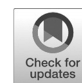
Research Article

Cancer Epidemiology, Biomarkers & Prevention



Identification of Novel Circulating miRNA Biomarkers for the Diagnosis of Esophageal Squamous Cell Carcinoma and Squamous Dysplasia

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Abstract

Background: Circulating miRNAs have been identified as diagnostic biomarkers for esophageal squamous cell carcinoma (ESCC), but their efficacy in discovering early-stage ESCC is still unsatisfying. Esophageal squamous dysplasia (ESD) is the precursor lesion of ESCC. Notably, little is known about the role(s) of circulating miRNAs in identifying ESD. In this study, we, therefore, aimed to identify serum miRNAs as novel diagnostic markers for detecting ESD and ESCC.

Methods: The genome-wide miRNA expression was profiled in 104 (52 ESCC and 52 controls) serum samples using microarray. Seven candidate miRNAs from the microarray assay were evaluated for their diagnostic performance in another cohort of 266 participants (96 ESCC, 92 ESD, and 78 healthy controls).

Results: The serum levels of miR-16-5p, miR-197-5p, miR-451a, and miR-92a-3p were associated with ESCC; the biomarker based on the panel of these four miRNAs could efficiently distinguish patients with ESCC from the controls [AUC = 0.856; 95% confidence interval (CI), 0.794–0.905; $P < 0.001$]. The serum levels of miR-16-5p, miR-320c, miR-638, and miR-92a-3p were significantly higher in patients with ESD than in controls, and this four-miRNA signature could efficiently differentiate patients with ESD from the controls (AUC = 0.842; 95% CI, 0.778–0.893; $P < 0.001$). In addition, compared with serum carcinoembryonic antigen and carbohydrate antigen 199, miRNA-based panels had a better diagnostic performance in distinguishing patients with ESCC and ESD from healthy controls.

Conclusions: Our study identified two novel panels of circulating miRNAs with high efficiency in detecting ESCC and ESD, suggesting that circulating miRNAs, in particular the combination of them, might serve as noninvasive biomarkers for the early detection of ESCC.

Impact: This study suggests the feasibility of using circular miRNA-based blood tests to aid in the detection of ESD and ESCC.

Introduction

Esophageal cancer ranks the seventh most common malignancy and the sixth leading cause of cancer-related mortality in the world, with an estimation of 572,000 new cases and 509,000 deaths in 2018 (1). Esophageal squamous cell carcinoma (ESCC) is the main histopathologic subtype of esophageal cancer, accounting for 90% of all cases, due largely to its high incidence rate in many developing countries. The Taihang Mountain region, mainly including Linzhou in Henan Province and Cixian in Hebei Province, a rural area of north-central China, has the highest ESCC incidence in the world (>100 per 100,000 population; ref. 2). The survival of ESCC is very poor, largely due to the late development of symptoms and consequent late diagnosis. The 5-year survival rate of late-stage ESCC is less than 15%; however, early detection and treatment are associated with improved survival. In early-diagnosed patients, the 5-year survival rate may reach 85% (3, 4). Endoscopy with Lugol's iodine staining is the gold standard for the detection of esophageal cancer. Our recent 10-year prospective community assignment study showed that the use of endoscopic screening and treatment for cancer and dysplasia has contributed to the reduction in ESCC-associated mortality in high incidence areas of China (5). However, the diagnosis of dysplasia is difficult with intra- and interobserver variation (6). Furthermore, endoscopic screening is highly invasive, and the requirement for finely trained physicians and expensive equipment limit its application (7). Therefore, better adjunctive diagnostic biomarkers for ESCC are urgently needed. Acquisition of noninvasive biomarker

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from peripheral blood represents the most promising way in identifying biomarkers for early detection of esophageal cancer.

miRNAs are noncoding, single-stranded RNAs of about 17 to 25 nucleotides in length that regulate gene expression posttranscriptionally. They play important roles in oncogenesis and tumor metastasis (8, 9). More than 4,500 human miRNA sequences have been identified (http://www.mirbase.org/, Release 22: March 2018), and several specific miRNA profiles related to esophageal cancer tissues have been described (10–13). Furthermore, because of their high stability in circulation, blood miRNAs are ideal candidates as biomarkers for noninvasive diagnostic screening and for monitoring of cancer development (14). Although still an emerging area of research, our recent meta-analysis has highlighted the potential use of specific plasma/serum miRNAs in the detection of ESCC (15). However, most of these miRNAs were not further validated in independent case–control studies, and most studies only focused on a single miRNA rather than their combination. Advances in bioinformatics have facilitated the analysis of large, complex miRNA microarray datasets, and future studies are likely to use combined approaches for miRNA analysis. Furthermore, none of these studies have analyzed the expression of serum miRNAs in patients with esophageal squamous dysplasia (ESD), the precancerous lesion of ESCC, to explore their potential as early diagnostic biomarkers for ESD.

Therefore, in this study, we aimed to identify novel serum miRNA biomarkers for the detection of ESD and ESCC in adjuvant to pathologic diagnostic approaches.

