Plasma miRNA-506 as a Prognostic Biomarker for Esophageal Squamous Cell Carcinoma

Shu-Ping Li
Hong-Xin Su
Da Zhao
Quan-Lin Guan

Background:
MicroRNAs (miRNAs) are responsible for regulating proliferation, differentiation, apoptosis, invasion, and metastasis in tumor cells. miRNA-506 is abnormally expressed in multiple tumors, indicating that it might be oncogenic or tumor-suppressive. However, little is known about the association between miRNA-506 expression and esophageal squamous cell carcinoma (ESCC).

Material/Methods:
We examined the expression of miRNA-506 in the plasma of ESCC patients using quantitative real-time polymerase chain reaction (qRT-PCR) to determine the association between miRNA-506 expression and clinicopathological features of ESCC. ROC curves were produced for ESCC diagnosis by plasma miRNA-506 and the area under curve was calculated to explore its diagnostic value.

Results:
Average miRNA-506 expression levels were remarkably higher in the plasma of ESCC patients than in healthy volunteers (P<0.001). The expression of miRNA-506 in the plasma was closely associated with lymph node status (P=0.004), TNM stage (P=0.031), and tumor length (P<0.001). According to ROC curves, the area under the curve for plasma miRNA-506 was 0.835, indicating statistical significance for ESCC diagnosis by plasma miRNA-506 (P<0.001). Kaplan-Meier analysis showed that patients with high miRNA-506 expression had significantly shorter survival time than those with low miRNA-506 expression. Cox regression analysis demonstrated that T stage, N stage, tumor length, and miRNA-506 expression levels were significantly correlated with prognosis in ESCC patients.

Conclusions:
miRNA-506 can serve as an important molecular marker for diagnosis and prognostic prediction of ESCC.

MeSH Keywords: Diagnosis • Esophageal Neoplasms • MicroRNAs • Prognosis

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Background

Esophageal cancer, which originates in the esophageal mucosal epithelium, is characterized by strong invasiveness and high mortality [1]. Approximately, 300 000 people die from esophageal cancer in the world annually [2]. There is significant variability in morbidity and mortality rates of among different countries. China has one of the highest frequencies of esophageal cancer, with an average annual death rate of 150 000 [3]. The pathology in more than 90% of these patients is esophageal squamous cell carcinoma (ESCC). Postoperative recurrence and metastasis are the main causes of death in patients with ESCC. Factors that lead to postoperative recurrence, invasion, and metastasis include activation of proto-oncogenes, inactivation of tumor suppressor genes, and down-regulation or abnormal expression of multiple proteins.

MicroRNAs (miRNAs) are post-transcriptional expression products of a series of regulatory genes that have both oncogene and tumor-suppressor roles. These micromolecules are crucial for the genesis and development of tumors [4]. ESCC tissues have been shown to contain abnormally expressed miRNAs, which are closely related to the signaling pathways required for the initiation and development of the disease [5]. miRNAs are responsible for regulating proliferation, differentiation, apoptosis, invasion, and metastasis in tumor cells. Approximately 1000 different miRNAs have been identified, of which miRNA-506 is an important regulator of tumorigenesis [6]. miRNA-506 is abnormally expressed in multiple tumors, indicating that it is potentially oncogenic or tumor-suppressive. Currently, little is known about the association between miRNA-506 and ESCC. Therefore, we examined the expression of miRNA-506 in the plasma of ESCC patients using quantitative real-time polymerase chain reaction (qRT-PCR), and investigated the association between miRNA-506 expression and clinicopathological features of ESCC.

Material and Methods

Patients and blood samples

The subjects in this study were 110 ESCC patients treated in The First Hospital of Lanzhou University from January 2009 to December 2011. All patients were diagnosed with ESCC, and complete clinical data for these patients were available. Among the 110 ESCC cases, 45 were female and 55 were male, with ages ranging from 37 to 74 years and an average age of 59.2±10.3 years. Fasting peripheral blood (5 mL) was drawn from each patient and placed in anticoagulant tubes at room temperature for 30 min, followed by centrifugation at 4000×g for 5 min at 4°C. The plasma supernatant was collected and stored at −80°C until use. None of the patients received anti-cancer treatment before the blood specimen collection and all provided informed consent. The blood samples were processed in accordance with medical ethics standards. The research was approved by the Ethics Committee of The First Hospital of Lanzhou University. The control group consisted of 40 healthy volunteers.

