

Original Article
Gastroenterology &
Hepatology



MicroRNA Expression in Plasma of Esophageal Squamous Cell Carcinoma Patients

Dong Hwahn Kahng ,¹ Gwang Ha Kim ,^{1,2} Su Jin Park ,² Sora Kim ,³ Moon Won Lee ,¹ Bong Eun Lee ,¹ and Hoseok I ⁴

¹Department of Internal Medicine, Pusan National University College of Medicine, Busan, Korea

²Biomedical Research Institute, Pusan National University Hospital, Busan, Korea

³Department of Convergence Medical Sciences, Pusan National University Graduate School of Medicine, Yangsan, Korea

⁴Department of Thoracic Surgery, Pusan National University College of Medicine, Busan, Korea



Received: Jan 8, 2022

Accepted: May 16, 2022

Published online: Jun 8, 2022

Address for Correspondence:

Gwang Ha Kim, MD, PhD

Department of Internal Medicine, Pusan National University College of Medicine, and Biomedical Research Institute, Pusan National University Hospital, 179 Gudeok-ro, Seo-gu, Busan 49241, Korea.
Email: doc0224@pusan.ac.kr

© 2022 The Korean Academy of Medical Sciences.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<https://creativecommons.org/licenses/by-nc/4.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

ORCID iDs

Dong Hwahn Kahng
<https://orcid.org/0000-0003-1772-5406>
Gwang Ha Kim
<https://orcid.org/0000-0001-9721-5734>
Su Jin Park
<https://orcid.org/0000-0001-9880-9825>
Sora Kim
<https://orcid.org/0000-0001-7697-5849>
Moon Won Lee
<https://orcid.org/0000-0002-8411-6398>
Bong Eun Lee
<https://orcid.org/0000-0003-2734-2134>

ABSTRACT

Background: Patients with esophageal squamous cell carcinoma (ESCC) have a poor prognosis and there are no effective clinical biomarkers. Recently, stable microRNAs detected in the blood have been suggested as potential biomarkers in various cancers. Therefore, we investigated whether plasma microRNAs could be feasible biomarkers for ESCC.

Methods: Peripheral blood samples were obtained from 16 healthy volunteers and 66 ESCC patients before treatment between May 2016 and April 2021. Plasma miR-18b, miR-21, miR-31, and miR-375 expression levels were measured using reverse transcription-quantitative polymerase chain reaction.


Results: Compared with those in healthy controls, the expression levels of plasma miR-21 were significantly higher ($P = 0.022$) and those of plasma miR-31 and miR-375 were significantly lower in ESCC patients (both $P < 0.001$). Plasma miR-18b expression levels increased in ESCC patients, but the difference was not significant ($P = 0.164$). The sensitivities and specificities of miR-21, miR-31, and miR-375 for differentiating ESCC patients from healthy controls were 87.5% and 61.9%, 87.5% and 98.4%, and 87.5% and 100%, respectively. There was no difference in expression levels of plasma miR-21, miR-31, and miR-375 according to clinicopathological characteristics of sex, age, tumor size and location, histologic grade, and tumor-node-metastasis stage.

Conclusion: Our study demonstrated that plasma miR-21, miR-31, and miR-375 could be potential biomarkers for the diagnosis of ESCC. Particularly, plasma miR-31 and miR-375 showed high sensitivity and specificity for differentiating ESCC patients from healthy controls.

Keywords: Esophageal Neoplasms; MicroRNA; Plasma; Squamous Cell Carcinoma

INTRODUCTION

Esophageal cancer is the 10th most common cancer worldwide and the 6th leading cause of cancer-related mortality.¹ Histopathologically, esophageal cancer is mainly classified as squamous cell carcinoma (SCC) and adenocarcinoma. SCC constitutes > 90% of all esophageal

Hoseok I <https://orcid.org/0000-0001-8930-8148>**Funding**

This study was supported by the Korean College of Helicobacter and Upper Gastrointestinal Research Foundation Grant (KCHUGR-202002503). The biospecimens and data used in this study were provided by the Biobank of Pusan National University Hospital, a member of the Korea Biobank network.

