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Xiaoling Wu

Xiaohui (Jane) Tan
George Washington University

Sidney W. Fu
George Washington University

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Review

May Circulating microRNAs be Gastric Cancer Diagnostic Biomarkers?

Xiaoling Wu^{1,2}, Xiaohui Tan² and Sidney W. Fu²✉

1. Department of Gastroenterology, Chengdu Military General Hospital, Chengdu, China
2. Department of Medicine (Division of Genomic Medicine), The George Washington University School of Medicine and Health Sciences, Washington, DC

✉ Corresponding author: Sidney W. Fu, M.D., Ph.D. Department of Medicine, Division of Genomic Medicine, The George Washington University School of Medicine and Health Sciences, 2300 Eye Street, N.W. Ross Hall 402C Washington, DC 20037. Email: sfu@gwu.edu; Tel: 202-994-4767

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Abstract

Gastric cancer (GC) is the third leading cause of cancer-related deaths. More than 80% of the diagnosis was made at the advanced stages of the disease, highlighting the urgent demand for novel biomarkers that can be used for early detection. Recently, a number of studies suggest that circulating microRNAs (miRNAs) could be potential biomarkers for GC diagnosis. Cancer-related circulating miRNAs, as well as tissue miRNAs, provide a hopeful prospect of detecting GC at early stages, and the prospective participation of miRNAs in biomarker development will enhance the sensitivity and specificity of diagnostic tests for GC. As miRNAs in blood are stable, their potential value as diagnostic biomarkers in GC has been explored over the past few years. However, due to the inconsistent or sometimes conflicting reports, large-scale prospective studies are needed to validate their potential applicability in GC diagnosis. This review summarizes the current development about potential miRNA biomarkers for GC diagnosis and the obstacles hindering their clinical usage.

Key words: gastric cancer, circulating miRNA, diagnosis, biomarkers

Introduction

Gastric cancer (GC) is the third most common cause of cancer-related mortality worldwide, and has a particularly high incidence in Asian countries including China and Japan. Although the incidence of gastric cancer has been reduced in certain developed countries over the past few decades, there are still over 1 million newly diagnosed cases and 850,000 deaths globally each year. More than 70% of the cases occurs in developing countries, with 50% occur in Eastern Asia [1].

Due to the absence of specific early symptoms, most GCs are diagnosed at advanced stages with no effective treatment. As the prognosis for GC patients differs depending on the disease stage at the time of diagnosis, early detection of GC is crucial. Endoscopic biopsy is the best way to find GC before presenting clinic symptoms, however, few patients would like to undergo endoscopy due to the potential side effects,

including aspiration pneumonia, bleeding, and perforation. Hence, there is an urge need for the discovery of non-invasive early detection biomarkers for GC patients.

Nowadays, several serum tumor markers have been used in clinic for several years, such as α -fetoprotein (AFP), carcinoembryonic antigen (CEA) and carbohydrate antigens 125 and 19-9 (CA 125 and CA 19-9). However, none of these markers are specific or sensitive in GC, especially in the early stage. Even though the combined use of these tumor markers improves the sensitivity to some extent for the advanced GC, these markers yield inconsistent results when used for early GC detection [2]. Therefore, identifying novel sensitive biomarkers for early GC diagnosis have become a hot research topic worldwide [3, 4, 5].

Despite extensive studies, the exact molecular

mechanisms of GC are still poorly understood. GC development is a complex, multi-step process involving deregulation of several oncogenic pathways. These oncogenic signaling pathways can be activated by some genetic and epigenetic alterations, such as the aberrant gene methylation, histone modifications and microRNA regulation. Increasing evidence indicates that GC is largely governed by complex interactions between multiple pro- and anti-oncogenic signaling pathways [6].

MiRNAs are small non-coding RNAs, about 18-24 nucleotides in length that control various biological processes through binding to the 3' untranslated region (UTR) of mRNAs, and affecting the stability and translation of their target genes. miRNAs function through miRNA-RNA induced silencing complex, which binds to specific mRNA targets via base pairing at the 3'-UTR, leading to translational repression or degradation of these mRNAs. In addition, miRNAs have also been shown to target mRNAs in the coding region, 5'-UTR as well as promoter region. This sparked great interests in elucidating the dynamic roles of miRNA that may play in the development and progression of cancer, and led to the rapid expansion of this field [7] [8] [9]. According to miRBase Release 21 (June 2014), 2,588 unique mature human miRNAs have been identified [5, 6].

