

Original Article

MiR-21-3p and miR-21-5p in tumor tissue as diagnostic biomarkers for gastric cancer

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Abstract: Background: MicroRNAs (miRNAs) are a group of small non-coding RNAs, which modulate the expression of certain target genes and thereby control multiple biological processes. In this study, we selected miR-21-3p and miR-21-5p to evaluate their diagnostic value for Gastric Cancer (GC). Methods: A total of 50 GC patients were recruited. Normal gastric, paracancerous, and GC tissues were collected from all these participants during the operation. The levels of miR-21-3p and miR-21-5p were determined by quantitative real-time PCR (qPCR) with U6snRNA expression used as the internal control. Nonparametric tests were employed for the further statistical analyses, where relative expression of miR-21-3p or miR-21-5p in tumor or paracancerous tissues to that in normal tissues were employed to rule out the individual difference. Results: Higher expression of both miR-21-3p and miR-21-5p were identified in GC tissue than in paracancerous tissue. Significant differences of miR-21-3p or miR-21-5p level were detected among groups subdivided by depth of tumor invasion, lymph node metastasis and clinical tumor node metastasis (TNM) stage. But when it came to tumor differentiation, the difference of miR-21-3p was not statistically significant, while miR-21-5p was. Both of them showed moderate diagnostic value (miR-21-3p: AUC=0.710, sensitivity =82%, specificity =64%; miR-21-5p: AUC =0.726, sensitivity =82%, specificity =66%). Conclusion: Both miR-21-3p and miR-21-5p may serve as potential biomarker for GC diagnosis, while miR-21-5p is more potent in distinguishing the differentiation of the tumor.

Keywords: MicroRNA (miRNA), gastric cancer, miR-21-3p, miR-21-5p

Introduction

Due to lower infection rate of *Helicobacter pylori* and more intake of fresh vegetables and fruits, the incidence of GC has dramatically decreased during the past decades [1]. However, with nearly 1 million new patients diagnosed per year, GC still remains the fifth most common malignancy worldwide and represents a severe public health issue [2]. Most GC cases are initially diagnosed at advanced stage [3]. Although the standard D2 lymphadenectomy and feasible chemotherapies has largely improved the overall survival of GC, the prognosis of advanced patients is still poor. Identification of novel diagnostic biomarkers for GC is thus of great necessity.

MicroRNAs (miRNAs), a group of small non-coding RNAs, exist in a variety of creatures and

negatively regulate the expression of target genes at post-transcription level [4]. A great deal of research has reported that miRNAs play a significance role in multiple biological processes including cell differentiation, proliferation, and apoptosis [5-7]. Aberrant expression of certain miRNAs has been found to be involved in the genesis and development of human cancer [8-11]. Therefore, identifying miRNAs as novel diagnostic biomarkers has been of great interest. Specifically, Hsa-miR-21 has been widely studied, which showed a consistent over-expression in, hepatocarcinoma [12], breast cancer [13], and bladder cancer [14]. Previous studies have suggested that miR-21 could promote the development of cancer via multiple ways, including regulating mitochondrial apoptosis [15], mediating life activities of stem cells [16], controlling proliferation of tumor cells [17],

