying GC and develop novel biomarkers for its early diagnosis, accurate assessment, targeted therapy, and prognosis evaluation.

MicroRNAs (miRNAs) are short (about 22 nucleotides in length), highly conserved small non-coding RNA molecules that negatively regulate gene expression by binding to target message RNAs (mRNAs) at their 3'-untranslated region, leading to mRNA degradation or translation suppression [5]. Deregulation of miRNA expression has been identified in many human diseases, including cancers [6, 7]. Some highly expressed miRNAs could function as oncogenes by repressing tumor suppressor genes, whereas low-expressed miRNAs could function as tumor suppressors by negatively regulating oncogenes. Emerging evidence suggests that miRNAs act as key regulators in a wide variety of biological processes that contribute to tumorigenesis and development, such as tumor cell differentiation, proliferation, apoptosis, invasion, angiogenesis, and epithelial mesenchymal transition [8-12]. Since circulating miRNAs are well protected from RNase digestions and highly stable in plasma/serum [13], they have been regarded as novel potential biomarkers in cancer detection and monitoring. For example, serum miRNA-30e and miRNA-223 are useful diagnostic biomarkers for hepatocellular carcinoma [14]. Serum miRNA-365 expression correlates with overall survival of patients with non-small cell lung cancer [15]. Serum expression levels of miRNA-17, miRNA-21, and miRNA-92 predict recurrence after adjuvant chemotherapy in colon cancer patients [16]. Serum miRNA-183 can be used to predict the response of renal cell carcinoma cells to the cytotoxicity induced by natural killer cells [17]. However, the use of circulating miRNAs as blood-based, minimally invasive biomarkers in GC is still relatively less explored.

Previous studies have suggested the tumor-suppressive function of miRNA-206 and decreased miRNA-206 expression has been reported in various solid tumors, including oral squamous cell carcinoma [18], anaplastic thyroid cancer [19], non small cell lung cancer cell [20], breast cancer [21], hepatocellular carcinoma [22], pancreatic adenocarcinoma [23], gastric cancer [24], colorectal cancer [25], renal cell carcinoma [26], and cervical cancer [27]. Low level of serum miRNA-206 may be unfavorable prognostic biomarker for patients with melanoma and osteosarcoma [28, 29]. Tissue miRNA-206 has been reported to be down-regulated in patients with GC, and its down-regulation was correlated with tumor progression and poor overall survival [24, 30]. However, the diagnostic and prognostic values of serum miRNA-206 in GC patients remains unknown. In the present study, we investigated serum miRNA-206 levels in patients with GC, and evaluated its association with clinicopathological features and survival time.

Materials and Methods

Patient samples

A total of 150 consecutive patients (mean age 59.8 ± 7.2 years; range 39-73 years) who received curative resection of GC in the Zhumadian Central Hospital (Zhumadian, Henan Province, China) between January 2007 and March 2011 were included in this study. The diagnosis of GC was confirmed by histological evaluation.

All patients underwent contrast-enhanced CT (CECT) prior to operation, and none of them had pre-resection metastases. None of the patients had previously undergone chemotherapy, radiation therapy, or immunotherapy. Thirty two of these patients received D1 lymph node dissection, and the others received D2 lymph node dissection. Paired peripheral blood samples (5ml) were collected at the time of primary

diagnosis and 4 weeks after surgery. For recurrence group, blood samples were also collected at time of recurrence. Blood samples from 150 healthy age- and sex-matched volunteers were used as control. All samples were centrifuged at 3000g for 10 min at 4 °C and the supernatant serum were stored at –80 °C until further analysis. Follow-up data for all GC patients were acquired. Overall survival (OS) was calculated from the date of diagnosis to death or last follow-up. Disease-free survival (DFS) was defined as the time from diagnosis to the first evidence of recurrence or metastasis. This study was approved by the Ethics Review Board of our hospital and informed consent was obtained from each patient.

