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Original Article

Hemolysis-free plasma miR-214 as novel biomarker of gastric cancer and is correlated with distant metastasis

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Abstract: Circulating miRNAs gains popularity for its potential ability to serve as biomarkers of cancer. The aim of present study was to evaluate the usefulness of plasma miR-214 as novel biomarkers for gastric cancer (GC) detection. Attempts were made to address several pitfalls in sample processing and study design in previous studies. We conducted a two-step analysis: (1) in pilot study comprising of 30 patients and 30 controls, levels of miR-214 were significantly higher in primary GC tissues than normal tissues ($P = 0.0215$). Plasma miR-214 was significantly higher in patients with GC than in controls ($P < 0.0001$). (2) In test of larger cohort, there was significantly decreasing tendency of plasma miR-214 from patients before, 14 days and 1 month after surgical resection ($P < 0.0001$). There were significantly higher levels of miR-214 in 80 GC patients than in 70 controls ($P < 0.0001$). Receiver operating characteristics (ROC) curves yielded area under the curve (AUC) value of 0.845. Moreover, high plasma miR-214 had significant correlation with distant metastasis ($P = 0.038$). Thus, our data suggest that plasma miR-214 was novel hemolysis-free markers of gastric cancer.

Keywords: Plasma miR-214, gastric cancer, biomarkers, distant metastasis

Introduction

Gastric cancer (GC) is the fourth most common malignance and one of the leading causes of cancer-related death worldwide [1, 2]. Early detection of this malignance allows for early treatment and is of vital importance to reduce mortality [3]. Currently, endoscopy is the most reliable technique for confirmation of diagnosis, however, its invasiveness as well as weakness of depending heavily on the skills of endoscopist hinders wide utilization for screening procedure [4]. On the other hand, many of the well-known gastric cancer-related tumor marker, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), lacks sufficient sensitivity and specificity to serve as screening tools [4, 5]. Hence, there is urgent need for identification of novel biomarkers to improve gastric cancer detection.

Recent studies have revealed that microRNAs (miRNAs), a class of noncoding RNAs, play pivotal roles in the regulation of mRNA expression post-transcriptionally [6]. The discovery that miRNAs expression is frequently dysregulated in a cancer-specific way renders possibility of developing these RNAs as biomarkers for cancer detection [7-11]. Tumor-derived miRNAs can be present in blood and appear to be stable after being subject to ribonuclease digestion as well as some harsh conditions such as extreme temperature and pH, and numerous studies have shown diagnostic and prognostic potential of circulating miRNAs [12-16]. The identification of disease-specific miRNAs was hindered by the fact that blood also contains miRNAs originating from hemolysis of cells, whose levels associate with blood cell count [17, 18]. Some of these cellular miRNAs in circulation have been considered as tumor-related biomarkers in previous study, however their different levels might be more correlated to blood cell events rather than the presence of cancer. One possible assay to avoid misidentification of non-specific miRNAs might be the determination of hemoglobin levels as markers of hemolysis in plasma samples to judge whether they are suitable for further miRNAs analysis [18].

Table 1. Demographic and clinical characteristics of overall GC patients and controls

Variables	Patients (n = 110)		Controls (n = 100)		*P*-value
	No.	%	No.	%	
Age (years)	55.8 ± 6.6		55.5 ± 6.7		0.781
BMI (kg/m^2)	23.3 ± 3.1		22.7 ± 3.4		0.147
Gender					
Male	62	56.4	58	58	0.811
Female	48	43.6	42	42	
Alcohol consumption					
Ever/current	58	52.7	52	52	0.434
Never	34	30.9	37	37	
Unkown	18	16.4	11	11	
Smoking status					
Ever/current	68	61.8	55	55	0.457
Never	35	31.8	40	40	
Unknown	7	6.4	5	5	
Location					
Cardia	24	21.8			
Body	15	13.6			
Antrum	71	63.6			
TNM stage					
I	22	20.0			
II	41	37.3			
III	32	29.1			
IV	15	13.6			
Differentiation					
Well and moderate	62	56.4			
Poor	48	43.6			