Author Contributions

Conceptualization: Kim GH, I H. Data curation: Kahng DH, Kim GH, Kim S, Lee MW, I H. Formal analysis: Kim GH, Park SJ, Lee MW. Investigation: Kim S, Lee MW, Lee BE. Methodology: Park SJ, Kim S. Project administration: Kim GH. Software: Kahng DH, Park SJ. Supervision: Kim GH. Validation: Lee BE, I H. Visualization: Lee MW. Writing - original draft: Kahng DH, Kim GH. Writing - review & editing: Kahng DH, Kim GH, Park SJ, Kim S, Lee MW, Lee BE, I H.

cancer cases in East Asian countries, including Korea, Japan, and China and the overall 5-year survival rate remains 25–30%.^{2,3} However, patients with mucosal or submucosal cancer without lymph node metastasis have a > 80% survival rate.⁴ Therefore, many attempts have been made to ensure early detection and intervention to increase the curability of treatment, which prolongs the survival of patients with esophageal squamous cell carcinoma (ESCC). Currently, ESCC is diagnosed by examination of endoscopic forceps biopsy or resection specimens, but these approaches cannot be easily performed because of their invasiveness and high cost.^{5,6} These specimens do not represent the intra-tumoral heterogeneity and mechanisms of tumor progression and resistance to treatment.⁷ Although non-invasive tumor markers such as SCC antigen and carcinoembryonic antigen have been used for early detection and monitoring tumor dynamics of ESCC in clinical settings, they do not have sufficient sensitivity and specificity for early diagnosis and tumor progression.⁸ Therefore, the identification of novel and effective biomarkers for early detection and prediction of tumor progression is important to improve clinical outcomes for ESCC patients.

MicroRNAs (miRNAs) are small non-coding RNAs (18–25 nucleotides long) that can function as oncogenes or tumor suppressors by translational repression or degradation of the target mRNAs.⁹ miRNAs are stably detectable in plasma and serum,¹⁰ and tumor-derived miRNAs are resistant to endogenous ribonuclease activity because they are packaged by several secretory particles such as exosomes and apoptotic bodies in blood.^{11,12} Accordingly, circulating miRNAs can be present in a stable form, and their expression level is reproducible and consistent among subjects.¹³ miRNAs contribute to carcinogenesis and could provide new therapeutic strategies such as biomarkers and therapeutic targets for cancer. Numerous studies have demonstrated the role of miRNAs in the tumorigenesis and progression of various cancers and the potential of non-invasive biomarkers for diagnosis or prognosis of cancers.^{14,15} Several studies have reported the potential application of circulating miRNAs in ESCC^{16,17}; however, the results are inconsistent because of the differences in research methods and included populations among studies.

Therefore, in order to find miRNAs that can be generalized for use in clinical practice, we selected four miRNAs (miR-18b, miR-21, miR-31, and miR-375) from the published academic literature as candidate targets for the plasma miRNA assay.^{18–22} We hypothesized that these plasma miRNAs would be identified as potentially useful biomarkers in ESCC patients. Therefore, we investigated whether a plasma miRNA assay would be useful for differentiating ESCC patients from healthy controls. We then determined whether the plasma miRNA levels were associated with the clinicopathological characteristics of ESCC patients.

METHODS

Patients

Between May 2016 and April 2021, 16 healthy volunteers and 66 patients histopathologically diagnosed with ESCC who had not been diagnosed with other past or current malignancies were enrolled in the study at Pusan National University Hospital (Busan, Korea). The patients' medical history, physical examination, complete blood count and biochemistry tests, endoscopic ultrasonography, and imaging studies using chest and abdominal computed tomography (CT) and/or positron-emission tomography with CT for tumor staging were investigated. Staging was assessed according to the American Joint Committee on Cancer tumor-node-metastasis (TNM) staging for esophageal cancer (8th edition).²³

Treatment modalities

Treatment was individualized according to the initial clinical TNM stage at the time of diagnosis. Five of the 66 patients underwent neoadjuvant chemotherapy and additional surgical resection, whereas 12, 14, 18, 15, and 2 patients underwent surgical resection, concurrent chemoradiation, palliative chemotherapy, endoscopic submucosal dissection (ESD), and conservative treatment, respectively. In appropriately selected superficial ESCC patients, ESD was performed under conscious sedation.²⁴ In patients with resectable esophageal cancer that was unsuitable for ESD, the Ivor Lewis esophagogastrectomy or 3-hole minimally invasive esophagectomy was performed.²⁴ In patients with locally advanced or metastatic cancer, neoadjuvant or palliative chemotherapy with 5-fluorouracil and cisplatin was administered. Some patients underwent concurrent chemoradiation comprising two cycles of combined 5-fluorouracil and cisplatin with 50 Gy of radiotherapy in the same period.

