RESEARCH ARTICLE

Downregulated MicroRNA-133a in Gastric Juice as a Clinicopathological Biomarker for Gastric Cancer Screening

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Abstract

Circulatory miR-133a is a marker shared by several types of cancer. In this study we evaluated the feasibility of using miR-133a levels in gastric juice to screen for gastric cancer. A total of 204 samples of gastric juice and mucosa from gastric cancer, atrophic gastritis, gastric ulcer, superficial gastritis and healthy cases were collected by gastroscopy. The results showed that miR-133a levels in gastric juice and carcinoma tissues of patients with gastric cancer were significantly downregulated and positively correlated. Moreover, miR-133a in gastric juice has high operability, high reliability, high sensitivity, high specificity and relative stability, fit for clinical diagnosis of gastric cancer.

Keywords: MicroRNA-133a - down-regulation - gastric juice - gastric cancer

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Introduction

Gastric cancer (GC) is clearly a primary cause of cancer-related deaths, respectively representing the 2nd and 4rd in males and females worldwide (Siegel et al., 2012). In China, GC accounts 25.53 per 10 million in the average annual mortality rate (Lin et al., 2011). Currently, surgical resection is still the main strategy for treatment of GC. After surgical resection patients diagnosed with early stage GC may reach about 90% postoperative 5 year survival rate. However, despite advances in surgery and other treatment modalities, the prognosis of patients with GC remains poor as the majority of patients with GC are diagnosed at an advanced stage, resulting in a great decrease in the 5 year survival rate after surgical resection. The common blood-based tumor markers, including carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA), can't satisfactorily detect GC. Endoscopy followed by pathologic biopsy specimen examination of a gastric mucosa continues to be the most reliable method of diagnosing GC. Therefore, exploring novel indicators for screening and diagnosis of early cancer is an important topic of GC research.

MicroRNAs are a class of non-coding tiny nucleotides with a length of 21-25 nt, that can regulate protein coding gene expression at the post-transcriptional level via targeting the 30 untranslated region of specific mRNAs to regulate mRNA degradation or translational inhibition (Calin et al., 2004). A growing evidence demonstrates that microRNAs are aberrantly expressed in many cancers to play significant roles in initiation, development and metastasis of cancers (Nohata et al., 2012). MicroRNA-133a (MiR-133a) has been validated as a tumor suppressor in relation to colorectal cancer (Dong et al., 2013), ovarian cancer (Guo et al., 2014), breast cancer (Cui et al., 2013), prostate cancer and bladder cancer (Wang et al., 2014). Specially, ectopic expression of miR-133a plays a crucial role in initiation and progression of GC via suppress of proliferation, invasion and migration of GC cells (Wu et al., 2010; Qiu et al., 2014). As the miR-133a levels in peripheral blood are shared by several types of cancer, therefore, it is necessary to identify novel way to link miR-133a levels with GC specially. Gastric juice may provide plenty of useful information about gastric condition and helicobacter pylori status (Tucci et al., 2007). To date, it is unknown if miR-133a exists in gastric juice and how much its value is in diagnosis of GC. The aim of this study was to investigate the miR-133a expression in gastric juice and to evaluate its clinical significance in screening of GC.

Materials and Methods

Sample Collection

A total 204 subjects, including 62 patients with primary GC (mean age, 61.5±12.7 years), 32 patients with atrophic gastritis (58.4±14.7 years), 36 cases with gastric ulcer (55.4±3.2 years), 40 cases with superficial gastritis (54.6±15.1 years) and 34 cases with normal mucosa (57.2±13.5 years) were obtained from the files of the Department of Surgery, Clinical Medical College,

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Yangzhou university from the year 2012 to 2015. None of these patients had received chemotherapy prior to surgery. Each clinical specimen was histopathologically confirmed by endoscopic examination followed by diagnosis of biopsies. The biopsy specimens from GC tissue, pathologic and normal mucosa, as well as three milliliters of gastric juice were collected from the patients and volunteers. Gastric juice was centrifuged at 2000 g for 30 min at 4°C. The supernatant and specimens were frozen at -80°C until further analysis. Informed consent was obtained from all participants for sample and ongoing data collection. The project was approved by the Ethics Committee of Yangzhou University.