In this study, we focused on miR-214 which has been reported to be over-expressed in GC tissues compared with normal gastric tissues [19-22]. We speculated that circulating miR-214 could be detected in plasma and predict tumor characteristics, which could facilitate early treatment of GC. Consequently, our results demonstrated that hemolysis-free miR-214 levels in plasma could distinguish GC patients from healthy controls, monitor tumor dynamics and predict distant metastasis. Our studies provided evidence that plasma miR-214 could serve as a useful biomarker for detection of gastric cancer.

Materials and methods

Patients and samples

The study design was approved by Chinese People's Liberation Army General Hospital Research ethics committee, and each individual provided written informed consent. Between 2013 and 2014, 110 preoperative blood samples of GC patients and 100 age and sex matched healthy control samples were collected at People's Liberation Army (PLA) of General Hospital (Beijing, China). Healthy controls were patients with a benign disease such as hernia and had no evidence of malignancy. Fasting blood was collected in 5 ml K2-EDTA Vacutainer (BD, Franklin Lakes, NJ) the day after admission. None of the patients underwent radiotherapy and chemotherapy before blood sampling. Paired blood samples were collected from a subgroup of 40 patients before, 14 days and 1 month after surgical operation. The first couple of ml of blood was discarded to remove potentially tissular and cellular contaminations from the puncture site [23]. Attempts were made to maintain consistent protocols in processing and storage within first 4 hours from collection in Clinical Specimen Bank of PLA. The plasma was separated from cell debris by centrifugation at 3000 g for 10 min at room temperature and stored at -80°C. A further centrifugation (1200 g for 15 min at 4°C) was performed before RNA extraction to remove cell debris. The level of hemolysis in plasma samples was assessed by spectrophotometric analysis [18]. The absorbance peak at 414 nm, indicating the presence of free hemoglobin, was used for quantitative measurement of hemolysis. Plasma samples with A_{414} reading exceeding 0.2 were considered hemolysis and were excluded. The demographic and clinical features of overall enrolled cohorts were summarized in **Table 1**. Frozen fresh tissue samples were collected from 10 patients to compare miRNA expressions between primary tumors and matched non-tumorous tissues. The matched "normal gastric tissue" was obtained from a 5 centimeter distance from the tumor margin, which was further confirmed by pathologist that they do not have

