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-22, miR-126, miR-148b, miR-185, miR-221, miR-223, 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/m^2/day) on Days 1-5 in combination with cisplatin (30 mg/m^2/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). The
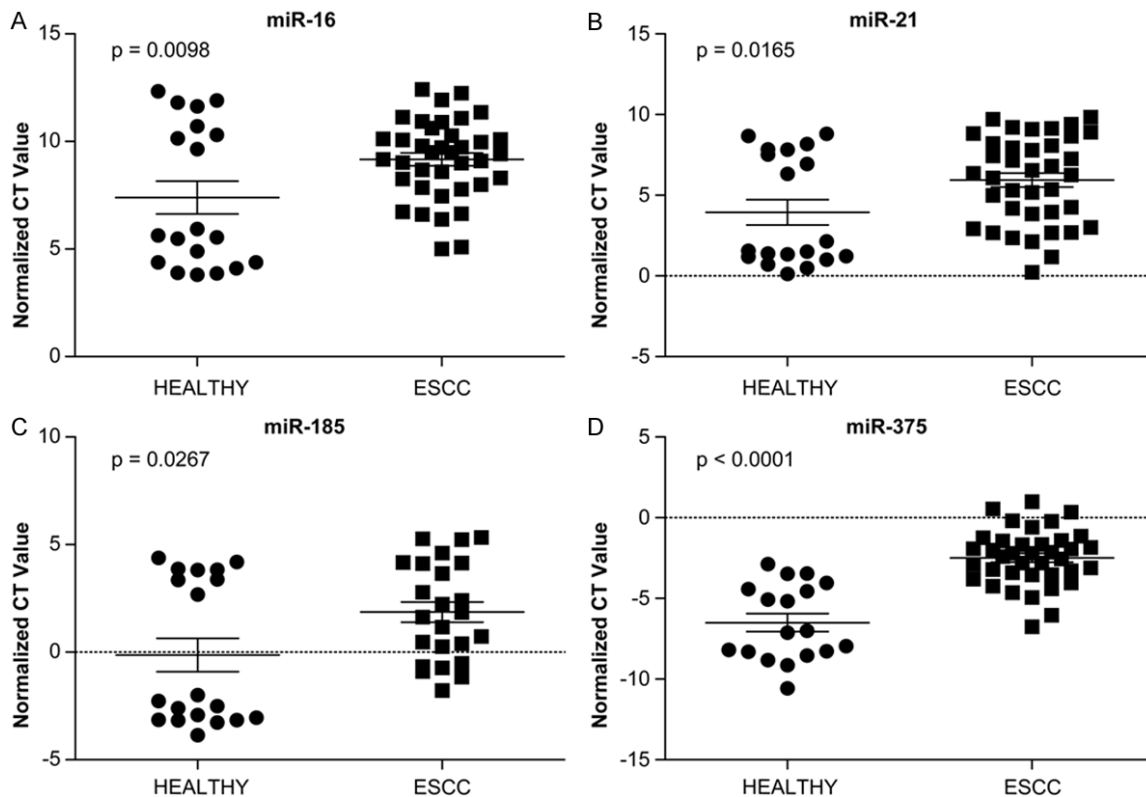
Figure 1. Comparison of plasma miRNA levels in ESCC patients and healthy volunteers. The plasma levels of miR-16 (A), miR-21 (B), miR-185 (C), and miR-375 (D) were significantly higher in patients with ESCC than in healthy volunteers ($P = 0.010$, $P = 0.017$, $P = 0.027$, and $P < 0.001$, respectively). ESCC: esophageal squamous cell carcinoma.

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

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

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

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 (ΔCt = Ct_{1228} miRNA - Ct_{target} miRNA). A lower ΔCt value referred to a lower expression of target miRNA. These reactions were run in triplicate.

