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Original Article

Development of a novel circulating miRNA panel for early detection of esophageal cancer

Yingying Chen[1*], Yunhan Xu[2,4*], Li Li[1], Xunlei Pang[1], Hui Li[5], Guodong Zhao[3,4], Jun Yang[1]

[1]Department of Gastroenterology, Affiliated Hospital of Xuzhou Medical University, Xuzhou 221002, Jiangsu, China; [2]School of Medicine, Biomedical Engineering, Chinese University of Hong Kong (Shenzhen), Shenzhen 518100, Guangdong, China; [3]Zhejiang University Kunshan Biotechnology Laboratory, Zhejiang University Kunshan Innovation Institute, Kunshan 215300, Jiangsu, China; [4]Suzhou VersaBio Technologies Co. Ltd., Kunshan 215300, Jiangsu, China; [5]Department of Gastroenterology, The First People's Hospital of Xuzhou, The Affiliated Xuzhou Municipal Hospital of Xuzhou Medical University, Xuzhou 221002, Jiangsu, China. [*]Equal contributors.

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Abstract: Objectives: Esophageal cancer (EC) is a highly aggressive malignancy with a low survival rate. Current detection methods like endoscopy are invasive and expensive, resulting in low compliance rates. This study aimed to develop and validate a novel circulating microRNA (miRNA)-based panel for early EC detection and compare its effectiveness in serum versus plasma samples. Methods: The study developed a multiplexed miRNA assay to detect eight EC-related miRNAs in both plasma (n=45) and serum (n=148) samples. Expression levels of these miRNAs were compared between EC patients and control subjects. The serum-based panel was further validated in an independent cohort and compared with carcinoembryonic antigen (CEA), a conventional tumor marker. Results: The multiplexed miRNA panel demonstrated excellent PCR amplification efficiency with no cross-interference between miRNAs. Six of the eight miRNAs showed significantly higher expression in EC patients compared to controls in serum samples (area under the curve (AUC) >0.900), while plasma samples showed poor discrimination (AUC<0.700). The optimized 6-miRNA panel in serum achieved perfect discrimination (AUC=1.0) between EC and controls in both training and validation cohorts. This panel also effectively distinguished EC from gastric and colorectal cancers and significantly outperformed CEA (AUC=0.619) in diagnostic accuracy. Conclusion: Serum is more suitable than plasma for EC detection using miRNA biomarkers. The optimized 6-miRNA panel demonstrated exceptional accuracy for EC detection, offering a promising non-invasive approach for early diagnosis. This method requires smaller sample volumes than traditional techniques, potentially improving clinical applicability and patient compliance.

Keywords: miRNA, plasma, serum, esophageal cancer, non-invasive testing

Introduction

Esophageal cancer (EC) is a highly aggressive malignancy originating from the esophageal epithelium [1]. In 2022, global epidemiological data reported 510,716 new cases and 445,129 deaths due to EC [2]. Despite a declining incidence rate ranking seventh, EC remains a significant concern due to its remarkably low 10% survival rate, necessitating a rigorous approach to its management [3].

Barium swallow, a form of X-ray imaging utilizing a contrast dye, enhances esophageal visibility for detecting anomalies in its lining [4]. However, its efficacy in discerning subtle esophageal wall changes, including precancerous lesions, is limited. Currently, endoscopy remains the gold-standard screening modality for EC [5], employing a flexible endoscope equipped with a high-resolution camera to visualize esophageal mucosa and detect suspicious lesions or premalignant changes. During endoscopy, a physician can extract a small tissue sample (biopsy) from suspicious areas for microscopic examination, making it the gold standard for early EC diagnosis [6]. Nevertheless, due to invasiveness, dietary restrictions, and high costs, endoscopy exhibits a low compliance rate [7]. Therefore, a cost-effective, non-invasive and sensitive approach is urgently needed for EC diagnosis.

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With the rapid advancements in biotechnology, particularly in high-throughput sequencing and multi-omics technologies, an increasing number of markers are being discovered [8, 9]. Simultaneously, the development of liquid biopsy has seen the utilization of numerous non-invasive markers in clinical diagnostics, including blood- or stool-based DNA methylation tests [10-12]. Currently, DNA methylation stands as the most widely used and stable marker for cancer diagnosis [13], with several literature reports highlighting its application in early EC diagnosis [14-16]. Nonetheless, DNA methylation presents notable limitations in EC diagnosis: 1) lower sensitivity in detecting early-stage EC [14, 17]. 2) The lower amount of circulating free DNA (cfDNA) in blood typically requires 3-4 mL of plasma for detection, leading to a complex process and high cost [17, 18]. 3) Additionally, cfDNA has a short half-life, necessitating prompt testing after sampling to ensure result accuracy [19].

MicroRNAs (miRNAs) constitute a class of small noncoding RNAs, typically 19-25 nt in length, playing a crucial role in post-transcriptional silencing of target genes [20, 21]. These miRNAs are widely distributed in various human tissues and can be consistently detected in bodily fluids such as saliva, urine, blood, and stool [21], positioning miRNAs as potentially effective non-invasive markers for cancer diagnosis [22]. Currently, several studies focusing on the early diagnosis of EC based on circulating free miRNA (cf-miRNA) in blood have been reported [23, 24]. Notably, cf-miRNA exhibits a more abundant payload than cfDNA methylation and requires only 200-300 μL of serum or plasma for detection [25, 26]. Furthermore, the stability of serum or plasma miRNAs extends preservation for up to one year or even 5-10 years [25, 26]. Consequently, miRNAs emerge as promising markers for the early diagnosis of EC. For example, Miyoshi et al. identified eight EC-related miRNAs and developed an 8-miRNA panel through integrated analyses of tumor tissue, serum samples, and bioinformatics data [26]. This panel was further validated in two retrospective cohorts and one prospective cohort, demonstrating robust sensitivity and specificity for EC detection, making it one of the most comprehensive and clinically relevant miRNA panels reported to date. Compared with individual miRNAs such as miR-375 or miR-143 that have been proposed in smaller-scale studies [27, 28], the Miyoshi panel offers stronger evidence and greater potential for clinical application [26]. However, its evaluation has so far been limited to a single research group, and independent validations are still lacking. Moreover, the costly and operationally complex single-plex qPCR detection method raises concerns. In addition, it remains unclear whether these EC-related miRNAs exhibit consistent expression in both plasma and serum. To address these issues, we established a novel multiplex miRNA panel, and compared miRNA signatures in plasma and serum. Simultaneously, we assessed the diagnostic performance of a 6-miRNA panel in serum samples.