RNA extraction

After obtaining informed consent, 3 mL peripheral blood was collected from healthy controls and ESCC patients before initial treatment with surgery, ESD, chemotherapy, or chemoradiation. Cell-free plasma was separated from blood samples within 2 hours after collection using a one-spin protocol (800 ×g force for 10 minutes at 4°C) to prevent contamination by cellular nucleic acids. Plasma samples were stored at -80°C until miRNA analysis.

Total RNA was extracted from 400 µL plasma samples using the mirVana PARIS™ (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Briefly, to isolate miRNA from the blood samples, an equal volume of 2× denaturing solution was added to 400 µL plasma at 16°C, and the mixture was incubated for 5 minutes on ice. Next, 650 µL acid-phenol was added, and the tube was vigorously agitated for 10 seconds. After centrifugation at 10,000 ×g for 10 minutes at 4°C, the supernatant was transferred to a new tube and 1.25 times of ethanol was added. Following incubation for 5 minutes at 16°C, for each sample, a filter cartridge was placed into one of the collection tubes, 600 µL of the mixture was added into the filter cartridge, and the sample was centrifuged at 10,000 ×g for 30 seconds at 4°C. Then, 600 µL of wash solution 1 was added to the filter cartridge and the sample was centrifuged at 10,000 ×g for 30 seconds at 4°C. Next, 500 µL of wash solution 2/3 was added and the sample was centrifuged at 10,000 ×g for 30 seconds at 4°C. Then, 500 µL of wash solution 2/3 was added and the sample was centrifuged at 10,000 ×g for 1 minute at 4°C. The filter cartridge was then transferred to a new collection tube. Finally, total miRNA was eluted into 50 µL elution solution according to the manufacturer's protocol, and was stored at -80°C after centrifugation at 10,000 ×g for 1 minute.

Reverse transcription (RT)-quantitative polymerase chain reaction (qPCR) of miRNAs

RT was conducted using a TaqMAN miRNA RT kit (Applied Biosystems, Foster City, CA, USA). The reaction system was 15 µL, containing 5 µL RNA extract, 1.5 µL 10× RT buffer, 0.2 µL RNase inhibitor (20 U/µL), 1 µL reverse transcriptase (50 U/µL), 0.15 µL 100 mM deoxyribonucleotide triphosphates, 3 µL specific miRNA primer (Table 1: miR-18b, miR-21, miR-31, miR-375, and miR-191), and 4.15 µL nuclease-free water. For the complementary DNA (cDNA) synthesis, the reaction mixtures were incubated at 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes.

Subsequently, RT-qPCR was performed using the RG-6000 Real Time PCR (Corbett Research, Sydney, Australia) with TaqMan® Universal PCR Master Mix II, no UNG (Thermo Fisher

Table 1. MicroRNA sequences and internal reference

miRNA/gene	Sequences
miR-18b	UAAGGUGCAUCUAGUGCAGUUAG
miR-21	CAACACCAGUCGAUGGGCUGU
miR-31	UGCUAUGCCAACAUUUGCCAU
miR-375	UUUGUUCGUUCGGCUCGCGUGA
miR-191	CAACGGAAUCCCAAAGCAGCUG

Scientific). The 20 μ L reaction mixtures for the RT-qPCR contained 2 μ L cDNA, 10 μ L master mix, 1 μ L assay primer, and 7 μ L nuclease-free water. The reactions were incubated at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min. The cycle threshold (Ct) values were calculated using Rotor-Gene 6000 series software 1.7 (Corbett Research). The expression levels of miRNAs from plasma samples were normalized to those of miR-191 and calculated using the equation $2^{-\Delta\Delta Ct}$.²⁵ $\Delta\Delta Ct$ was calculated by subtracting the average Ct value of the corresponding reference miRNA from that of the miRNA of interest.