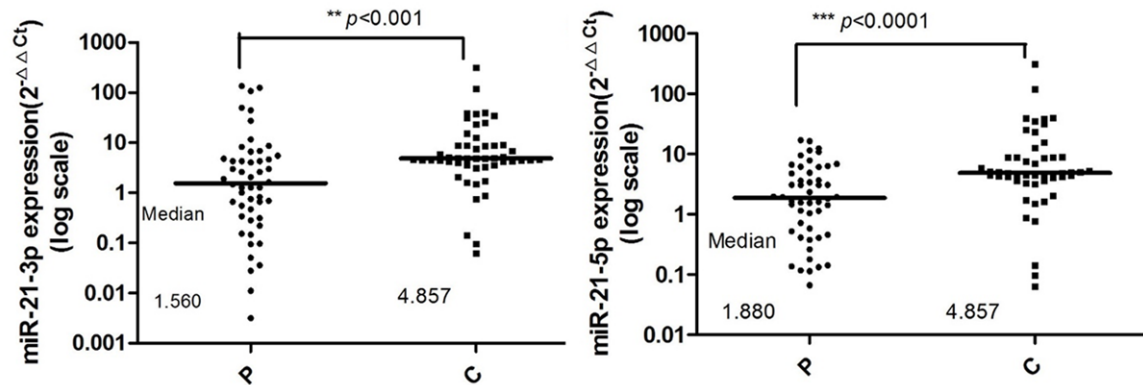


Figure 1. MiR-21-3p and miR-21-5p relative expression in paracancerous tissues (P) and GC (C) tissues from GC patients (*P*-values were calculated using the Mann-Whitney U-test).

and promoting tumor cell invasion [18]. However, few has been reported so far regarding the expression levels of two mature isoforms of miR-21, miR-21-3p and miR-21-5p, in GC tissues and their relationship with clinicopathological parameters of GC patients.

In this study, we determined the expression of miR-21-3p and miR-21-5p in normal gastric, paracancerous, and tumor tissues from GC patients, and analyzed their possible relationship with the clinicopathological factors of GC patients. Our results pave the way for employing miR-21-3p or miR-21-5p as the diagnostic biomarkers for GC in clinical practice.

Materials and methods

Ethical statement

Use of all gastric tissues from GC patients in the study were approved and confirmed by the Ethical Committee of Xinhua Hospital of Chongming branch on the basis of the Declaration of Helsinki. Each patient who participated in this study has signed an informed consent.

Sample collection

50 patients were recruited in this study, which had been diagnosed to have GC and received surgical operation at the General Surgery Department, Xinhua Hospital and its Chongming Branch Hospital, Affiliated to School of Medicine, Shanghai Jiaotong University during January to September, 2015. None of the 50 patients had previous history of chemotherapy, radiation therapy or immunotherapy. Tumor tis-

sues, paracancerous tissues (3 cm from the tumoral macroscopic margins), normal gastric tissues (5 cm from the tumoral macroscopic margins) were harvested at the surgical operation [19], frozen in liquid nitrogen immediately and then stored at -80°C . Clinicopathologic information of each patient including age, gender, tumor size, depth of tumor invasion, lymph node metastasis, tumor differentiation and clinical tumor node metastasis (TNM) stage were recorded for statistical analyses. TNM stages were listed in accordance with the TNM classification from American Joint Committee on Cancer (AJCC) 7th edition [20].

RNA extraction and reverse transcription PCR (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, 1 ml TRIzol reagent was added to 100 mg tissue from each sample in an RNase-free triturator. Tissues were grinded into homogenate and 0.2 ml chloroform was added for phase separation. After centrifugation at the speed of $12,000\times g$ for 15 min, the upper aqueous phase was collected and 0.5 ml isopropanol was added to precipitate RNA. The RNA precipitate was then swept with 75% ethanol and dissolved in diethyl pyrocarbonate-treated water. The quantity of RNA was measured with a BioPhotometer plus (Eppendorf, German). The integrity of the RNA sample was checked by agarose gel electrophoresis to ensure the quality. RT-PCR was performed using Transcript MiRNA First-Strand cDNA Synthesis SuperMix (TransGen Bi., China) according to the manufacturers' instructions.