Materials and methods

Sample collection

This study was approved by the Institutional Review Boards of Affiliated Hospital of Xuzhou Medical University (Ethics Committee reference number: XYFY2021-KL311 and XYFY2022-KL390) in accordance with the principles of the Helsinki Declaration. The informed consent was obtained from all participating subjects. Blood samples were collected from 14 patients diagnosed with EC and 31 non-cancer controls using 5 mL K_2EDTA tubes in accordance with the instructions of the miRNA extraction kit (**Figure 1**). The use of K_2EDTA tubes was required to minimize potential interference with subsequent miRNA detection, which may occur when plasma was obtained from tubes containing other anticoagulants such as sodium citrate or heparin. Samples were kept at 4°C and processed within 8 hours. Plasma (1 mL) was isolated via a two-step centrifugation at 1,300 g, then immediately stored at -80°C. In a separate serum cohort, 5 mL blood samples were obtained using Gel & Clot Activator Tubes from 38 EC patients, 7 individuals with gastric cancer (GC), 8 with colorectal cancer (CRC), and 95 healthy controls. Serum samples were then randomly allocated into a training set (n=68) and a validation set (n=80) for further analysis (**Figure 1**). These samples were left at room temperature for 30 min, after which serum (1 mL) was separated by centrifugation at 1,800 rpm, followed by immediate freezing and storage at -80°C. The inclusion criteria for all participants were: (1) age ≥18 years; (2) non-pregnant status; (3) availability of endoscopic examination results; and (4) histopathological

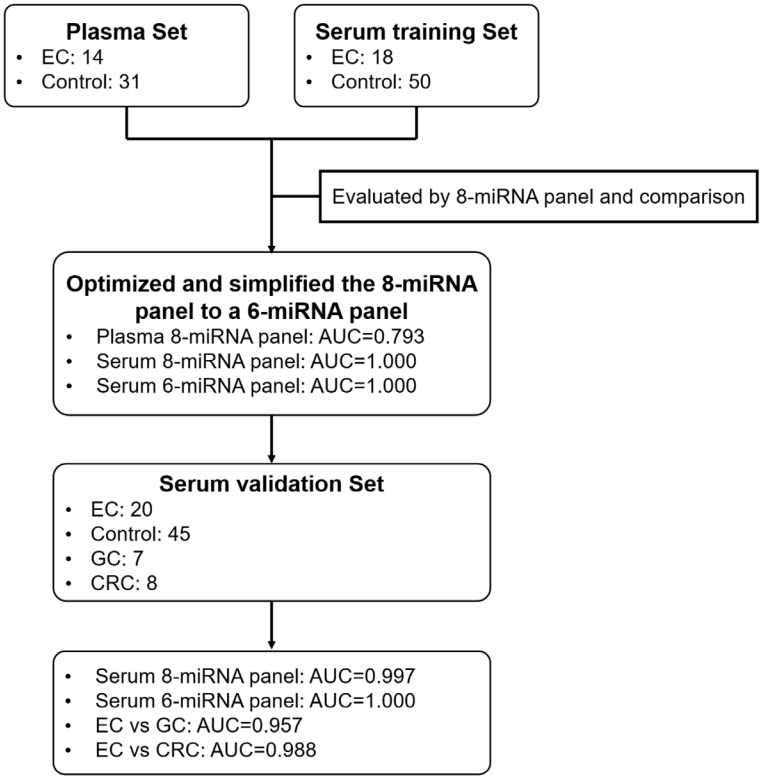

Figure 1. Schematic representation of the study design.

confirmation for cancer patients. Exclusion criteria were: (1) hemolytic or lipemic serum/plasma samples; and (2) insufficient serum/plasma volume. All participants underwent endoscopic examination. For participants with abnormal findings, the final diagnosis was confirmed by histopathological analysis. Participants without abnormal findings on endoscopy were considered as controls. Blood samples were collected prior to any surgical or therapeutic intervention.

Cell free circulating miRNA extraction

Cf-miRNAs from both plasma and serum were isolated from 200 µL aliquots using the Versamedx® miRNA Extraction Kit (Suzhou VersaBio Technologies Co., Ltd, Jiangsu, China), in accordance with the protocol provided by the manufacturer. Briefly, 1 mL of lysis buffer was added to each sample, and the mixture was vortexed and incubated at room temperature for 5 min. Subsequently, 3.5 µL of spike-in control (cel-miR-39-3p) was added and thoroughly mixed. Then, 200 µL of chloroform was added, mixed by vortexing, and incubated at room temperature for 3 min. The mixture was centrifuged for 15 min at 12,000 ×g at 4°C, and 750 µL of the upper aqueous phase was transferred to a new tube. To this, 1,125 µL of ethanol was added and thoroughly mixed by vortexing. Then, 700 µL of the sample was pipetted into a spin column and centrifuged at 8,000 ×g for 15 s at room temperature. The flow-through was discarded, and this step was repeated twice. Subsequently, the sample was washed with Wash Buffer R1, R2, and 80% ethanol. Finally, miRNA was eluted in 30 µL elution buffer and stored at -80°C until use.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Stem-loop reverse transcription quantitative PCR (RT-qPCR) was employed to quantify miRNA expression according to previously published methods [29]. For the reverse transcription step, a 30 µL reaction mixture was prepared containing 1 µL of 1 µM stem-loop RT primer, 1 µL of 10× RT mix, 1 µL of Hiscript II Enzyme Mix (Vazyme, Jiangsu, China), 4 µL of miRNA sample, and 22 µL of DEPC-treated water. The thermal profile for reverse transcription was as follows: 16°C for 15 minutes, 42°C for 40 minutes, 85°C for 5 minutes, and finally held at 4°C for 1 minute. Subsequently, qPCR amplification was carried out using an ABI7500 Real-Time PCR System (Applied Biosystems, USA). Each 20 µL PCR reaction contained 0.8 µL each of 10 µM forward and reverse primers, 0.2 µL of 10 µM TaqMan probe, 3 mM $MgCl_2$ solution (NuHigh Biotechnologies, Jiangsu, China), 0.1 mM dNTPs mixtures (Sangon Biotech, Shanghai, China), 4 µL of PCR buffer, 0.4 µL of Taq DNA Polymerase (NuHigh Biotechnologies), 4 µL of reverse-transcribed product, and 5.62 µL of DEPC-treated water. The qPCR cycling conditions included an initial denaturation at 94°C for 10 min, followed by 45 cycles of 95°C for 15 s, 56°C for 30 s (annealing and fluorescence acquisition), and 72°C for 10 s. The sequences of miRNAs, primers, and probes utilized are

provided in Table S1. Primer and probe synthesis was performed by GENEWIZ (Jiangsu, China), while miRNAs and plasmids were synthesized by Sangon Biotech (Shanghai, China).