Materials and Methods

Study population and study design

In this study, the participants were composed of 370 Chinese Han people, including 148 unrelated patients with ESCC, 92 patients with ESD, and 130 healthy controls. All ESCC cases were pathologically diagnosed for the first time and were recruited consecutively from the Endoscopy Center of Cancer Hospital, Linzhou City, Henan Province, China, from October 2014 to October 2015. During the same period, ESD cases and healthy controls were randomly selected from an early esophageal cancer screening program with 10,221 subjects from 27 villages in Hejian County and Chengjiao County of Linzhou City, which represented 40% of eligible residents ages 40 to 69 years in these two counties. The demographic data and the information of related risk factors were obtained by in-person interview using a structured questionnaire, including tobacco smoking (current/former or never). The smokers refer to continuous smoking for more than 6 months with a self-estimate daily consumption of at least one cigarette. The nonsmokers have no current or previous history of tobacco use. Pathologic information was collected from medical records, including histologic grade, differentiation degree, and lesion location. This study protocol was approved by the Institutional Review Board of Capital Medical University (Approval No. 2014SY31), and the written informed consent was obtained from each subject.

This study was divided into two phases of case–control studies (Fig. 1). In the first stage, serum samples from 52 ESCC patients and 52 gender- and age-matched healthy controls were used to profile miRNA expression with a microarray assay. Differential miRNAs were identified. The extremely upregulated miRNAs or those with moderate upregulation but having functional or biomarker-related reports in ESCC were selected for further analysis. In the second phase, candidate miRNAs were validated using quantitative real-time polymerase chain reaction (qRT-PCR) in an independent cohort of 266 subjects comprising 96 patients with ESCC, 92 patients with ESD, and 78 healthy controls. ROC curve-based risk assessment analysis was then performed among the ESCC, ESD, and control groups to evaluate the diagnostic performance of candidate miRNAs in ESCC and ESD. Each subject was assigned to either high- or low-risk group by comparing the expression levels of miRNA biomarkers to the corresponding cut-off values derived from the ROC curves. In addition, we also compared the diagnostic performance of the novel miRNA biomarkers with that of the serum carcinoembryonic antigen (CEA) and carbohydrate antigen 199 (CA199).

Sample processing and RNA extraction

We collected five mL peripheral blood from each subject and transferred the blood into a tube without anticoagulants such as EDTA or citrate before endoscopy examination. None of the patients enrolled in this study had received any treatment when the specimens were collected. The blood samples were centrifuged at $820 \times g$, 4°C for 10 minutes to spin down the blood cells. The supernatant was then transferred to a 1.5 mL Eppendorf tube followed by a second centrifugation at $16,000 \times g$, 4°C for 10 minutes to completely remove additional cellular nucleic acids attached to cell debris. The serum samples were separated within 1 hour and stored at −80°C until further utilization. Total RNA was extracted and purified from 500 μL of serum using the mirVana-PARIS Kit (Ambion), following the manufacturer's instructions. The integration of extracted RNA samples was inspected with an Agilent Bioanalyzer 2100 (Agilent Technologies).

miRNA microarray

miRNA microarray assay was performed using the Agilent Human miRNA (8*60K) V19.0 (Agilent Technologies). miRNA molecules in 100 ng total RNA was labeled by miRNA Complete Labeling and Hyb Kit (Agilent Technologies) according to the manufacturer's instructions. Each slide was hybridized with 100 ng Cy3-labeled RNA in hybridization Oven (Agilent Technologies) at 55°C, and spin at 20 rpm for 20 hours according to the manufacturer's instructions. After hybridization, slides were washed in staining dishes (Thermo Shandon) with Gene Expression Wash Buffer Kit (Agilent Technologies). Slides were scanned by Agilent Microarray Scanner (Agilent Technologies) and Feature Extraction software 10.7 (Agilent Technologies) with default settings. Raw data were normalized by the median of hsa-miR-1228-3p and then analyzed by Gene Spring Software 12.6 (Agilent Technologies; ref. 16).

qRT-PCR of miRNAs

The levels of the seven candidate miRNAs (miR-16-5p, miR-197-5p, miR-320c, miR-451a, miR-486-5p, miR-638, and miR-92a-3p) selected from the microarray were further quantified by qRT-PCR using human TaqMan MicroRNA Assay Kits (Applied Biosystems), with hsa-miR-1228-3p as the reference 17. Reverse transcription of the total RNA was performed using the TaqMan MicroRNA Reverse Transcription Kit (ABI). The RT reaction system (25 μL) comprised 5 μL of RNA extract, 0.25 μL of 100 mmol/L dNTPs, 1.6 μL of multiscribe reverse transcriptase, 2.5 μL of 10 × reverse transcription buffer, 0.3 μL of RNase inhibitor, 12.8 μL of 5 × RT primer, and 2.55 μL of nuclease-free water. To synthesize cDNA, the reaction mixture was incubated at 16°C for 30 minutes,