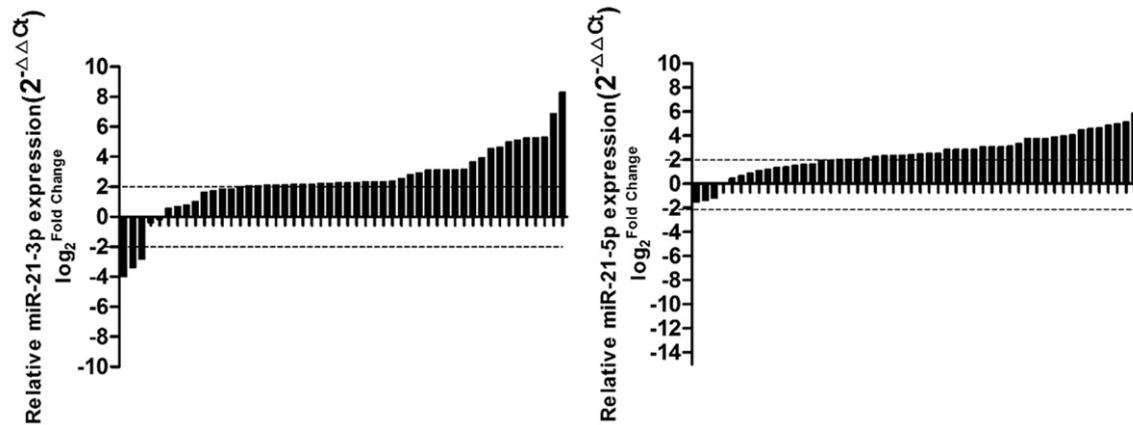


Figure 2. Fold change of miR-21-3p and miR-21-5p in GC tissues from 50 GC patients.

Table 1. Clinical features of investigated GC patients

Characteristics		miR-21-3p ($2^{-\Delta\Delta C_t}$)	P value	miR-21-5p ($2^{-\Delta\Delta C_t}$)	P value
Ages(years old)	<60	2.44±2.19	0.83	2.56±1.38	0.92
	≥60	2.41±2.26		2.56±1.59	
Gender	Female	2.11±2.42	0.41	2.41±1.64	0.56
	Male	2.64±2.21		2.55±1.62	
Tumor size (cm)	<5	2.44±2.11	0.58	2.31±2.11	0.51
	≥5	2.23±1.93		2.78±1.75	
Depth of tumor invasion	T1-T2	1.85±0.83	0.03	1.70±1.27	0.04
	T3-T4	2.44±2.19		2.85±1.97	
Lymph node metastasis	No	1.51±0.84	<0.01	1.53±0.59	<0.01
	Yes	2.06±0.62		2.94±2.03	
Tumor differentiation	Low	2.37±2.17	0.2	2.94±1.82	<0.01
	Middle or high	0.95±1.66		1.07±1.45	
TNM stage	I	0.61±1.46	<0.01	0.93±0.93	<0.01
	II	1.35±1.74		1.51±1.20	
	III	3.17±1.72		3.50±1.76	

P-values were calculated using the Mann-Whitney U-test, and the difference was regarded as significant if the *p* value was less than 0.05.

Quantitative real-time PCR (qPCR)

The technique of qPCR was performed using CFX96 real time PCR (Bio-Rad, USA) and SY-BR® Premix Ex Taq™ II (Perfect Real Time) (TaKaRa, China) in according with manual instructions. Expression of U6snRNA was used as an internal control. $2^{-\Delta\Delta C_t}$ method was employed to determine the relative expression of miRNAs in tumor and paracancerous tissues.

Statistical analysis

Statistical analyses were conducted using SPSS Statistics software (version 22, IBM Inc, USA). Graphs were drawn in GraphPad Prism

5.0 (Graphpad Software Inc., California). The differences of miRNAs expression between subcategories were tested by Mann-Whitney U test and were regarded as significant if the *P* value was less than 0.05 [21].

Results

miR-21-3p and miR-21-5p expression in GC and paracancerous tissues

The relative expression of miR-21-3p and miR-21-5p in all paracancerous and GC tissues was described in **Figure 1**. As shown, both miR-21-3p and miR-21-5p levels were higher in tumor tissues than those in paracancerous tissues