Blood CEA detection

A 2 mL blood sample was collected from each participant, and serum carcinoembryonic antigen (CEA) levels were quantified using the Cobas e801 electrochemiluminescence platform (Roche Diagnostics, Mannheim, Germany). To assess the diagnostic value of CEA in comparison with the 6-miRNA panel, all samples for which both CEA and miRNA data were available - 33 patients with EC and 86 non-cancer controls - were included in the analysis. Samples lacking CEA measurements were excluded.

Statistical analysis

The expression of miRNAs was normalized to hsa-miR-16-5p alone in both serum and plasma specimens, while cel-miR-39-3p was used to assess extraction efficiency. All miRNA levels were represented as $2^{-\Delta\Delta Ct}$. For control subjects with no amplification signals, the Ct values were set to 50, a value higher than the maximum PCR cycle. The $2^{-\Delta\Delta Ct}$ values were used for plotting receiver operating characteristic (ROC) curves for miRNAs, and the values of the area under the curve (AUC) were calculated, when the AUC value is greater than 0.8 and the *p*-value is less than 0.05, we consider the diagnostic performance to be satisfactory [30]. For combined diagnostic analysis, the relative expression levels ($2^{-\Delta\Delta Ct}$ values) of the selected miRNAs were simultaneously entered into a multivariable regression model. The predicted probability generated by the model was then applied to construct ROC curves and to calculate the corresponding AUC values. These AUCs were used to compare the diagnostic performance of individual miRNAs and the combined panel. Statistical comparisons of miRNA expression levels between groups were conducted using the Mann-Whitney U test, with significance defined as a *p*-value less than 0.05. All data analyses were carried out using MedCalc (version 19.6.0), GraphPad Prism (version 8.0), and IBM SPSS Statistics (version 22.0).

Results

The novel panel developed in this study incorporated a spike-in control (cel-miR-39-3p) to monitor the extraction efficiency of plasma and serum, as well as an endogenous miRNA control (hsa-miR-16-5p) to normalize the samples. Considering the number of fluorescence channels commonly used in the qPCR instrument and cost considerations, we detected 10 miRNAs in three tubes, each labeled with a different fluorophore: Tube-1 included cel-miR-39-3p (FAM), hsa-miR-16-5p (JOE), hsa-miR-21-5p (ROX), and hsa-miR-106b-5p (CY5); Tube-2 included hsa-miR-93-5p (FAM), hsa-miR-16-5p (JOE), hsa-miR-181a-5p (ROX), and hsa-miR-151a-3p (CY5); Tube-3 included hsa-miR-17-5p (FAM), hsa-miR-16-5p (JOE), hsa-miR-25-3p (ROX), and hsa-miR-103a-3p (CY5). The above results indicate that each tube had an endogenous miRNA control (hsa-miR-16-5p), which is used to monitor the effectiveness of each reaction, preventing false negatives. Firstly, to validate whether there was interference among the miRNAs in the multiple reactions in each tube, we compared the Ct values of each miRNA between the single-plex assay and the 4-plex assay. As shown in **Figure 2A**, there was no significant difference in Ct values between single-plex and multiplex reactions, demonstrating that there was no significant mutual interference in the multiplex assay and indicating good stability. Next, we assessed the amplification efficiency of the 3-tube multiplex RT-qPCR assay using plasmids containing cDNA sequences of miRNAs. These plasmids were diluted with a concentration gradient, with copy numbers ranging from 10 to 100,000 per reaction. As shown in **Figure 2B-D**, all reactions exhibited excellent linear ranges (R^2>0.99). Meanwhile, the amplification efficiency of all miRNAs fell within the range of 90%-100%, demonstrating the accuracy of the PCR reaction. This also indirectly confirms the absence of interference or competitive reactions among different miRNAs, ensuring precise quantification across varying miRNA concentrations and guaranteeing accuracy in subsequent blood sample testing.

To compare miRNA signatures between plasma and serum, we included 45 cases of plasma and 68 cases of serum (training set). The characteristics of plasma and serum samples are detailed in **Table 1**. As shown in **Figure 3**, no significant differences were observed in the Ct values of the spike-in control (cel-miR-39-3p) between EC and control in plasma or serum samples, and the standard deviation was minimal. This ensures that the extraction method had no preference for either serum or plasma,

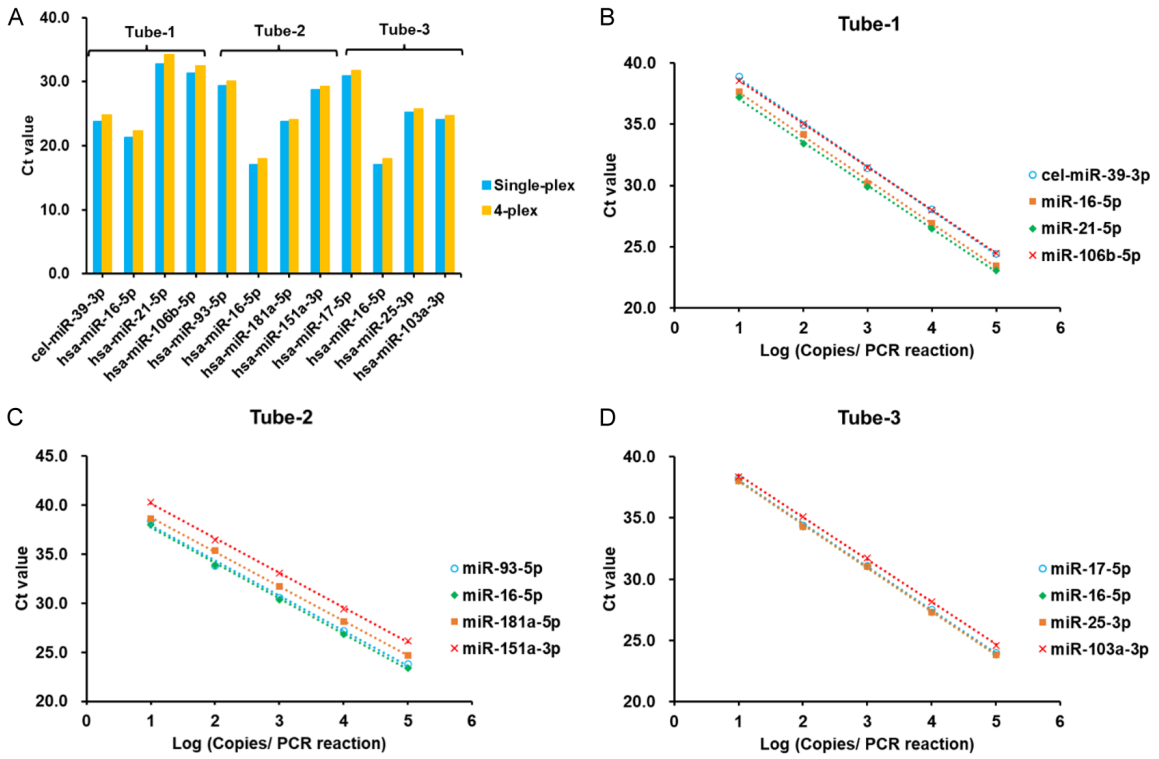

Figure 2. The amplification performance of the multiplexed miRNAs assay. (A) the comparison between single-plex and 4-plex assay, (B-D) the liner ranges of cDNA for each miRNA in three tubes.