Statistical analysis

Data are expressed as median, range or interquartile range (IQR). The Mann-Whitney test was used to compare the miRNA expression levels between ESCC patients and healthy controls. Receiver-operating characteristic (ROC) curves and the area under the curve (AUC) were used to further evaluate the levels of the four miRNAs to differentiate ESCC patients from healthy controls. The sensitivity, specificity, and positive and negative predictive values of the miRNAs for differentiating ESCC patients from healthy controls were expressed using 95% confidence intervals (CIs). Differences in the four miRNAs according to clinicopathologic characteristics were analyzed using the Mann-Whitney test or Kruskal-Wallis test. All statistical analyses were performed using IBM statistical package for the social sciences (SPSS) version 22.0, for Windows (IBM Co., Armonk, NY, USA). All *P* values were two-sided, and statistical significance was set at $P < 0.05$.

Ethics statement

The study protocol was reviewed and approved by the Institutional Review Board of Pusan National University Hospital (2012-017-098), and written informed consent was obtained from all patients prior to blood sampling.

RESULTS

Baseline clinicopathologic characteristics of ESCC patients

The clinicopathologic characteristics of the 66 ESCC patients including 62 men and 4 women with a median age of 68.5 (range, 37–87) years are summarized in **Table 2**. Thirteen tumors were located in the upper third of the esophagus, whereas 31 and 22 were in the middle and lower third, respectively. The median tumor size was 4.0 (range, 0.4–10.0) cm and histopathologically, 14, 43, and 9 tumors were well, moderately, and poorly differentiated, respectively. With regard to T and N stages, 26, 2, 32, and 6 tumors were T1, T2, T3, and T4, respectively and 26, 15, 11, and 14 tumors were N0, N1, N2, and N3, respectively. Distant metastases were observed in 11 patients. For TNM stage, 24, 4, 19, and 19 patients were diagnosed with stage I, II, III, and IV disease, respectively.

Table 2. Baseline clinicopathologic characteristics of 66 patients with esophageal cancer

Characteristics	Values
Median age (range), yr	68.5 (37–87)
Sex	
Male	62
Female	4
Tumor location	
Upper third	13
Middle third	31
Lower third	22
Median tumor size (range), cm	4.0 (0.4–10.0)
Histologic grade	
Well differentiated	14
Moderately differentiated	43
Poorly differentiated	9
T stage	
T1	26
T2	2
T3	32
T4	6
N stage	
N0	26
N1	15
N2	11
N3	14
M stage	
M0	55
M1	11
TNM stage	
I	24
II	4
III	19
IV	19

Data are presented number of patients.
TNM = tumor-node-metastasis.

Expression of four plasma miRNAs in ESCC patients

The expression level of plasma miR-21 was significantly higher in ESCC patients than in healthy controls (0.95 [IQR, 0.39–3.02] vs. 0.40 [IQR, 0.27–0.77], $P = 0.022$; **Table 3, Fig. 1**). In contrast, the expression levels of plasma miR-31 and miR-725 were significantly lower in ESCC patients than in healthy controls (0.30 [IQR, 0.09–1.88] vs. 3878.76 [IQR, 1,367.05–5,294.04], $P < 0.001$ and 1.73 [IQR, 0.39–5.06] vs. 240.14 [IQR, 170.20–264.05], $P < 0.001$, respectively). The expression level of plasma miR-18b was higher in ESCC patients than in healthy controls, but the difference was not statistically significant (0.79 [IQR, 0.29–3.02] vs. 0.39 [IQR, 0.27–0.77], $P = 0.164$).

Table 3. Expression levels of four plasma microRNAs between healthy controls and patients with esophageal cancer

MicroRNA	Healthy control (n = 16)	Esophageal cancer (n = 66)	P value
miR-18b	0.39 (0.27–0.77)	0.79 (0.29–3.02)	0.164
miR-21	0.40 (0.26–0.62)	0.95 (0.42–3.35)	0.022
miR-31	3,878.76 (1,367.05–5,294.04)	0.30 (0.09–1.88)	< 0.001
miR-375	240.14 (170.20–264.05)	1.73 (0.36–5.06)	< 0.001

Values are presented as median (interquartile range).

