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. 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. 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, non-coding RNAs that post-transcriptionally regulate gene expression. 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. 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. For example, plasma miR-92a-2 has been identified as a noninvasive diagnostic biomarker for small cell lung cancer. Serum miR-503 was more effective than carcinoembryonic antigen in discriminating patients with gastric cancer from healthy individuals. Serum miR-424 in patients with hepatocellular carcinoma was associated with the blood alpha-fetoprotein concentration, vein invasion, and TNM stage. Increased circulating miR-21 concentrations predicted poor overall survival in patients with gastric cancer, lung cancer, and colorectal cancer.

MiR-9 has been shown to play important roles in the development of several types of cancer, including lung cancer, thyroid carcinoma, breast cancer, ovarian cancer, gastric cancer, and ESCC. 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. 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 \(-80^\circ C\) 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.
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 $2^{-\Delta\Delta C_{t}}$.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

| 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)  |    |
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%.
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. Another showed that upregulated miR-483-5p expression was correlated with lymph node metastasis and advanced clinical stage and predicted poor overall and disease-free survival in patients with ESCC. The miR-200c level was associated with tumor response to platinum-based chemotherapy and clinical outcomes of patients with advanced ESCC. Depletion of miR-205 sensitized ESCC cells to ionizing radiation.

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. 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, osteosarcoma, and acute ischemic stroke. 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. The serum miR-9 concentration was also correlated with lymph node metastasis and TNM stage in patients with OSCC. Furthermore, serum miR-9 was a prognostic biomarker for osteosarcoma and OSCC. 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, MAPK14, PI3K/AKT, JAK-STAT, and Hippo signaling pathways. In terms of ESCC, Song et al. 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, 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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References
1. Napier KJ, Scheerer M and Misra S. Esophageal cancer: a review of epidemiology, pathogenesis, staging workup and treatment modalities. World J Gastrointest Oncol 2014; 6: 112–120.
2. Tang WR, Chen ZJ, Lin K, et al. Development of esophageal cancer in Chaoshan region, China: association with environmental, genetic and cultural factors. Int J Hyg Environ Health 2015; 218: 12–18.
3. He L and Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004; 5: 522–531.
4. Xie R, Wu SN, Gao CC, et al. MicroRNA-30d inhibits the migration and invasion of human esophageal squamous cell carcinoma cells via the posttranscriptional regulation of enhancer of zeste homolog 2. Oncol Rep 2017; 37: 1682–1690.
5. Fendereski M, Zia MF, Shafiee M, et al. MicroRNA-196a as a potential diagnostic biomarker for esophageal squamous cell carcinoma. *Cancer Invest* 2017; 35: 78–84.

6. Zhao Y, Song Y, Yao L, et al. Circulating microRNAs: promising biomarkers involved in several cancers and other diseases. *DNA Cell Biol* 2017; 36: 77–94.

7. Shigeyasu K, Toden S, Zumwalt TJ, et al. Emerging role of MicroRNAs as liquid biopsy biomarkers in gastrointestinal cancers. *Clin Cancer Res* 2017.

8. Yu Y, Zuo J, Tan Q, et al. Plasma miR-92a-2 as a biomarker for small cell lung cancer. *Cancer biomark* 2017; 18: 319–327.

9. Wu D, Cao G, Huang Z, et al. Decreased miR-503 expression in gastric cancer is inversely correlated with serum carcinoembryonic antigen and acts as a potential prognostic and diagnostic biomarker. *Onco Targets Ther* 2016; 10: 129–135.

10. Yao H, Liu X, Chen S, et al. Decreased expression of serum miR-424 correlates with poor prognosis of patients with hepatocellular carcinoma. *Int J Clin Exp Pathol* 2015; 8: 14830–14835.

11. Komatsu S, Ichikawa D, Tsujiura M, et al. Prognostic impact of circulating miR-21 in the plasma of patients with gastric carcinoma. *Anticancer Res* 2013; 33: 271–276.

12. Zhao W, Zhao JJ, Zhang L, et al. Serum miR-21 level: a potential diagnostic and prognostic biomarker for non-small cell lung cancer. *Int J Clin Exp Med* 2015; 8: 14759–14763.

13. Toyama Y, Takahashi M, Hur K, et al. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. *J Natl Cancer Inst* 2013; 105: 849–859.