Table 1. The characteristics of subjects enrolled in this study

	Plasma cohort		Serum cohort					
			Training set		Validation set			
	EC (n=14)	Control (n=31)	EC (n=18)	Control (n=50)	EC (n=20)	Control (n=45)	GC (n=7)	CRC (n=8)
Age								
Range (min-max)	55-85	18-82	56-82	33-85	50-82	35-90	57-80	51-86
Median	71.5	29	69.5	58.5	67.5	61	73	60.5
Mean±SD	72.3±7.1	36.5±16.1	68.8±6.7	59.0±10.8	68.4±7.9	60.8±11.6	70.4±7.7	63.8±11.1
Gender (n/%)								
Male	13 (92.9)	4 (12.9)	14 (77.8)	20 (40.0)	16 (80.0)	20 (44.4)	5 (71.4)	7 (87.5)
Female	1 (7.1)	27 (87.1)	4 (22.2)	30 (60.0)	4 (20.0)	25 (55.6)	2 (28.6)	1 (12.5)

EC, esophageal cancer; GC, gastric cancer; CRC, colorectal cancer.

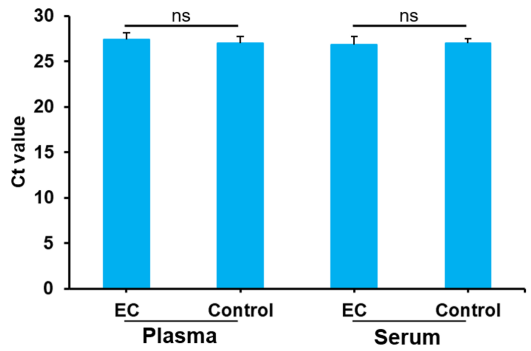

Figure 3. Ct values of spike-in control (cel-miR-39-3p) in plasma and serum. ns, not significant.

meaning that the extraction process does not affect the subsequent comparison of cf-miRNAs between plasma and serum.

Based on the ROC curve analysis (**Figure 4A**), the AUC values of EC-related miRNAs in plasma are all <0.700, especially for hsa-miR-181a-5p, hsa-miR-151a-3p, and hsa-miR-103a-3p, the AUC values for these miRNAs are all below 0.6, and all eight miRNAs in plasma had *P*-values >0.05, indicating a poor ability to distinguish between EC and the control group. In serum samples (**Figure 4B**), except for hsa-miR-106b-5p and hsa-miR-103a-3p, which showed no

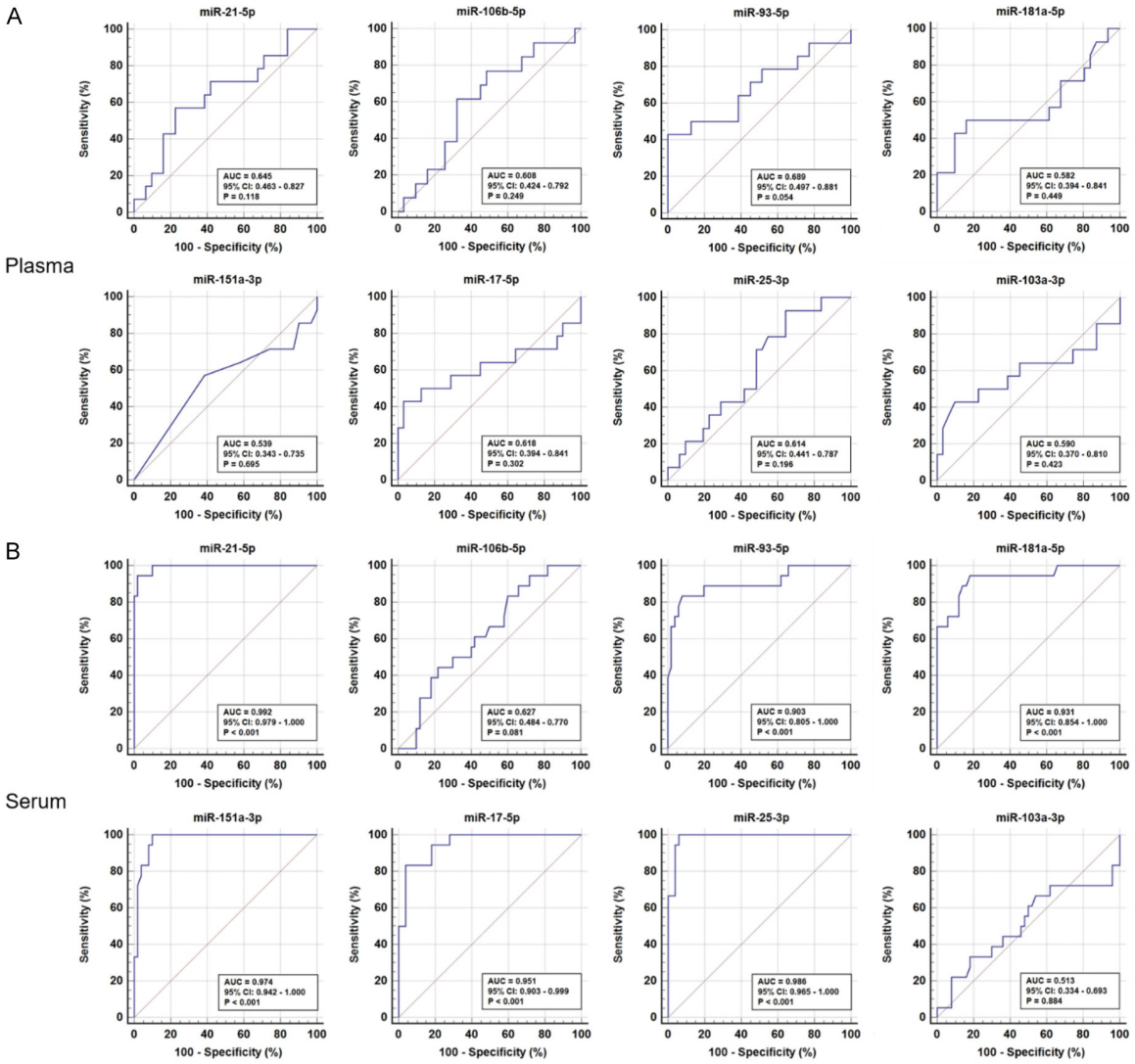

Figure 4. The ROC curves of 8 miRNAs in plasma (A) and serum (B).