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-
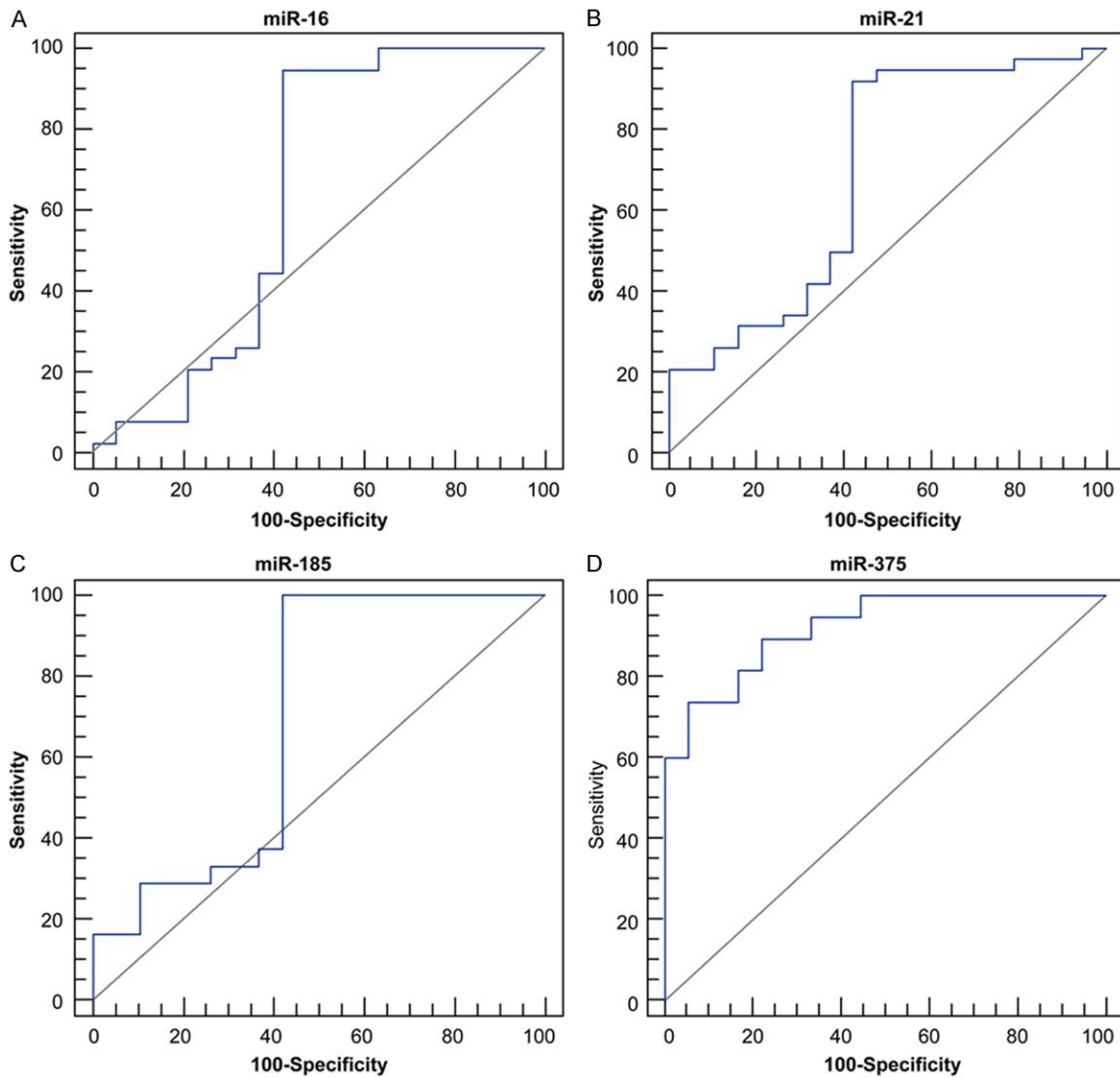
Figure 2. ROC curve analysis of the candidate miRNAs in discriminating the healthy group from ESCC patients. A-D: AUC estimation for plasma miR-16, miR-21, miR-185 and miR-375 levels, respectively, in discriminating the healthy group from ESCC patients.