Although the biological function of miRNAs has yet to be fully understood, miRNA analyses indicate that a wide range of cancers display significantly differential expression profiles compared to normal tissues [10] [11] [12] [13] [14] [15]. Since miRNAs are gene expression regulators, identification of aberrant tissue-specific miRNA expression in certain cancer may provide potential new biomarkers for clinical diagnosis, evaluation of prognosis, and choice of treatment [16] [17] [18] [19] [20]. Previous studies have revealed dozens of miRNAs that play important roles in gastric tumorigenesis, and might be potential biomarkers [21] [22] [23] [24] [25] [26]. As a potential biomarker that could be applied in clinical use, the selected cancer-associated markers, such as miRNA biomarkers, should be easy to detect, ideally from body fluids, such as blood. Thus researchers have devoted to identifying the sensitive miRNA markers in the blood for GC patients [27] [28] [29] [30] [31] [32].

Circulating miRNAs remain stable after incubation at room temperature for up to 24h or after up to eight cycles of freeze-thawing [32]. Their stability and easily testable length (about 22bp) make miRNAs suitable for being cancer biomarkers. Fortunately, several circulating miRNAs have been identified, which are significantly changed in their expression in GC patients compared to that of normal population [33] [34] [35].

Up-regulated circulating miRNAs in GC

In 2010, Tsujiura et al first reported the expression of a group of circulating miRNAs (miR-17-5p, miR-21, miR-106a, miR-106b and let-7a) were significantly aberrant in plasma of GC patients [36]. MiR-17-5p, miR-21, miR-106a and miR-106b expression were significantly upregulated whereas let-7a was downregulated in GC patients. Although the study only included 69 GC patients and 30 healthy volunteers, it indicated that detection of circulating miRNAs might provide new complementary tumor markers for GC patients.

Manuel Valladares-Ayerbes et al [37] analyzed the microarray expression profiles of the miR-200 family in 160 paired samples of non-tumor gastric mucosae and GC downloaded from ArrayExpress. Then they selected miR-200c for validation, and found a significantly increased blood miR-200c expression level compared to normal controls by qRT-PCR. Although the specificity was 100% but sensitivity was only about 65%. Increased miR-200c expression in blood were significantly associated with lymph node metastases and overall survival, but not with other clinical or pathological characteristics. These data suggest miR-200c has the potential to be a circulating diagnostic and prognostic biomarker.

In an E-cadherin / p53 double conditional knockout (DCKO) mouse model that recapitulates human diffuse-type GC (DGC) morphologically and molecularly, Rotkrue and his colleagues [38] demonstrated that DCKO mouse and human GCs are very similar in miRNA expression patterns. Among the reported 11 highly expressed serum miRNAs in human GC, four miRNAs, miR-103, miR-107, miR-194, and miR-210, were verified upregulated in sera of DCKO mice with both early- and advanced-stage DGC. Furthermore, the elevation of miR-103 and miR-194 occurred during the entire progression from the non-cancer status to the advanced-stage, implying they could be used as biomarkers in diagnosing the DGC stage. In addition, a high level of miR-210 may be a diagnostic biomarker for other cancers, including diffuse large B-cell lymphoma, pancreatic cancer and breast cancer.

There are several other reported up-regulated miRNAs that could be used as potential circulating biomarkers. For example, Li et al [39] [40] investigated the expression of miRNA-199a-3p between pre-operative plasma from EGC (early gastric cancer) patients and healthy controls; and between pre-operative and post-operative plasma using qRT-PCR. They found that the expression of plasma miRNA-199a-3p in EGC patients was significantly higher than that of healthy controls and gastric pre-cancerous diseases (GPD) patients. In addition, the

expression of miRNA-199a-3p in plasma was significantly decreased in the post-operative patients. The AUC of miRNA-199a-3p expression in plasma for EGC diagnosis was much higher than that of combined tumor markers. Therefore, plasma miRNA-199a-3p may serve as a prognostic biomarker for EGC. Furthermore, the expression of miRNA-199a-3p, miR-221, miR-106b and miR-20a are significantly elevated in plasma of GC patients.