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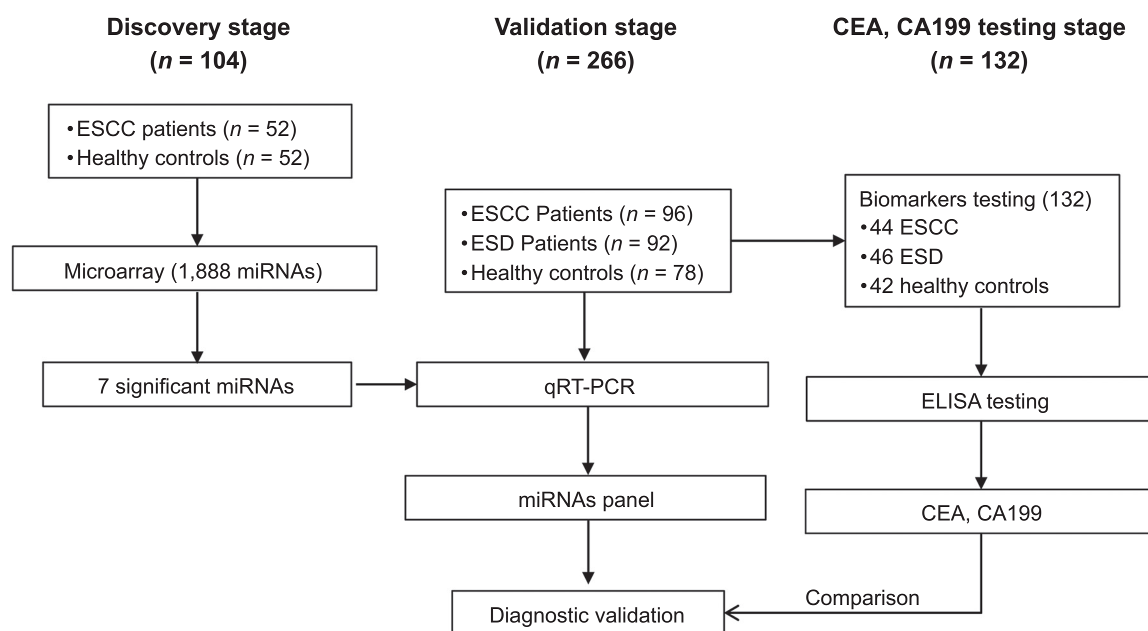

Figure 1.
The flowchart of the study design.

42°C for 30 minutes, 85°C for 5 minutes, and then held at 4°C. TapMan Universial PCR Master Mix (ABI) was used for RT-PCR. In the 10 μL reaction mixture, 0.75 μL of cDNA solution was amplified using 5 μL of 2 × TapMan Universial PCR Buffer (with UNG), 0.5 μL of 20 × TapMan MicroRNA Assay Mix, and 3.75 μL of nuclease-free water. qPCR was run on a 7900 HT Sequence Detection System (ABI), and the reaction mixtures were incubated at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Cycle threshold (Ct) values were calculated using the SDS 2.4 software (ABI). RT-PCR was performed in triplicate and the relative expression levels of miRNAs were calculated using the $2^{-\Delta Ct}$ method, $\Delta Ct = Ct$ (candidate miRNAs) − Ct (hsa-miR-1228-3p) (18). All primers used for qRT-PCR are listed in Supplementary Table S1.

ELISA assay for serum CEA and CA199

The CEA and CA199 levels were measured with corresponding ELISA Kit (Beckman Coulter) according to the manufacturer's instructions. The absorbance was measured in duplicate on the ACCESS 2 plate reader (Beckman Coulter).

Statistical analysis

Statistical analysis was carried out using SPSS (version 20.0), MedCalc (version 11.4.2.0), and *R* software (version 3.4.3). Demographic and clinical characteristics were compared among groups with Pearson χ^2 test or with one-way ANOVA. For the microarray assay, the Mann–Whitney test with the FDR algorithm was used to determine the differentially expressed miRNAs ($P <$ 0.05, fold change > 2.0) between the ESCC patients and healthy subjects. For the qRT-PCR data, miRNAs levels in the patients with ESCC, patients with ESD, and healthy controls were determined using the Kruskal–Wallis *H* test, due to the nonnormal distribution of the data. When multiple hypothesis test was performed, the *P* value for statistical significance was adjusted to 0.017 according to the Bonferroni correction in each analysis. Univariable and multivariable analyses were performed to select diagnostic miRNA markers based on patients with ESCC and ESD compared with healthy controls using the stepwise logistic regression model, adjusted for age, sex, and smoking (current/former or never). The cut-off for the entry and departure of the logistic regression model was 0.05 and 0.10, respectively. Leave-one-out cross-validation was also applied in multivariable logistic regression to obtain the predictive values of miRNA expression data. The area under the ROC curves (AUC) was used to evaluate the performance of the selected miRNAs in discriminating the patients with ESCC and ESD from healthy subjects. Sensitivity and specificity were calculated at an optimal cutoff point. A two-tailed *P* value of <0.05 was considered as statistically significant unless otherwise specified.

Results

Characteristics of the subjects in the study

The flowchart of this study is shown in Fig. 1. In the discovery phase, 52 patients with ESCC were frequency-matched to the same number of healthy individuals with similar distributions of age and gender. In the phase of qRT-PCR validation, a total of 266 subjects including 96 patients with ESCC, 92 patients with ESD, and 78 healthy controls were enrolled in the study. There was no significant difference in the distribution of age and gender among the control, ESD and ESCC groups. Detailed characteristics of the participants in this study were summarized in Table 1.

Selection of candidate miRNAs from the microarray data

A microarray containing probes for 1,888 human miRNAs was initially used to screen for significantly differentially expressed

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Table 1. Characteristics of the participants in the discovery and validating cohort

