Original Article
Circulating microRNAs in esophageal squamous cell carcinoma: association with locoregional staging and survival

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Abstract: Locoregional staging and prognostic information play a critical role in esophageal squamous cell carcinoma (ESCC) treatment strategies. Although microRNA (miRNA) is a promising marker for cancer detection, the relationship between circulating plasma miRNAs and ESCC remains unclear. Our study aims to investigate the association between circulating plasma miRNAs and tumor diagnosis or prognosis in ESCC patients. Plasma levels of miR-16, miR-21, miR-185, and miR-375 were evaluated by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays from 38 ESCC patients prior to treatment and 19 healthy subjects. Differences in selected miRNAs and their diagnostic and prognostic value were examined. Levels of four of the selected miRNAs were found to be significantly higher in ESCC patients than in controls; namely, miR-16, miR-21, miR-185, and miR-375 (P < 0.050). In addition, the area under the receiver operating characteristic (ROC) curve (AUC) for miR-375 was 0.921 (95% confidence interval [CI] 0.817-0.976). Moreover, the expression levels of miR-16 were higher in patients with T3-4 tumors than in patients with T1-2 tumors (P = 0.020). Kaplan-Meier survival analysis showed that high expression levels of miR-16 and miR-21 in the plasma correlated significantly with shortened progression-free survival (PFS; P = 0.031 and P = 0.038, respectively) and overall survival (OS; P = 0.022 and P = 0.041, respectively) in ESCC patients. Four plasma miRNAs were identified that could potentially serve as novel diagnostic biomarkers for ESCC. Moreover, specific miRNAs, such as miR-16 and miR-21, can predict poor survival in ESCC.

Keywords: MicroRNA, esophageal squamous cell carcinoma, locoregional staging, prognosis

Introduction

There are two major histological types of esophageal carcinoma: esophageal squamous cell carcinoma (ESCC) and adenocarcinoma. ESCC is the major type in China, where it accounts for more than 90% of cases of esophageal carcinoma; whereas adenocarcinoma is more common in the United States and in European countries [1]. ESCC is often diagnosed at a locally advanced stage and the outcomes for affected patients are poor. Esophagectomy, chemotherapy, and radiotherapy are currently the main treatments for ESCC, but accurate clinical staging and prognostic information is essential to direct appropriate treatment strategies. To develop new diagnostic methods and treatment strategies, investigators have focused on the potential of a particular class of microRNAs (miRNAs) to provide additional information about the characteristics and survival prospects of patients with ESCC.

miRNAs are small (22-24 nucleotides), noncoding RNA molecules that play important roles in regulating cell differentiation, proliferation, migration and apoptosis [2]. Altered miRNA expression in cancer tissue has been reported in most tumor types [3, 4], and there is increasing evidence that miRNA expression in cancer tissue is a useful prognostic marker [5-7]. In addition, the application of miRNA expression
levels as a blood biomarker has been explored in various types of cancer, including gastric, hepatocellular, and non-small cell lung cancer [8-10]. However, whether miRNA levels in plasma are a useful biomarker for patients with ESCC remains largely unexplored.

In this study, we analyzed the expression levels of nine selected miRNAs (miR-16, miR-21, miR-22, miR-126, miR-148b, miR-185, miR-221, miR-223, and miR-375) in plasma and their association with clinicopathological features and clinical outcomes.

Materials and methods

Patient population

A total of 38 patients with pathologically confirmed ESCC who were treated at the Sixth People’s Hospital of Jiao Tong University, Shanghai, China, between August 2009 and June 2013 were included in this study. All non-surgical patients were staged according to routine practice with air contrast barium esophagography, upper gastrointestinal endoscopy with histological biopsies and cervical, chest and abdominal contrast computed tomography (CT). All surgical patients were staged in accordance with the American Joint Committee on Cancer tumor-node-metastasis (TNM) staging system [11].