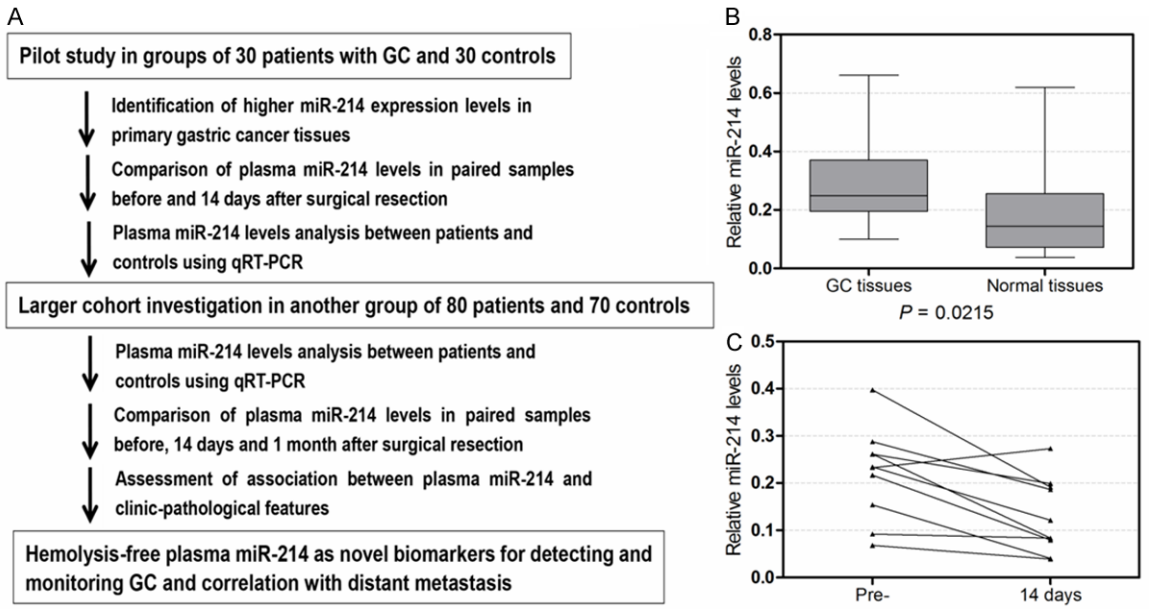

Figure 1. A. Study design to identify a novel candidate marker of plasma miR-214. B. Identification of higher expression levels of miR-214 in primary gastric cancer tissues ($P = 0.0215$). The lower and upper borders of the box indicate the 25th and 75th percentiles, respectively. The whiskers indicate the minimum and maximum values. C. Comparison of plasma miR-214 in paired samples from patients before and 14 days after surgical resection. The levels of miR-214 were reduced significantly ($P = 0.0098$).

tumor cells. Cancer staging was in accordance with the third edition of Japanese Classification of Gastric Carcinoma [24].

RNA extraction from plasma and tissue

Total RNA was extracted from 400 μl of hemolysis-free plasma according to a modified method [25]. The Trizol Reagent (Invitrogen, Carlsbad, CA) was used to denaturize plasma and the Qiagen miRNeasy Mini kit (Qiagen, Valencia, CA) was used to collect and purify RNA according to manufacturer's instructions. RNA was extracted from frozen fresh GC tissues using a standard Trizol protocol (Invitrogen, Carlsbad, CA).

Quantitative real-time RCR for detection of miRNAs

The quantity of miRNAs was quantified by duplicate qRT-PCR using the human TaqMan MicroRNA Assay Kits (Life Technologies). The reverse transcription reaction was conducted with TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) in 15 μl containing 5m μl of RNA extract, 0.15 μl of 100 mM dNTPs, 1 μl of Multiscribe Reverse Transcriptase (50 U/μl), 1.5 μl of 10× reverse transcription buffer, 0.19 μl of RNase inhibitor (20 U/μl), 3 μl of gene-specific stem-loop primer and 4.16 μl of nuclease-free water. For synthesis of cDNA, the reaction mixtures were incubated at 16°C for 30 min, at 42°C for 30 min, at 85°C for 5 min and then held at 4°C. Then, 1.33 μl of cDNA solution was amplified using 10 μl of TaqMan 2× Universal PCR Master Mix with no AmpErase UNG (Applied Biosystems), 1 μl of gene-specific probe and 7.67 μl of nuclease-free water in a final volume of 20 μl. Quantitative PCR was run on a 7900 Real-Time PCR system (Applied Biosystems) and the reaction mixtures were initiated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

The relative quality of plasma miRNA was calculated using the $2^{-\Delta Ct}$ method normalized to miRNA-16 which was used in previous study and was shown to be a stable reference gene in hemolysis-free plasma [18, 26-28]. Expression of miRNAs from tissue samples was normalized to U6 small nuclear RNA (RNU6B). ΔCt was calculated by subtracting the Ct values of miR-16 or RNU6B from the Ct values of the miRNAs of interest [29, 30].