14. Han L, Wang W, Ding W, et al. MiR-9 is involved in TGF-beta1-induced lung cancer cell invasion and adhesion by targeting SOX7. *J Cell Mol Med* 2017.

15. Chen Y, Zhang S, Zhao R, et al. Upregulated miR-9-3p promotes cell growth and inhibits apoptosis in medullary thyroid carcinoma by targeting BLCAP. *Oncol Res* 2016.

16. D’Ippolito E, Plantamura I, Bongiovanni L, et al. miR-9 and miR-200 Regulate PDGFRbeta-mediated endothelial differentiation of tumor cells in triple-negative breast cancer. *Cancer Res* 2016; 76: 5562–5572.

17. Sun C, Li N, Yang Z, et al. miR-9 regulation of BRCA1 and ovarian cancer sensitivity to cisplatin and PARP inhibition. *J Natl Cancer Inst* 2013; 105: 1750–1758.

18. Tsai KW, Liao YL, Wu CW, et al. Aberrant hypermethylation of miR-9 genes in gastric cancer. *Epigenetics* 2011; 6: 1189–1197.

19. Song Y, Li J, Zhu Y, et al. MicroRNA-9 promotes tumor metastasis via repressing E-cadherin in esophageal squamous cell carcinoma. *Oncotarget* 2014; 5: 11669–11680.

20. Sun L, Liu L, Fu H, et al. Association of decreased expression of serum miR-9 with poor prognosis of oral squamous cell carcinoma patients. *Med Sci Moni* 2016; 22: 289–294.

21. Fei D, Li Y, Zhao D, et al. Serum miR-9 as a prognostic biomarker in patients with osteosarcoma. *J Int Med Res* 2014; 42: 932–937.

22. Wu C, Li M, Hu C, et al. Clinical significance of serum miR-223, miR-25 and miR-375 in patients with esophageal squamous cell carcinoma. *Mol Biol Rep* 2014; 41: 1257–1266.

23. Song C, Lu P, Shi W, et al. MiR-622 functions as a tumor suppressor and directly targets E2F1 in human esophageal squamous cell carcinoma. *Biomed Pharmacother* 2016; 83: 843–849.

24. Xue L, Nan J, Dong L, et al. Upregulated miR-483-5p expression as a prognostic biomarker for esophageal squamous cell carcinoma. *Cancer Biomark* 2017.

25. Yu H, Duan B, Jiang L, et al. Serum miR-200c and clinical outcome of patients with advanced esophageal squamous cancer receiving platinum-based chemotherapy. *Am J Transl Res* 2013; 6: 71–77.

26. Pan F, Mao H, Bu F, et al. Sp1-mediated transcriptional activation of miR-205 promotes radioresistance in esophageal squamous cell carcinoma. *Oncotarget* 2017; 8: 5735–5752.

27. Ji Q, Ji Y, Peng J, et al. Increased Brain-Specific MiR-9 and MiR-124 in the Serum Exosomes of Acute Ischemic Stroke Patients. *PLoS One* 2016; 11: e0163645.
28. Huang X, Teng Y, Yang H, et al. Propofol inhibits invasion and growth of ovarian cancer cells via regulating miR-9/NF-kappaB signal. *Braz J Med Biol Res* 2016; 49: e5717.

29. Ben-Hamo R, Zilberberg A, Cohen H, et al. hsa-miR-9 controls the mobility behavior of glioblastoma cells via regulation of MAPK14 signaling elements. *Oncotarget* 2016; 7: 23170–23181.

30. Bing W, Pang X, Qu Q, et al. Simvastatin improves the homing of BMSCs via the PI3K/AKT/miR-9 pathway. *J Cell Mol Med* 2016; 20: 949–961.

31. Zhuang G, Wu X, Jiang Z, et al. Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. *EMBO J* 2012; 31: 3513–3523.

32. Deng J, Lei W, Xiang X, et al. Cullin 4A (CUL4A), a direct target of miR-9 and miR-137, promotes gastric cancer proliferation and invasion by regulating the Hippo signaling pathway. *Oncotarget* 2016; 7: 10037–10050.

33. Hashimoto Y, Akiyama Y and Yuasa Y. Multiple-to-multiple relationships between microRNAs and target genes in gastric cancer. *PLoS One* 2013; 8: e62589.