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

Table 2. Correlations between plasma miRNA levels and clinical characteristics

Characteristic	miR-16			miR-21			miR-185			miR-375		
	High	Low	P-value	High	Low	P-value	High	Low	P-value	High	Low	P-value
Age (years)												
≥ 65 years	10	11	0.744	11	10	0.744	10	11	0.744	11	10	0.744
< 65 years	9	8		8	9		9	8		8	9	
Gender												
Male	16	14	0.426	17	13	0.112	7	8	0.740	15	15	0.740
Female	3	5		2	6		12	11		4	4	
Tumor size												
≥ 5 cm	8	7	0.740	8	7	0.740	7	8	0.740	6	9	0.320
< 5 cm	11	12		11	12		12	11		13	10	
Clinical T-stage												
T1-T2	4	11	0.020*	5	10	0.100	11	7	0.194	7	8	0.740
T3-T4	15	8		14	9		8	12		12	11	
Clinical N-stage												
N0	10	8	0.516	10	8	0.516	10	9	0.746	11	7	0.194
N1	9	11		9	11		9	10		8	12	
Distant metastasis												
M0	17	13	0.112	17	13	0.112	10	8	0.519	16	14	0.426
M1	2	6		2	6		9	11		3	5	
Pathological stage												
I-II	9	10	0.746	10	9	0.746	11	8	1.000	10	9	0.746
III-IV	10	9		9	10		11	8		9	10	

*$P < 0.05$, as determined by Pearson's χ^2 test.

= 19). 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**) in

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

Clinical response	miR-16			miR-21			miR-185			miR-375		
	High	Low	P	High	Low	P	High	Low	P	High	Low	P
CR-PR	12	12	1.000	12	12	1.000	7	8	0.740	10	14	0.179
SD-PD	7	7		7	7		12	11		9	5	

CR, complete response; PR, partial response; SD, stable disease; PD, progress disease.
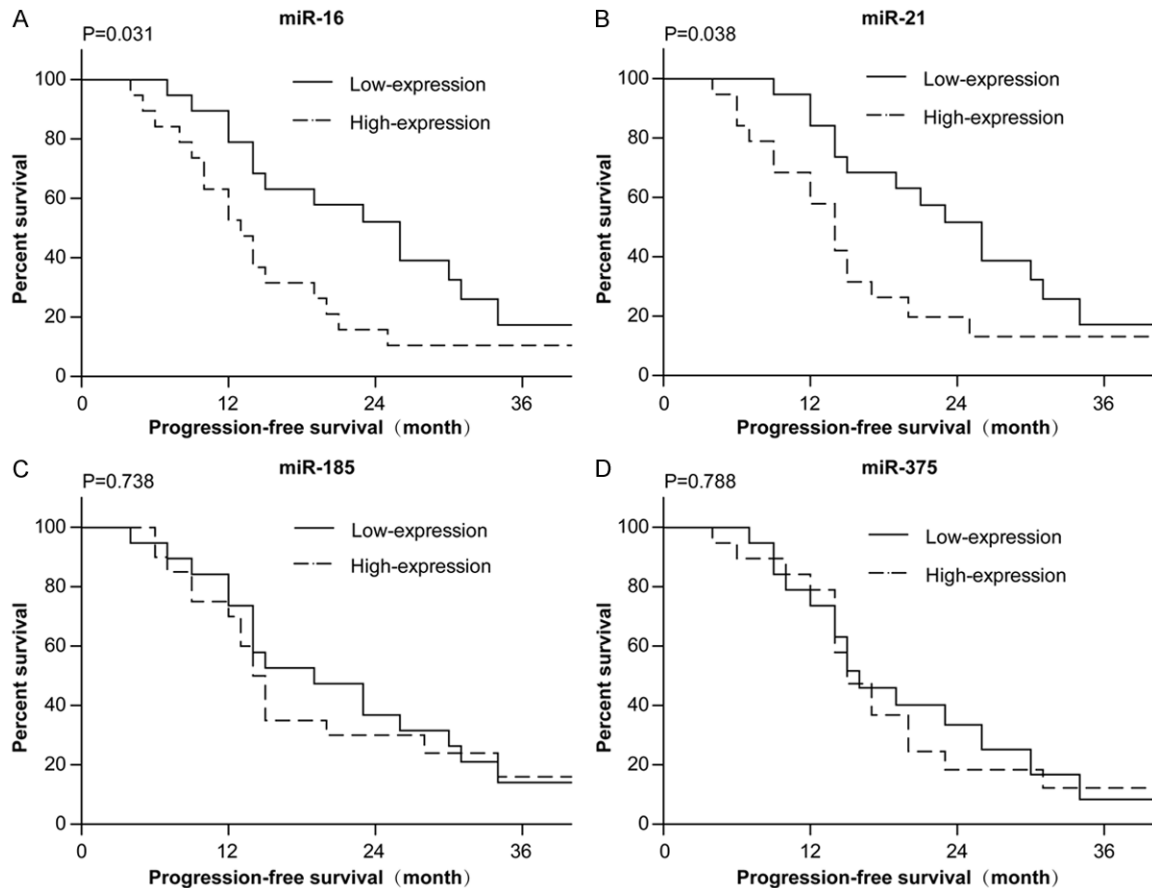
Figure 3. PFS curves for 38 patients with ESCC according to plasma levels of miR-16, miR-21, miR-185, and miR-375. A, B. Patients with low plasma levels of miR-16 and miR-21 demonstrated significantly longer PFS (P = 0.031 and P = 0.038, respectively) than other patients; C, D. Plasma expression of miR-185 and miR-375 was not significantly associated with PFS.