Statistical analysis

The nonparametric Mann-Whitney *U*-test and Kruskal-Wallis test were performed for compar-

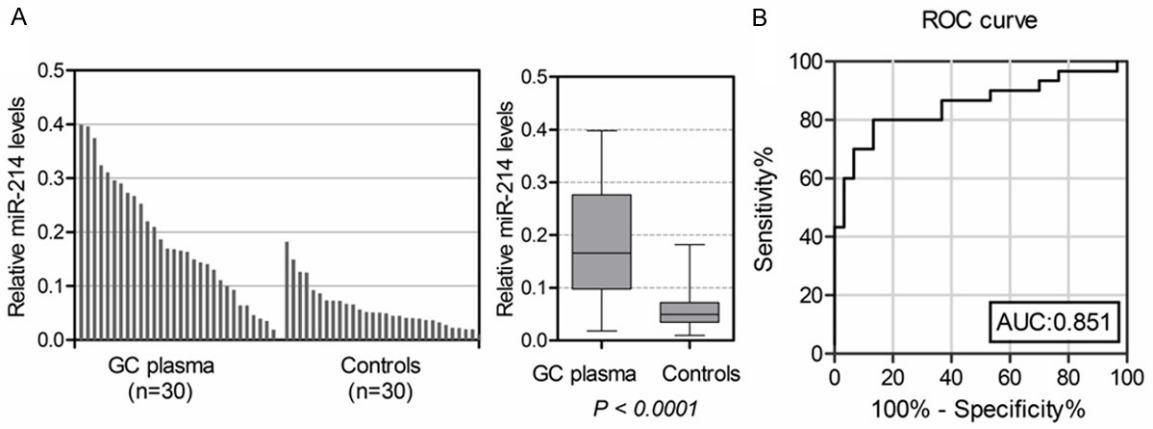

Figure 2. Differential expression of plasma miR-214 between patients with GC and healthy controls. A. MiR-214 was detectable in circulation by quantitative RT-PCR. Plasma miR-214 of patients was significantly higher than that of controls ($P < 0.0001$). B. ROC curves yielded AUC value of 0.851.

