RESEARCH ARTICLE

Plasma Post-operative miR-21 Expression in the Prognosis of Gastric Cancers

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Abstract

Tumor-associated microRNAs have been detected in serum or plasma, but whether plasma microRNA-21 (miR-21) could be a potential circulating biomarker for gastric cancer (GC) prognosis in Chinese is still uncertain. Real-time quantitative reverse transcription PCR (qRT-PCR) was employed in this study to compare the relative expression of miR-21 between pre-operative and post-operative paired plasmas from 42 patients with primary GCs. The results showed that the expression levels of miR-21 in the post-operative plasmas were significantly reduced by an average of 18.2 times in all patients when compared to the pre-operative plasmas, and by 22.1 times in the subgroup of patients without family history, while only 1.76 times in the subgroup of patients with a family history. With respect of clinicopathological characteristics, the plasma miR-21 expression was highly associated with differentiation degree and lymph node metastasis rate. The results suggested plasma miR-21 could be a novel potential biomarker for GC prognosis and evaluation of surgery outcomes, especially in patients without a family history.

Keywords: microRNA-21 - gastric cancer - quantitative PCR - plasma

Asian Pac J Cancer Prev, **14 (12)**, 7551-7554

Introduction

Gastroscopic screening for gastric cancer (GC) is currently the most reliable screening tool, but it is unsuitable as a first-line examination due to the invasive nature. There is a need for discovering some noninvasive biomarkers to improve the detection of GC. A recently discovered class of small noncoding RNAs, microRNAs (miRNAs) that regulate gene expression and serve multiple biological functions, has opened a new approach of tumor biomarkers for early cancer diagnosis (He et al., 2005). The stability of tumor-associated miRNA in blood allows it to be a novel noninvasive tumor biomarker for cancer detection. Circulating miRNAs have been detected in the plasma from patients suffering from breast, colon and gastric cancers (Huang et al., 2010; Chan et al., 2013). MicroRNA-21 (miR-21), one of the cancer-associated miRNAs, has been shown to have the proto-oncogene activity, which is associated with human tumors (Liu et al., 2011; Ma et al., 2011). MiR-21 expression in a variety of solid tumor tissues was significantly higher than that in normal tissues, and was associated with local invasion and lymph node metastasis (Pan et al., 2010). Several studies on the expression of plasma miR-21 in Chinese has been reported (Chan et al., 2008; Li et al., 2012; Wang et al., 2012). Meta-analysis showed that miR-21 has potential diagnostic value with a moderate sensitivity and specificity for GC (Zeng et al., 2013), but the plasma miR-21 as the biomarker of diagnosis and prognosis of GC is still uncertain. In this study we collected plasmas from patients with primary gastric cancer, and used quantitative reverse transcription PCR (qRT-PCR) to compare circulating miR-21 expression in pre- and post-operative paired plasmas, and studied its clinical significance in gastric carcinogenesis.

Materials and Methods

Patients and samples

A total of 42 plasma samples were collected from the GC patients, who underwent gastrectomy between November 2011 and March 2012 at Jiangsu Cancer Hospital, China. The collections were performed before and 7th day after gastrectomy. Relevant clinical and survival data were available for all patients. Written informed consent was obtained from all patients after approval by the hospital's ethics committee. No patient received chemotherapy or radiotherapy before blood sampling. All clinicopathological variables including gender, age, tumor size, tumor location, the depth of tumor invasion, status of lymph node metastasis, and histological differentiation were defined according to Japanese Classification of Gastric Cancer and collected from gastric cancer database of our hospital. Tumors were

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Table 1. Plasma miR-21 Avg Ct Value Fold Changes in Gastric Cancer

	n	miR-21Avg Ct		U6 Avg Ct		RQ	*P*
		Preoperative	Postoperative	Preoperative	Postoperative		
Gastric cases	42	27.05±3.94	27.97±3.21	35.52±1.77	38.47±2.18	18.20	—
Family history	8	28.65±5.17	27.34±5.22	35.77±1.22	35.07±4.45	1.76	<0.05
No family history	34	26.67±5.27	28.29±4.90	35.46±1.87	35.57±4.96	22.11	

*Avg Ct, average threshold cycle number, RQ value, relative expression fold changes. U6 as an internal control; *Avg Ct, average threshold cycle number, RQ value, relative expression fold changes. U6 as an internal control