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 form normal controls, while specific miRNAs such as miR-16 and miR-21 could predict poor survival.
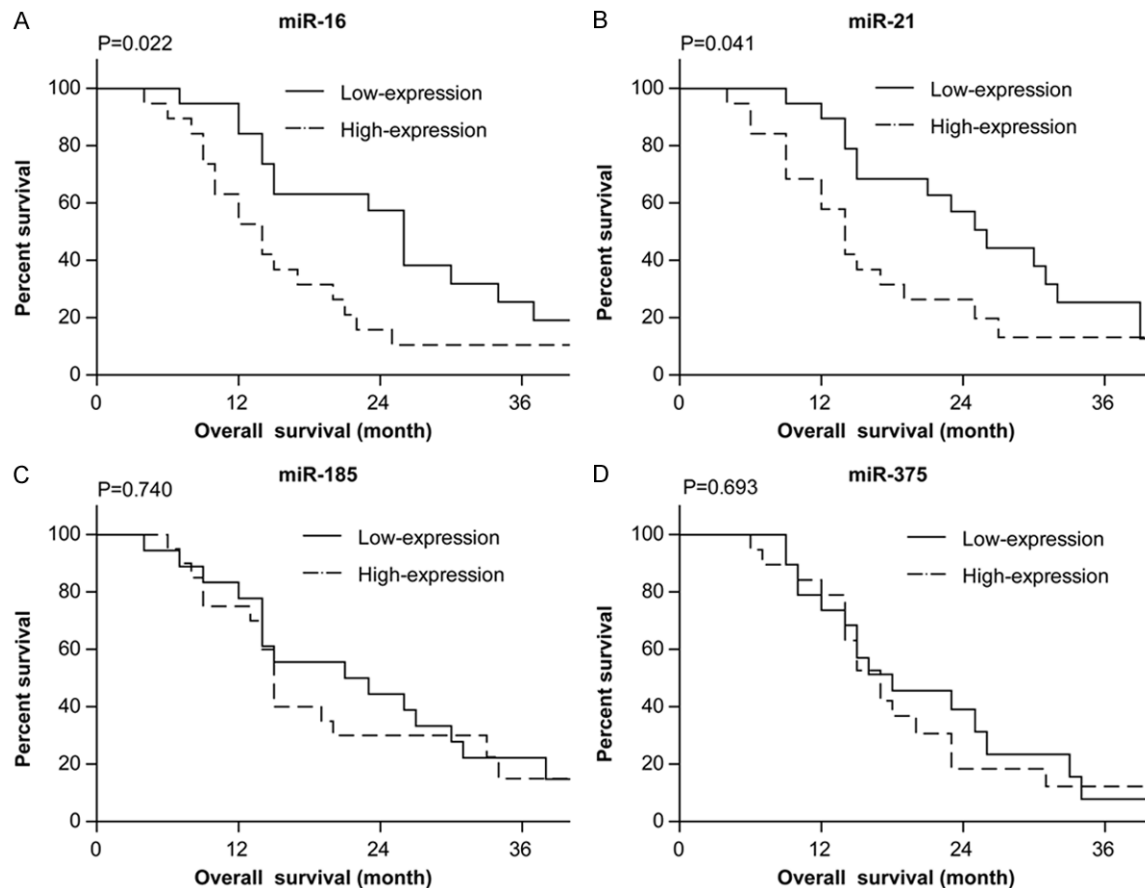
Figure 4. OS curves for 38 patients with ESCC according to plasma levels of miR-16, miR-21, miR-185, and miR-375. A, B. Patients with low plasma levels of miR-16 and miR-21 demonstrated significantly longer OS (P = 0.022 and P = 0.041, respectively) than other patients. C, D. Plasma expression of miR-185 and miR-375 was not significantly associated with the OS.