significant difference between EC and control (P>0.05), the AUC values of the remaining miRNAs were all >0.900, indicating outstanding discrimination between EC and control subjects. Furthermore, the comparison of the expression levels of these eight miRNAs in plasma and serum (**Figure 5**) revealed that EC miRNA levels for all miRNAs were higher in serum than in plasma (P<0.0001). In control samples, six miRNAs showed significantly higher levels in serum than in plasma (P<0.0001), except for hsa-miR-25-3p, which displayed a significantly higher expression level in plasma than in serum (P<0.05). Additionally, one miRNA (hsa-miR-151a-3p) did not exhibit a significant difference between plasma and serum. This is because hsa-miR-151a-3p could be detected in EC, but most control samples showed no Ct values, so they were assigned Ct values of 50.0 for analysis.

When a combined analysis of the eight EC-related miRNAs was performed, it was observed that the ability to distinguish between EC and control is further enhanced in both plasma and serum samples (**Figure 6A**, **6B**). However, the AUC value in plasma was only 0.793 (95% CI: 0.618-0.968, sensitivity: 64.3%, specificity: 96.8%, **Figure 6A**), while in serum samples, it reached 1.0 (95% CI: 1.000-1.000, sensitivity: 95.0%, specificity: 100.0%, **Figure 6B**). In the serum sample analysis, the effectiveness of hsa-miR-106b-5p and hsa-miR-103a-3p in distinguishing between EC and control in serum

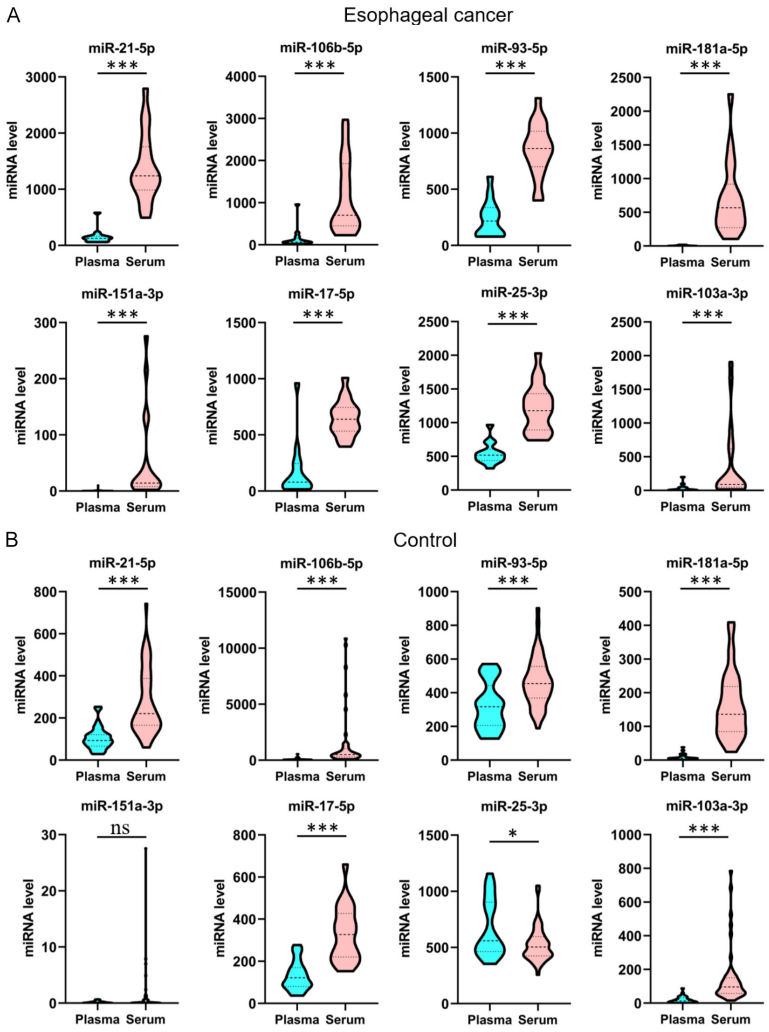

Figure 5. The comparison of 8 miRNA expression levels in plasma and serum. (A) Esophageal cancer, (B) control subjects. *, $P<0.05$, **, $P<0.01$; ***, $P<0.0001$.

was not significant (**Figure 4B**). Consequently, we excluded these two miRNAs to replot the serum ROC curves. The combined analysis of the remaining six miRNAs (hsa-miR-21-5p, hsa-miR-93-5p, hsa-miR-181a-5p, hsa-miR-17-5p, hsa-miR-25-3p, and hsa-miR-151a-3p) in serum still achieved an AUC value of 1.0 (95% CI: 1.000-1.000, sensitivity: 95.0%, specificity: 100.0%, **Figure 6C**).

To further validate the diagnostic capability of the serum miRNA panel for EC, we included 20 EC cases, 45 controls, eight cases of GC, and seven cases of CRC in the validation set (**Figure 1**; **Table 1**). In the validation set, the mean Ct values of EC and control subjects showed no significant difference (Figure S1), indicating consistency in cf-miRNA extraction. The 8-miRNA panel demonstrated an AUC value of 0.997 (95% CI: 0.989-1.000, sensitivity: 95.0%, specificity: 100.0%) for distinguishing EC and control, while the 6-miRNA panel exhibited a higher AUC value of 1.0 (95% CI: 1.000-1.000, sensitivity: 100.0%, specificity: 100.0%), consistent with the results in the training cohort (**Figure 7A**, **7B**). Additionally, the 6-miRNA panel also demonstrated excellent discriminatory ability between EC and GC (AUC=0.957, 95% CI: 0.887-1.000) or CRC (AUC= 0.988, 95% CI: 0.958-1.000) (**Figure 7C**, **7D**). As the most commonly used blood tumor marker in clinical diagnosis, the performance of CEA was compared with that of the 6-miRNA panel in the samples from both the training and validation cohorts that underwent both tests. The results in **Figure 7E** revealed that the AUC of CEA in distinguishing EC from control was only 0.619 (95% CI: 0.503-0.735), while the AUC value of the 6-miRNA panel reached 1.0 (95% CI: 0.999-1.000), demonstrated significant superiority in distinguishing EC from control subjects. Meanwhile, we compared the ROC curves between different age groups and genders and found no significant difference between each group (Figure S2).