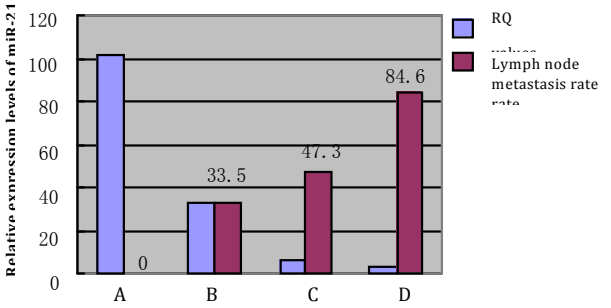

Figure 1. Correlation of Relative Expression of miR-21 in Gastric Cancer Patients and Lymph Node Metastasis Rate in Four Subgroups of Tumor Differentiation Degrees. A: well differentiated; B moderately differentiated; C: mediate-poorly differentiated; D: poorly differentiated

Table 2. Clinicopathological Features in Patients with Gastric Cancer

Factor	
Age (years)	61.4 (41-80)
Gender (male\female)	33\19
Location (lesser curvature/gastric angle/ antrum/cardia/gastric body)	10\6\12\11\3
*Pathological type (UL/SD/T\ UT/UTP \O)	9\6\4\8\8\7
Metastasis (with/no)	22\20
Differentiation (poor/mediate-poor/mediate\well)	14\18\6\4
Family history (with/no)	8\34

*Ulcer type low differentiated carcinoma/Surface depressed cell carcinoma/Tubular adenocarcinoma/Ulcer type tubular adenocarcinoma/Ulcer type tubular papillary adinocarcinoma/ Other

staged according to the seventh edition of the UICCTNM classification system.

Three milliliters of whole blood was collected in EDTA Vacutainer (BD Company, USA) for plasma. Sample collections were centrifuged at 2, 500 rpm and 4°C for 10 min. The supernatant fluids were then stored at -80°C until total RNA extraction.

Total RNA extraction from tissues

RNA was extracted from frozen fresh GC tissues using miRNeasy Mini Kit (QIAGEN Company, Germany) according to the manufacture's instructions. The RNA pellets were air-dried and dissolved in 20μl of nuclease-free water.

Real-time fluorescence qRT-PCR

Reverse transcription (RT) was carried out in 20μl reaction master mix with RT-PCR kit (QIAGEN Company, Germany) to transcript total RNA to cDNA according to the manufacturer's instructions. Reverse transcription reaction was prepared on ice, and the conditions used involved incubations at 16°C for 30 min; 37°C for 60 min and then 72°C for 10 min, and the product was preserved at -20°C.

Quantification of differentially expressed miRNAs was conducted with Rotor Gene Q real-time PCR instrument (Qiagen Company, Germany) and the cycle threshold (Ct) values were calculated as the number of cycles required for the fluorescent signal to cross the threshold in qPCR.

Real-time PCR primers: miR-21: F: 5'-GCCCGC TAGCTTATCAGACTGATG-3'; R: 5'-GTGCAGGGTCC GAGGT-3'; U6: F: 5'-GCGCGTCGTGAAGCGTTC-3'; R: 5'-GTGCAGGGTCCGAGGT-3.

Each reaction was performed in a final volume of 25 μl containing 2.0 μl of the cDNA, 0.5 μl of each primer, and 22 μl of SYBR (TaKaRa Company) miRNA assay mix. The amplification profile was: denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 5 sec and 60°C for 30 sec. Each sample was run in triplicates for analysis. The expression of miRNAs from tissue samples was normalised using the △CT method relative to U6 small nuclear RNA (RNU6B) by subtracting the Ct values of internal control from the Ct values of the miRNA of interest. △△Ct was then calculated by subtracting △Ct of the control from △Ct of disease. Fold change of miRNA (RQ) was calculated by the equation of $2^{-\triangle\triangle Ct}$.

Statistical analysis

SPSS11.0 software was used for data analysis. The Wilcoxon test was used to compare the paired plasma samples obtained before and after operation. *P*-value <0.05 was considered statistically significant.

Results

MiR-21 expression between the pre-operative and post-operative plasmas in gastric carcinoma

It was found that the expression levels of miR-21 (27.93±3.21) in the post-operative plasmas from 42 cases were significantly reduced by 18.20 times when compared to the pre-operative paired plasmas (Table 1). Moreover, the post-operative plasma miR-21 expression was decreased by an average of 22.11 times in the subgroup of patients without family history, while only by 1.76 times in the subgroup of patients with family history when compared to the pre-operative (Table 1).