All patients received radiotherapy alone or post-operative radiotherapy or radiochemotherapy according to local practice. Radiotherapy was started on Day 1 and delivered at 2 Gy/day for 5 days a week to a total radiation dose of 60-70 Gy for non-surgical patients, and a total radiation dose of 50 Gy for surgical patients. Chemotherapy and radiotherapy were initiated on the same day. Chemotherapeutics consisted of the protracted infusion of 5-fluorouracil (750-1,000 mg/m²/day) on Days 1-5 in combination with cisplatin (30 mg/m²/day) with adequate hydration and continuous intravenous infusion of antiemetics between Days 1-3. A total of two cycles of chemotherapeutics were performed during radiotherapy at 4-week intervals. This was followed by two more periods of chemotherapeutics at the same doses performed at 3-weekly intervals, 3 weeks following the completion of radiotherapy.

Follow-up data were collected until death or December 2013. All patients had a regular follow-up schedule including a complete history and physical examination every 3 months during the first 2 years, every 6 months during the first 3-5 years and every year thereafter.

The study was approved by the Institutional Ethics Board, and signed consent forms were obtained from 38 patients and 19 healthy donors who volunteered to join the study.

RNA extraction

Peripheral blood (10 ml) was obtained from each patient before treatment and from healthy volunteer controls. Immediately after collection, blood samples were subjected to isolation of cell-free nucleic acids using a 3-spin protocol (1500 rpm for 30 min, 3000 rpm for 5 min, 4500 rpm for 5 min) to prevent contamination by cellular nucleic acids. Then plasma samples were frozen in liquid nitrogen and stored at -80°C until further processing.

Total RNA used for quantification of miRNA levels was extracted from plasma samples using a mirVana PARIS kit (Ambion, Austin, TX) according to the manufacturer’s instructions. miR-1228 was chosen as the reference for normalization of the expression of plasma miRNA. The extracted RNA was eluted in 100 μl of preheated nuclease-free water and measured on a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA), then immediately stored at -80°C.

Reverse transcriptase reactions and qRT-PCR

TaqMan miRNA Assays (Applied Biosystems, Foster City, CA) were used for determining miRNA levels in plasma according to the manufacturer’s instructions. These assays target only a mature form of the specific miRNA, which ensures a biologically relevant result. Reverse transcription (RT) was performed using Taqman miRNA RT kits according to the instructions from Applied Biosystems. Briefly, the cDNA was synthesized from total RNA (100 ng) using miRNA-specific primers in a 40-μl reaction volume. The RT reaction was performed using the following thermal cycling program: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. The RT product was diluted 10-fold, and 4 μl of the product was used in a total reaction volume of 10 μl for relative quantification by real-time PCR using an ABI 7900HT fast system (Applied Biosystems, Foster City, CA).
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The thermal cycling program used for the quantification was as follows: 96°C for 5 min and followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. The cycle threshold (Ct) number is defined as the cycle number at which the fluorescence crossed the fixed threshold. The Ct number was calculated using the second derivative method in the ABI software. The miRNA level was normalized to endogenous plasma miR-1228 expression in each sample and presented as the ΔCt value ($\Delta C_t = C_{target} - C_{miRNA}$). A lower ΔCt value referred to a lower expression of target miRNA. These reactions were run in triplicate.

### Table 1. Comparison of miRNA levels in the healthy group and ESCC group

<table>
<thead>
<tr>
<th>miRNA</th>
<th>P-value</th>
<th>Fold change</th>
<th>AUC</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-16</td>
<td>0.010</td>
<td>3.436</td>
<td>0.643</td>
<td>0.505 to 0.765</td>
</tr>
<tr>
<td>miR-21</td>
<td>0.017</td>
<td>4.001</td>
<td>0.690</td>
<td>0.553 to 0.806</td>
</tr>
<tr>
<td>miR-126</td>
<td>0.072</td>
<td>2.982</td>
<td>0.644</td>
<td>0.506 to 0.766</td>
</tr>
<tr>
<td>miR-223</td>
<td>0.234</td>
<td>2.320</td>
<td>0.589</td>
<td>0.450 to 0.717</td>
</tr>
<tr>
<td>miR-375</td>
<td>0.000</td>
<td>16.181</td>
<td>0.921</td>
<td>0.817 to 0.976</td>
</tr>
<tr>
<td>miR-22</td>
<td>0.127</td>
<td>2.421</td>
<td>0.675</td>
<td>0.516 to 0.810</td>
</tr>
<tr>
<td>miR-148b</td>
<td>0.156</td>
<td>2.861</td>
<td>0.678</td>
<td>0.518 to 0.812</td>
</tr>
<tr>
<td>miR-185</td>
<td>0.027</td>
<td>3.973</td>
<td>0.697</td>
<td>0.538 to 0.828</td>
</tr>
<tr>
<td>miR-221</td>
<td>0.084</td>
<td>4.293</td>
<td>0.715</td>
<td>0.557 to 0.842</td>
</tr>
</tbody>
</table>

AUC, Area under the curve; CI, confidence interval.