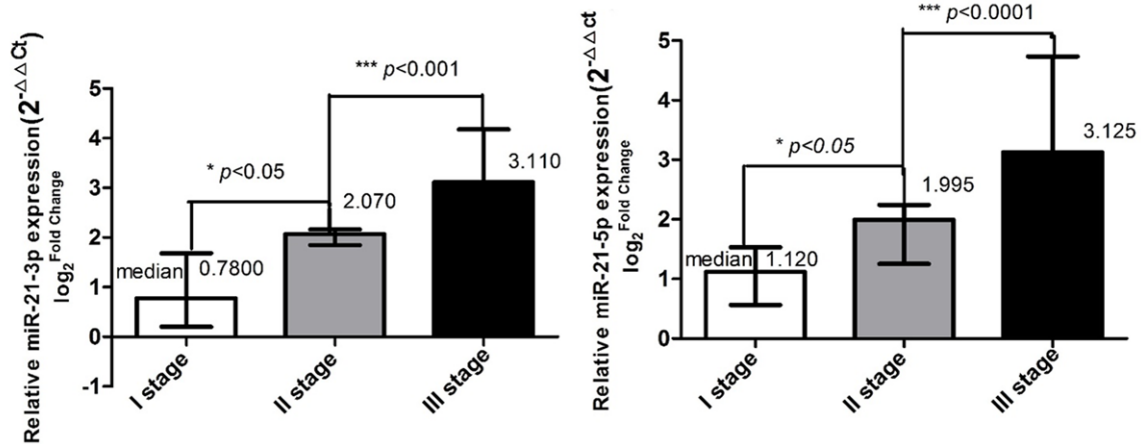


Figure 3. The difference of miR-21-3p and miR-21-5p expression in subgroups divided by TNM stage.

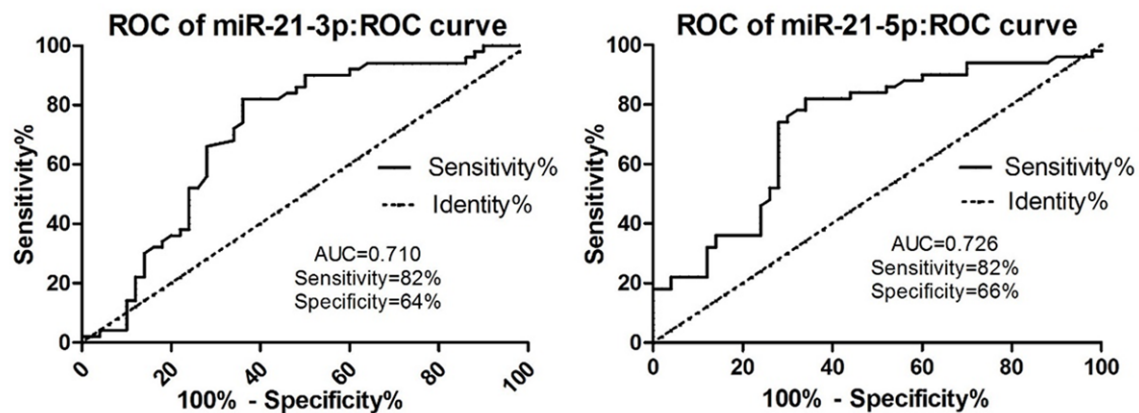


Figure 4. ROC curve analyses of miR-21-3p and miR-21-5p in GC tissues and paracancerous tissues from 50 GC patients. *p*-values were calculated using the Mann-Whitney U-test. (*P*<0.0001 for miR-21-3p and miR-21-5p).

(*P*=0.0003 for miR-21-3p and *P*<0.0001 for miR-21-5p). As is shown in Figure 2, the fold change was 3.113 (4.847/1.560) and 2.584 (4.857/1.880) for miR-21-3p and miR-21-5p, respectively. Further comparison between paired paracancerous and tumor tissues revealed that 90% of the patients (45/50) showed elevated expression of miR-21-3p and 94% (47/50) of the patients were with up-regulated expression of miR-21-5p. If the cut-off change was set to 2-fold, 74% of the patients (37/50) still showed an increase in miR-21-3p expression and 68% of the patients (34/50) had up-regulated miR-21-5p.

Relationship between clinicopathological factors and miR-21-3p or miR-21-5p expression in GC patients