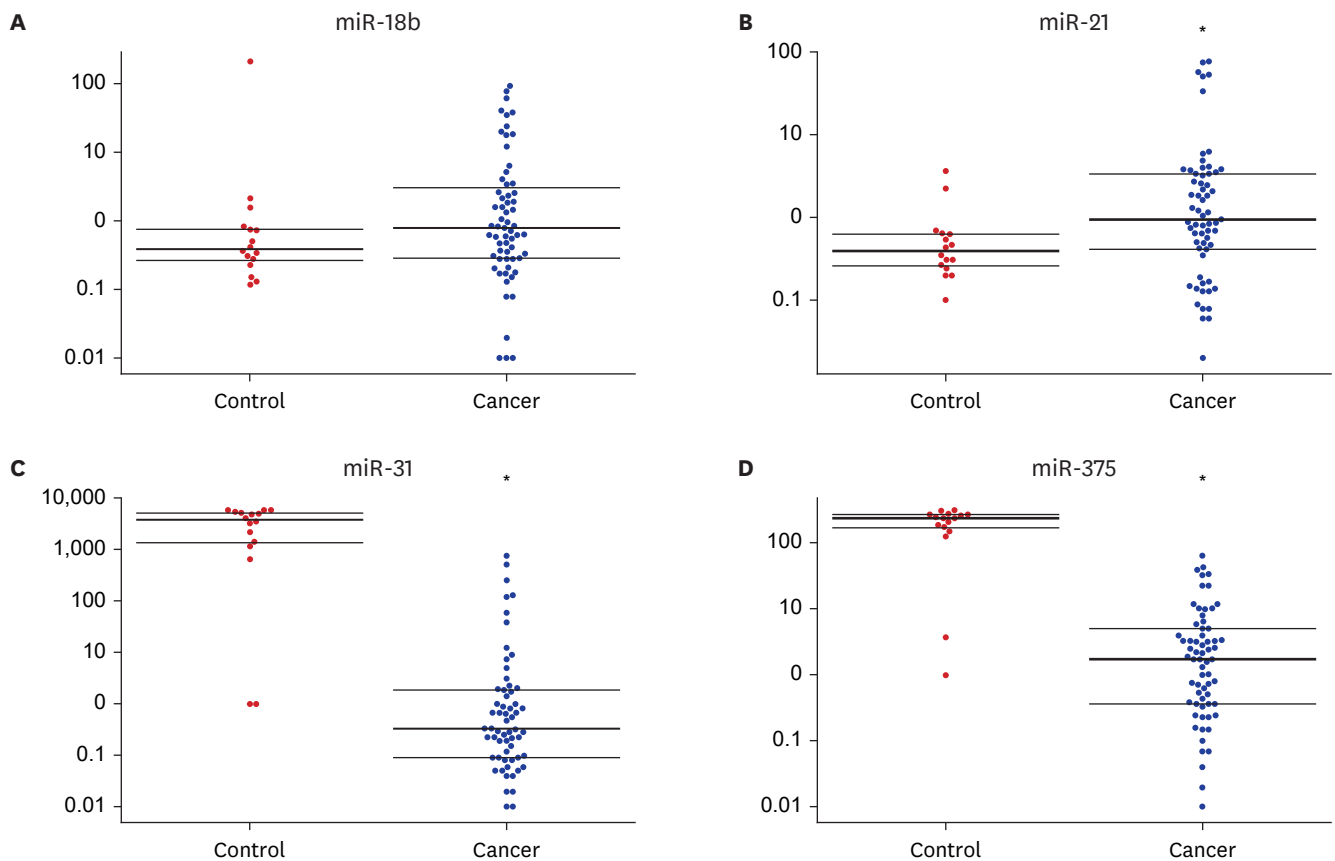


Fig. 1. Expression levels of four plasma microRNAs in 16 healthy controls and 66 patients with esophageal cancer. (A) miR-18b. (B) miR-21. (C) miR-31. (D) miR-375. Horizontal line: median with interquartile range. *P value < 0.05.

The ROC curves were analyzed to determine whether the plasma miRNAs could be used as potential diagnostic biomarkers for differentiating ESCC patients from healthy controls (Fig. 2). The AUCs were 0.613 (95% CI, 0.497–0.721) for miR-18b, 0.687 (95% CI, 0.572–0.786) for miR-21, 0.876 (95% CI, 0.781–0.940) for miR-31, and 0.920 (95% CI, 0.838–0.968) for miR-375. At the cut-off values of 0.83, 0.69, 508.46, and 63.12, the sensitivities and specificities in the diagnosis of ESCC were 81.3% and 47.6% for miR-18b, 87.5% and 61.9% for miR-21, 87.5% and 98.4% for miR-31, and 87.5% and 100% for miR-375, respectively (Table 4).