	Discovery cohort			Validating cohort			
Variables	**Controls**	**ESCC**	***P* value**	**Controls**	**ESD**	**ESCC**	***P* value**
Number	*n* = 52	*n* = 52		*n* = 78	*n* = 92	*n* = 96	
Age, mean ± SD	57.83 ± 5.49	59.19 ± 4.12	0.07[a]	59.06 ± 2.83	59.42 ± 6.63	60.10 ± 7.74	0.53[b]
Gender, *n* (%)			1.00[c]				0.20[c]
Male	24 (46.15)	24 (46.15)		35 (44.90)	37 (40.20)	51 (53.10)	
Female	28 (53.85)	28 (53.85)		43 (55.10)	55 (59.80)	45 (46.90)	
Smoking status, *n* (%)			0.52[c]				0.003[c]
Never	39 (75.00)	35 (67.31)		61 (78.20)	78 (84.80)	61 (63.50)	
Current/former	13 (25.00)	17 (32.69)		17 (21.80)	14 (15.20)	35 (36.50)	
Dysplasia stage, *n* (%)							
Mild					60 (65.20)		
Moderate					19 (20.70)		
Severe					13 (14.10)		
Tumor stage, *n* (%)							
I		4 (7.69)				12 (12.50)	
II		6 (11.54)				38 (39.60)	
III		17 (32.69)				33 (34.40)	
IV		1 (1.93)				13 (13.50)	
Unspecified		24 (46.15)				0	
Differentiation, *n* (%)							
Well		6 (11.54)				24 (25.00)	
Moderate		21 (40.38)				58 (60.40)	
Poor		1 (1.93)				14 (14.60)	
Unspecified		24 (45.15)				0	

[a]*t* test.
[b]ANOVA.
[c]χ^2 test.

serum miRNAs between the patients with ESCC and healthy controls. The raw data of the microarray have been deposited in the Gene Expression Omnibus (GEO) database and is accessible through the GEO accession number GSE112840 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112840). Seven miRNAs, including miR-16-5p, miR-197-5p, miR-320c, miR-451a, miR-486-5p, miR-638, and miR-92a-3p, with significantly upregulated expression levels in the ESCC groups compared with the controls (fold change was 2.8–12.2; all $P < 0.05$; Supplementary Table S2), were selected as candidates for further validation with qRT-PCR.

Validation of candidate miRNAs in a new cohort

We then measured the levels of the seven selected miRNAs in a larger cohort of participants (named as validation cohort) that included 96 patients with ESCC, 92 patients with ESD, and 78 healthy controls using qRT-PCR. The levels of these miRNAs were then compared in pairwise among the three groups, including the comparisons between ESCC and healthy controls, between ESCC and ESD, and between ESD and healthy groups. As shown in Table 2 and Fig. 2, four miRNAs including miR-16-5p ($P < 0.001$), miR-197-5p ($P < 0.001$), miR-320c ($P < 0.001$), and miR-486-5p ($P < 0.001$) were significantly upregulated in patients with ESCC compared with the healthy participants, consistent with the results of the miRNA array. The other three candidates, miR-451a ($P = 0.042$), miR-638 ($P = 0.022$), and miR-92a-3p ($P = 0.388$) showed no significant difference in the expression levels between the ESCC and control groups according to the criteria of $P < 0.017$. When we compared the ESD and control groups, all the seven candidate miRNAs had significantly upregulated levels in the patients with ESD than in the controls (fold change ranges from 1.786 to 5.194, all $P < 0.001$; Table 2; Fig. 2). Regarding the comparison between the patients with ESCC and ESD, patients with ESCC showed significantly decreased expression levels of miR-197-5p ($P = 0.002$), miR-320c ($P = 0.004$), miR-638 ($P < 0.001$), and miR-92a-3p ($P = 0.009$), whereas no significant difference was observed in miR-16-5p ($P = 0.878$), miR-451a ($P = 0.158$), and miR-486-5p ($P = 0.320$) levels between the ESCC and ESD groups (Table 2; Fig. 2). In addition, we compared the serum concentrations of these candidate miRNAs in patients with ESCC at different stages or with different differentiation degrees; however, no significant association between miRNA levels and these clinical characteristics was found (both $P > 0.017$; data not shown). We also assessed the expression levels of the seven miRNAs in the serum from patients with ESD with mild, moderate, or severe dysplasia. Similarly, no significant difference was discovered in the levels of these miRNAs in the patients at different dysplasia stages ($P > 0.017$; data not shown).

Table 2. Validation of candidate miRNAs by qRT-PCR in ESCC, ESD, and healthy controls groups

	ESCC vs. Control		ESD vs. Control		ESCC vs. ESD	
miRNA	**Fold change[a]**	***P* value[b]**	**Fold change[a]**	***P* value[b]**	**Fold change[a]**	***P* value[b]**
miR-16-5p	5.164	<0.001	5.194	<0.001	0.994	0.878
miR-197-5p	1.636	<0.001	2.182	<0.001	0.750	0.002
miR-320c	1.928	<0.001	2.513	<0.001	0.767	0.004
miR-451a	1.401	0.042	2.384	<0.001	0.587	0.158
miR-486-5p	1.980	<0.001	2.662	<0.001	0.744	0.320
miR-638	0.588	0.022	2.000	<0.001	0.294	<0.001
miR-92a-3p	0.747	0.388	1.786	<0.001	0.418	0.009

[a]Fold change was analyzed by calculating the ratio of the median levels of candidate miRNAs in the two indicated groups.
[b]Kruskal–Wallis *H* test, *P* value for significance was corrected to 0.017 for multiple comparisons.