Further, we probed the possible relationship between the expression levels of miR-21-3p or

miR-21-5p and clinicopathological features of the patients. Because the data did not obey normal distribution, nonparametric tests were used to analyze the difference. As was shown in the Table 1, we found no statistically significant difference of miR-21-3p or miR-21-5p expression between subcategories divided by age, gender and tumor size. Nonetheless, higher miR-21-3p and miR-21-5p expression were observed in the patients with tumor of T3 and T4 invasion than those of T1 and T2 invasion (*P*=0.03 for miR-21-3p and *P*=0.04 for miR-21-5p). Elevated levels of both miR-21-3p and miR-21-5p were also detected in the patients with lymph node metastasis (*P*<0.01 for both miRNAs). The difference of miR-21-5p expression was statistically significant between subgroups divided by the differentiation status of the tumor (*P*<0.01), while the difference of miR-21-3p expression was not (*P*=0.20). In addition

both miR-21-3p and miR-21-5p expression were related with the TNM stage of the patients. ($P < 0.01$ for miR-21-3p, $P < 0.01$ for miR-21-5p). Pairwise comparison was depicted in **Figure 3** and showed the expression of both investigated miRNAs was higher in tumors at more advanced stage. The difference between II and III stage ($P = 0.0001$ for miR-21-3p, $P < 0.0001$ for miR-21-5p) was more obvious than the difference between I and II stage ($P = 0.0334$ for miR-21-3p, $P = 0.0465$ for miR-21-5p).

Analysis of diagnostic value

The diagnostic value of miR-21-3p and miR-21-5p expression level was represented by their ability to distinguish GC samples from paracancerous samples. The Receiver-operator characteristic (ROC) curves were depicted in **Figure 4**. The area under the curve (AUC) for miR-21-3p was 0.710 (95% CI: 0.610-0.796, $P < 0.0001$) and for miR-21-5p was 0.726 (95% CI: 0.628-0.811, $P < 0.0001$). The sensitivity and specificity of miR-21-3p were 82% and 64% respectively, and 82% and 66% for miR-21-5p respectively.

Discussion

MiRNAs represent an important post-transcriptional regulation mechanism of gene expression. It was frequently reported that they played a significant role in tumorigenesis by acting as oncogenes or anti-oncogenes [22], or by indirectly regulating the expression of oncogenes and anti-oncogenes [23]. Therefore, aberrant expression of certain miRNAs has been widely implicated in the genesis and development of human cancer. Specifically, miR-21, one of the most studied, is upregulated in most human cancers, especially gastric cancer [24]. This altered miR-21 expression pattern provided a new opportunity for developing novel biomarkers for diagnosing gastric cancer at early stage. However, most of the studies has been focused on the role of miR-21 in gastric cancer lines; few has been reported regarding the level of its two mature isoforms, miR-21-3p and miR-5p, in GC patients and their association with clinicopathological factors.

In this research, clinicopathological features of GC including pathological staging, depth of tumor invasion and lymph node metastasis

showed a strong association with both the expression of miR-21-3p and miR-21-5p. However, no significant difference was observed between the expression of miR-21-3p and miR-21-5p. To our surprise, the difference of miR-21-5p level, but not that of miR-21-3p level, between subgroups of samples divided by tumor differentiation is significant. Notably, in our study, relative expression of the investigated miRNAs in the tumor and paracancerous tissues to the normal tissues was employed to exclude individual difference, which made our results more accurate and representative.

The diagnostic value of miR-21-3p and miR-21-5p was also analyzed in this study. We found that the area under the ROC curve, sensitivity and specificity for miR-21-3p in GC diagnosis were 0.71, 0.82, and 0.64 respectively; while those for miR-21-5p were 0.73, 0.82, and 0.66 respectively, which were both lower than previous results of 0.78, 0.89 and 0.91 respectively reported by a meta-analysis of miR-21 [25]. The reason might be that we compared the tumor tissues with the paracancerous tissues but not the normal tissues.

It should be noted that this study may have two main limitations. First, the distribution of relative miRNAs expression in investigated samples, especially in paracancerous tissues were not exceedingly concentrated and did not obey normal distribution, so that the statistical methods used in this study was nonparametric test, which showed lower power. The main cause was lack of sufficient samples. In addition, for ethical reasons, we could not collect normal gastric tissues from healthy controls. However, this problem could be solved by using serum from GC patients and healthy controls.

In summary, our results proved that both miR-21-3p and miR-21-5p may serve as potential diagnostic biomarkers for GC, while miR-21-5p is more potent in distinguishing the degree of tumor differentiation. Further studies with larger sample pool and healthy controls, and those on their bio-functions are currently underway.

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Disclosure of conflict of interest

None.

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