RNA isolation and qRT-PCR

Total RNA was isolated from the plasma samples in accordance with the instructions on the miRNAVana™ PARIST™ kit (Ambion, Austin, TX). The OD of total RNA was measured using the NanoDropND-1000 ultraviolet photometric machine (Thermo Scientific Wilmington, DE). The extracted RNA was reverse transcribed into cDNA according to the instructions of TaqMan miRNA reverse transcription reagent kit (Qiagen, Valencia, CA). The reverse transcription conditions were as follows: 30 min at 16°C, 60 min at 42°C, 5 min at 85°C, and a termination reaction at 4°C. PCR amplification was subsequently performed, and primers specific for miRNA-506 and an internal reference were used. The PCR conditions were as follows: 1 cycle at 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s, and 1 cycle of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. A total volume of 10 μL was used and the reactions were performed in triplicate with U6 as an internal reference. Each experiment was repeated 3 times and data were quantitatively analyzed by comparing threshold cycle (Ct) values (2−DDCt) [7]. The relative expression (RQ) of the target gene in the sample was expressed as ΔΔCt, which was calculated using the equation:

\[
\Delta\Delta Ct = \Delta Ct - \text{Avg} (Ct_{\text{miRNA-506}} - Ct_{\text{U6}}) - \text{Avg} (Ct_{\text{miRNA-506}} - Ct_{\text{U6}}).
\]

Statistical methods

The clinical stage of patients was determined using the tumor node metastasis (TNM) stage system developed jointly by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) [8]. The Mann-Whitney test was adopted to compare differences in plasma miRNA-506 expression between ESCC patients and healthy volunteers. Differences in plasma miRNA-506 levels among patients with ESCC of different stages were analyzed using the Mann-Whitney test (for 2 groups). The receiver operating characteristic curve (ROC curve) was produced to evaluate the utility of miRNA-506 for diagnosing ESCC. A chi-square test was applied to assess the relationship between the expression of plasma miRNA-506 and clinicopathological features of ESCC. The primary outcome indicators for ESCC patients were disease-free survival (DFS) and overall survival (OS). The Kaplan-Meier method was utilized for survival analysis, whereas the log-rank test was used to compare survival rates between the 2 groups. The joint effect analysis of all covariates was conducted using the Cox proportional hazard model. SPSS 19.0
software (SPSS Inc., Chicago, IL) was used for statistical analysis. A value of $P<0.05$ was considered statistically significant.

**Results**

**miRNA-506 expression in plasma of ESCC patients**

Based on qRT-PCR results, the average miRNA-506 expression was significantly higher in the plasma of ESCC patients than in healthy volunteers ($P<0.001$; Figure 1). We conducted subgroup analysis of the 100 ESCC cases. Compared to that of patients with stage I or II ESCC, or with a tumor length of $<4$ cm, the expression of miRNA-506 was higher in the plasma of patients with stage III ESCC or with a tumor length $>4$ cm ($P<0.001$, $P=0.031$, respectively; Figures 2, 3). The 100 ESCC patients were divided into a high-expression group and a low-expression group based on the median expression level of plasma miRNA-506 (median $2^{\Delta\text{Ct}}$ value). We then analyzed the relationship between the expression of plasma miRNA-506 and the clinicopathological features of ESCC; the results indicated that the expression level of plasma miRNA-506 was closely associated with lymph node status ($P=0.004$), TNM stage ($P=0.031$), and tumor length ($P<0.001$). However, the expression of plasma miRNA-506 had no significant relationship with other clinicopathological features in ESCC patients (Table 1).

