Six Serum-Based miRNAs as Potential Diagnostic Biomarkers for Gastric Cancer

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Abstract

Background: Circulating miRNAs in serum may serve as promising diagnostic biomarkers for patients with gastric cancer. Methods: Using qRT-PCR-based Exiqon panel, we identified 58 differentially expressed miRNAs from three gastric cancer pool samples and one normal control (NC) pool in the initial screening phase. Identified miRNAs were further validated in the training (49 gastric cancer vs. 47 NCs) and validation phases (154 gastric cancer vs. 120 NCs) using qRT-PCR. The expression levels of the miRNAs were also determined in tissues, arterial serum, and exosomes. Results: Consequently, six serum miRNAs (miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR20a-3p, and miR296-5p) were significantly overexpressed in gastric cancer compared with NCs. The areas under the receiver operating characteristic curve of the six-miRNA panel were 0.764 and 0.702 for the training and validation phases, respectively. miR10b-5p and miR296-5p were significantly upregulated in gastric cancer tissues (n = 188). In addition, patients who did not receive adjuvant chemotherapy with high expression of miR10b-5p or miR296-5p in tissues tended to suffer worse overall survival. Furthermore, the expression levels of miR10b-5p, miR195-5p, miR20a-3p, and miR296-5p were significantly elevated in exosomes from gastric cancer serum samples (n = 30). Conclusions: We identified a six-miRNA panel in serum for the detection of gastric cancer. Impact: Our findings provide a novel serum miRNA signature for gastric cancer diagnosis, and will serve as the basis of the application of circulating miRNAs in clinical for the detection of gastric cancer in the future.

Introduction

Gastric cancer is still a severe public health problem worldwide, particularly in Eastern Asia (1). According to the updated cancer statistics in China, gastric cancer is still the second leading cause of cancer mortality (2). Recently, gastric cancer has been divided into four molecular subtypes according to The Cancer Genome Atlas (TCGA) project, which further elucidated the immense heterogeneity of gastric cancer (3). Because of the lack of early diagnosis and late presentation, a fair number of gastric cancer patients were diagnosed at advanced stages (4). Currently, gastroscopy is still the criterion standard test for diagnosing gastric cancer. In Japan and Korea, a screening program based on endoscopy has been carried out to detect early gastric cancer for years (5). However, this cancer screening is too expensive and invasive to be popularized in China. Moreover, noninvasive or minimally invasive markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are widely used in clinical, but these similar markers are not sensitive and specific enough to facilitate early detection of gastric cancer (6). With the development of genomics, proteomics, and metabolomics, an increasing number of biomarkers have been identified and studied (7). Promisingly, novel noninvasive markers with potential clinical value will be discovered to detect early gastric cancer and then improve the prognosis for gastric cancer patients.

miRNAs are endogenous ~22 nt noncoding RNAs that post-transcriptionally regulate gene expression and function as oncogenes or tumor suppressors by degrading target miRNAs or blocking their translation (8, 9). Accumulating evidence has indicated that miRNAs are specific for different tumor types and microRNA expression profiles can distinguish between normal and tumor tissues, including gastric cancer (10, 11). Meanwhile, miRNAs can be stably detected in circulating plasma or serum. Circulating miRNAs have emerged as excellent noninvasive biomarkers for the early diagnosis and the prediction prognosis of cancer (12). Recently, miRNA expression profiles have become more heated.
and accurate than mRNA expression profiles (10). Up to date, several studies have screened out some circulating miRNA biomarkers with diagnostic and prognostic value for gastric cancer from numerous differentially expressed miRNAs (13–15). However, because of different research methods and populations (source, sample size, and clinical factors), these selected miRNAs showed inconsistency between laboratories. In our previous study, a five-miRNA signature in the peripheral plasma for the detection of gastric cancer was identified (13). Herein, we characterized the serum miRNA expression profiles in gastric cancer patients and healthy controls via miRCURY LNA microRNA Array followed by two phases of qRT-PCR validation. Furthermore, dysregulated serum miRNAs were assessed in tissue samples. Finally, we detected serum exosomal miRNAs to confirm the potential form of the identified miRNAs in the circulation. We aimed to identify a serum miRNA expression profile that could be a useful biomarker for gastric cancer diagnosis.