RNA Extraction

The method for extraction of total RNA, including miRNAs, is similar to that described previously (Zhang et al., 2012). Briefly, gastric juice and mucosa biopsies were placed at room temperature for 1 h. Then 10 mg mucosa biopsies mixed with 1 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA) were homogenized. While 250 μl gastric juice were mixed with 750 μl Trizol LS reagent by vortex for 30 s. After 200 μl chloroform was added, it was vortex-mixed for 15 s and centrifuged at 12,000 g for 15 min at 4°C. Finally, RNA was extracted according to the manufacturer's instructions.

RT-PCR for MiR-133a levels

We examined the miR-133a expression level by quantitative real-time PCR (RT-PCR). In brief, cDNA was reversely transcripted with MicroRNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Then quantitative PCR for miR-133 and glyceraldehyde-3-phosphate dehydrogenase (GAPDHa), which was an endogenous control, were performed on an ExicyclerTM 96 PCR machine (LG company, Korea). The primers were as follows: For miR-133a: F-UUUGGUCCCCUUCAACCAGCUG-3' and R-UAAACCAAGGUAAAAUGGUCGA.For GAPDH: F-GAAATCCCATCACCATCTTCCAGG, R-GAGCCCCAGCCTTCTCCATG.

Amplification condition was: an initial denaturation at 95°C for 10 min; 95°C for 15 s, 62°C for 60 s, 40 cycles. All samples were run in duplicate. The 2−ΔCT method was use to analyze the real-time PCR data. Results were normalized with reference to GAPDH (Wu et al., 2010).

Receiver operating characteristic (ROC) curve analysis

ROC analysis is a useful tool to connect the data points (sensitivity, 1 specificity) for evaluating the performance of diagnostic tests. The area under the ROC curve (AUC) is described as a simple and convenient overall measure of diagnostic test accuracy, and represents probability and correspondence between ROC and the tested factor. AUC values higher than 0.9 are considered to have high diagnostic value.

Statistical analysis

All statistic analyses were performed using SPSS software. Comparisons of miR-133a expression among the groups were analyzed by one-way ANOVA followed by Tukey's multiple-range tests. The correlation of miR-133a expression between gastric juice and mucosa biopsies was analyzed by Pearson's correlation test. Data were presented as mean±SEM with $P<0.05$ as the limit for statistical significance.

Results

MiR-133a levels in endoscopic mucosa biopsies and gastric juice

As shown in Figure 1a and 1b, miR-133a generally exhibited lower expression in carcinoma tissues ($F[4, 204]=56.5$, $P<0.001$) and gastric juice of patients with GC ($F[4, 204]=52.5$, $P<0.001$) than those with other benign-diseases and normal controls. The average expression levels of miR-133a in GC tissues were reduced by 57.6% ($p<0.01$), 58.6% ($p<0.01$), 56.5% ($p<0.01$) and 52.7% ($p<0.01$) compared with healthy controls, superficial gastritis group, gastric ulcer group and atrophic gastritis group, respectively (Figure 1a). Also, the gene expression in gastric juice of patients with GC was respectively decreased by 45.6% ($p<0.01$), 51.1% ($p<0.01$), 51.9% ($p<0.01$) and 43.5% ($p<0.01$) compared with healthy, superficial gastritis, gastric ulcer and atrophic gastritis groups (Figure 1b).

We examined whether the miR-133a levels in gastric juice related to those in mucosa biopsies. The Pearson's correlation test revealed that the gene levels in gastric juice