### Statistical analysis

Overall survival (OS) was defined as the time interval from the date of diagnosis to the date of cancer-related death or the end of follow-up. Progression-free survival (PFS) was defined as the time interval from the date of diagnosis to the date of tumor recurrence or tumor metastasis.

Graphpad 5 (GraphPad Software, San Diego, CA) was used for statistical analysis. Differences between variables were examined for statistical significance using the Student’s t-test and chi-square test. The area under the receiver operating characteristic ROC curve (AUC) was used as an accuracy index for evaluating the diagnostic performance of the selected microR-
NA panel. Survival curves of the patients were calculated using the Kaplan-Meier method and analyzed by the log-rank test. Two-sided significance levels of $P < 0.05$ were considered to indicate a statistically significant difference.

Results

Plasma miRNAs are potential diagnostic markers for ESCC

The plasma levels of nine miRNAs (miR-16, miR-21, miR-22, miR-126, miR-148b, miR-185, miR-221, miR-223, and miR-375) were examined by qRT-PCR in 38 patients with ESCC and 19 healthy volunteers. The levels of miR-16, miR-21, miR-185, and miR-375 in plasma were significantly higher in patients with ESCC than in healthy volunteers ($P = 0.010, P = 0.017, P = 0.027$ and $P < 0.001$, respectively; Figure 1). Among the nine candidate targeted miRNAs, miR-375 had the highest level in terms of both area under the curve (AUC) and statistical difference (16.18-fold change; AUC = 0.921). The results are shown in Table 1 and Figure 2.

The 38 ESCC patients were divided into two groups on the basis of the median value of the expression level of each miRNA: high-expression group ($n = 19$) and low-expression group ($n = 19$).
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Table 2. Correlations between plasma miRNA levels and clinical characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>miR-16</th>
<th>miR-21</th>
<th>miR-185</th>
<th>miR-375</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>P-value</td>
<td>High</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 65 years</td>
<td>10</td>
<td>11</td>
<td>0.744</td>
<td>11</td>
</tr>
<tr>
<td>&lt; 65 years</td>
<td>9</td>
<td>8</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>14</td>
<td>0.426</td>
<td>17</td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td>5</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 5 cm</td>
<td>8</td>
<td>7</td>
<td>0.740</td>
<td>8</td>
</tr>
<tr>
<td>&lt; 5 cm</td>
<td>11</td>
<td>12</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Clinical T-stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>4</td>
<td>11</td>
<td>0.020*</td>
<td>5</td>
</tr>
<tr>
<td>T3-T4</td>
<td>15</td>
<td>8</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Clinical N-stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>10</td>
<td>8</td>
<td>0.516</td>
<td>10</td>
</tr>
<tr>
<td>N1</td>
<td>9</td>
<td>11</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>17</td>
<td>13</td>
<td>0.112</td>
<td>17</td>
</tr>
<tr>
<td>M1</td>
<td>2</td>
<td>6</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Pathological stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>9</td>
<td>10</td>
<td>0.746</td>
<td>10</td>
</tr>
<tr>
<td>III-IV</td>
<td>10</td>
<td>9</td>
<td></td>
<td>9</td>
</tr>
</tbody>
</table>

*P < 0.05, as determined by Pearson’s χ² test.

The relationship between the expression of these four plasma miRNAs (miR-16, miR-21, miR-185, and miR-375) and the clinical characteristics of patients with ESCC was analyzed. Only miR-16 level was found to be associated with T-stage; being higher in patients with T3-4 tumors than in patients with T1-2 tumors (P = 0.020, Table 2). There were no other significant relationships between plasma miRNA levels and other clinical characteristics.

