RESEARCH ARTICLE

Upregulation of MicroRNA 181c Expression in Gastric Cancer Tissues and Plasma

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Abstract

Objective: To test the microRNA-181c (miR-181c) expression in tissues and plasma of gastric cancer (GC) cases, analyze any correlations, and explore the possibility of miR-181c as a potential molecular marker for GC diagnosis. Materials and Methods: Relative miR-181c expression levels in cancers and plasma from 30 GC patients was tested using reverse transcription–real-time fluorescent quantitation PCR and compared to that in samples from 30 gastric ulcer and 30 chronic gastritis patients. Results: The miR-181c expression level in the GC tissues was significantly higher than that in the gastric ulcer and chronic gastritis tissues ($P = 0.000$), as was the miR-181c expression level in the GC plasma ($P = 0.000$). We determined that miR-181c expression in GC plasma was positively correlated to its expression in the GC tissues ($P = 0.000$). Conclusions: The expression of miR-181c is upregulated in GC tissues and plasma, and the miR-181c expression level in GC plasma is positively correlated to that in the corresponding cancer tissues. Plasma miR-181c is possibly a new serological marker for GC diagnosis.

Keywords: MicroRNA - 181c (miR-181c) - gastric cancer - plasma - cancer tissue

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Introduction

MicroRNAs (miRNAs) are tiny, non–protein-coding RNA molecules composed of 19-26 nucleotides that regulate the level of posttranscriptional gene expression and are associated with a variety of tumors when expressed abnormally (Jay et al., 2007). Recently, there have been concerns regarding the correlation of miRNA-181c (miR-181c) to tumors. Studies have shown that abnormal miR-181c expression is closely related to glioma, squamous cell carcinoma of the tongue, breast cancer, and other tumors (Wong et al., 2008; Lowery et al., 2009; Slaby et al., 2010). Scholars have studied the correlation of miR-181c to gastric cancer (GC) at cellular level and found that the miR-181c expression varies according to the GC cell line: it is downregulated in the KATO-III, MKN45, MKN74, AGS cell lines, and so on, and upregulated in the HSC58, MKN7, and GCIY cell lines, and the like (Hashimoto et al., 2010). However, whether miR-181c expression in the tissue and plasma samples from GC patients is increased or decreased currently remains unclear.

GC is one of the most common malignant tumors in China, ranked first in terms of morbidity and mortality rates. Early diagnosis and treatment can significantly improve the GC cure rate and prognosis. Nevertheless, effective methods for the early diagnosis of GC are lacking. Recent studies have found that tumor-related miRNA can also be detected in the peripheral blood of patients with cancer (Mitchell et al., 2008). This means that it is possible to use miRNA as a novel, noninvasive molecular marker for cancer diagnosis. We tested the expression of miR-181c in the tissue and plasma of patients with GC, analyzed the correlation between them, and evaluated the possibility of using miR-181c as a potential serological molecular marker for GC diagnosis.

Materials and Methods

Subjects

Thirty patients each with GC, gastric ulcer, and chronic gastritis as confirmed at our hospital from June 2010 to March 2011 based on gastrointestinal symptoms were included. All the patients were examined and their diagnosis confirmed by gastroscopy and biopsy pathology, and we excluded those with other malignant tumor histories. The age range of the 24 men and 6 women with gastric adenocarcinoma was 37-83 years; the mean age was 68.5 ± 10.4 years. The 21 men and 9 women with gastric ulcer were 34-78 years old; the mean age was 63.7 ± 12.6 years. The 20 men and 10 women with chronic gastritis were 34-83 years old; the mean age was 62.4 ± 12.2 years. The sexes and ages in the three groups were not significantly different ($P > 0.05$). The biopsy sites examined with gastroscopy were cancer tissue, gastric

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ulcer tissues, and the antral mucosa in the GC, gastric ulcer, and chronic gastritis patients, respectively. The biopsy lesion tissues were underwent routine pathological examination when all the patients were examined by gastroscopy, two pieces of the appropriate biopsy tissues were additionally obtained with clippers, and 3 mL venous blood was obtained before or after the gastroscopy examination. The Medical Ethics Committee of Aerospace Center Hospital ratified the study program and all patients signed informed consent forms. The study conformed to The Code of Ethics of the World Medical Association (Declaration of Helsinki) printed in the British Medical Journal (18 July 1964).