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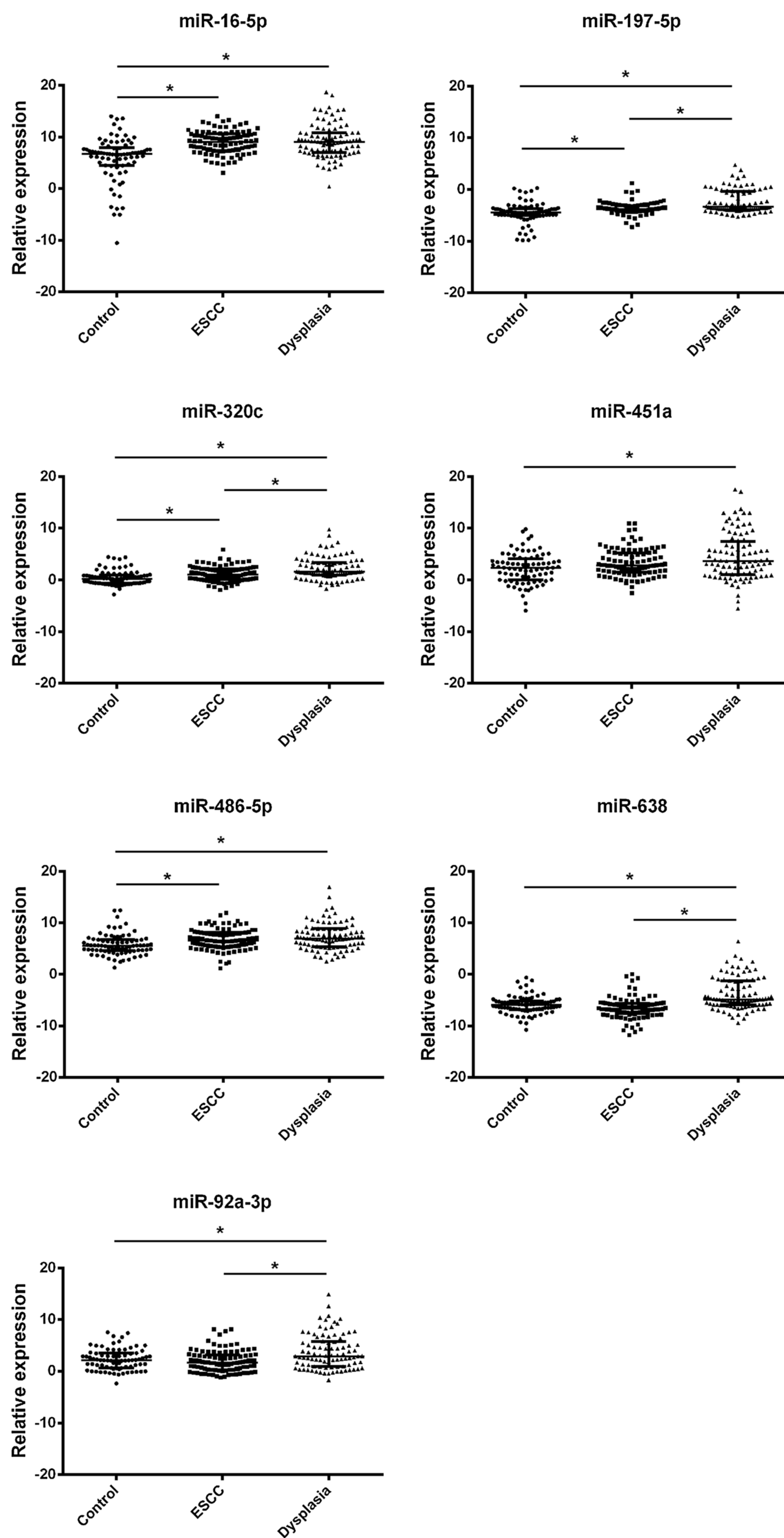

Figure 2.

Expression levels of miR-16-5p, miR-197-5p, miR-320c, miR-451a, miR-486-5p, miR-638, and miR-92a-3p in the serum of 96 patients with ESCC, 92 patients with ESD, and 78 healthy subjects (validating cohort). The lines marked the median and quartile values. Kruskal–Wallis *H* test was used to determine the statistical significance. *, *P* value for statistical significance was corrected to 0.017 for multiple comparisons.

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Diagnostic performance of the candidate miRNAs

The diagnostic accuracy of these seven candidate miRNAs in discriminating the patients with ESCC and healthy controls was evaluated by the AUC, which ranged from 0.553 to 0.759 (Supplementary Fig. S1). The multivariable *P* values for four of them (miR-16-5p, miR-197-5p, miR-451a, and miR-92a-3p) were all <0.05 (Table 3). We, therefore, used the panel composed of these four miRNAs to predict the risk of being diagnosed with ESCC and constructed the ROC curve. The AUC of this four-miRNA panel was 0.856 (95% CI, 0.794–0.905), with a sensitivity of 89.6% and a specificity of 76.3% in detecting ESCC (Fig. 3A).

To test the diagnostic values of the seven candidate miRNAs in detecting ESD, the precursor lesion of ESCC, we further examined the diagnostic performance of them in distinguishing patients with ESD from healthy controls. The ROC analysis revealed that the AUC ranged from 0.621 to 0.789 (Supplementary Fig. S2). The multivariable logistic regression analyses showed that four miRNAs (miR-16-5p, miR-320c, miR-638, and miR-92a-3p) turned out to be significant predictors of ESD (all $P < 0.01$; Table 3). Similarly, the predicted probability was used to construct the ROC curve. The corresponding AUC of the combination of these four miRNAs for detecting ESD was 0.842 (95% CI, 0.778–0.893; the sensitivity was 82.6% and the specificity was 80.8%; Fig. 3B). These results indicated that the combined miRNA signature could differentiate patients with ESD from healthy controls and had a better diagnostic performance than any single miRNA. Notably, miR-16-5p and miR-92a-3p were included in the combined miRNA panels for both ESCC and ESD, suggesting that they may play a more important role in the early detection of ESD and ESCC.