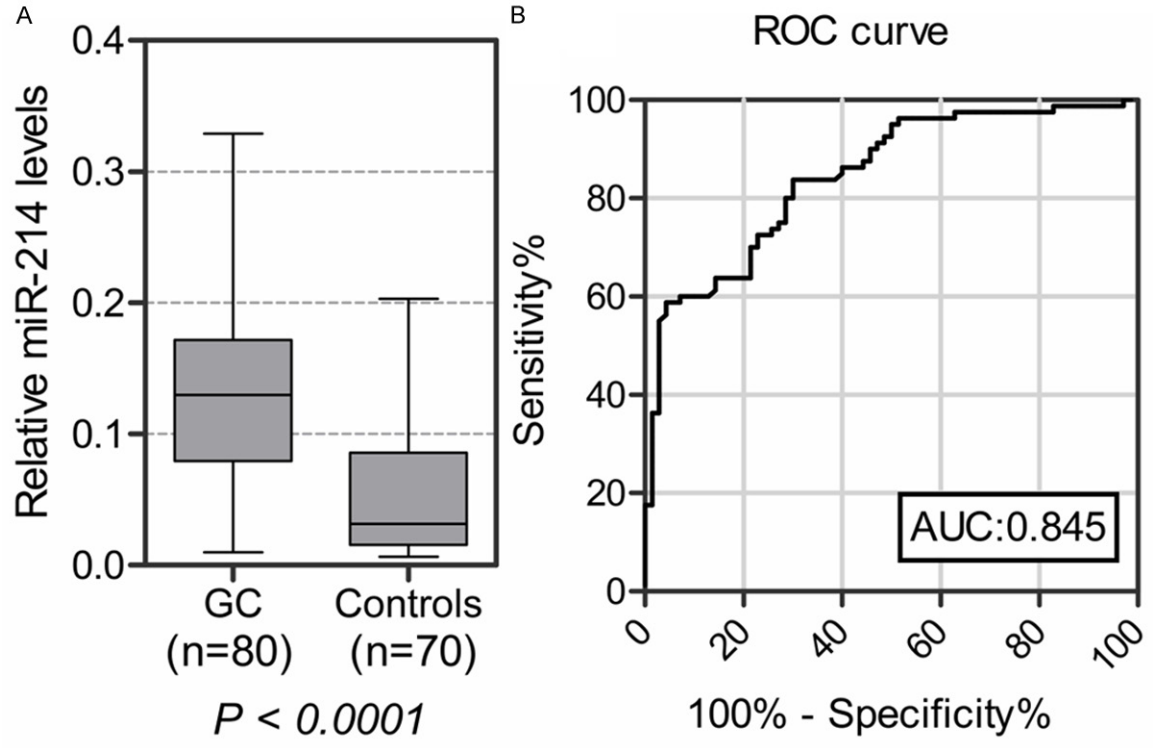

Figure 3. Larger cohorts investigation in another 80 cancer patients and 70 controls. Box and whisker plot indicated expression levels of miR-214 were significantly elevated in GC patients (A) and ROC analysis in plasma miR-214 assay yielded AUC 0.845 (B).

ing the miRNA levels of plasma, and the Wilcoxon's signed rank test was performed for paired data. The Mantel-Haenszel x^2-test was performed to compare clinical and demographic characteristics among groups and was used to evaluate the correlation between the level of miRNAs expression and the clinical factors. Receiver-operating characteristic (ROC) curves were constructed and the area under the ROC curve (AUC) was used to assess the diagnostic potential of plasma miRNAs for detecting GC. The Youden's Index was applied to determine

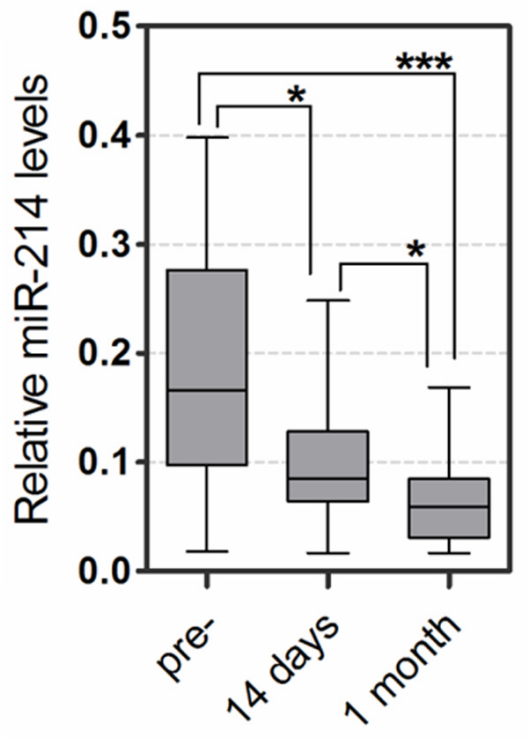

Figure 4. Comparison of samples from patients before, 14 days and 1 month after surgical operation. There was significantly decreasing tendency of levels of plasma miR-214. Kruskal-Wallis test was used to determine statistical significance. *P < 0.05. ***P < 0.0001.

cut-off value for plasma miR-214 [31]. P value < 0.05 was considered significant. Statistical computations were calculated by GraphPad Prim 5.0.

Results

Study design to develop a novel biomarker of plasma miRNA

Prior to this study, we reviewed previous reports regarding consistently over-expressed miRNAs in GC tissues compared with normal tissues. Then, we chose miR-214 as a candidate in this plasma miRNA assay as it has been found to be frequently over-expressed and have an oncogenic role in GC [19, 32]. Therefore, as illustrated in **Figure 1A**, we conducted a two-step study to investigate the diagnostic potential of plasma miR-214: (1) confirmation of higher miR-214 levels in primary GC tissues than adjacent normal tissues, investigating whether there might be distinct levels of plasma miR-214 between 30 patients with GC and 30 healthy controls in pilot test; (2) evaluating the diagnostic usefulness of plasma miR-214 by large-scale test in another cohort of 80 patients with GC and 70 healthy controls.