Fu Z et al [29] analyzed the level of circulating miR-222 in plasma of GC patients using qRT-PCR in 114 GC patients, compared to 36 chronic atrophic gastritis (CAG) patients and 56 healthy controls. The results showed that the expression of circulating miR-222 in plasma was significantly upregulated in GC compared with either CAG or healthy controls. And its expression level was significantly correlated with clinical stages and lymph nodes metastasis. The ROC curve analysis revealed that miR-222 had considerable diagnostic accuracy, yielded an AUC of 0.850 with 66.1 % sensitivity and 88.3 % specificity in discriminating GC from healthy controls. Moreover, Kaplan-Meier analysis demonstrated a correlation between increased circulating miR-222 level and reduced disease-free survival and overall survival. These findings suggested that circulating miR-222 in plasma might be a potential and useful non-invasive biomarker for the early detection and prognosis of GC.

MiR-106b-25 has been studied in several cancers. Zhang R et al [41] tested miR-106b-25 expression in 40 post-operative and 20 pre-operative plasma samples of GC patients and explored the correlation between MiR-106b-25 and related clinical pathological factors. The expression of miR-106b-25 cluster in plasma was significantly correlated with tumor size, pathological type and TNM stage in GC patients. What's more, the three members of miR-106b-25 cluster expressed consistently at a high level both in tissue and plasma. Considering the relationship between three miRNAs and some clinical pathological factors, it was implied that miR-106b-25 could be the potential tumor biomarker for diagnosis and prognosis for GC patients.

In another study, circulating miR-122 and -192 expression levels were analyzed in GC patients with qRT-PCR [42]. They found that plasma miR-122 expression was significantly decreased while miR-192 was increased in GC/DM samples compared to normal or benign controls. The similar expression pattern of these two miRNAs was observed in patients with post-distant metastases compared to those with pre-distant metastases. . Dysregulation of miR-122 expression but not miR-192 expression in plasma was associated with more favorable prognosis for GC. This suggests that that evaluation of circulating miR-122

and miR-192 expression levels could potentially help early detection of DM in GC.

Tsujiura M et al [43] hypothesized that miR-18a, which is a member of miR-17-92 cluster and has been reported as highly expressed in GC tissues, could be used as a novel plasma biomarker in GC patients. The study showed that miR-18a expression in both biopsy tissues and plasma was significantly higher in GC patients than in healthy controls, with AUC value of 0.8059. The plasma miR-18a levels were significantly reduced in post-operative plasma compared to that of preoperative samples. In a miR-18a overexpressing cell line, the miR-18a concentration in cultured medium was increased, suggesting that miRNA-18a might be released from cancer cells into the surrounding environment. Therefore, circulating miR-18a could be a useful biomarker for screening GC and monitoring tumor dynamics.

There were many other circulating miRNAs reportedly increased in GC, such as miR-21 [44] [45] [46] [47] [48] [49] [50] [51], miR-221 [52], miR-378 [53], miR-451 and miR-486 [54]. Several studies found that miR-421 was overexpressed in GC [55] [56] [57], and the positive detection rate of miR-421 is higher than that of serum CEA. Since the serum concentration of miR-421 has higher sensitivity and specificity than CEA [58], miR-421 may serve as an efficient early diagnostic biomarker. Along with the in-depth study about the circulating miRNAs associated with GC, more miRNAs would be found to have the potential of being diagnosis biomarkers in GC.

Down-regulated circulating miRNAs in GC

Besides the up-regulated miRNA, there were also down-regulated miRNA in GC.

Multiple groups reported and validated that miR-375 was down-regulated in GC [59] [60, 61] [62]. miR-375 was significantly down-regulated in distal gastric adenocarcinoma tissues as well as in serum [63]. At a normalized cutoff of 0.218, miR-375 yielded a receiver operating [62] characteristic (ROC) area under the curve (AUC) of 0.835 with a specificity of 80% and a sensitivity of 85%, in the discrimination of distal gastric adenocarcinoma from control tissues, while the level of miR-31 was significantly lower [64] [65]. Another study found that miR-195-5p was significantly down-regulated in GC, indicating miR-195-5p may serve as a novel tumor suppressor miRNA involving gastric carcinogenesis [66].

There were over 20 down-regulated circulating miRNAs reported till today, yet their clinical utility needs to be validated [67].