Compare the diagnostic performance of miRNA panels with that of serum CEA and CA199

We measured the serum levels of CEA and CA199 in 132 (50%) serum samples of the validation cohort (44 ESCC, 46 ESD, and 42 controls). However, no significant difference was found in CEA ($P = 0.052$) and CA199 ($P = 0.429$) levels among the three groups (Supplementary Table S3). Regarding the ability in discriminating patients with ESCC and healthy controls, the ROC analyses showed that the AUC for CEA, CA199, and the four-miRNA panel (composed of miR-16-5p, miR-197-5p, miR-451a, and miR-92a-3p) was 0.633 (95% CI, 0.522–0.734), 0.532 (95% CI, 0.422–0.641), and 0.885 (95% CI, 0.798–0.944), respectively (Supplementary Fig. S3). In the binary regression analysis, the combination of CEA, CA199, and the miRNA panel did not significantly increased the AUC of the miRNA panel (from 0.885 to 0.898; 95% CI, 0.814–0.953; Supplementary Fig. S3). Although this combination slightly improved the specificity of the four-miRNA panel (from 80.95% to 83.33%), the decline in the sensitivity was observed (from 95.45% to 93.18%) in distinguishing patients with ESCC from healthy controls (Supplementary Table S4). In the patients with ESD, the ROC curve area for CEA, CA199, and the four-miRNA panel (miR-16-5p, miR-320c, miR-638, and miR-92a-3p) was 0.494 (95% CI, 0.386–0.603), 0.579 (95% CI, 0.469–0.683), and 0.881 (95% CI, 0.795–0.941), respectively (Supplementary Fig. S4). Furthermore, the combination with CEA and CA199 only slightly increased the AUC of the four-miRNA panel from 0.881 to 0.883 (Supplementary Fig. S4), increased the sensitivity from 78.26% to 84.78% (not significant), but decreased the specificity from 85.71% to 80.95% (Supplementary Table S4).

Discussion

Early diagnosis of ESCC presents a challenge due to the lack of sensitive and specific biomarkers, hence highlighting the need for new early diagnostic tools. In this study, we systematically determined the expression levels of serum miRNAs in patients with ESCC by a genome-wide miRNA microarray and quantified the expression levels of seven differential miRNAs, miR-16-5p, miR-197-5p, miR-320c, miR-451a, miR-486-5p, miR-638, and miR-92a-3p in patients with ESCC, patients with ESD, and healthy controls via qRT-PCR. All these seven miRNAs were significantly elevated in patients with ESCC or with ESD compared with healthy controls. In addition, the levels of serum miR-197-5p, miR-320c, miR-638, and miR-92a-3p were significantly higher in patients with ESD than in patients with ESCC (Fig. 2). Moreover, we revealed that the serum miR-16-5p, miR-197-5p, miR-451a, miR-92a-3p, miR-320c, and miR-638 were potential circulating biomarkers for early diagnosing ESCC. The combination of miR-16-5p, miR-197-5p, miR-451a, and miR-92a-3p from the multivariable logistic regression model showed high accuracy in discriminating patients with ESCC from healthy controls (Fig. 3). In patients with ESD, the diagnostic value of the miRNAs panel composed of miR-16-5p, miR-320c, miR-638, and miR-92a-3p was also evaluated, with an AUC of 0.842, a sensitivity of 82.6%, and a specificity of 80.8%. To our knowledge, this study is the first to evaluate the association of serum miRNA expression profile with ESD.

Table 3. Univariable and multivariable analysis of candidate miRNAs for the risk of ESCC and ESD

	ESCC vs. Control				ESD vs. Control			
	Univariable analysis		Multivariable analysis[a]		Univariable analysis		Multivariable analysis[b]	
Variable	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age	1.029 (0.979–1.081)	0.261	1.065 (0.994–1.142)	0.074	1.013 (0.956–1.074)	0.654	1.046 (0.964–1.135)	0.277
Gender	0.718 (0.394–1.308)	0.279	1.045 (0.356–3.070)	0.936	1.210 (0.657–2.228)	0.541	0.948 (0.347–2.592)	0.917
Smoking	2.059 (1.044–4.062)	0.037	2.784 (0.845–9.176)	0.092	0.644 (0.294–1.409)	0.271	0.495 (0.137–1.786)	0.283
miR-16-5p	1.411 (1.227–1.623)	<0.001	1.722 (1.234–2.402)	0.001	1.324 (1.176–1.491)	<0.001	1.567 (1.145–2.145)	0.005
miR-197-5p	1.455 (1.158–1.827)	0.001	1.540 (1.017–2.332)	0.041	1.799 (1.418–2.282)	<0.001	0.927 (0.534–1.611)	0.789
miR-320c	1.478 (1.172–1.864)	0.001	1.099 (0.621–1.946)	0.746	1.854 (1.464–2.349)	<0.001	1.956 (1.269–3.013)	0.002
miR-451a	1.144 (1.025–1.278)	0.016	1.728 (1.096–2.723)	0.018	1.173 (1.074–1.281)	<0.001	1.074 (0.657–1.755)	0.777
miR-486-5p	1.277 (1.090–1.496)	0.002	0.878 (0.567–1.358)	0.558	1.350 (1.158–1.573)	<0.001	1.258 (0.800–1.980)	0.320
miR-638	0.847 (0.721–0.995)	0.043	1.037 (0.715–1.502)	0.849	1.442 (1.226–1.695)	<0.001	2.134 (1.507–3.022)	<0.001
miR-92a-3p	0.934 (0.809–1.079)	0.355	0.252 (0.148–0.428)	<0.001	1.219 (1.079–1.376)	0.001	0.329 (0.205–0.529)	<0.001

[a]$\text{Logit}(P = \text{ESCC}) = -0.996 + 0.543 \times \text{miR-16-5p} + 0.432 \times \text{miR-197-5p} + 0.547 \times \text{miR-451a} - 1.379 \times \text{miR-92a-3p}$.