MiR-214 levels in primary GC tissues and pilot test of 30 pairs of participants

To confirm previously reported high expression levels of miR-214 in primary GC tissues, we measured its expression in 10 paired GC tissues and adjacent normal tissues by quantitative RT-PCR. After normalization to RNU6B expression, the expression levels of miR-214 was significantly elevated in cancer tissues (P = 0.0215; **Figure 1B**). We speculated that higher levels of miR-214 in tissues might influence the levels of plasma miR-214 of GC patients. Thus, we carried out pilot test by determining plasma miR-214 in a small group of 30 pairs of participants. Interestingly, the plasma miR-214 levels were significantly higher in GC patients than in normal controls (P < 0.0001; **Figure 2A**) and ROC plots showed a clinically satisfactory discrimination between these two groups with AUC value of 0.851 (95% CI 0.749-0.953; P < 0.0001) (**Figure 2B**). To investigate whether there could be a decline after resection of primary GC tissues, plasma miR-214 levels of 10 GC patients 14 days after surgical operation were also determined. **Figure 1C** showed the plasma miR-214 expression levels were significantly reduced after gastrectomy (P = 0.0098).

Large-scale test in another group of 80 patients with GC and 70 healthy controls

Based on findings in pilot test, we examined the plasma miR-214 using quantitative RT-PCR in a larger cohort comprised of 80 patients and 70 matched controls. The miR-214 expression pattern alternation in this test was consistent with that of the pilot test. The levels of miR-214 were significantly higher in GC patients compared with controls (**Figure 3A**; P < 0.0001). ROC curves were then constructed to estimate the sensitivity and specificity of this miRNA. The AUC value was 0.845 (95% CI 0.784-0.906; P < 0.0001) (**Figure 3B**), implying relatively high diagnostic potential. We also determined miR-214 expression in paired 40 plasma samples from patients before surgical operation, 14 days and 1 month after curative gastrectomy. As shown in **Figure 4**, the plasma miR-214 levels were found to be significantly reduced gradually (P < 0.0001).

Table 2. Correlation between miR-214 levels and clinicopathological parameters in 80 GC patients in step 2

Variables	No.	Low levels of plasma miR-214	High levels of plasma miR-214	*P*-value
Age (years)				0.389
≤ 55	28	13 (41%)	15 (31%)	
≥ 56	52	19 (59%)	33 (69%)	
Gender				0.637
Male	50	19 (59%)	31 (65%)	
Female	30	13 (41%)	17 (35%)	
Tumor size (mm)				0.714
≥ 50	43	18 (56%)	25 (52%)	
< 50	37	14 (44%)	23 (48%)	
Location				0.909
Cardia	12	5 (16%)	7 (15%)	
Body	9	3 (9%)	6 (13%)	
Antrum	59	24 (75%)	35 (72%)	
Depth of invasion				0.325
T1/2	25	12 (38%)	13 (27%)	
T3/4	55	20 (62%)	35 (73%)	
Lymph node metastasis				0.465
Positive	39	14 (44%)	25 (52%)	
Negative	41	18 (56%)	23 (48%)	
Distant metastasis				0.038
Positive	10	1 (3%)	9 (19%)	
Negative	70	31 (97%)	39 (81%)	
Differentiation				0.926
Well and moderate	33	13 (41%)	20 (42%)	
Poor	47	19 (59%)	28 (58%)	
TNM stage				0.228
I	12	5 (16%)	7 (14%)	
II	22	10 (31%)	12 (25%)	
III	36	16 (50%)	20 (42%)	
IV	10	1 (3%)	9 (19%)	

Correlation between plasma miR-214 levels and clinicopathological characteristics in 80 GC patients

Finally, we evaluated the correlation between plasma miR-214 levels and clinicopathological characteristics in 80 GC patients. From the results, patients with high plasma miR-214 levels, with a cut-off value of 0.058 calculated by Youden's Index, showed significant association with distant metastasis ($P = 0.038$; **Table 2**).