**Plasma miRNA-506 as diagnostic biomarker for ESCC**

We then produced ROC curves for ESCC diagnosis by plasma miRNA-506 and calculated the area under the curve, as well as the sensitivity and specificity of all thresholds. The area under the curve for plasma miRNA-506 was 0.835, indicating that there was a statistically significant difference in ESCC diagnosis by plasma miRNA-506 ($P<0.001$). At the best cutoff point, the sensitivity and specificity were 81.2% and 87.3%, respectively (Figure 4).

**miRNA-506 expression in plasma and prognosis of patients with ESCC**

To probe the relationship between the expression of plasma miRNA-506 and prognosis for ESCC patients, we conducted long-term follow-up of the 100 ESCC patients. We plotted the survival curves for these ESCC patients. Kaplan-Meier analysis showed that patients with high miRNA-506 expression had significantly shorter survival time (DFS and OS) than that of patients with low miRNA-506 expression. The survival curves for ESCC patients are shown in Figures 5 and 6. Univariate analysis showed that $T$ stage, $N$ stage, tumor length, and high miRNA-506 expression levels were significantly correlated with DFS and OS ($P<0.05$; Tables 2, 3). Multivariate analysis using the Cox regression model suggested that high miRNA-506 expression was an independent indicator of poor patient prognosis [DFS: $\text{HR}=2.647$, 95% CI=(1.529–4.582), $P=0.001$; OS $\text{HR}=2.351$, 95% CI=(1.317–4.195), $P=0.004$]. Other clinical and pathological features, as well as prognosis of ESCC patients, are summarized in Table 3.
Table 1. The plasma of miRNA-506 expression status and clinicopathological characteristics of patients with ESCC.

| Features        | miRNA-506 | P value |
|-----------------|-----------|---------|
|                 | Low (n=58) | High (n=42) |
| Age             |           |          |
| <65             | 25        | 20      |
| ≥65             | 33        | 22      |
| Sex             |           |          |
| Male            | 30        | 25      |
| Female          | 28        | 17      |
| Smoking         |           |          |
| Never or light  | 30        | 23      |
| Heavy           | 28        | 19      |
| Drinking        |           |          |
| Never or light  | 33        | 22      |
| Heavy           | 25        | 20      |
| Differentiation |           |          |
| Well            | 19        | 11      |
| Moderate        | 25        | 19      |
| Poor            | 14        | 12      |
| T stage         |           |          |
| T1–2            | 31        | 17      |
| T3–4            | 27        | 25      |
| N stage         |           |          |
| N0–1            | 39        | 16      |
| N2–3            | 19        | 26      |
| TNM stage       |           |          |
| I–II            | 32        | 14      |
| III             | 26        | 28      |
| Tumor length    |           |          |
| <4 cm           | 44        | 17      |
| ≥4 cm           | 14        | 25      |
| Site of tumor   |           |          |
| Cervical        | 6         | 4       |
| Upper thoracic  | 3         | 9       |
| Middle thoracic | 24        | 17      |
| Low thoracic    | 25        | 12      |
The miRNA-506 gene is located on the X chromosome (Xq27.3) [9]. An increasing amount of evidence suggests that miRNA-506 inhibits the expression of its target genes through binding to the target mRNAs, and is involved in regulating and controlling cell proliferation, apoptosis, cell cycle arrest, cell aging, cell differentiation, epithelial-mesenchymal transition, cell invasion, and metastasis, as well as initiation and development of tumors [10–12].