Reagents

We used TRI Reagent BD (mrcgene; Cincinnati, OH), Total RNA Isolation Kit (Ambion; Austin, TX), reverse transcription (RT) kit (Qiagen; Hilden, Germany), and a SYBR Green Real-Time PCR Master Mix (Takara; Dalian, China). All the other chemicals used were of analytical reagent grade.

Samples

The GC, gastric ulcer, and chronic gastritis mucosa tissue samples were harvested during the gastroscopy, quickly frozen in liquid nitrogen, and stored at -80°C. Three milliliters of peripheral blood was collected before and after the gastroscopy, and the plasma was harvested after centrifugation and stored at -80°C.

RNA isolation and real-time RT–PCR

For the real-time RT–PCR, RNAs were extracted from homogenized tissues or plasma using TRIzol according to the manufacturer's instruction. Genomic DNA contaminants were removed using DNA-free DNase Treatment and Removal (Ambion). The RNA purity was assessed by spectrophotometry (A260/A280 > 1.8). Single-stranded cDNAs were generated using the RT kit according to the manufacturer's directions. The real-time quantitative PCR experiments were performed with an ABI Prism 9700 Sequence Detection System (Applied Biosystems; Foster City, CA) using the SYBR Green PCR Master Mix according to the manufacturer's protocol. The primer sequences were as follows: hsa-miR-181c sense: 5'-AACATTCAACCTGTCGGTGAGT-3'; internal control U6 sense 5'-CAAGGATGACACGCAAATTCG-3'. All anti-sense primers were universal primers provided in the kit. Each dilution was amplified with triplicate PCR and plotted as the mean values. Fluorescence measurements were made in every cycle. The following cycling conditions were used: 95°C for 30 s, and 40 cycles of 95°C for 5 s and 60°C for 34 s. The fold-change in the relative gene expression to that of the control was determined by the standard $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The experimental data were analyzed using SPSS13.0 statistical software and expressed as mean ± standard deviation. The difference in the relative levels of miR-181c expression in the GC, gastric ulcer, and chronic gastritis tissues and plasma were compared using one-way ANOVA. The correlation of miR-181c expression in the GC tissues and plasma was defined using bivariate correlation analysis and linear regression analysis. The test level at $\alpha = 0.05$, $P < 0.05$ was considered significantly different.

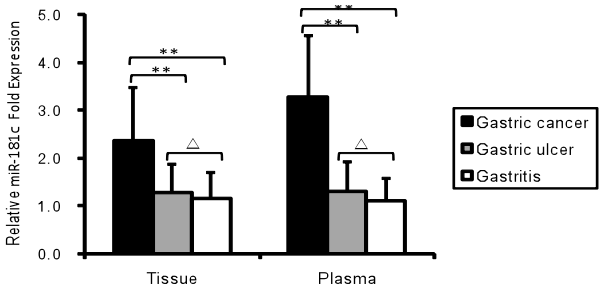

Figure 1. The Differential Expression of miRNA-181c in the Tissues and Plasma of Gastric Cancer, Gastric Ulcer, and Chronic Gastritis ($P < 0.01$; $^{\Delta}P > 0.05$)**

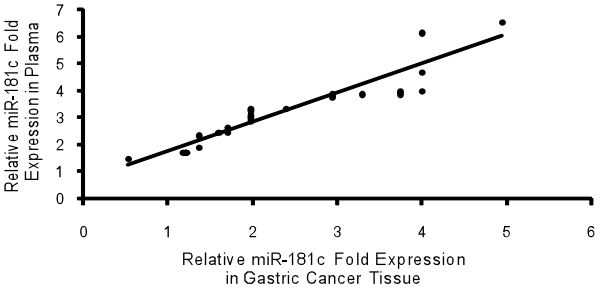

Figure 2. Relative Level of miR-181c Expression in the Plasma of Gastric Cancer Patients Positively Correlated with That in Tissue ($y = 1.084\chi + 0.693$, $R^2 = 0.866$)

Results

Difference of miR-181c expression in GC, gastric ulcer, and chronic gastritis tissues

The relative levels of miR-181c expression in the GC, gastric ulcer, and chronic gastritis tissues were 2.37 ± 1.11, 1.29 ± 0.59, and 1.16 ± 0.55, respectively. The level of miR-181c expression in the GC tissues was significantly higher than that in the gastric ulcer and chronic gastritis tissues ($P = 0.000$), whilst the expression levels in the gastric ulcer and chronic gastritis tissues were not significantly different ($P = 0.536$, Figure 1).