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.

Acknowledgements

Disclosure of conflict of interest

None.

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References

[1]   Lu SH. Alterations of oncogenes and tumor suppressor genes in esophageal cancer in China. Mutat Res 2000; 462: 343-353.

[2]   Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[3]   Zhang B, Pan X, Cobb GP and Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol 2007; 302: 1-12.

[4]   Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR and Golub TR. MicroRNA expression profiles classify human cancers. Nature 2005; 435: 834-838.

[5]   Hamano R, Miyata H, Yamasaki M, Sugimura K, Tanaka K, Kurokawa Y, Nakajima K, Takiguchi S, Fujiwara Y, Mori M and Doki Y. High expression of Lin28 is associated with tumour aggressiveness and poor prognosis of patients in oesophagus cancer. Br J Cancer 2012; 106: 1415-1423.

[6]   Liu N, Chen NY, Cui RX, Li WF, Li Y, Wei RR, Zhang MY, Sun Y, Huang BJ, Chen M, He QM, Jiang N, Chen L, Cho WC, Yun JP, Zeng J, Liu LZ, Li L, Guo Y, Wang HY and Ma J. Prognostic value of a microRNA signature in nasopharyngeal carcinoma: a microRNA expression analysis. Lancet Oncol 2012; 13: 633-641.

[7]   Gao G, Gay HA, Chernock RD, Zhang TR, Luo J, Thorstad WL, Lewis JS Jr and Wang X. A microRNA expression signature for the prognosis of oropharyngeal squamous cell carcinoma. Cancer 2013; 119: 72-80.

[8]   Konishi H, Ichikawa D, Komatsu S, Shiozaki A, Tsujiura M, Takeshita H, Morimura R, Nagata H, Arita T, Kawaguchi T, Hirashima S, Fujiwara H, Okamoto K and Otsuji E. Detection of gastric cancer-associated microRNAs on microRNA microarray comparing pre- and post-operative plasma. Br J Cancer 2012; 106: 740-747.

[9]   Tomimaru Y, Eguchi H, Nagano H, Wada H, Kobayashi S, Marubashi S, Tanemura M, Tomokuni A, Takemasa I, Umeshita K, Kanto T, Doki Y and Mori M. Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. J Hepatol 2012; 56: 167-175.

[10]   Heegaard NH, Schetter AJ, Welsh JA, Yoneda M, Bowman ED and Harris CC. Circulating micro-RNA expression profiles in early stage non-small cell lung cancer. Int J Cancer 2012; 130: 1378-1386.

[11]   Rice TW, Blackstone EH and Rusch VW. 7th edition of the AJCC Cancer Staging Manual: esophagus and esophagogastric junction. Ann Surg Oncol 2010; 17: 1721-1724.

[12]   Tanaka K, Miyata H, Yamasaki M, Sugimura K, Takahashi T, Kurokawa Y, Nakajima K, Takiguchi S, Mori M and Doki Y. Circulating miR-200c levels significantly predict response to chemotherapy and prognosis of patients undergoing neoadjuvant chemotherapy for esophageal cancer. Ann Surg Oncol 2013; 20 Suppl 3: S607-615.

[13]   Li X, Jiang C, Wu X, Sun Y, Bu J, Li J, Xiao M, Zheng Y and Zhang J. A systems biology approach to study the biology characteristics of esophageal squamous cell carcinoma by integrating microRNA and messenger RNA expression profiling. Cell Biochem Biophys 2014; 70: 1369-1376.

[14]   Ye SB, Li ZL, Luo DH, Huang BJ, Chen YS, Zhang XS, Cui J, Zeng YX and Li J. Tumor-derived exosomes promote tumor progression and T-cell dysfunction through the regulation of enriched exosomal microRNAs in human nasopharyngeal carcinoma. Oncotarget 2014; 5: 5439-5452.

