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Plasma microRNA-9 as a diagnostic and prognostic biomarker in patients with esophageal squamous cell carcinoma

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Abstract

Purpose: Emerging evidence indicates that circulating microRNAs (miRs) might act as noninvasive biomarkers for cancer diagnosis and prognosis. We examined the expression pattern and clinical significance of plasma miR-9 in patients with esophageal squamous cell carcinoma (ESCC).

Methods: Venous blood samples (6 mL) were collected from 131 patients with ESCC and 131 healthy controls, and the plasma miR-9 concentration was detected by reverse transcription polymerase chain reaction. The association of plasma miR-9 expression with clinicopathologic factors and survival of patients with ESCC was evaluated. Receiver operating characteristic (ROC) curve analysis was applied to evaluate the clinical value of plasma miR-9 for ESCC diagnosis.

Results: The plasma miR-9 expression levels in patients with ESCC were significantly upregulated compared with normal controls. High plasma miR-9 concentrations were significantly correlated with poor tumor differentiation, large tumor size, deep local invasion, lymph node metastasis, advanced clinical stage, and poor survival. ROC curve analysis showed that the plasma miR-9 concentration could efficiently distinguish patients with ESCC from healthy controls. Multivariate survival analysis confirmed plasma miR-9 as an independent prognostic factor for ESCC.

Conclusions: Plasma miR-9 expression was upregulated in ESCC and might act as a novel diagnostic and prognostic biomarker.

Keywords

microRNAs, esophageal squamous cell carcinoma, biomarkers, prognosis

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Introduction

Esophageal cancer is the eighth most common cancer worldwide, and esophageal squamous cell carcinoma (ESCC) accounts for about 90% of all cases of esophageal cancer in China.[1] Despite the development of diagnostic technologies and treatment modalities, most patients with ESCC are diagnosed at the advanced stage, and the prognosis of ESCC is still quite poor.[2] Thus, identification of noninvasive biomarkers with high sensitivity and specificity for ESCC is urgently needed to improve early detection and prognostic assessment.

MicroRNAs (miRs) are short (about 22 nucleotides in length), single-stranded, noncoding RNAs that post-transcriptionally regulate gene expression.[3] Growing evidence shows that some miRs have a tumor-promoting or -suppressing function, and miR expression is aberrant in many kinds of malignant tumors, including ESCC.[4,5] Additionally, some cancer-related miRs are detectable and stable in body fluids, indicating the potential of circulating miRs as a new class of biomarkers for the early diagnosis, prognosis prediction, and therapeutic evaluation of patients with cancer.[6,7] For example, plasma miR-92a-2 has been identified as a noninvasive diagnostic biomarker for small cell lung cancer.[8] Serum miR-503 was more effective than carcinoembryonic antigen in discriminating patients with gastric cancer from healthy individuals.[9] Serum miR-424 in patients with hepatocellular carcinoma was associated with the blood alpha-fetoprotein concentration, vein invasion, and TNM stage.[10] Increased circulating miR-21 concentrations predicted poor overall survival in patients with gastric cancer,[11] lung cancer,[12] and colorectal cancer.[13]

MiR-9 has been shown to play important roles in the development of several types of cancer, including lung cancer,[14] thyroid carcinoma,[15] breast cancer,[16] ovarian cancer,[17] gastric cancer,[18] and ESCC.[19] MiR-9 is overexpressed in primary ESCC tumor tissues and can be detected in the serum of patients with oral squamous cell carcinoma (OSCC) and osteosarcoma.[20,21] However, circulating miR-9 expression in patients with ESCC remains unclear. In the current study, we investigated the plasma miR-9 concentration in patients with ESCC and assessed its clinical value as a novel biomarker for ESCC diagnosis and prognosis.

Materials and methods

Study population

The study protocol was approved by the Research Ethics Committee of Tianjin Medical University General Hospital (No. 2011009), and all participants provided written informed consent.