Discussion

In this study, we developed a multiplexed miRNA panel for early detection of EC and compared miRNA expression between plasma and serum. The results demonstrated that serum was a more suitable sample for EC detection. The miRNA panel exhibited high sensitivity and specificity for detecting EC, particularly in distinguishing EC from GC and CRC, two other gastrointestinal cancers that may cause interfer-

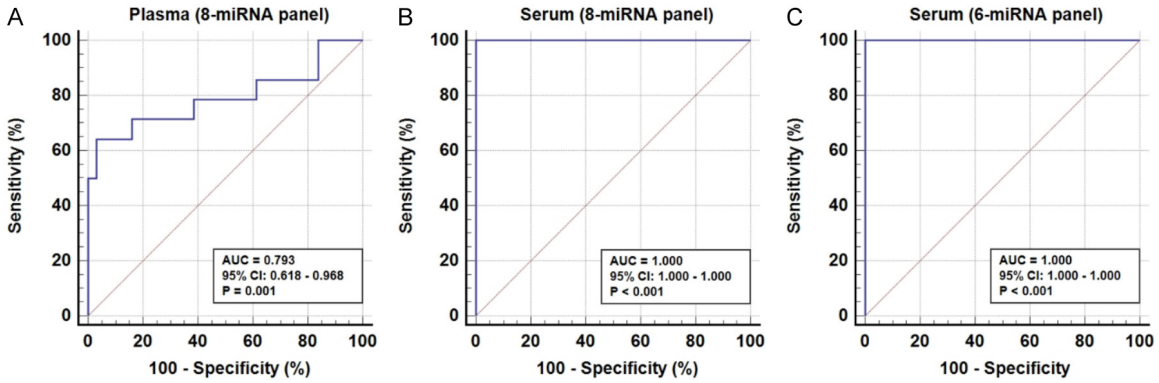

Figure 6. The ROC curve for 8-miRNA panel in plasma (A), the ROC curves for 8-miRNA (B) and 6-miRNA panels in serum training set (C).

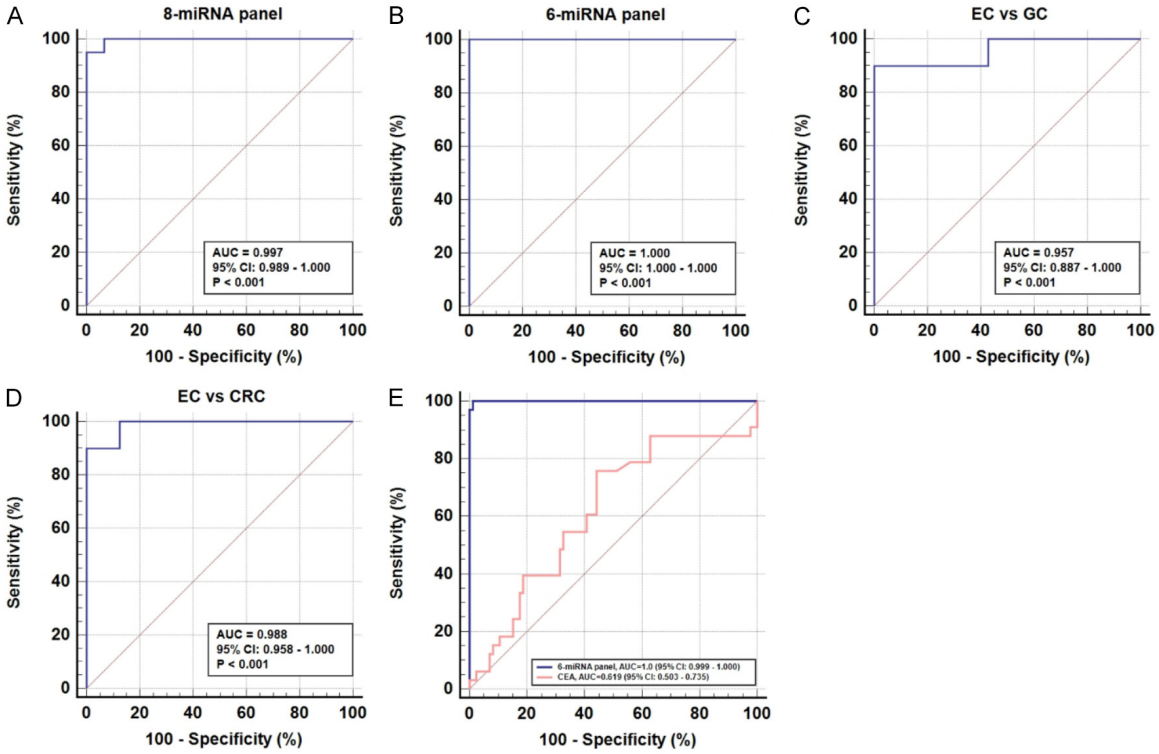

Figure 7. The ROC curves of serum 8-miRNA panel for distinguish EC and control in validation set (A), the ROC curves of serum 6-miRNA panel for distinguish EC and control in validation set (B), the ROC curves of serum 6-miRNA panel serum 6-miRNA panel for distinguish EC and GC in validation set (C), the ROC curves of serum 6-miRNA panel serum 6-miRNA panel for distinguish EC and CRC in validation set (D), and the ROC curves of the serum 6-miRNA panel were compared with those of CEA (E).

ence in serum. The serum-based 6-miRNA panel showed superior diagnostic performance compared to plasma-based assays and conventional biomarkers like CEA. Given that endoscopic screening is invasive, costly, and has low public compliance, the proposed non-invasive blood-based assay offers a promising alternative for large-scale screening, especially among high-risk populations. Its high AUC, ease of sample collection, and potential to distinguish EC from other gastrointestinal tumors indicate strong clinical utility. The overall cost per test of the miRNA panel is substantially lower than that of endoscopy, and it allows high-throughput processing without the need for patient scheduling or waiting time, suggesting poten-

tial cost-effectiveness in clinical practice. This approach could help triage individuals for endoscopy more effectively, thereby improving early detection rates, reducing unnecessary invasive procedures, and ultimately enhancing patient outcomes.