Difference of miR-181c expression in GC, gastric ulcer, and chronic gastritis plasma

The relative levels of miR-181c expression in the GC, gastric ulcer, and chronic gastritis plasma were 3.27 ± 1.30, 1.31 ± 0.62, and 1.12 ± 0.45, respectively. The level of miR-181c expression in the GC plasma was significantly higher than that in the gastric ulcer and chronic gastritis plasma ($P = 0.000$), whilst the levels of miR-181c expression in the gastric ulcer and chronic gastritis plasma were not significantly different ($P = 0.404$, Figure 1).

Relation of relative levels of miR-181c expression in the GC plasma and tissues

After they were defined using bivariate correlation

analysis and linear regression analysis, we determined that the relative levels of miR-181c expression in the GC plasma and tissues were positively correlated. The regression equation was $y = 1.084\chi + 0.693$, the correlation coefficient $r = 0.931$, and the determination coefficient $R^2 = 0.866$ ($P = 0.000$, Figure 2).

Discussion

Numerous studies have revealed that a variety of miRNA are correlated to GC (Wang et al., 2012), and have explored the possibility of using miRNAs as a molecular biomarker for GC diagnosis based on their expression in plasma or serum (Liu et al., 2012). This study detected the expression of miR-181c in the tissue and plasma of GC patients and compared it with the corresponding tissue samples from patients with gastric ulcer and chronic gastritis. The levels of miR-181c expression in the GC tissue and plasma were significantly higher than that in the gastric ulcer and chronic gastritis tissue and plasma. The level of miR-181c expression in the GC plasma was positively correlated to that in the GC tissues, suggesting the possibility of using miR-181c as a potential marker for GC diagnosis.

GC is one of the most common malignancies of the digestive system, posing a serious threat to human health. Surgical resection is an effective treatment for early GC; however, most GC is diagnosed at the late stage, and the patient loses the chance of a cure through surgery or faces the prospect of a higher postoperative recurrence rate. The prognosis is worse after radiotherapy and chemotherapy. The 5-year survival rate is 20-25% in America, Europe, and China (Hartgrink et al., 2009; Ferlay et al., 2010). Hence, improving the level of early GC diagnosis is important to increase the cure rate of GC patients, prolong survival time, and improve the prognosis. Nevertheless, there is still a lack of effective noninvasive methods for the early diagnosis of GC. The traditional serum markers such as carbohydrate antigen 199 and carcinoembryonic antigen are not effective methods for the early screening and diagnosis of GC due to their lack of sensitivity and specificity.

miRNA inhibits target mRNA translation and regulates posttranscriptional gene expression by directly cleaving the target mRNA, or completely/incompletely and complementarily combining with the 3' untranslated region of the target mRNA, consequently regulating cell proliferation, differentiation, and apoptosis and participating in the individual development, metabolism, and tumor development process (Osman, 2012). Recent studies have discovered that the expression of miRNA in normal tissue and tumor tissue is different, suggesting a close relation with tumorigenesis (Mattie et al., 2006; Yang et al., 2008; Habbe et al., 2009); it has also been discovered that miRNA expression profiles in tumor tissue are more accurate than the encoded protein mRNA, thus indicating a possible means of early tumor diagnosis (Criscitiello et al., 2010; Cheng et al., 2011). In a review on GC postoperative wax specimens, we found that miR-181c is upregulated in GC tissue and is closely associated with cancer tissue differentiation, clinical stage, and survival time, hinting at the possibility of using miR-181c as a molecular biomarker for the diagnosis and prognosis evaluation of GC (research results to be published). In the present study, we detected the expression of miR-181c in fresh biopsy tissues obtained by gastroscopy, determining that its level of expression in GC tissues is significantly higher than that in the tissues of benign gastric ulcer and chronic gastritis. This finding revalidates the correlation between miR-181c and GC and the specificity of cancer tissues, and further supports the feasibility of using miR-181c as a molecular marker for the diagnosis of GC.