[b]$\text{Logit}(P = \text{ESD}) = 2.922 + 0.449 \times \text{miR-16-5p} + 0.671 \times \text{miR-320c} + 0.758 \times \text{miR-638} - 1.112 \times \text{miR-92a-3p}$.

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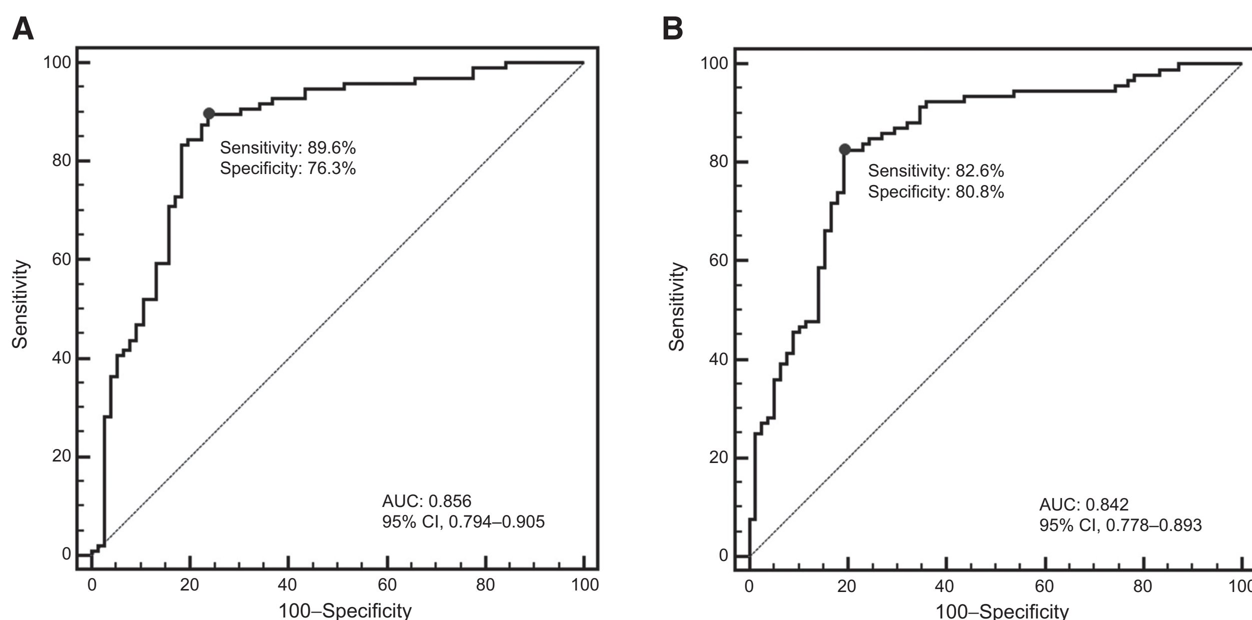

Figure 3.
The ROC curve analysis for the diagnostic performance of serum miRNA panels in discriminating patients with ESCC and ESD from healthy controls. **A,** Patients with ESCC and healthy controls. **B,** Patients with ESD and healthy controls. The optimal cut-off points (dots) were marked on the ROC curves.

As traditional tumor biomarkers, serum CEA and CA199 have been used for ESCC screening and diagnosis. However, it has been recognized that CEA and CA199 both have poor sensitivity in the detection of ESCC and ESD as well (19). In our study, both miRNA panels demonstrated significantly higher diagnostic accuracy than CEA and CA199 in discriminating patients with ESCC or ESD from healthy controls. In addition, no significant differences in the diagnostic performance were observed between the four-miRNA panels and the combinations of miRNA panel with CEA and CA199 (Supplementary Table S4; Supplementary Figs. S3 and S4). These results implicated that these miRNAs may serve as reliable early biomarkers for the detection of ESCC and its precancerous lesions ESD.

Among the seven candidate miRNAs, miR-16-5p and miR-92a-3p consistently showed significantly differential expression in ESCC and dysplasia, and this encouraging result increasingly demonstrated potentially important roles of miR-16 and miR-92a in tumorigenesis and development of ESCC. It has been reported that miR-16 was one of the key oncogenic components of the miR-15 cluster. At the circulating blood level, it has been shown that serum miR-16 have a relatively high value as promising biomarkers in diagnosing oral cancer (20) and liver cancer (21). The plasma level of miR-16 was also found to be significantly higher in patients with ESCC than in controls in a study with small sample size (22). In this study, we found higher levels of serum miR-16 in patients with ESCC and dysplasia than that in healthy controls, which suggest that miR-16 may be a universal circulating biomarker for cancer. Aberrantly increased expression levels of miR-16 were previously reported in ESCC tissue samples and could target the 3′UTR of targeting mRNAs to suppress ESCC cell apoptosis (23). Our recent study found that transfection of miR-16 mimics significantly reduced the luciferase activity of reporter plasmids with the *FBXW7* 3′UTRs ($P < 0.05$; data not shown). The further functional study is needed to confirm the role of miR-16 in ESCC carcinogenesis.