Table 1. Up-regulated circulating miRNA in GC

Author	miRNA	Samples	Methods	Proportion	Usage
Tsujiura [36]	miR-17-5p miR-21 miR-106a miR-106b	69 GC patients, 30 healthy volunteer	qRT-PCR	miR-17-5p(P=0.006,),miR-21 (P=0.05), miR-106a (P=0.008) and miR-106b (P<0.001, AUC=0.721) higher in GC, let- 7a (P=0.002) lower, ratio of miR-106a/let-7a AUC= 0.879	Early diagnosis
Manuel Val-ladares-Ayerbe s [37]	miR-200c	67 blood samples (52 stage I-IV GC patients and 15 controls	qRT-PCR	AUC-ROC was 0.715(P=0.012), Sensitivity 65.4%, specificity 100%, accurate rate 73.1%	Diagnostic, prognostic biomarker
Rotkrua [38]	miR-103, -107, -194, -210	DCKO rat model, 5 DGC-bearing mice vs 5 controls	TaqMan q RT-PCR, miRNA microarrays	miR-103, miR-107, miR-194, miR-210 (P=0.045, 0.004, 0.004 and 0.030) was higher in DCKO mouse sera	Early diagnosis of DGC
Rotkrua [39,40]	miR-199a-3p	30 EGC patients and 70 controls	miRNA microarray, qRT-PCR	AUC for EGC 0.818,sensitivity 76%, specificity 74%, and accuracy 75%,P<0.001	Diagnostic biomarker for EGC
Fu Z [29]	miR-222	114 GC patients , 36 CAG patients, 56 healthy controls	qRT-PCR	AUC 0.850, sensitivity 66.1 %, specificity 88.3 %	Early detection and prognosis of GC
Zhang R [41]	miR-106b-25 cluster(miR-106b, -93, -25)	40 post-operative and 20 pre-operative plasma samples	TaqMan qRT-PCR	Change fold were 2.51(miR106b), 2.32(miR-93), 2.10(miR-25)respectively, correlated with tumor size, bomman type and TNM stage(P<0.05)	Diagnosis and prognosis biomarker
Chen Q [42]	miR-192	12 pairs of samples	qRT-PCR	AUC for plasma miR-192 was 0.732(95% CI 0.623-0.841,P<0.01)	Early diagnosis
Tsujiura M [43]	miR-18a	104 GC patients, 65 healthy volunteers	qRT-PCR	Higher miR-18a in GC than controls, P<0.0001,ROC value 0.8059	Screening GC and monitoring tumor dynamics
Wu J [58]	miR-421	90 GC patients and 90 controls	qRT-PCR	Higher sensitivity and specificity than CEA and CA-125 in GC diagnosis	Early diagnosis
Song J [48]	miR-21	103 GC patients	qRT-PCR	Increased level of miR-21 associated with tumor size and pT stages(r=0.2633,P=0.0072)	Indicator for tumor burden in GC patients, biomarker for therapy

Table 2. Down-regulated circulating miRNA in GC

Author	miRNA	Samples	Methods	Proportion	Usage
Chen Q[42]	miR-122	12 pairs of blood samples	qRT-PCR	AUC for plasma miR-122 was 0.808, 95% CI 0.712-0.905,P<0.01	miR-122 down-regulated in GC, higher miR-122 may indicate a favorable prognosis
Zhang WH[68]	miR-375		Microarray, qRT-PCR	miR-375 ROC 0.835, specificity 80%, sensitivity 85%	Biomarker for distal GC
Gorur A[71]	miR-195-5p	20 blood samples from GC patients and 190 volunteers	q RT-PCR	miR-195-5p down-regulated 13.3 fold, P<0.05	miR-195-5p was a tumor suppressor miRNA and may contribute to gastric carcinogenesis

Difficulties in the application of circulating miRNAs as GC diagnostic biomarkers

1. Suitable internal reference genes

The prospective studies about circulating miRNAs suggested a bright future for the development of non-invasive biomarkers for GC molecular diagnosis. However, the accurate identification of miRNA-based biomarkers has been a significant challenge and is still difficult in clinical settings. Measurement of circulating miRNAs may be influenced by a number of factors, including amount of starting materials, methods of sample collection, RNA extraction, etc. . Among the reported potential circulating miRNAs, some of them were detected in serum, others in plasma. One of the meta-analyses suggested that plasma-based miRNA assay reached a higher accuracy compared to serum-based one for GC [68]. Meanwhile, technical challenges exist in measuring circulating miRNA.