In total, 131 patients with newly diagnosed and histologically confirmed ESCC, treated at Tianjin Medical University General Hospital from March 2008 to November 2011, were retrospectively enrolled in this study. None of the patients had undergone chemotherapy or radiotherapy before surgery. Venous blood samples (6 mL) from each patient were drawn into tubes containing EDTA-K2 prior to any treatment and centrifuged at $3000 \times g$ for 15 min at 4°C. The supernatant was then stored in RNase-free tubes at −80°C until further analysis. Blood samples simultaneously obtained from 131 age- and sex-matched healthy individuals were used as the control group. The clinicopathologic information of the patients with ESCC is listed in Table 1. All of these patients underwent postoperative follow-up, and overall survival was defined as the time from the date of diagnosis to the date of death or last follow-up.

Table 1. Association of plasma miR-9 expression with clinicopathologic features of esophageal squamous cell carcinoma.

		Plasma miR-9 expression		
Clinicopathological features	Patients (n)	High (n, %)	Low (n, %)	*P*
Age in years				
<60	64	33 (51.6)	31 (48.4)	0.378
≥60	67	32 (47.8)	35 (52.2)	
Sex				
Male	86	40 (46.5)	46 (53.5)	0.212
Female	45	25 (55.6)	20 (44.4)	
Tumor differentiation				
Well + moderate	71	29 (40.8)	42 (59.2)	0.022
Poor	60	36 (60.0)	24 (40.0)	
Tumor size				
<4 cm	52	18 (34.6)	34 (65.4)	0.007
≥4 cm	79	47 (59.5)	32 (40.5)	
T classification				
T_{1-2}	72	27 (37.5)	45 (62.5)	0.003
T_{3-4}	59	38 (64.4)	21 (35.6)	
N classification				
Positive	87	51 (58.6)	36 (41.4)	0.005
Negative	44	14 (31.8)	30 (68.2)	
TNM stage				
I + II	55	16 (29.1)	39 (70.9)	<0.001
III	76	49 (64.5)	27 (35.5)	

RNA extraction and real-time quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from 500 μL of plasma from each sample using an mirVana miRNA isolation kit (Applied Biosystems, Foster City, CA, USA) and dissolved in 100 μL of RNase-free water. Next, 1 μg of total RNA was used for reverse transcription to synthesize cDNA using a PrimeScript reverse transcription (RT) reagent kit (Takara, Shiga, Japan) in a 20-μL reaction system. The RT products were 1:5 diluted and subjected to quantitative polymerase chain reaction (PCR) using a SYBR Green PCR Kit (Takara) on a Roche 480 Real-Time PCR System (Roche, Basel, Switzerland). The reaction conditions were as follows: 95°C for 5 min followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. U6 RNA was used as an internal reference for normalization. The expression levels of miR-9 were calculated using the equation $2^{-\Delta\Delta Ct}$.[22]

Statistical analysis

The miR-9 levels between the patients with ESCC and healthy controls were compared with the Mann–Whitney U-test. The chi-square test was performed to determine the relationship between plasma miR-9 expression and clinical pathological variables. Receiver operating characteristic (ROC) curve analysis was applied to evaluate the value of plasma miR-9 for ESCC diagnosis. The correlation between plasma