Materials and Methods

Study design and clinical samples

A total of 441 histopathologically confirmed gastric noncardia adenocarcinoma patients and 233 normal controls (NC) were enrolled in this study. All samples were collected from First Affiliated Hospital of Nanjing Medical University, 458 serum samples were collected between 2013 and 2015, and 216 tissue samples were collected between 2010 and 2011. All the procedures were approved by Institutional Review Boards of the First Affiliated Hospital of Nanjing Medical University, and the written informed consent was obtained from each participant. Our study was conducted according to the approved guidelines by the Hospital Ethics Committee.

The study was separated into three stepwise phases (Fig. 1). In the initial screening phase, serum samples were collected from 30 gastric cancer patients and 10 NCs and pooled as 3 gastric cancer pool samples and 1 NC pool sample (10 serum samples were pooled as 1 pool sample). These microarray data have been deposited in the Gene Expression Omnibus (GEO) database (accession number GSE86226) to comply with Minimum Information About a Microarray Gene Experiment (MIAME) guidelines. Differentially expressed miRNAs were assessed by Exiqon miRNA qPCR panel. Serum samples were collected from 30 gastric cancer patients and 10 NCs and pooled as 3 gastric cancer pool samples and 1 NC pool sample (10 serum samples were pooled as 1 pool sample). Differentially expressed miRNAs were assessed by Exiqon miRCURY-Ready-to-Use PCR-Human-panel-I+II-V1.M (Exiqon miRNA qPCR panel). Approximately 20 to 25 ng RNA isolated from each pool of serum samples was reverse transcribed to cDNA by using the miRCURY Locked Nucleic Acid (LNA) Universal Reverse Transcription (RT) microRNA PCR, polyadenylation, and cDNA Synthesis Kit (Exiqon miRNA qPCR panel) following the manufacturer’s protocol. Microarrays were scanned on 7900HT real-time PCR system (Applied Biosystems) with Exiqon miRCURY-Ready-to-Use PCR-Human-panel-I + II-V1.M (Exiqon miRNA qPCR panel), which could detect 168 miRNAs in plasma/serum to identify differently expressed miRNAs. Melting curve analyses were performed at the end of the PCR cycles. Detectable miRNAs were those with a Ct < 37 and five Ct less than the negative control (no template control, NTC). An RNA spike-in (UniSp6) and a DNA spike-in (Sp3) were used as technical controls to evaluate if the technical performance of all samples is similar. The Ct values were normalized based on the average of the normalizer assays in the panel and this included miR191-5p, miR423-5p, miR425-5p, and miR93-5p. The formula used to calculate the normalized Ct values is: normalized Ct (ΔCt) = average Ct (assay) − average Ct (normalizer assays). The relative expression levels of miRNAs between colorectal cancer patients and NCs were calculated using 2−ΔΔCt method. In the training phase, the dysregulated miRNAs identified via screening phase were confirmed using qRT-PCR in 49 gastric cancer samples and 47 NCs. After that, in the validation phase, the validated miRNA though training phase were further examined in 154 gastric cancer samples and 120 NCs. The identified miRNAs were further validated in 188 gastric cancer tissue specimens which were obtained from gastric cancer patients with radical resection of gastric cancer and 28 normal gastric mucosa tissues which were obtained from healthy people with gastroscopy. Serum exosomal miRNAs were also assessed in 30 gastric cancer patients and 28 NCs. In addition, we have followed up these gastric cancer patients who provided tissue samples to assess the prognostic value of the identified miRNAs. The median postoperative follow-up period was 50.2 ± 14.6 months.

Sample preparation and exosome isolation

Briefly, 5 mL of venous blood sample was collected from each participant before initial treatment. The whole blood was...
Huang et al.

separated into serum and cellular fractions by centrifugation at 1,500 rpm for 10 minutes and 12,000 rpm for 2 minutes within 12 hours after collection. The serum sample was stored at −80°C for further processing. Tissue specimens were collected from surgical patients without preoperative chemoradiotherapy and kept in liquid nitrogen.