Table 5 summarizes the expression levels of the four plasma miRNAs according to the clinicopathologic characteristics of the 66 ESCC patients. There was no difference in the expression levels of the four miRNAs according to sex, age, tumor location, histologic grade, and TNM stage, except for the lower expression level of miR-18b in patients with a large tumor (> 3 cm) than in those with a small tumor (≤ 3 cm).

Table 4. Sensitivity, specificity, and positive and negative predictive values of four plasma microRNAs for differentiation of patients with esophageal cancer from healthy controls

MicroRNA	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Positive predictive value, % (95% CI)	Negative predictive value, % (95% CI)
miR-18b	81.3 (54.4–96.0)	47.6 (34.9–60.6)	28.3 (22.0–35.5)	90.9 (77.7–96.6)
miR-21	87.5 (61.7–98.4)	61.9 (48.8–73.9)	36.8 (28.8–45.7)	95.1 (84.0–98.6)
miR-31	87.5 (61.7–98.4)	98.4 (91.2–100.0)	93.3 (66.5–99.0)	96.8 (89.1–99.1)
miR-375	87.5 (61.7–98.4)	100.0 (94.5–100.0)	100.0 (not available)	97.0 (89.9–99.2)

CI = confidence interval.

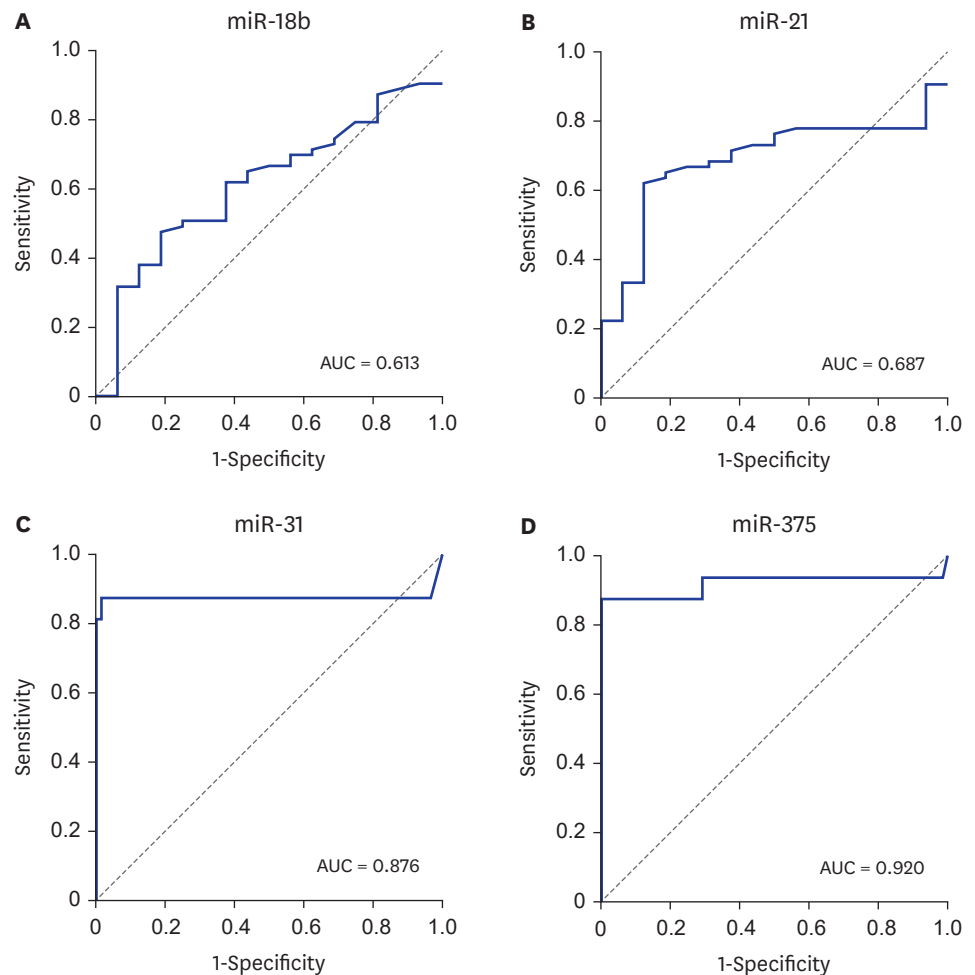


Fig. 2. Receiver-operating characteristic curve analyses of four plasma microRNAs to discriminate patients with esophageal cancer from healthy controls. (A) miR-18b. (B) miR-21. (C) miR-31. (D) miR-375. AUC = area under the curve.