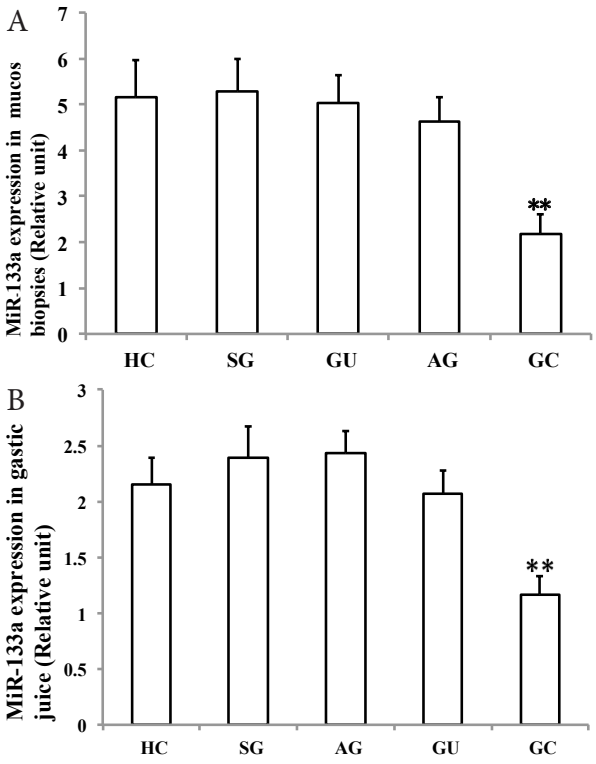

Figure 1. Downregulation of miR-133a Expression in Endoscopic Mucosa Biopsies and Gastric Juice. MiR-133a expression in carcinoma tissues (A) and gastric juice (B) of patients with gastric cancer (GC) was reduced compared with those in normal and other pathologic mucosa tissues, respectively. All data shown are the means ± SEM. ** $P < 0.01$ vs. healthy control (HC), superficial gastritis (SG), gastric ulcer (GU) and atrophic gastritis (AG) groups, respectively

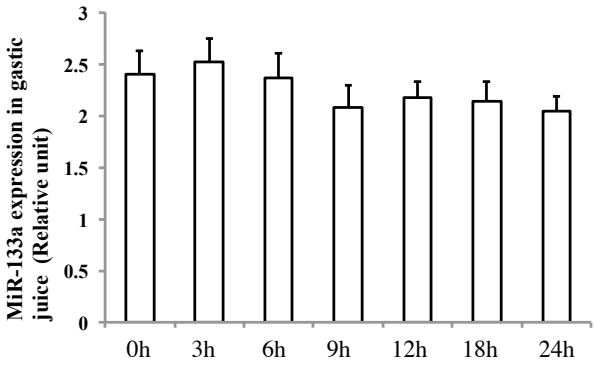

Figure 2. Measurement of the Stability of miR-133a in Gastric Juice. MiR-133a in gastric juice could be robustly and reliably examined for 24 h under the storage conditions of temperature 4 °C. All data shown are the means ± SEM

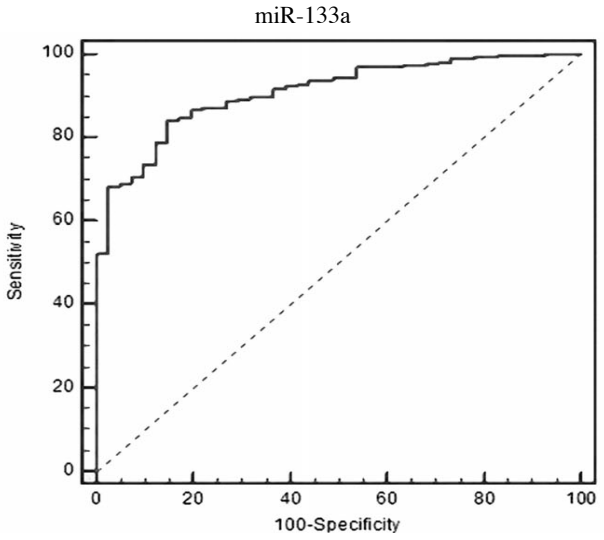

Figure 3. The Receiver Operating Characteristic (ROC) Curve of miR-133a in Gastric Juice for the Diagnostic Value of Gastric Cancer. The ROC curve of miR-133a in gastric juice was plotted to discriminate gastric cancer and gastric benign patients with sensitivity of 85.9 % and specificity of 84.8 %, AUC = 0.907

was significantly directly correlated with those in mucosa biopsies with Pearson's correlation 0.972 (p<0.0001). (Figure 1 is here)

Assessment of miR-133a stability in gastric juice

To evaluate the stability of miR-133a in gastric juice, we kept gastric juice under the condition of temperature 4±1°C and tested it at 0, 3, 6, 9, 12, 18 and 24 h. The results showed that no significant difference of the miR-133a level was found (Figure 2; F[4, 204]=2.49, P=0.032). The gene level at 24 h was reduced by 14.9% (p>0.05), 19.1% (p>0.05), 13.6% (p>0.05), 1.9% (p>0.05), 5.9% (p>0.05) and 4.7% (p>0.05) compared with those at 0, 3, 6, 9, 12 and 18 h, respectively. These results suggested that gastric juice-based microRNAs were stable with a highly reproducible detection (Figure 2).