To isolate serum exosome, 200 μL serum was processed with ExoQuick (System Biosciences) according to the manufacturer’s protocol. All serum samples were incubated with ExoQuick exosome precipitation solutions for 30 to 60 minutes at 2 to 8°C, followed by centrifugation at 13,000 rpm for 2 minutes. After the supernatants were removed, the exosome pellets were retained for further RNA extraction.

RNA extraction
Total RNA was extracted from 200 μL serum or exosome using the mirVana PARIS Kit (Ambion) following the manufacturer’s protocol. Then 5 μL of synthetic Caenorhabditis elegans miR-39 (5 nmol/L; Ribobio) was spiked into each sample after the addition of denaturing solution (Ambion) for normalization of the sample-to-sample variation. TRizol (Invitrogen) was used to extract total RNA from tissue samples. Total RNA was lysed in 100 μL RNase-free water and stored at −80°C for further use. The ultraviolet spectrophotometer was used to evaluate the concentration and purity of the extracted total RNA.

qRT-PCR and data normalization
The amplification of miRNAs was conducted using the specific primers of reverse transcription (RT) and PCR from Bulge-Loop mirRNA qRT-PCR Primer Set (Ribobio). The quantification of PCR product was evaluated by the level of fluorescence in emitted by SYBR Green (SYBR Premix Ex Taq II, TaKaRa). RT reactions were carried out at 42°C for 60 minutes followed by 70°C for 10 minutes. The qRT-PCR was conducted on LightCycler 480 Real-Time PCR System (Roche Diagnostics) in 384-well plates at 95°C for 20 seconds, followed by 40 cycles of 95°C for 10 seconds, 60°C for 20 seconds, and then 70°C for 10 seconds. The specificity of PCR products was evaluated by the melting curve analysis.

The expression of miRNAs in serum was determined using the 2−ΔΔCt method relative to the combination of exogenous reference miRNA (cel-miR-39) and endogenous reference miRNA (miR16). ΔCt = CtmiR-16 − (Ctcel-miR-39 + Ctmir-16). The relative levels of miRNAs in tissue specimens and exosomes were calculated using the comparative 2−ΔΔCt method relative to RNU6B (U6) and cel-miR-39.

Statistical analysis
Mann–Whitney test was used to analyze differential miRNAs expression between gastric cancer patients and NCs. The association between miRNAs and the clinical characteristics was evaluated by the one-way ANOVA or χ2 test. Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to estimate the diagnostic value of the candidate miRNAs for gastric cancer. Logistic regression model for gastric cancer prediction was applied on the data from the training and validation phases. Univariate survival analysis was performed using the Kaplan–Meier method. Survival curves were compared with the log-rank test. All the statistical analyses were performed using SPSS software (version 20.0, IBM). A two-sided P value <0.05 was defined as statistical significance.

Results
Characteristics of the subjects
Our experiment was divided into two stages after the screening phase: the training phase and the validation phase as showed in the flow chart (Fig. 1). The clinical and demographics features of the gastric cancer patients and NCs are listed in Table 1. A total of 370 participants, including 203 gastric cancer patients and 167 NCs, were enrolled in our study to assess the differently expressed miRNAs in the serum of gastric cancer patients. In each stage, there was no significant difference in the distribution of gender or age between gastric cancer patients and NCs.

miRNA profiling in the screening phase
To identify candidate serum miRNAs for gastric cancer diagnosis, the Exiqon miRCURY-Ready-to-Use PCR-Human-panel-L-I+II-V1.1 was conducted based on the qRT-PCR platform. A total of 179 miRNAs were initially screened in 3 gastric cancer and 1 NC pooled serum samples and 51 upregulated miRNAs and 7 downregulated miRNAs showed at least a 1.5-fold altered expression (Supplementary Table S1). In addition, we included five miRNAs (miR185-5p, miR20a-5p, miR210, miR25-3p, and miR92b-3p) that were previously identified by our research team in plasma of gastric cancer patients (13).