MicroRNA Isolation and Real-Time RT-PCR Assay

Total RNA was extracted from 400 µL of serum samples using a miRVana PARIS Kit (Ambion, Austin, TX, USA), and eluted into 100 µL of pre-heated (95°C) RNase-free water according to the manufacturer's instructions. The concentration of all RNA samples were quantified by Biophotometer (Eppendorf, USA). Reverse-transcription was carried out with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) in 15 µL containing 5 µL of RNA extract, 0.15 µL of 100 mM dNTPs, 1 µL of Multiscribe Reverse Transcriptase (50 U/µL), 1.5 µL of 10 × Reverse Transcription Buffer, 0.19 µL of RNase inhibitor (20 U/µL), 1 µL of gene-specific primer and 6.16 µL of nuclease-free water. The reaction mixtures were incubated at 16°C for 30 min, followed by 42°C for 30 min, then 85°C for 5 min before being held at 4°C. Then, 1.33 µL of cDNA solution was amplified using 10 µL of TaqMan 2 × Universal PCR Master Mix with no AmpErase UNG (Applied Biosystems), 1 µL of gene-specific primer and 7.67 µL of nuclease-free water in a final volume of 20 µL. Quantitative PCR was run on a 7300 Real-Time PCR system (Applied Biosystems) and the reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantification of miRNA-206 expression was calculated with the $2^{-\Delta Ct}$ method. Due to the lack of universal endogenous controls for serum samples, synthetic cel-miRNA-39 was spiked into each sample as an internal control as previously described [13, 31].

Patient follow-up

Follow-up was performed at 3-month intervals for 1 year, then at 6-month intervals for 3 years, and then yearly for the next years. It consisted of a physical examination, a complete blood count, liver function tests, and serum tumor markers. The patient also underwent chest X-rays, abdominal CT scan, and gastroscopy every 6 months.

Statistics

Statistical analyses were carried out using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) or MedCalc 9.3.9.0 (MedCalc, Mariakerke, Belgium) software. P values < 0.05 were considered statistically significant. The Mann-Whitney U test was performed to compare the expression level of serum miRNA-206 between GC patients and healthy controls. Wilcoxon signed rank test was performed to compare serum miRNA-206 levels between samples obtained before and after operation. Associations between clinicopathological parameters and serum miRNA-206 expression were evaluated using chi-square test. Survival curves were constructed with the Kaplan-Meier method and compared by log-rank tests. Cox regression analysis was performed to analyze prognostic significance of each variable. Receiver-operating characteristic (ROC) curve was constructed and the area under the ROC curve (AUC) was calculated to assess the potential value of serum miRNA-206 for GC diagnosis.

Results

Decreased serum miRNA-206 in GC samples and its diagnostic value

Serum miRNA-206 levels in 150 GC patients and 150 healthy controls were detected by RT-PCR. The results showed that serum miRNA-206 was significantly down-regulated in GC patients compared to healthy controls ($P < 0.001$, Fig. 1). The median expression level of miRNA-206 was used as a cut-off point to divide all the 150 patients into two groups: high serum miRNA-206 expression group ($n = 75$) and low serum miRNA-206 expression group ($n = 75$).

Fig. 1. The pre-operative serum concentrations of miRNA-206 were significantly lower in gastric cancer patients than in normal controls ($P < 0.001$, Mann-Whitney U test). There was no significant difference in pre-operative serum miRNA-206 expression between recurred and non-recurred patients. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median, respectively.

ROC curve analysis showed that serum miRNA-206 was a useful marker for discriminating GC patients from healthy controls, with the AUC value of 0.89 (95% CI, 0.82-0.95; Fig. 2). The optimal sensitivity and specificity were 78% and 86%, respectively.

Serum miRNA-206 correlates with clinicopathological features of GC

Table 1 displayed the associations between serum miRNA-206 expression and the clinicopathological features. Low serum miRNA-206 levels were significantly associated with deep local invasion ($P = 0.007$), positive lymphatic metastasis ($P = 0.009$), and advanced Tumor-Node-Metastasis (TNM) stage ($P = 0.001$). There were no significant correlation between serum miRNA-206 expression and other clinical features such as patient's gender, age, tumor size, tumor site, and cancer differentiation.

Serum miRNA-206 correlates with tumor recurrence and patient's prognosis

During follow-up, relapse was observed in 110 patients in total, with a median follow-up time of 38 months. Among them, 71 developed metastases at first sign of relapse, and 39 got relapse based on identified locoregional recurrence. We found that pre-operative serum miRNA-206 levels were significantly lower in both recurred and non-recurred GC patients than in healthy controls (Fig. 1). In addition, low serum miRNA-206 was associated with high recurrence rate ($P < 0.001$, Table 1). ROC analysis revealed that serum miRNA-206 could discriminate between recurred and non-recurred patients, with the AUC value of 0.85 (95% CI, 0.78-0.91, Fig. 3).

Fig. 4 showed that the expression of serum miRNA-206 was significantly

Fig. 2. Receiver-operating characteristic (ROC) curve analysis of the serum miRNA-206 to detect gastric cancer patients. The AUC of 0.89 (95% CI, 0.82-0.95) indicates good diagnostic value ($P < 0.001$).