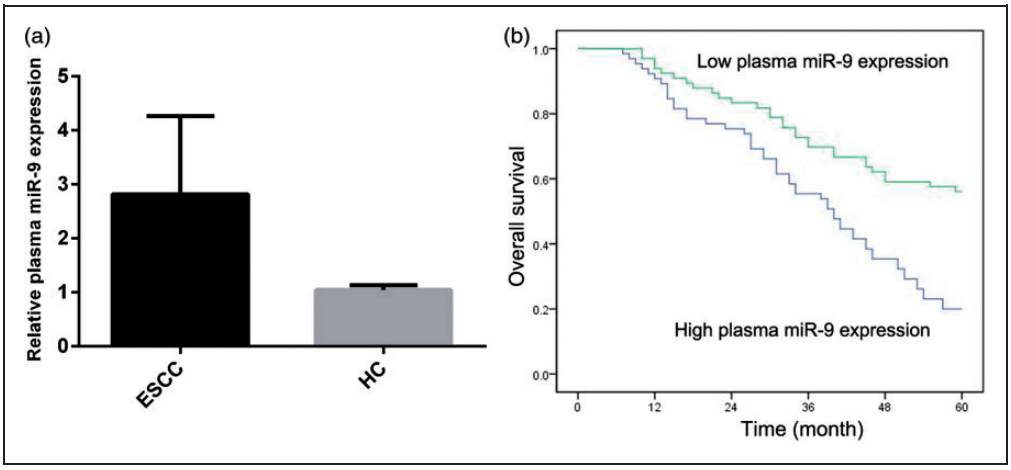

Figure 1. Plasma miR-9 expression and prognostic significance in patients with esophageal squamous cell carcinoma (ESCC). (a) miR-9 levels in plasma from patients with ESCC were significantly higher than those in healthy controls (HC) ($P < 0.01$). (b) Kaplan–Meier overall survival curves for patients with ESCC with high versus low plasma miR-9 expression.

miR-9 and the survival of patients with ESCC was estimated by Kaplan–Meier and log-rank analyses. A Cox regression model was carried out to test the independence of each variable. All statistical analyses were performed using SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA), and statistical significance was set at $P < 0.05$.

Results

Expression and prognostic significance of plasma miR-9 in patients with ESCC

We examined the expression of miR-9 in plasma from 131 patients with ESCC and 131 healthy volunteers, and the results showed that plasma miR-9 was upregulated in patients with ESCC ($P < 0.01$) (Figure 1(a)). Next, we demarcated high and low plasma miR-9 groups by the median value. High expression of plasma miR-9 was found to be significantly associated with poor tumor differentiation ($P = 0.022$), large tumor size ($P = 0.007$), deep local invasion ($P = 0.003$), lymph node metastasis ($P = 0.005$), and advanced clinical stage ($P < 0.001$) (Table 1). Kaplan–Meier analysis indicated that the overall survival of patients with a high plasma miR-9 level was significantly shorter than those with a low plasma miR-9 level (log-rank test, $P < 0.001$) (Figure 1(b)). Moreover, the multivariate analysis identified the plasma miR-9 level ($P = 0.009$), tumor invasion ($P = 0.032$), lymph node metastasis ($P = 0.015$), and TNM stage ($P = 0.006$) as independent prognostic factors for ESCC (all $P < 0.05$) (Table 2).

Diagnostic potential of plasma miR-9 in patients with ESCC

An ROC curve was drawn to evaluate the diagnostic value of plasma miR-9 in patients with ESCC. The area under the curve was 0.913 (95% confidence interval, 0.873–0.953) (Figure 2). At the optimal cut-off point (relative expression of 2.11), plasma miR-9 had a sensitivity of 85.5% and specificity of 98.5%.

Table 2. Cox regression analysis of factors associated with overall survival of patients with esophageal squamous cell carcinoma.

	Univariate analysis		Multivariate analysis	
Variables	Hazard ratio	*P*-value	Hazard ratio	*P*-value
Age (≥60/<60 years)	0.89	0.569	——	——
Sex (Male/female)	1.07	0.614	——	——
Differentiation (well+moderate/poor)	4.41	0.003	1.12	0.097
Lymphatic metastasis (negative/positive)	3.95	0.011	3.56	0.015
T classification (T_{1-2}/T_{3-4})	3.49	0.027	2.93	0.032
TNM stage (I + II/III)	4.93	<0.001	4.36	0.006
Plasma miR-9 expression (low/high)	4.76	<0.001	4.15	0.009