Because of the relatively low expression of RNA isoforms in serum and plasma, conventional methods of spectrophotometry are not reliable to control for total amount of RNA extracted from samples; and the high concentration of miRNAs in blood cells can easily confound results if these are not removed carefully and/or cellular lysis is not prevented [69].

In a research on internal control small RNA U6 expression, Xiang M et al [70] found that the expression level of U6 was gradually decreased after 1, 2, and 4 cycles of freezing and thawing. It is reasoned to doubt that the expression of miRNA markers may not be stable and could be fluctuant under different cycles of freezing and thawing. More thorough research and standard procedure of sample collection should be established before these novel biomarkers being used in practice.

Furthermore, age and sex may be confounding variables when miRNAs were measured from blood samples. Meder B et al [71] explored the impact of age and sex on miRNAs in a cohort of 109 physiologically

unaffected individuals whose blood miRNAs was characterized by microarray technology. They also investigated an independent cohort from a different institution consisting of 58 physiologically unaffected individuals with similar mean age, but with a smaller age distribution. The samples were measured with high-throughput sequencing. They detected 318 miRNAs that were significantly correlated with age and, after adjustment for multiple testing, 35 of them remained statistically significant. Regarding sex, 144 miRNAs showed significant dysregulation; however, no miRNA remained significant after adjustment for multiple testing. In the high-throughput datasets, they observed a smaller number of significant associations, mainly as an effect of the smaller cohort size and age distribution. They concluded that age distribution of individuals recruited for case-control studies needs to be carefully considered, whereas sex may be less confounding. Thus the possible influence of age or sex should be considered when the expression of circulating miRNAs is being assessed.

The main method of miRNA detection was real-time quantitative Polymerase Chain Reaction (qPCR). This effective technology could significantly amplify the tiny content of miRNAs to an easily detectable level. There are numerous challenges to achieve sufficient sensitivity and specificity in the measuring circulating miRNAs, particularly in selecting appropriate normalization controls. There is however currently no agreement on this issue, which makes it impossible to compare the results from different labs or platforms. Therefore, it is essential to develop an effective and uniformed normalization strategy in evaluating circulating miRNA expression levels. One of the popular and relatively accurate strategies is to use a stable reference gene or a set of gene signatures (s) to evaluate circulating miRNA expression. Otherwise the data may not be reliable. One example is to use the same amount of the starting total RNA, however, due to the low yield of total RNA from serum or plasma, it is hard to measure the RNA yield and quality accurately with the available methods [72].

Despite the most common controls, U6 and miR-16 are used as internal control for miRNA quantification, Xiang M et al [70] found large fluctuations in U6 expression from their microarray-based serum miRNA profiling on a number of cancer patients and normal controls. They also demonstrated that the expression of U6, miR-16 and miR-24 in serum was subjected to different freeze-thaw cycles. Therefore, U6 may not be an ideal internal control for circulating miRNAs.

In order to identify stable controls for circulating miRNA quantification, a global circulating miR-

NA profiling study was performed using healthy controls, chronic hepatitis B, cirrhosis and a variety of cancer patients. The most stable controls were selected by a number of programs, such as GeNorm, NormFinder, and coefficient of variability (CV). Seven candidates miRNAs, miR-1225-3p, miR-1228, miR-30d, miR-939, miR-940, miR-188-5p, and miR-134 were identified, and validated by qPCR, along with the commonly used four other controls, miR-16, miR-223, let-7a, and RNU6B using an independent cohort. miR-1228 (CV = 5.4%) turns out to be the most stable one among all tested. Interestingly, miR-1228 is involved in metabolism-related signal pathways and organ morphology by Ingenuity Pathway Analysis (IPA), which suggests that miR-1228 may be a housekeeping miRNA gene. The stable expression of miR-1228 in the blood may qualify it as a promising control for circulating microRNAs quantification [73]. Another study indicated that miR-93 may be a useful internal control for serum miRNA analysis in GC [34].

Chen X et al. [74] screened and validated the reference genes using a strategy by combining the Illumina's sequencing by synthesis (SBS) technology, qPCR, literature screening and statistical analysis. A panel of let-7d, let-7g and let-7i was developed as a reference set for circulating miRNA normalization, and subsequently validated across numerous healthy controls and patients, and was demonstrated to be statistically better than the commonly used reference set, such as U6, RNU44, RNU48 and miR-16. Because of the variations of the internal control genes, special attention should be paid when comparing the diagnostic value of the circulating miRNAs.