Table 1. Correlation between serum miRNA-206 expression and different clinicopathological features in patients with gastric cancer

	Low serum miRNA-206	High serum miRNA-206	P-value
Age			
≥60	45(57.7%)	33(42.3%)	0.072
<60	30(41.7%)	42(58.3%)	
Gender			
Male	46(46.9%)	52(53.1%)	0.391
Female	29(55.8%)	23(44.2%)	
Differentiation			
Well-moderate	34(51.5%)	32(48.5%)	0.435
Poor	41(48.8%)	43(51.2%)	
Location			
Middle and proximal third	38(52.1%)	35(47.9%)	0.372
Distal third	37(48.1%)	40(51.9%)	
Tumor size			
≥5cm	42(57.5%)	31(42.5%)	0.102
<5cm	33(42.9%)	44(57.1%)	
Invasion depth			
T1, T2	21(35.6%)	38(64.4%)	0.007
T3, T4	54(59.3%)	37(40.7%)	
TNM stage			
I/II	18(31.6%)	39(68.4%)	0.001
III	57(61.3%)	36(38.7%)	
Lymphatic metastasis			
Negative	14(33.3%)	28(66.7%)	0.009
Positive	61(56.5%)	47(43.5%)	
Recurrence			
Yes	65(59.1%)	45(40.9%)	<0.001
No	10(25.0%)	30(75.0%)

Fig. 3. Receiver-operating characteristic (ROC) curve analysis of the serum miRNA-206 to discriminate between recurred and non-recurred patients. The AUC of 0.85 (95% CI, 0.78-0.91) indicates good discriminative power ($P < 0.001$).

Fig. 4. Serum miRNA-206 levels in the pre-operative ($n = 150$), post-operative ($n = 150$), and recurrence ($n = 110$) groups. Serum miRNA-206 was significantly increased after surgery ($P < 0.001$), but decreased to a level close to the preoperative concentration when recurrence occurred.

Fig. 5. Kaplan-Meier survival curves of gastric cancer patients based on serum miRNA-206 expression level. Low serum miRNA-206 expression was significantly associated with poor overall survival and disease-free survival (both $P < 0.001$, log-rank test).

increased after surgery. Moreover, serum miRNA-206 levels in the recurrence group were significantly reduced and similar to the pre-operative levels. Kaplan-Meier analysis with the log-rank test indicated that GC patients in low serum miRNA-206 group had a significantly shorter OS and DFS (both $P < 0.001$; Fig. 5) than those in high serum miRNA-206 group.

Univariate and multivariate analyses were used to analyse the prognostic value of serum miRNA-206 and other clinical parameters for GC. In the univariate survival analysis, tumor size, depth of infiltration, lymph node metastasis, TNM stage, lymph node dissection pattern, and serum miRNA-206 levels were associated with OS and DFS. In the multivariate Cox regression model, low-level of serum miRNA-206 expression was an unfavorable prognostic factor for OS and DFS of GC patients independent of other clinicopathological factors, including local invasion, lymph nodes status, clinical stage, and method of lymph node dissection (Table 2).

Discussion

GC is still a major public health problem of worldwide concern. Up to now, the exact mechanisms underlying GC are not fully understood. The identification of genetic alterations would be important for the screening, diagnosis and treatment of GC. The discovery of miRNAs has broadened our understanding of carcinogenesis. In terms of GC, abnormal

Table 2. Univariate and multivariate survival analysis in patients with gastric cancer

Factors	Univariate analysis		Multivariate analysis	
	R	P-value	R	P-value
Overall survival				
Age	1.08	0.215	—	—
Gender	1.31	0.126	—	—
Differentiation	1.44	0.094	—	—
Tumor location	0.87	0.238	—	—
Tumor size	2.46	0.02	1.42	0.082
Invasion depth	3.05	0.005	3.46	0.003
Lymphatic metastasis	2.72	0.012	2.64	0.015
TNM stage	3.89	<0.001	2.95	0.006
D1/D2 lymph node dissection	2.57	0.016	2.73	0.011
Serum miRNA-206	3.73	<0.001	2.39	0.018
Disease-free survival				
Age	1.14	0.257	—	—
Gender	1.42	0.173	—	—
Differentiation	1.56	0.119	—	—
Tumor location	0.95	0.305	—	—
Tumor size	2.06	0.023	1.13	0.091
Invasion depth	3.17	0.002	4.25	0.001
Lymphatic metastasis	2.93	0.016	3.01	0.009
TNM stage	4.19	<0.001	3.78	0.002
D1/D2 lymph node dissection	3.05	0.008	3.46	0.006
Serum miRNA-206	3.98	<0.001	2.57	0.024

expression of several miRNAs and their function has been reported. For example, miRNA-451 showed decreased expression in GC tissues, and its down-regulation was correlated with positive lymph node metastasis, advanced clinical stage, and poor prognosis [32]. Ectopic expression of miRNA-133a inhibited GC cell proliferation, migration, and invasion, and induced cell apoptosis [33]. Decreased plasma miRNA-940 may serve as a novel diagnostic biomarker for GC [34]. Overexpression of miRNA-23b-3p reversed GC cell resistance to multiple chemotherapeutics *in vitro* [35]. Thus, functional miRNAs may be applied for GC diagnosis and prognosis, and also act as potential novel therapeutic targets.