Evaluation of candidate miRNAs by qRT-PCR
The selected miRNAs in the screening phase were validated in the training stage including 49 gastric cancer patients and 47 NCs using qRT-PCR analysis. After that, 14 differentially
expressed miRNAs were identified and then were confirmed in the validation phase with a larger sample set. Ultimately, six miRNAs (miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR20a-3p, and miR296-5p) showed significantly elevated expression levels in gastric cancer serum and were selected for the following analysis. The differential expression patterns of the six miRNAs between gastric cancer patients and NCs were concordant between the training and the validation phase (Supplementary Fig. S1). In addition, all the six miRNAs had significantly higher expression levels in serum of gastric cancer patients as compared with NCs when the results of the two stages were combined (Fig. 2).

Diagnostic value of the candidate miRNAs
ROC curve analysis was utilized to evaluate the diagnostic value of the six miRNAs in discriminating gastric cancer patients from NCs. The AUCs were 0.627, 0.652, 0.637, 0.683, 0.637, and 0.652 for miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR20a-3p, and miR296-5p, respectively (Supplementary Fig. S2). Furthermore, when the six miRNAs were combined together as a panel, it showed a higher accuracy than any individual miRNA in discriminating gastric cancer patients from NCs (AUC: 0.703; 95% CI, 0.651–0.756; Fig. 3A). Meanwhile, the diagnostic value of the six-miRNA panel was also assessed in the training and validation phases separately and the AUCs were 0.764 (95% CI, 0.651–0.876; Fig. 3B).
We further analyzed the expression levels of the six miRNAs in serum from patients with different TNM stages in the total training and validation phases. All six miRNAs were found to be elevated, miR10b-5p, miR185-5p, and miR296-5p were significantly upregulated in patients with stage III + IV compared to those with stage I + II (Supplementary Fig. S3).

The candidate miRNAs in tissue samples
To validate the consistency of the six miRNAs in serum and tissue of gastric cancer patients, we detected the expression levels of the six miRNAs in 188 gastric cancer tissue specimens and 28 normal gastric mucosa tissues. The clinical characteristics of these patients are listed in Supplementary Table S2. As shown in Fig. 4, only the expression of miR10b-5p and miR296-5p was significantly higher in tumor samples than in normal tissues (Fig. 4).

Next, to evaluate whether the six candidate miRNAs were associated with the prognosis of gastric cancer patients, 188 gastric cancer patients were followed up. The median overall survival and disease-free survival (DFS) times were 50.5 ± 14.5 and 48.3 ± 17.6 months, respectively. At the end of follow-up, Aug 2015, 145 (77.1%) patients were still alive. The cutoff value for high or low miRNA expression was the median expression levels of each miRNA. As a consequence of the Kaplan–Meier survival analysis, either the OS or DFS showed no significant difference between high miRNA expression and low miRNA expression of individual six miRNAs (Supplementary Fig. S4A and S4B). Taking into account the use of adjuvant chemotherapy, subgroup analysis was performed to further explore the association between miRNA and gastric prognosis. For patients who did not receive adjuvant chemotherapy, high expression of miR10b-5p and miR296-5p might lead to worse OS and DFS (Supplementary Fig. S5A and S5B). The clinical characteristics of 55 gastric cancer patients without adjuvant chemotherapy were showed in Supplementary Table S3. However, the expression levels of the six miRNAs showed no significant difference in patients who underwent adjuvant chemotherapy (Supplementary Fig. S6A and S6B).

Comparison of miRNAs in arterial and venous serum
Next, we explored the expression levels of the six miRNAs in six arterial serum samples and six matched peripheral serum samples to identify the differences in miRNA expression between peripheral and arterial serum. All the six miRNAs showed higher expression levels in peripheral serum, but due to the relatively small sample size, the results were not statistically significant (Supplementary Fig. S7).

Exploration of miRNAs in serum exosomes
For further research, exosomal miRNAs extracted from 30 gastric cancer and 28 NC serum samples were explored to assess the potential form of the identified miRNAs in peripheral serum. Compared to NCs, all the six miRNAs were upregulated in gastric cancer serum exosomes, but only miR10b-5p, miR195-5p, miR20a-3p, and miR296-5p had significance in statistics (Fig. 5).