The six miRNAs included in our final panel - hsa-miR-21-5p, hsa-miR-93-5p, hsa-miR-181a-5p, hsa-miR-151a-3p, hsa-miR-17-5p, and hsa-miR-25-3p - have all been reported to be involved in various oncogenic processes, and many have been specifically implicated in EC or related gastrointestinal malignancies. hsa-miR-21-5p, hsa-miR-93-5p, and hsa-miR-17-5p are most frequently upregulated oncomiRs in solid tumors, including EC. They promote tumor progression by targeting multiple tumor suppressors such as PTEN, and are associated with enhanced proliferation, invasion, and resistance to apoptosis [31-33]. In EC, hsa-miR-181a-5p has been associated with tumor proliferation and invasion via modulation of the Wnt/β-catenin pathway and targeting of tumor suppressors such as RASSF6 [34]. hsa-miR-151a-3p has been reported to enhance cancer cell migration and metastasis in cancers by regulating genes involved in cytoskeletal remodeling and EMT [35]. hsa-miR-25-3p targets multiple apoptotic regulators such as Bim and is upregulated in several gastrointestinal cancers, including EC [36]. Collectively, the biological functions of these miRNAs support their involvement in EC pathogenesis and justify their selection for inclusion in a diagnostic panel. Their consistent upregulation in EC and known oncogenic roles enhance the clinical relevance and potential utility of our findings.

The elevated serum levels of these six miRNAs may be attributed to their secretion via exosomes derived from EC cells [37]. Exosomes are small extracellular vesicles that facilitate intercellular communication and are known to carry various biomolecules, including miRNAs. Studies have shown that tumor cells release exosomes containing oncogenic miRNAs, which can be detected in the bloodstream and reflect the tumor's molecular characteristics [38]. Specifically, hsa-miR-21-5p has been identified in exosomes from head and neck squamous cell carcinoma (HNSCC) cells, suggesting its potential role in mediating communication between tumor cells and the surrounding microenvironment [39]. Similarly, hsa-miR-93-5p has been found in exosomes from CRC cells, indicating its involvement in tumor progression and potential as a biomarker [40]. hsa-miR-181a-5p, hsa-miR-151a-3p, and hsa-miR-25-3p have also been reported to be present in exosomes from various cancer types, including breast and oral squamous cell carcinoma, highlighting their potential roles in tumor biology and as diagnostic indicators [41]. The presence of these miRNAs in exosomes suggests that their elevated levels in serum may reflect active secretion by EC cells, contributing to the observed high expression levels.

Plasma and serum are the two most commonly used sample types for miRNA detection [42, 43]. However, due to differences in pre-analytical methods for plasma and serum, miRNA levels can vary, and even opposite results can be obtained in applications. In this study, we found that the previously reported eight EC-related miRNAs showed no significant difference between EC and control in plasma, while six of the eight miRNAs exhibited excellent discriminatory ability between EC and control in serum, with the majority of miRNA levels being significantly higher in serum than in plasma. Wang et al. also observed higher miRNA concentrations in serum samples compared to the corresponding plasma samples [44]. They speculated that this difference arises from the release of miRNAs from blood cells caused by the coagulation process, thereby affecting the spectrum of extracellular miRNAs [44]. Conversely, Foye et al. found that the concentration and diversity of some miRNAs were greater in plasma than in serum samples [45]. They postulated that this reflected extracellular miRNA release from white blood cell contamination in plasma [45]. Although the conclusions drawn from these studies may seem contradictory, they all agree that the differences in miRNA between plasma and serum originate from extracellular miRNA contamination caused by pre-processing steps. Therefore, in blood miRNA detection, standard procedures for sampling, storage, and pre-processing are crucial [46].

The eight miRNAs used in this study were selected from Miyoshi's report [26]. Their 8-miRNA panel showed AUC values ranging from 0.80 to 0.89 in retrospective cohorts, and demonstrated AUC values ranging from 0.92 to 0.93 in two prospective cohorts [26]. In comparison with the previous report by Miyoshi et al., two of the

eight miRNAs in our study (hsa-miR-106b-5p and hsa-miR-103a-3p) did not show significant differences in distinguishing EC from controls, whereas the remaining six miRNAs maintained the same trend and diagnostic performance as reported previously. This discrepancy may be attributed to several factors: (1) population differences, as our study was conducted in a smaller Chinese cohort compared with the larger Japanese cohorts in Miyoshi et al.; (2) sample type and processing differences, since miRNA stability can be affected by serum choice and handling conditions; (3) technical differences, because Miyoshi et al. used single-plex qPCR while our study employed a multiplex RT-qPCR panel, which may influence the sensitivity of certain miRNAs; and (4) biological variability, as the expression of hsa-miR-106b-5p and hsa-miR-103a-3p may be more heterogeneous across EC subtypes and clinical stages. These considerations highlight the need for further validation in larger and diverse cohorts to fully assess the performance of these miRNAs across populations. Fortunately, the remaining six miRNAs in this study maintained the same trend as the previous study. Moreover, excluding these two miRNAs not only reduced detection costs but also achieved AUC values consistent with or even surpassing those of the 8-miRNA panel. Meanwhile, our study revealed that the optimized 6-miRNA panel had AUC values as high as 1.0 in two retrospective cohorts and demonstrated higher accuracy than CEA. The improvement in sensitivity may be attributed to the highly sensitive RT-qPCR method established in this study (**Figure 2**), capable of detecting trace amounts of miRNAs. Furthermore, in this study, the previously used single-plex miRNA assays were consolidated into three-tubes 4-plex assays, and there was no interference among each miRNA, this is important for clinical applications as it reduces costs and increases throughput.

Qin et al. developed a 5-methylation marker panel for EC detection by using 3-4 mL plasma, achieving an AUC of 0.93, but the sensitivity for stage I EC was only 43% [17]. In 2021, Qiao et al. introduced a bisulfite sequencing-based method for early EC detection using 8-10 mL of blood. They demonstrated sensitivities of 74.7-76.2% with specificities of 94.1-95.9%, but the sensitivity for stage I-II EC was only 58.8% [47] Pei et al. utilized 0.5-1 mL of plasma to develop a 2-methylation marker panel for EC detection, achieving AUC values of 0.661-0.845 [14]. The DNA methylation panels mentioned above utilized 5-15 times more samples for EC detection, yet their diagnostic efficacy still falls short of the data achieved in this study. At the same time, serum as the most commonly used sample type in clinical diagnosis [48], has advantages such as easy acquisition and strong stability. Serum cf-miRNA testing requires a small amount of sample, so in future clinical applications, it only needs to use the remaining serum from routine blood tests and co-analysis with other biomarkers, without the need for re-sampling, reducing patient discomfort. In contrast, contemporary plasma-based cfDNA liquid biopsy techniques frequently demand the collection of 10-20 mL of blood from individuals, potentially heightening patient discomfort and compromising compliance. This demonstrates that the serum-based EC diagnostic method is a more economical and accurate approach than DNA methylation markers in clinical application.