Relationship between plasma miRNA levels and immediate response to radiotherapy

Two weeks after completion of radiotherapy, all patients were restaged through endoscopy and CT to evaluate the clinical response to radiotherapy, as assessed according to the World Health Organization Response Criteria for Measurable Diseases. Complete response (CR) represented total regression of the tumor. Partial response (PR) consisted of more than 50% reduction in primary tumor size on the CT scan. Progressive disease (PD) was defined as more than 25% increase in the primary tumor or the appearance of a new lesion. Stable disease (SD) represented cases that did not meet the criteria for PR or PD. For evaluation, CR and PR cases were grouped together into a response group, while the SD and PD cases were combined as a no response group. The relationship between plasma miRNA levels and response to radiotherapy was examined. There was no significant relationship between expression level of miR-16, miR-21, miR-185, or miR-375 and immediate treatment response to radiotherapy (P = 1.000, P = 1.000, P = 0.740, and P = 0.179, respectively; Table 3).

Impact of Plasma Circulating MiRNA on OS and PFS

After a median 22 months (range, 4-95 months) of follow-up, the 3-year OS and PFS rates were 18.11% and 13.78%, respectively. Our data showed that high levels of miR-16 and miR-21 in the plasma correlated significantly with shortened PFS (P = 0.031 and P = 0.038, respectively; Figure 3A, 3B) and OS (P = 0.022 and P = 0.041, respectively; Figure 4A, 4B)
patients with ESCC who received radiotherapy. However, plasma levels of miR-185 and miR-375 did not correlate with prognosis (Figures 3C, 3D and 4C, 4D).

**Discussion**

miRNA expression profiling has been shown to classify tissue and tumor type accurately. While analysis of miRNA expression in biopsy samples may be associated with sampling errors and invasiveness, the quantitation of plasma miRNA levels is less invasive, simpler and can monitor tumor dynamics. Some recent reports have focused on the potential of miRNA levels in the plasma as a diagnostic and prognostic marker for malignancy [12, 13]. In this study, we examined selected plasma miRNA profile in ESCC using qRT-PCR assays. A new panel of four miRNAs (miR-16, miR-21, miR-185, and miR-375) was identified that could clearly differentiate ESCC patients from normal controls, while specific miRNAs such as miR-16 and miR-21 could predict poor survival.

### Table 3. Relationship between plasma miRNA levels and immediate response to radiotherapy

<table>
<thead>
<tr>
<th>Clinical response</th>
<th>miR-16</th>
<th></th>
<th>miR-21</th>
<th></th>
<th>miR-185</th>
<th></th>
<th>miR-375</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>P</td>
<td>High</td>
<td>Low</td>
<td>P</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>CR-PR</td>
<td>12</td>
<td>12</td>
<td>1.000</td>
<td>12</td>
<td>12</td>
<td>1.000</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>SD-PD</td>
<td>7</td>
<td>7</td>
<td></td>
<td>7</td>
<td>7</td>
<td></td>
<td>12</td>
<td>11</td>
</tr>
</tbody>
</table>

CR, complete response; PR, partial response; SD, stable disease; PD, progress disease.
Several reports have demonstrated that miRNA is consistently detectable in the plasma. Tumor-derived exosomes serve as vehicles of intercellular communication and contain resourceful miRNA, the circulating exosome constituting the main portion of plasma miRNA [14, 15]. Therefore, it may be considered that the circulating miRNA level in the plasma may reflect tumor tissue miRNA expression. Furthermore, blood sampling can overcome the problems associated with biopsy sampling, and collecting blood samples can be less invasive than performing a biopsy.

In the current study, we focused on plasma miRNA expression levels to determine their utility as markers for predicting the clinical characteristics, response to treatment, and prognosis in patients with ESCC who received radiotherapy. We based our selection of the nine miRNAs (miR-16, miR-21, miR-22, miR-126, miR-148b, miR-185, miR-221, miR-223, and miR-375) on our interpretation of the published literature.

There is a consensus that changes in the expression of miR-21 are important in cancer development. miR-21 is widely accepted to be an oncogenic miRNA, and is up-regulated in a variety of human tumors. miR-21 has anti-apoptotic activity, and other reports have demonstrated that its expression is correlated with poor clinical outcomes in esophageal cancer [12, 16]. The targets of miR-21 are tumor and metastasis suppressor genes, including tumor suppressor gene tropomyosin 1 (TPM1) [17], phosphatase and tensin homologue (PTEN) [18] and programmed cell death-4 (PDCD4) [19], which are involved in tumor growth, invasion, and metastasis. Our study also revealed that the level of plasma miR-21 is higher in ESCC patients than in healthy volunteers (P = 0.017, Figure 1B) and that high plasma miR-21
levels predicted significantly poorer PFS and OS (P = 0.038 and P = 0.041, Figures 3B and 4B), which is concordant with the consensus.