2. High specificity and sensitivity of circulating miRNAs

As ideal diagnostic biomarkers, circulating miRNAs should be specific enough to distinguish GC from other diseases, and sensitive to be detectable. However, most of the reported circulating miRNAs with aberrant expression profile in GC were also observed in other cancers. For example, the expression of miR-200 family was dysregulated not only in GC [75], but also in colorectal carcinoma [76] [77] [78], hepatocellular carcinoma [79], anaplastic thyroid cancer [80], ovarian cancer [81] [82], breast cancer [83] [84], prostate cancer [85], and pancreatic cancer [86]. MiR-222 was classified as oncomiR both in GC and hepatitis B-related hepatocellular carcinoma [87]. Even the contradictory results were found on the same miRNA in GC. For example, miR-199a-3p was found both up-regulated [40] and down-regulated [88] in GC in different studies.

Since there is no single circulating miRNA that could be used to diagnose GC till today, the combina-

tion of several miRNAs has been presented by some researchers. In a meta-analysis research, a total of 107 studies from 42 articles were included about the potential circulating miRNAs in GC and colorectal cancer (CRC). The overall analysis of all GI cancers showed that circulating miRNAs have a relatively good diagnostic performance. In addition, single-miRNA assay displayed a relatively low diagnostic performance, while multiple-miRNA assay significantly improved the diagnostic accuracy for GC and CRC [68]. Therefore, a signature of miRNAs may serve as promising biomarkers for the diagnosis of GC.

In another meta-analysis conducting to assess the diagnostic value of circulating miRNAs for GC in Asian population, the literature search was carried out in databases (PubMed, Embase, Web of Science, The Cochrane Library, and CNKI) and other sources using combinations of keywords relating to GC, miRNAs, and diagnosis. The values of sensitivity, specificity, positive likelihood ratios (PLR), negative likelihood ratios (NLR), and diagnostic odds ratio (DOR) reported in individual studies were pooled using random-effects models. Potential sources of heterogeneity were assessed with subgroup and meta-regression analyses. The summary receiver operating characteristic (SROC) curve and the AUC were used to assess the diagnostic accuracy of miRNAs. This meta-analysis included 1,279 patients with GC and 954 healthy controls from 20 publications. The pooled sensitivity, specificity, PLR, NLR, DOR, and AUC were 0.78, 0.80, 4.0, 0.28, 14, and 0.86, respectively. Subgroup analyses showed that early stages (I and II) GC were more easily detected than later stages, and that multiple miRNAs assays were more accurate than single miRNA assays [89]. This meta-analysis concluded that miRNAs had a higher diagnostic value for GC in early stages (I and II) than in late stages, and multiple miRNAs assays had a better diagnostic value than single miRNA assays.

Besides the meta-analysis, a panel of five miRNAs (miR-16, miR-25, miR-92a, miR-451 and miR-486-5p) seemed to show consistently high level expression in GC patients' plasma [31]. This 5-miRNA panel may serve as a potential non-invasive biomarker in early GC detection.

The combination of miRNA markers could consist of both up-regulated and down-regulated miRNAs. For example, overexpressed miR-223, miR-21 and underexpressed miR-218 in GC patients yield the AUC values of 0.9089, 0.7944, and 0.7432, respectively, while the combined ROC analysis reveals the highest AUC value of 0.9531 in discriminating GC patients from healthy controls [44]. This suggests that a cluster of miRNAs would be a better diagnostic biomarker

than single miRNA, with much higher sensitivity, specificity, and accuracy.

Conclusions

The search for better non-invasive biomarkers for the diagnosis of GC has led to the investigation of aberrant circulating miRNAs in plasma or serum. The expression level of miRNAs could be easily detected using qRT-PCR. The studies demonstrated that higher sensitivity and specificity can be achieved by circulating miRNAs as compared to other commonly used biomarkers. Furthermore, the combination of several miRNAs reached higher specificity and sensitivity than single miRNA. However, the standard protocol of sample treatment and the suitable internal controls should be further established to make the detection comparable before the miRNA biomarkers being fully utilized in clinical practice.

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Competing Interests

The authors have declared that no competing interest exists.

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