This study still has some limitations, including a relatively small sample size, a limited number of EC cases, and lack of multicenter validation. Additionally, most of the EC samples lacked complete clinical staging information, preventing stratified analysis by stage to evaluate the diagnostic performance of the miRNA panel in early-stage EC. Another limitation is the lack of information on potential confounding factors, such as smoking, alcohol consumption, and comorbidities, which may influence miRNA expression. Future studies with larger, well-characterized cohorts, complete staging information, and comprehensive clinical data are needed to confirm the findings and to assess the performance and generalizability of the miRNA panel across different EC stages and clinical contexts.

Conclusion

This study evaluated the expression difference of eight EC-related miRNAs between plasma and serum, revealing that serum is the suitable sample type for EC early detection, and the optimized 6-miRNA panel demonstrated high accuracy for EC detection. We anticipate that non-invasive panel will have an important role in EC early detection in the future.

Disclosure of conflict of interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Address correspondence to: Guodong Zhao, Zhejiang University Kunshan Biotechnology Laboratory, Zhejiang University Kunshan Innovation Institute, Kunshan 215300, Jiangsu, China. E-mail: gddn89429@hotmail.com; guodong.zhao@versabiotech.com; Jun Yang, Department of Gastroenterology, Affiliated Hospital of Xuzhou Medical University, Xuzhou 221002, Jiangsu, China. E-mail: 18796249248@163.com

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Table S1. Oligonucleotides used in Stem-loop RT-qPCR

miRNA	oligonucleotides	Sequence (5'-3')
cel-miR-39-3p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAGCT
	Primer-F	AACAGTGTCACCGGGTGTAAA
	Primer-R	CAGTGCAGGGTCCGAGGTA
	Probe	CAGCTTGGTCGTAT
miR-16-5p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCCAA
	Primer-F	AACCGGTAGCAGCACGTAAA
	Primer-R	CAGTGCAGGGTCCGAGGTA
	Probe	ACGACCGCCAATAT
hsa-miR-21-5p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACAT
	Primer-F	TGTCCGCCTAGCTTATCAGAC
	Primer-R	CAGTGCAGGGTCCGAGGTA
	Probe	ATGTTGAGTCGTAT
hsa-miR-106b-5p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATCTGC
	Primer-F	AGACGACCTAAAGTGCTGACA
	Primer-R	CAGTGCAGGGTCCGAGGTA
	Probe	TGCAGATGTCGTAT
hsa-miR-93-5p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCTG
	Primer-F	CACGTCCAAAGTGCTGTTCG
	Primer-R	CAGTGCAGGGTCCGAGGTA
	Probe	GCAGGTAGGTCGTA
hsa-miR-181a-5p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCACC
	Primer-F	ACACGCCTAACATTCAACGCTG
	Primer-R	CAGTGCAGGGTCCGAGGTA
	Probe	GGTGAGTGTCGTAT
hsa-miR-151a-3p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCTCAA
	Primer-F	AACGCACCTAGACTGAAGCT
	Primer-R	CAGTGCAGGGTCCGAGGTA
	Probe	CTTGAGGGTCGTAT
hsa-miR-17-5p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCTG
	Primer-F	CCTCGAGCCAAAGTGCTTACAG
	Primer-R	CAGTGCAGGGTCCGAGGTA
	Probe	GCAGGTAGGTCGTA
hsa-miR-25-3p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGACC
	Primer-F	ACCACCGCATTGCACTTGTC
	Primer-R	CAGTGCAGGGTCCGAGGTA
	Probe	GGTCTGAGTCGTAT
hsa-miR-103a-3p	RT Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATAGC
	Primer-F	ACTCCGGAAGCAGCATTGTACA
	Primer-R	CAGTGCAGGGTCCGAGGTA
	Probe	GCTATGAGTCGTAT

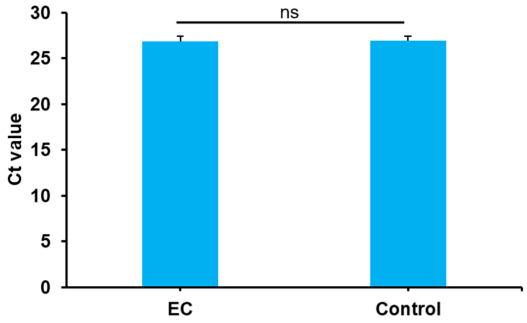

Figure S1. Ct values of spike-in control (cel-miR-39-3p) in validation cohort. ns, not significant.

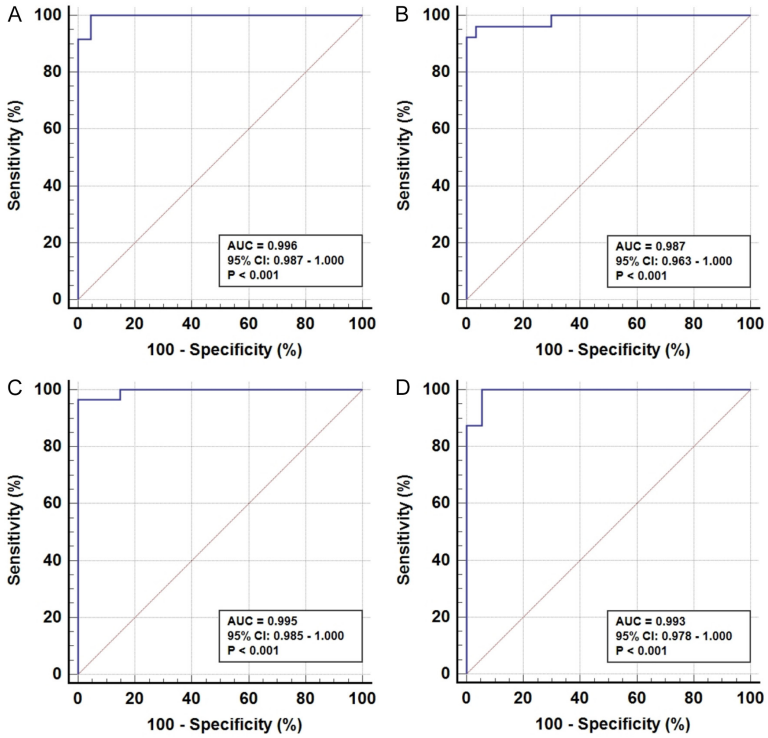

Figure S2. The ROC curves of different groups detected by serum 6-miRNA panels. (A) <65 years old, (B) ≥65 years old, (C) male, (D) female.