Although it has been reported that in some malignancies such as non-small cell lung cancer and prostate cancer [20, 21], the expression level of miR-21 may be used as a biomarker to predict chemotherapy response, whether it is also a useful biomarker for response to radiotherapy in ESCC remains controversial. Our preliminary analysis of the available data did not show any association between the level of plasma miR-21 or other selected miRNA and radiotherapy response. However, considering our limited number of patients, further studies are warranted to confirm the association of plasma miRNA level with radiotherapy response in patients with ESCC.

In contrast, the role of miR-16 in cancer is somewhat unclear. Several studies have suggested that the expression of miR-16 is altered in human malignancies, such as ovarian cancer, non-small cell lung cancer, and bladder cancer [22-24]. Furthermore, miR-16 has been shown to participate in multiple pathways by regulating different genes; possible downstream targets include reversion-inducing-cysteine-rich protein with kazal motifs (RECK) and SOX6, Vacuolar Protein Sorting 4a, B-cell lymphoma 2 (BCL2) and the nuclear factor-kappa B1/matrix metallopeptidase 9 (MMP9) [25-27], which could suppress cell apoptosis while promoting growth. Our current study demonstrated that patients with ESCC have higher plasma levels of miR-16 than healthy subjects. Furthermore, patients’ miR-16 plasma levels correlated significantly with the degree of cancer invasion, with plasma miR-16 levels being higher in patients with T3-4 tumors than in those with T1-2 tumors (P = 0.020, Table 2). All the data described are consistent with the current literature. In addition, to the best of our knowledge, there are no reports confirming the plasma level of miR-16 as a circulating blood biomarker for ESCC. Our current study is the first to demonstrate that high levels of miR-16 in the plasma correlated significantly with shortened PFS and OS (P = 0.031 and P = 0.022, Figures 3A and 4A) in ESCC. This suggests that plasma levels of miR-16 not only show satisfactory diagnostic performance in early detection, but are a useful blood prognosis biomarker for patients with ESCC who have undergone radiotherapy.

There are some limitations inherent in estimating plasma miRNA. First, the origin of circulating miRNA in the blood is controversial. Usually, miRNA is considered to be released from the cancer cell by exosomes serving as vehicles. In our study, we found that plasma miR-16 levels were higher in patients with T3-4 tumors than T1-2 tumors, which suggests that circulating miR-16 levels may reflect tumor burden and that circulating miR-16 may originate from cancer cells. On the other hand, noncancerous tissue can also derive miRNA. A recent study has shown that miR-21 expression level in tumor stroma was a predictor for squamous cell carcinoma prognosis [28], suggesting that circulating plasma miRNA may be an indirect tumor predictor. Furthermore, some previous studies have demonstrated that circulating miRNA levels do not always correlate with miRNA expression from the primary tumor [29, 30]. Further studies are warranted to confirm the association of circulating miRNA levels with primary tumor miRNA expression in ESCC. A second limitation is that we only evaluated nine selected miRNAs based on the published literature, meaning that other significant miRNAs might be overlooked by this approach. Thus, array-based studies on a larger patient cohort are warranted to fully evaluate the impact. Nevertheless, our study has shown that four of our selected circulating miRNAs do correlate with ESCC.

In conclusion, we found that the level of plasma miR-16, miR-21, miR-185, and miR-375 correlated with patients who presented with ESCC. The plasma level of miR-16 has the potential to support tumor staging, while a higher level of plasma miR-16 and miR-21 suggests a poor prognosis in ESCC patients who have received radiotherapy. These blood biomarkers might provide additional information to complement conventional clinical staging and improve the rationality of treatment programs. Future work should include array studies and the evaluation of a broader range of miRNAs in larger patients cohorts to identify possible plasma miRNA markers and possible downstream gene targets.

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Disclosure of conflict of interest

None.

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