Discussion
Emerging evidence shows that stable circulating miRNAs play an increasingly important role in the diagnosis of gastric cancer. Recently, researchers continually focus on finding out specific miRNA panels for the detection of gastric cancer. Microarray profiling followed by qRT-PCR validation is currently acknowledged as the standard method for miRNA quantification (18). In this study, Exiqon miRNA qPCR panels were used to analyze differential expression profiling of serum miRNAs in three gastric cancer and one NC pooled samples in the screening phase. This platform was proved to be of better sensitivity and linearity than TaqMan platform whereas less abundant miRNAs were measured (19). In the following validation stages conducted by qRT-PCR, the key was to choose proper endogenous reference miRNAs for data normalization. Ideal reference miRNAs should belong to different functional classes, significantly reducing the possibility of confounding coregulation (20). In addition, reference miRNAs should be stable and detectable in circulation. However, to date, there is still no consensus endogenous reference for miRNA quantification has been established. Currently, miR16-5p has been selected as endogenous reference in accumulating studies and is relatively stable in the circulation (21–23). In our previous study, the specificity of PCR products was evaluated by the melting curve analysis and the expression level of plasma miRNAs was assessed as absolute concentration based on a standard curve constructed with the use of synthetic miRNAs. Considering these synthetic miRNAs were exogenous references, which could not fully reflect the degradation degree of different samples. Thus, we added miR16 as an internal reference to further normalize the sample-to-sample variation. After validation in the training and validation phases, miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR20a-3p, and miR296-5p were confirmed to be significantly upregulated in gastric cancer serum, whereas miR10b-5p, miR185-5p, and miR296-5p were significantly upregulated in patients with advanced stage (III and IV). The diagnostic value of the six miRNAs was verified in gastric cancer tissues and serum exosomes, and the encouraging results increasingly demonstrated the important roles for the six miRNAs in tumorigenesis and progression.

Among the six identified miRNAs, miR185-5p has been reported as a reliable biomarker for discriminating gastric cancer patients from NCs in our previous study (13). This result suggested that there was uniformity between the expression of miRNAs in serum and plasma. Even so, miR185-5p demonstrated inconsistent function in gastric cancer in previous studies (24, 25).

It is reported that miR185-5p was upregulated in three gastric cancer molecular subtypes (tumors positive for Epstein–Barr virus, microsatellite unstable tumors, and tumors with chromosomal instability) defined by TCGA project compared to normal controls (3). In addition, miR20a-5p was identified in our previous study (13), whereas in this study, another mature form of miR20a, miR20a-3p, was upregulated in serum of gastric cancer patients. miR20a is a member of the miR17-92a cluster, which modulates tumor formation and function as an oncogene by influencing the translation of E2F transcription factor 1 (E2F1; ref. 26). miR10b-5p has been identified as a prognostic biomarker in gastric cancer (27) and proved to function as an oncogene in various cancers such as breast cancer (28), esophageal cancer (29), and colorectal cancer (30). As a driver of metastasis, miR10b-5p could enhance cell migration and invasion by binding HOXD10 gene (31). Moreover, both circulating miR10b-5p and miR132-3p were discovered for the first time to be valuable biomarkers of gastric cancer in our study. As for miR132-3p, Li and colleagues (32) suggested that miR132-3p might promote cell growth through suppression of FoxO1 translation. Besides, miR132-3p...
was highly expressed in the endothelium of human tumors and hemangiomas, and could function as an angiogenic switch by suppressing endothelial p120RasGAP expression, leading to Ras activation and the induction of neovascularization (33). Coincidentally, miR296-5p, another angiogenesis-related angiomiRs, was upregulated in both serum and tissue samples from gastric cancer patients. miR296-5p promotes angiogenesis in tumors by directly targeting the hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), and thereby reducing HGS-mediated degradation of VEGFR2 and PDGFR-β (34). When it comes specifically to gastric cancer, the overexpression of miR296-5p could promote gastric cancer cell growth through repressing
Caudal-related homeobox 1 (CDX1), which has been reported to have vital roles in gastric intestinal metaplasia as an intestinal-specific transcription factor (35). It is reported that miR195-5p was upregulated in the tissue of gastric cancer patients and could predict the recurrence of gastric cancer (36). But another study demonstrated that miR195-5p was a tumor suppressor miRNA in the circulation of gastric cancer patients (37). Thus, the conflicting function of miR195-5p is needed to be further investigated. Of course, further research should be conducted to uncover the detail mechanism for the deregulation of these miRNAs in gastric cancer formation and development.