Correlation of relative expression of miR-21 and histological differentiation and lymph node metastasis

In four differentiation degree subgroups consisting of high (4 cases), medium (16 cases), mediate-poor (18 cases) and poor (14 cases), the average post-operative miR-21

expressions were decreased by 101.4 times, 33.2 times, 6.3 times and 3.1 times, respectively with comparison to the pre-operative. Correspondingly, lymph node metastasis rate in four subgroups were 0 (0/4), 33.5% (2/6), 47.3% (9/19) and 84.6% (11/13), respectively. RQ was decreased along with the increase of differentiation degrees, and reversely correlated with lymph node metastasis rate ($r = -0.8925$, $P < 0.05$) (Figure 1).

Relationship between plasma miR-21 expression and other clinicopathological characteristics

The clinical pathological characteristics of 42 cases of gastric cancer were shown in Table 2. Data analysis showed that the plasma miR-21 expression changes was not associated with age, gender and pathological types ($P>0.05$).

Discussion

Early detection of gastric cancer is the key approach to improve the prognosis of gastric cancer. Much effort has been made to develop preliminary screening tests in easily accessible specimens. Accumulating reports suggest the potential of microRNAs in the early detection of patients with several malignancies (Wong et al, 2008; Ng et al, 2009). These findings prompted us to investigate the usefulness of miRNAs in patients with GCs.

RNA from apoptotic or necrotic tumor cells and circulating cell lyses can be released to the circulating blood in various forms (Thery et al., 2002; Cocucci et al., 2009), but the RNA molecules in body fluids were limited as biomarker applications due to generally lower circulating levels. Researches found that circulating miRNA in serum and plasma is usually combined with the protein stablely, not in free form (Chen et al., 2008; Mitchell et al., 2008), thus resistant to RNase degradation (Arroyo et al., 2011). Therefore, circulating RNA may act as the potential biomarker in cancer diagnosis and prognosis (Deddens et al., 2013; Tambyah et al., 2013).

MiR-21 is one of the most frequently studied oncomiRNAs. It has been proved that phosphatase and tensin homologue is the direct target of miR-21 whose expression is elevated in GC tissues (Chan et al., 2008; Zhang et al., 2008). The expression of miR-21 has been reported to be remarkably increased in breast cancer, liver cancer, lung cancer, ovarian cancer and other malignant tumors, and was associated with tumor invasion, metastasis and prognosis (Hiyoshi et al., 2009; Zhang et al., 2010; Zhang et al., 2012). Several studies on gastric cancer demonstrated that miR-21 expression in tumor tissues was significantly higher than non-tumor tissues, and was closely related to tumor size and depth (Motoyanma et al., 2010).

In this observational study, we detected circulating miR-21 expression by qRT-PCR. The results showed that the overall expression of plasmas miR-21 was significantly decreased after operation and was highly associated to differentiation degrees and lymph node metastasis rate. More than 1/3 of the gastric cancer patients have family history, which was reported as an important factor for gastric cancer (OR=2.254, P=0.007) (Gong et al., 2013). Tracz study also found that in gastric cancer patients with family history, c-myc gene expression was significantly higher than those without family history. After tumor resection, c-myc gene expression was significantly reduced in patients without family history, but not in patients with family history (Tracz et al., 2013).

We measured circulating miR-21 in paired plasma before and 7 days after surgical removal of the tumors, to confirm tumor release of the circulating miRNAs. As a result, the concentrations of miR-21 were significantly reduced postoperatively in patients with high pre-operative plasma miR-21. Although the kinetics and metabolism of the plasma miRNAs have not yet been clearly elucidated, 7 days seems to be sufficient time for clearance of the circulating miR-21 (Li et al., 2013).

Some study reported that stomach cancer predisposition was closely associated with a family history of GC (Zuk et al., 2012). Our results showed that the post-operative plasma miR-21 expression was significantly decreased only in the subgroup of patients without family history. We speculate that the specific genetic background and signal transduction pathway, such as G-protein pathway in patients with family history may contribute to the observed differences (Marcon et al., 2010; Zuk et al., 2012).

Although our results are promising, there still are some limitations in this study: (1) the sample size of GC is small, further large samples are necessary; (2) absolute quantification approach would be preferable for further validations because internal control for plasma miRNA measurement is not generally accepted.

In conclusion, our study provided a potential biomarker for evaluation of surgery outcomes of GC. By combining plasma miR-21 expression with conventional clinicopathological factors, we may be able to predict GC outcome more accurately, leading to additional therapeutic intervention.

Acknowledgements

This work was supported by the Program for the Talents in Science and Education of Jiangsu Province (No. RC2011092) and the Fund of Jiangsu Cancer Hospital Project (No. ZM201002).

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