As a member of the miR17-92 cluster, miR-92a has been observed dysregulated in human serum and plasma, making it possible to be a noninvasive molecular marker for the diagnosis of many types of cancer, such as hepatocellular carcinoma (HCC) (24, 25), colorectal cancer (26, 27), breast cancer (28), and gastric cancer (29). The association between ESCC and miR-92a has been previously reported in tissues, and the upregulation of miR-92a was associated with poor survival of patients with ESCC. Furthermore, previous investigations suggest that miR-92a could repress *CDH1* expression and promote ESCC cell growth, migration, and invasion (30, 31).

Although the differential expression of several candidate miRNAs in this study has been identified previously, to our knowledge, our study is the first to reveal that circulating miR-197-5p, miR-320c, and miR-638 significantly contribute to distinguishing ESCC and ESD cases from the controls. The aberrant expression of miR-197-5p has been previously reported in esophageal cancer tissues, which paved the way for using circulating miR-197-5p as a biomarker for ESCC diagnosis (32). Although miR-320c was rarely studied in ESCC, the diagnostic utility of circulating miR-320 has been investigated in glioblastoma (33). Another study also revealed the association of the serum levels of the miR-320 family members with clinical parameters and their diagnostic potential in prostate cancer (34). As for miR-638, no study evaluated the expression level of circulating miR-638 in ESCC. But it is reported that decreased level of serum exosomal miR-638 predicted poor prognosis in HCC (35). In ESCC and breast cancer cells, miR-638 acts as an oncogene and has significantly higher expression in ESCC and breast cancer tissues compared with normal tissues (36). Thus, miR-638 might play distinct roles in various types of cancer and need to be further investigated.

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Circulating miRNAs are considered to be closely associated with cancer occurrence and progression. However, the mechanisms of these circulating miRNAs in ESCC carcinogenesis are not entirely understood. Current evidence suggests that circulating miRNAs might be produced or released from cancer-related cells and eventually affect the tumorigenesis or specific pathologic process of recipient cells. Our results showed that the median levels of serum miR-197-5p, miR-320c, miR-638, and miR-92a-3p were significantly higher in patients with ESD than in patients with ESCC (Fig. 2). This finding might indicate the larger alteration of miRNA changes during the initial transformation toward premalignant lesion (37). We therefore hypothesized that increased expression of certain miRNAs might result from abnormal tissue formation, such as esophageal dysplasia. Evaluation of specific miRNAs in the serum or tissues has also been carried out in the premalignant states of HCC (38) and esophageal adenocarcinoma (37, 39). In addition, it has been demonstrated that the expression levels of miR-16, miR-92a, and miR-638 were aberrantly increased in ESCC tissues (23, 31, 36), which might support the notion that these circulating miRNAs were packaged and released from ESCC tissues. However, a recent study has shown that miR-451 was upregulated in serum samples from patients with ESCC compared with the normal group, but it was downregulated in ESCC tumor tissues compared with their adjacent normal counterparts (40–42). This inconsistency may be due to sample selection as well as the specific release or uptake pattern of circulating miRNAs during the tumorigenesis (43).

Compared with other studies of circulating miRNAs in diagnosing ESCC (40, 44–46), our study is unique for the following reasons: First, serum miRNAs were screened by microarrays based on a large number of samples in the initial stage, which enabled us to better identify potential diagnostic biomarkers. Second, the relatively large cohort of samples was randomly selected from a well-defined, high-incidence population, which allowed us to better evaluate the association between miRNAs expression and the severity of esophageal lesions (ESD and ESCC). At last, previously, little attention has been paid to the association between miRNA expression and precancerous esophageal lesions. This study included not only ESCC and healthy groups but also the ESD group, emphasizing the applications of serum miRNAs in early detection of ESCC.

Several limitations of the study should be considered. First, the microarray was performed only in patients with ESCC and healthy controls, the ESD cases were not included in the screening stage. In fact, we wished to determine the extreme difference to differentiate patients with ESCC and high tumor burden from these healthy individuals, it would be difficult to develop a diagnostic miRNA panel from patients with small tumors and early-stage cancer. Second, the diagnostic miRNA panels have not been validated in another independent cohort. However, we performed internal validation using the leave-one-out cross-validation method to estimate the diagnostic accuracy of miRNAs panels, which still showed high power to differentiate ESCC and ESD from healthy controls. Third, cigarette smoking is known to alter miRNA expression (47). Because of non-availability of the data of detailed smoking patterns between the cases and controls, there may exist residual confounding from smoking, thus reducing the sensitivity and specificity of the miRNA markers in detecting the ESD and ESCC. In addition, the limited sample size may not be sufficient for us to identify the association between the miRNAs and the clinical variables in stratified analyses. The relevant hypotheses proposed in this study still need further investigation in future studies.

In conclusion, this study identified novel serum miRNA panels that could differentiate patients with ESCC and ESD from healthy controls with a high degree of accuracy. Moreover, these identified miRNAs potentially function as early diagnostic biomarkers of ESCC even at dysplasia stage. However, to confirm the clinical relevance of these miRNAs, long-term follow-up studies with large-scale cohorts and further mechanism researches are necessary.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: W. Wei, F. Liu
Development of methodology: Yi Shen, Y. Ding, Q. Ma
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Ding, Q. Ma
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Yi Shen, Y. Ding, Y. He, F. Zhang, D. Zheng
Writing, review, and/or revision of the manuscript: Yi Shen, Y. Ding, L. Zhao, W. Wei, F. Liu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Niu, X. Guo, Y. Shao, Y. He, F. Zhang
Study supervision: W. Wei, F. Liu

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