In the histological diagnosis of GC, however, tissue specimens must be obtained through a difficult, invasive procedure. This procedure is not suitable for regular follow-up after GC treatment, and largely limits the clinical application of histological diagnosis. Recent studies have revealed that tumor-related miRNAs can present in the serum or plasma in stable form, and the possibility of using miRNA as a new, noninvasive molecular marker for tumor diagnosis has strengthened considerably. As plasma and serum can be retrieved easily through noninvasive methods, the examination of stably present miRNA in the circulating blood is a promising means of tumor diagnosis. Past studies have concluded that RNA is not stable in peripheral blood, being susceptibly degraded by RNase and difficult to test. However, recent studies (Mitchell et al., 2008) have discovered that miRNA is very stable in the peripheral blood, is detectable with little change to its abundance even following the addition of RNase, DNase, hydrochloric acid, and sodium hydroxide to the serum, or following repeated freeze–thawing. These findings strongly indicate that peripheral blood miRNA may be used as a new biomarker for cancer diagnosis. Mitchell et al. (2008) discovered that miR-141, whose expression level is increased in prostate cancer tissue, also increases significantly in the plasma; the diagnostic sensitivity of miR-141 in the plasma of 25 patients with prostate cancer was 60%, the specificity was up to 100%, and its diagnostic accuracy was superior to that of prostate-specific antigen. Ng et al. (2009) reported that miR-92 was significantly higher in the plasma of patients with TNM stage I colon cancer; the diagnostic sensitivity was up to 89%, specificity was up to 70%, and its diagnostic accuracy was significantly better than that of the fecal occult blood test. Resnike et al. (2009) determined that the expression levels of eight miRNAs in the serum of patients with ovarian cancer were significantly different from that of healthy serum; serum miRNA detection can be used for the early diagnosis of ovarian cancer, and its clinical value is superior to that of cancer antigen 125. In the field of GC research, a number of serum or plasma miRNA molecular markers of potential value have been identified and applied for GC testing (Tsujiura et al., 2010; Liu et al., 2011). Li et al. (2012) found that the levels of miR-223 and miR-21 expression in Stage I GC plasma were significantly higher than that of the healthy control group; Liu et al. (2012) reported that miR-378 was significantly increased in the serum of early GC. This study discovered that the level of miR-181c expression in the plasma of GC patients was significantly higher than that in the plasma of gastric ulcer and chronic gastritis patients; further, bivariate linear

regression and correlation analysis approaches revealed that the relative levels of miR-181c expression in the GC plasma and tissue were positively correlated. This suggested that if there were a higher level of expression in GC tissues, the level of miR-181c expressed in the plasma would increase. Plasma specimens are obtained more easily than tissue specimens are. This suggests that plasma miR-181c has the potential to be a new serum marker for the diagnosis and follow-up of GC.

It is still unclear why miR-181c expression in the peripheral blood plasma of patients with GC is upregulated. Current studies have conjectured that the increased plasma miRNA level is due to the release of overexpressed miRNA from tumor tissues into the peripheral blood. There are two theories regarding the release mechanism: the injury theory and the super-micro vesicle theory. The former states that miRNA is released into the peripheral blood during the proliferation and dissolving of cancer cells, while the latter states that the miRNA is transmitted intercellularly through super-micro vesicles and is released extracellularly and to the peripheral blood when the super-micro vesicles shed off the cell membrane during transmission (Ji et al.,2009; Brase et al., 2010). Additional studies have found that many tumor cells present in the microenvironment of GC, such as macrophages, myeloid cells, dendritic cells, and T cells, and so on, can release a secretory product (exosomes) functionally. The secretory product transmits the miRNA to other cells or the circulating blood (Valadi et al., 2007). Based on the above hypotheses, it is believed that the increased level of miR-181c expression in GC plasma may be due to the release of overexpressed miR-181c from cancer tissue into the peripheral blood somehow, or that miR-181c is upregulated in some tumor-associated cells and transmits the miRNA to the peripheral blood through the secretory product.

To conclude, the upregulated miR-181c expression in the plasma of patients with GC was positively correlated with its level of expression in GC tissues; plasma miR-181c has the potential to be a new, noninvasive serological marker for GC diagnosis. Further studies must be performed to explore its clinical value, for example, by increasing the sample size to identify the diagnosis thresholds and analyzing GC patients hierarchically according to tumor stage to identify the plasma miR-181c values in early GC diagnosis and to prospectively understand the function of plasma miR-181c on the follow-up and prognostic evaluation after GC.

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