Although many miRNAs are expressed in tissues and tumor cells, their development as biomarkers requires tissue collection by invasive methods as opposed to the more convenient approach of studying peripheral blood. The stability and easy detectability make circulating miRNAs an ideal candidate to serve as a biomarker for cancer detection. In this study, we showed decreased serum miRNA-206 levels in GC patients compared with healthy controls. Down-regulation of serum miRNA-206 was correlated with various important clinicopathological parameters. Moreover, serum miRNA-206 expression was found to be significantly elevated in paired post-operative samples and reduced in patients with recurrence. ROC curve analysis revealed that serum miRNA-206 had a moderate diagnostic value for GC, and could discriminate between recurred and non-recurred patients. Multivariate analysis confirmed serum miRNA-206 expression as an independent prognostic factors for both OS and DFS. These results suggest that serum miRNA-206 may not only serve as a useful diagnostic biomarker for GC, but also predict cancer recurrence and patient's prognosis.

Our results are consistent with previous findings. Tian et al. reported that expression levels of miRNA-206 in serum samples from patients with melanoma were significantly lower than those in healthy controls [28]. Patients with low serum miRNA-206 levels had higher clinical stage and shorter overall survival and disease-free survival. Zhang et al. showed the association between decreased serum miRNA-206 expression and high tumor grade, positive metastasis, tumor recurrence, and poor survival in human osteosarcoma [29]. The potential diagnostic and prognostic values of serum miRNA-206 in other human malignancies would be an interesting and important topic of future investigations.

Despite recent improvements in experimental and clinical oncology, about 70% of GC patients are firstly diagnosed at late stages with locally advanced or metastatic disease [36], and D2 lymphadenectomy has been widely used in Asia [37]. Lustosa et al reported that D2

or D3 lymphadenectomy showed lower incidence of recurrence and lower mortality with recurrent disease compared to D1 lymph node dissection [36]. Degiuli et al compared D2 and D1 lymphadenectomy in the treatment of GC and showed a 5-year disease-specific survival benefit for patients with pT2-4 status and positive lymph nodes in the D2 group [38]. In our study, GC patients treated with D2 lymphadenectomy also showed prolonged OS and DFS, which is consistent with the results of the above mentioned studies. However, D2 lymph node dissection might lead to higher postoperative mortality and morbidity [39]. Yarema et al claimed that D2 lymphadenectomy could improve the prognosis in European populations of GC patients, but only when the surgical quality of lymphadenectomy execution is adequate [40]. Thus, it is still debatable whether D2 lymphadenectomy should be performed routinely or selectively.

It is now clear that miRNAs exhibit oncogenic or tumor suppressive properties by regulation of target gene expression. Zhang et al. demonstrated that miRNA-206 could suppress GC cells proliferation at least partially through targeting the cyclin D2 [41]. Zheng et al. revealed that introduction of miRNA-206 inhibited GC cell migration and invasion through the c-Met pathway [42]. In addition, upregulation of miRNA-206 suppressed clear cell renal carcinoma proliferation and invasion by targeting VEGF-A [43]. MiRNA-206 functioned as a tumor suppressor gene in pancreatic adenocarcinoma by targeting ANXA2 and KRAS genes [23]. EGFR was involved in miRNA-206-associated non-small cell lung cancer progression [44]. Thus, the potential regulatory circuitry afforded by miRNA-206 may be enormous, and the complex molecular mechanisms on how miRNA-206 suppresses cancer formation and development need further clarification.

In summary, the current study showed that level of serum miRNA-206 was down-regulated in GC patients, and associated with aggressive clinicopathological characteristics. More importantly, serum miRNA-206 might serve as a minimally invasive biomarker for GC diagnosis, and decreased serum miRNA-206 could predict GC recurrence and poor patient's survival. Future studies with larger sample size and longer follow-up time should be carried out to confirm the present conclusion.

Disclosure Statement

The authors had no conflicts of interest to declare in relation to this article.

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