Discussion

Tumor-specific alternations in nucleic acids in the plasma of cancer patients are promising diagnostic tools and biomarkers for monitoring of various cancers [33]. Tumor-derived miRNAs have been demonstrated to be detectable and stable in circulation compared with circulating DNA and mRNA [12], showing its potential ability to serve as new biomarkers for identifying cancer patients. In fact, numerous studies have reported that miRNAs could exert quite accurate performance for cancer detection [26, 34-38]. Nevertheless, few studies took into consideration hemolysis as well as other factors such as sampling time, which could remarkably influence the quantity of circulating miRNAs [25, 39]. With regard to this issues, in present study, we made several attempts to exclude factors potentially affecting expression levels of plasma miRNAs: (1) blood were all collected in fasting conditions; (2) all blood have been processed to generate plasma within 4 h in consistent protocols; (3) plasma samples were subject to spectrometric analysis at A_{414} to exclude hemolysis.

Additionally, we conducted a two-step experiment to identify whether plasma miR-214 could be suitable biomarkers for detecting of GC. Initially, we confirmed the high expression levels of miR-214 in primary tissues, which were consistent with previous studies [19-21]. Then, in a pilot study composing of 30 patients with GC and 30 healthy controls, we found that plasma miR-214 was significantly elevated in patients compared with healthy controls. The comparison of pre- and postoperative plasma samples showed that the levels of miR-214 decreased after surgical resection. In the absence of hemolysis, namely plasma samples with A_{414} reading < 0.2, these findings suggested that miR-214 might be released by primary tumors. In line with this suggestion, studies

have shown that tumor-overexpressed miRNA in plasma are significantly reduced at least 10 days [40] to 14 days [41] after surgery. ROC curves were constructed to estimate diagnostic performance of this miRNA and yielded AUC value of 0.851. These interesting findings prompted us to validate the diagnostic potential of plasma miR-214 in a larger cohort.

Thus, we enrolled another group of 80 GC patients and 70 healthy controls to test the validity of plasma miR-214 for cancer detection. In this step, we obtained a similar result that plasma miR-214 was significant higher in patients than in controls with AUC value of 0.845. We also measured plasma miRNAs in samples from patients before, 14 days and 1 month after surgical removal of primary tissues, to confirm tumor release of plasma miRNAs. As a result, there was significantly decreasing tendency of expression levels of plasma miR-214. Despite the fact that the metabolism of the plasma miRNAs has not yet been clearly understood, studies reported that 1 month appeared to be sufficient time for clearance of the circulating miRNAs [42, 43]. Regarding the miRNA release into circulation from tumor cells, there have been some reports in terms of miRNA mediated intercellular communication. Fabbri et al. [44] provided evidence that miRNA secreted from cancer cells could function as mediators to activate surrounding cells as cytokines do. MiR-126, in combination with apoptotic bodies, derived from endothelial cells is taken up by other cells in the vessel wall and could stimulate cytokine production [45-47]. However, in our study, the potentially functional role of high expression levels of plasma miR-214 has not yet been fully clarified. This issue is worthy of further investigation.

Finally, we investigated the correlations between plasma miR-214 and clinicopathological factors in patients with GC and found that preoperative plasma miR-214 might be suitable to predict distant metastasis which was often correlated with unfavorable outcome. In accordance with our findings, previous study has reported patients with high expression levels of miR-214 had shorter overall survival [48].

In summary, a non-invasive assay using circulating miRNAs provides new alternatives for cancer detection. Plasma miR-214 might serve as reliable hemolysis-free biomarkers for diagnosing and monitoring patients with GC. However, before we apply this assay in clinical routine, a prospective large-scale study was needed to further validate circulating miR-214 sensitivity and specificity in a wide range of diseases including gastric ulcer, colorectal cancer, colorectal polyps and other cancers. The study was currently under careful organization and we will report them in future.

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Disclosure of conflict of interest

None.

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