In vitro experiments confirmed that miRNA-506 can directly bind to the 3’UTR of SNAI2, which results in increased expression of E-cadherin through inhibiting the expression of SNAI2; this inhibits epithelial-mesenchymal transition in ovarian carcinoma cells and the invasive capacity of tumor cells [13,14]. Similarly, miRNA-506 has an anti-tumorigenic

### Table 2. Univariate and multivariate analyses of prognostic variables for disease-free survival.

| Variable          | Univariate analysis | Multivariate analysis |
|-------------------|---------------------|-----------------------|
|                   | P value             | HR (95% CI)           | P value   |
| Sex               | 0.897               | 1.038 (0.441–2.446)   | 0.932     |
| Age               | 0.577               | 1.007 (0.606–1.675)   | 0.978     |
| Smoking           | 0.945               | 0.830 (0.379–1.818)   | 0.641     |
| Drinking          | 0.697               | 1.086 (0.486–2.427)   | 0.841     |
| T stage           | 0.037               | 1.775 (1.147–2.746)   | 0.010     |
| N stage           | 0.032               | 1.753 (1.116–2.754)   | 0.015     |
| Differentiation   | 0.027               | 1.334 (1.142–1.790)   | 0.012     |
| Tumor length      | 0.477               | 0.884 (0.667–1.171)   | 0.390     |
| miRNA-506         | 0.001               | 2.647 (1.529–4.582)   | 0.001     |
role in various malignant tumors such as breast cancer, colorectal cancer, gastric cancer, and liver cancer [15–18]. However, there is also research showing that miRNA-506 has an oncogenic role in lung cancer [19]. The relationship between the expression of miRNA-506 and ESCC was previously unknown. In light of this conflicting evidence, we performed this research to probe the effects of miRNA-506 on the initiation and development of ESCC.

The plasma miRNA-506 level in ESCC patients was detected through qRT-PCR, and healthy volunteers served as control subjects. We discovered that the average miRNA-506 expression was remarkably higher in the plasma of ESCC patients compared to that of healthy volunteers. Subgroup analysis showed that the expression level of miRNA-506 was higher in the plasma of patients with stage III ESCC or those with a tumor length >4 cm. We also discovered that the expression level of miRNA-506 in the plasma was closely associated with clinicopathological features of ESCC patients, such as lymph node status, TNM stage, and tumor length. This suggests that miRNA-506 might promote the growth, proliferation, and invasion of ESCC tumor cells. However, this hypothesis is based on in vivo results, and thus need to be verified through in vitro experimentation. Long-term follow-up of ESCC patients showed that compared to ESCC patients with low miRNA-506 expression, those with high miRNA-506 expression had shorter survival. Multi-factor regression analysis revealed that high miRNA-506 expression was an important, independent indicator for predicting poor prognosis in ESCC patients. In addition, the ROC curves of ESCC diagnosis by plasma miRNA-506 showed an area under the curve of 0.835, indicating that determination of plasma miRNA-506 levels was of high diagnostic value for ESCC.

The results of the present work are contrary to those of numerous earlier studies. We reviewed, analyzed, and summarized the relevant literature regarding the relationship between miRNA-506 and malignant tumors. The mechanism of action of miRNA-506 during ESCC progression might be as follows. First, miRNA-506 could directly bind to cyclin-dependent kinase4/6 (CDK4/6), and then to cyclinD, regulating cell cycle progression from G1 to S stage [20]. Second, miRNA-506 would inhibit the expression of CDK4/6, which could stimulate proliferation of tumor cells in a feedback-mediated manner [19,20]. Third, miRNA-506 could inhibit the expression of SNAI2 and consequently increase the expression of E-cadherin, which would confer adhesiveness to mobile tumor cells [21]. Fourth, ETS1 protein is an important target of miR-506, and the combination of ETS1 and miR-506 could further influence tumor angiogenesis and the metastasis [22]. In addition, the direct binding of miR-506 to NF-kB could inhibit the NF-kB pathway, induce the production of reactive oxygen species (ROS), activate p35, and result in a mutual positive feedback loop with p53 [19,23].

**Conclusions**

High miRNA-506 expression was found to be associated with poor prognosis in patients with ESCC. Therefore, miRNA-506 could serve as an important molecular marker for diagnosis and prognosis in ESCC. However, the precise mechanism remains unclear and further in-depth research is required. We believe that it is possible to delay tumor growth, proliferation, infiltration, and metastasis by blocking miRNA-506-mediated signal transduction pathways.

**Conflicts of interest**

The authors report no conflicts of interest in this work.
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