The ROC analysis of miR-133a expression in gastric juice

To assess the diagnostic value of miR-133a in gastric juice, a ROC curve was constructed. The area under the ROC curve (AUC) of miR-133a in gastric juice was up to 0.907 (95 % CI=0.857-0.957, P<0.001; Figure 3). The sensitivity and specificity were 85.9 and 84.8 %, respectively.

Discussion

MiR-133a is a muscle-specific microRNA, so-called myomiR, and is highly conserved in musculatures of flies in mice and humans (Nohata et al., 2012). MiR-133a forms clusters in three different chromosomal regions in the human genome-6p12.2, 18q11.2 and 20q13.33 (Yamasaki et al., 2012). MiR-133a can directly regulated actin-related genes such as tropomyosin2 (TPM2), tropomyosin3 (TPM3), moesin (MSN) and fascin actin-bundling protein 1(FSCN1) (Kinoshita et al., 2012; Moriya et al., 2012) to regulate earliest differentiation of myogenic stem cells into myoblasts and to benefit muscle growing, maintaining and regenerating after skeletal muscle stress or injury (Wang et al., 2014).

Previous studies showed that miR-133a expression levels were significant downregulated in GC tissues and cell lines (Gong et al., 2015). Restoration of miR-133a expression can inhibit cancer cell proliferation, migration and invasion. MiR-133a can bind to the 3'-untranslated region of FSCN1 mRNA. Overexpression of miR-133a causes downregulation of FSCN1 expression to inhibit proliferation and invasion, but promoted apoptosis of gastric cancer cells, which may be reversed by upregulation of FSCN1. While downregulation of miR-133a results in an increase in FSCN1 expression to enhance proliferation and invasion, but suppressed apoptosis in GC cells (Lai et al., 2015).

In line with these, the results of current study showed that the miR-133a expression level was significantly reduced in GC tissues compared with normal or benign-disease mucosa, further demonstrated that the downregulated miR-133a level is a distinguishing characteristic of patients with GC. In addition, we assessed values of miR-133a in gastric juice for diagnosis of GC and found that the miR-133a level was significantly lower also in gastric juice from patients with GC than those from healthy and benign groups (Figure. 1a, b). The expression level of this gene in gastric juice is positively correlated with that in GC tissues. Therefore, the miR-133a level in gastric juice samples may serve as a specific marker for the diagnosis, evaluation and screening of GC.

There are several advantages of taking the miR-133a expression level in gastric juice as the potential surrogate material for the molecular genetic diagnosis of GC. Fist, gastric juice may be easily obtained during endoscopic examination. Hence, this operability in clinic is high. Next, it has been addressed that the miR-133a in peripheral blood is shared by several types of cancers as mentioned above (Cui et al., 2013; Dong et al., 2013; Guo et al., 2014; Wang et al., 2014). Our results in this study showed that miR-133a levels in gastric juice were positively correlative to those in GC tissue, i.e. the miR-133a levels in gastric juice were mainly mirrored the occurrence and development of GC. Therefore, the miR-133a level in gastric juice as the diagnosis material of GC has higher

reliability compared with that in serum/plasma. Moreover, in this study we found that miR-133a levels in gastric juice can be detected in a remarkably stable form within 24 h storage in ambient at 4 °C (Figure. 3), i.e. the stability of miR-133a in gastric juice is relatively high. This may fully meet the clinical test requirements. In contrast, some of intrinsic gastric juice peptides with fluorescence spectrum may be used in the diagnosis of GC (Zhang et al., 2012). But this method is not satisfactory enough, as fluorescent substances of these peptides in gastric juice are easily quenched. Last, the ROC curve analysis is widely used to evaluate effectiveness and specificity of a biomarker. In this study we found that the area under the ROC curve (AUC) of miR-133a in gastric juice was up to 0.907 with sensitivity of 85.9 % and specificity of 84.8 %. Thus, both of sensitivity and specificity of miR-133a in gastric juice are high as a biomarker for screening of GC.

In short, gastric juice presents only in the stomach. This virtue makes biomarkers in gastric juice has obvious advantages for the diagnosis of GC. MiR-133a in gastric juice has the characteristic of high operability, high reliability, high sensitivity, high specificity and relative stability, fairly suitable to be wide used in clinical diagnosis of GC. These advantages of miR-133a in gastric juice presented here offer the basis and rationale for using miR-133a as gastric juice-based biomarkers for GC screening.

Acknowledgements

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