These circulating miRNAs identified in our study were also involved in some other cancers. For instance, circulating miR132-3p and miR185-5p was evaluated as to be a diagnostic biomarker in hepatocellular carcinoma (38). miR10b-5p overexpression was found in serum of esophageal squamous cell carcinoma and could distinguish esophageal squamous cell carcinoma patients from cancer-free controls (39). Severino and colleagues (40) suggested that miR296-5p in circulation might act as a potential metastatic biomarker for oral squamous cell carcinoma. Obviously, more efforts need to be devoted to investigate the specificity of circulating miRNAs.

As blood flows from arterial to venous circulation, we speculated that circulating miRNAs released from tumor cells might present higher expression levels in arterial blood than those in peripheral serum. Although there were no significant differences, our result might support the hypothesis in some extent. Further researches containing larger samples are warranted.

Interestingly, some investigators found that circulating miRNAs were originated from the cell-derived exosomes, which could protect miRNAs against degradation by ribonuclease (42). Exosomes are small (40–100 nm) membrane vesicles secreted by most types of cell including cancer cell. We further explored serum exosomal miRNAs to better understand the potential form of the six miRNAs in serum. As a result, miR10b-5p, miR195-5p, miR20a-3p, and miR296-5p were significantly upregulated in serum exosomes from gastric cancer patients. Reportedly, exosomal miR10b-5p might play an important role in modulating tumor microenvironment, leading to outcome in favor of development and progression of breast cancer (43). Serum exosomal miR195-5p was used to distinguish hepatocellular carcinoma from patients with chronic hepatitis B (44). However, a representative study to characterize circulating miRNA complexes in human plasma and serum revealed that circulating miRNAs not

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**Figure 5.**
Expression of the six miRNAs in the serum exosomes of 30 GC patients and 28 NCs. A, miR-10b-5p; B, miR-132-3p; C, miR-185-5p; D, miR-195-5p; E, miR-20a-3p; F, miR-296-5p. Error bar: standard error. N: normal controls; T: tumor.
only cofractionated with vesicles like exosomes but also co-purified with the Ago2 ribonucleoprotein complex (45). miR15b-5p and miR132-3p might be co-purified with the Ago2 ribonucleoprotein complex other than exosomes in serum, which might be a possible explanation for the inconsistency of their expression levels between serum and serum exosomes. The mechanism of the generation of circulating miRNAs still needs additional studies.

Taken together, we identified a serum six-miRNA panel which could discriminate gastric cancer patients from healthy controls. In the future, circulating miRNAs will be to function as reliable biomarkers in diagnosis and prognosis of gastric cancer. Without doubt, the study on the mechanisms of the identified miRNAs in gastric cancer demands even greater effort.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Z. Huang, L. Wu, L. Zhang, H. Zhang, Y. Chen, W. Zhu, Y. Shu, P. Liu

References

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Grant Support
The authors are grateful to the support fund by the National Natural Science Foundation of China (Grant No. 81672400, to W. Zhu, 81672788, to P. Liu; 81201705, to W. Zhu, 81272532, to Y. Shu, 81302047, to D. Zhu); the Natural Science Foundation of Jiangsu Province (Grant No. BK20130243, to D. Zhu), and Jiangsu Province Clinical science and technology projects (Clinical Research Center. BL20120088, to Y. Shu).

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Received July 26, 2016; revised September 15, 2016, accepted September 21, 2016, published OnlineFirst October 18, 2016.