[15]   Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y and Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem 2010; 285: 17442-17452.

[16]   Lynam-Lennon N, Maher SG and Reynolds JV. The roles of microRNA in cancer and apoptosis. Biol Rev Camb Philos Soc 2009; 84: 55-71.

[17]   Zhu S, Si ML, Wu H and Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem 2007; 282: 14328-14336.

[18]   Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST and Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor

gene in human hepatocellular cancer. Gastroenterology 2007; 133: 647-658.

[19] Hiyoshi Y, Kamohara H, Karashima R, Sato N, Imamura Y, Nagai Y, Yoshida N, Toyama E, Hayashi N, Watanabe M and Baba H. MicroRNA-21 regulates the proliferation and invasion in esophageal squamous cell carcinoma. Clin Cancer Res 2009; 15: 1915-1922.

[20] Wei J, Gao W, Zhu CJ, Liu YQ, Mei Z, Cheng T and Shu YQ. Identification of plasma microRNA-21 as a biomarker for early detection and chemosensitivity of non-small cell lung cancer. Chin J Cancer 2011; 30: 407-414.

[21] Zhang HL, Yang LF, Zhu Y, Yao XD, Zhang SL, Dai B, Zhu YP, Shen YJ, Shi GH and Ye DW. Serum miRNA-21: elevated levels in patients with metastatic hormone-refractory prostate cancer and potential predictive factor for the efficacy of docetaxel-based chemotherapy. Prostate 2011; 71: 326-331.

[22] Ying H, Lv J, Ying T, Li J, Yang Q and Ma Y. MicroRNA and transcription factor mediated regulatory network for ovarian cancer: regulatory network of ovarian cancer. Tumour Biol 2013; 34: 3219-3225.

[23] Ke Y, Zhao W, Xiong J and Cao R. Downregulation of miR-16 promotes growth and motility by targeting HDGF in non-small cell lung cancer cells. FEBS Lett 2013; 587: 3153-3157.

[24] Jiang QQ, Liu B and Yuan T. MicroRNA-16 inhibits bladder cancer proliferation by targeting Cyclin D1. Asian Pac J Cancer Prev 2013; 14: 4127-4130.

[25] Zhu Y, Xia Y, Niu H and Chen Y. MiR-16 induced the suppression of cell apoptosis while promote proliferation in esophageal squamous cell carcinoma. Cell Physiol Biochem 2014; 33: 1340-1348.

[26] Adhikari N, Guan W, Capaldo B, Mackey AJ, Carlson M, Ramakrishnan S, Walek D, Gupta M, Mitchell A, Eckman P, John R, Ashley E, Barton PJ and Hall JL. Identification of a new target of miR-16, Vacuolar Protein Sorting 4a. PLoS One 2014; 9: e101509.

[27] Yang TQ, Lu XJ, Wu TF, Ding DD, Zhao ZH, Chen GL, Xie XS, Li B, Wei YX, Guo LC, Zhang Y, Huang YL, Zhou YX and Du ZW. MicroRNA-16 inhibits glioma cell growth and invasion through suppression of BCL2 and the nuclear factor-kappaB 1/MMP9 signaling pathway. Cancer Sci 2014; 105: 265-271.

[28] Mathe EA, Nguyen GH, Bowman ED, Zhao Y, Budhu A, Schetter AJ, Braun R, Reimers M, Kumamoto K, Hughes D, Altorki NK, Casson AG, Liu CG, Wang XW, Yanaihara N, Hagiwara N, Dannenberg AJ, Miyashita M, Croce CM and Harris CC. MicroRNA expression in squamous cell carcinoma and adenocarcinoma of the esophagus: associations with survival. Clin Cancer Res 2009; 15: 6192-6200.

[29] Pigati L, Yaddanapudi SC, Iyengar R, Kim DJ, Hearn SA, Danforth D, Hastings ML and Duelli DM. Selective release of microRNA species from normal and malignant mammary epithelial cells. PLoS One 2010; 5: e13515.

[30] Ohshima K, Inoue K, Fujiwara A, Hatakeyama K, Kanto K, Watanabe Y, Muramatsu K, Fukuda Y, Ogura S, Yamaguchi K and Mochizuki T. Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. PLoS One 2010; 5: e13247.