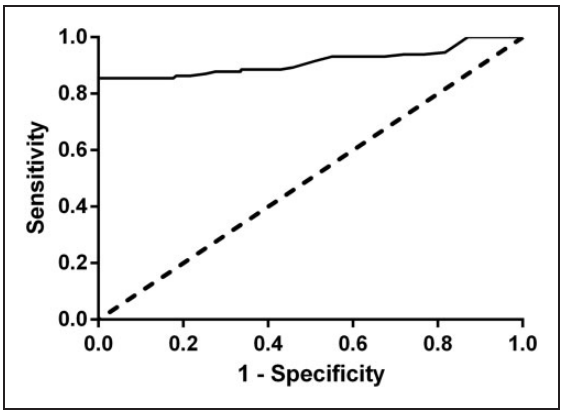

Figure 2. Receiver operating characteristic curve analysis illustrated that plasma miR-9 expression was a potential biomarker for discriminating patients with esophageal squamous cell carcinoma from healthy controls (area under the curve = 0.913).

Discussion

ESCC is a serious malignancy, and its exact molecular mechanisms remain largely unknown. Several studies have indicated the importance of miRs in ESCC tumor genesis and progression. For example, one study showed that overexpression of miR-622 reduced ESCC cell proliferation and invasion and enhanced cell apoptosis.[23] Another showed that upregulated miR-483-5 p expression was correlated with lymph node metastasis and advanced clinical stage and predicted poor overall and disease-free survival in patients with ESCC.[24] The miR-200c level was associated with tumor response to platinum-based chemotherapy and clinical outcomes of patients with advanced ESCC.[25] Depletion of miR-205 sensitized ESCC cells to ionizing radiation.[26]

The exploration of blood biomarkers is now a hotspot in cancer research because of the easy accessibility of such biomarkers. Circulating miRs have recently emerged as potential biomarkers for various cancers.[6,7] In the present study, we showed that miR-9 expression in the plasma of patients with

ESCC was significantly higher than that in the plasma of healthy individuals and was associated with tumor differentiation, tumor size, local infiltration depth, lymph node metastasis, and TNM stage. Overall survival of patients with high plasma miR-9 expression was dramatically shorter than in patients with low plasma miR-9 expression. At the optimal cut-off, plasma miR-9 had a sensitivity of 85.5% and specificity of 98.5% in discriminating ESCC from healthy volunteers. These findings imply that plasma miR-9 may serve as a valuable biomarker for the detection and prognosis prediction of ESCC.

Dysregulated circulating miR-9 expression has been reported in patients with other types of tumors and diseases, such as OSCC,[20] osteosarcoma,[21] and acute ischemic stroke.[27] An increased serum miR-9 concentration in patients with osteosarcoma was associated with advanced tumor stage, larger tumor size, and the presence of distant metastasis.[21] The serum miR-9 concentration was also correlated with lymph node metastasis and TNM stage in patients with OSCC.[20] Furthermore, serum miR-9 was a prognostic biomarker for osteosarcoma and OSCC.[20,21] Thus, the upregulation of circulating miR-9 is not specific to patients with ESCC, and the expression pattern and clinical significance of plasma miR-9 in other cancer types should be further investigated.

Several studies have also focused on the mechanisms of how miR-9 regulates cancer development, and various related pathways have been identified, such as the NF-kappaB,[28] MAPK14,[29] PI3K/AKT,[30] JAK-STAT,[31] and Hippo signaling pathways.[32] In terms of ESCC, Song et al.[19] reported that miR-9 promoted ESCC cell migration and tumor metastasis by targeting E-cadherin and inducing epithelial–mesenchymal transition. Because the relationships between miRs and their targets are not one-to-one but multiple-to-multiple,[33] future research is necessary to identify more downstream genes of miR-9 and further elucidate the oncogenic mechanisms of miR-9 in patients with ESCC.

In summary, our study showed that plasma miR-9 expression was significantly upregulated in patients with ESCC and that plasma miR-9 might serve as a noninvasive biomarker for ESCC diagnosis and prognosis. However, this was a single-institution retrospective study, and the sample size was relatively small. Large-scale, multicenter, prospective investigations are required to confirm our conclusions.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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