DISCUSSION

The reported expression profiles of various circulating miRNAs in ESCC are inconsistent.^{18-22,26} In the present study, we selected four miRNAs and investigated their expression levels in the plasma of ESCC patients based on previously published literature. We found that plasma miR-21, miR-31, and miR-375 could be potential biomarkers for ESCC diagnosis. Particularly, miR-31 and miR-375 showed high sensitivity and specificity for differentiating ESCC patients from healthy controls.

Presently, endoscopy with forceps biopsy remains a standard modality for diagnosing ESCC, but it has several limitations such as invasiveness, the possibility of missing small lesions, and sampling error. Although the use of advanced diagnostic technologies such as narrow-band imaging can increase the detection rate of ESCC, population-based screening is ineffective because of the relatively low incidence of ESCC. Thus, endoscopic screening for ESCC is limited to individuals who are at a high risk. Considering the low invasiveness of blood sampling, circulating miRNAs could be ideal biomarker candidates.

Table 5. Expression levels of the four plasma microRNAs according to clinicopathologic characteristics of 66 patients with esophageal cancer

Characteristics	miR-18	P value	miR-21	P value	miR-31	P value	miR-375	P value
Sex		0.888		0.272		0.062		0.058
Male	0.71 (0.29–3.51)		0.88 (0.38–3.27)		0.31 (0.09–1.76)		1.71 (0.36–3.95)	
Female	1.29 (0.73–1.74)		2.56 (0.95–4.62)		0.20 (0.01–0.34)		6.64 (3.35–10.39)	
Age, yr		0.161		0.051		0.110		0.535
≤ 65	0.53 (0.26–0.97)		0.75 (0.17–2.02)		0.23 (0.06–0.69)		1.30 (0.23–5.06)	
> 65	1.46 (0.32–3.79)		1.30 (0.57–3.97)		0.60 (0.11–2.99)		1.93 (0.52–4.24)	
Tumor location		0.797		0.779		0.468		0.687
Upper third	0.85 (0.62–1.58)		1.22 (0.69–2.60)		0.34 (0.06–0.69)		2.81 (0.23–9.92)	
Middle third	0.61 (0.35–3.56)		0.85 (0.41–3.34)		0.25 (0.08–1.56)		0.92 (0.36–3.35)	
Lower third	0.83 (0.15–4.11)		1.15 (0.15–3.51)		0.29 (0.12–5.03)		2.27 (0.47–3.94)	
Tumor size, cm		0.050		0.232		0.092		0.466
≤ 3	2.00 (0.55–8.98)		1.83 (0.88–3.51)		0.69 (0.16–4.25)		2.41 (0.63–3.36)	
> 3	0.58 (0.28–1.52)		0.72 (0.37–3.30)		0.23 (0.06–0.91)		1.38 (0.24–6.35)	
Histologic grade		0.985		0.928		0.418		0.659
Well differentiated	1.06 (0.15–18.38)		1.00 (0.27–3.15)		1.31 (0.12–6.90)		2.52 (0.46–5.16)	
Moderately differentiated	0.64 (0.35–2.14)		1.22 (0.35–3.71)		0.27 (0.08–0.89)		1.64 (0.23–4.77)	
Poorly differentiated	0.85 (0.28–3.56)		0.81 (0.67–1.34)		0.29 (0.12–0.83)		2.13 (0.75–3.95)	
T stage		0.569		0.678		0.277		0.143
T1	1.46 (0.38–13.57)		1.72 (0.60–3.62)		0.69 (0.16–7.88)		3.16 (0.75–5.10)	
T2	0.85 (0.46–1.23)		0.77 (0.63–0.91)		0.19 (0.12–0.26)		6.26 (4.42–8.09)	
T3	0.62 (0.31–3.08)		0.80 (0.27–3.28)		0.23 (0.08–0.69)		0.76 (0.23–3.30)	
T4	0.80 (0.23–1.52)		0.72 (0.26–2.25)		0.44 (0.05–1.94)		1.58 (0.59–17.70)	
N stage		0.144		0.329		0.198		0.691
N0	0.85 (0.44–10.81)		1.86 (0.60–3.58)		0.69 (0.22–6.43)		2.51 (0.44–5.06)	
N1	0.61 (0.24–1.72)		0.76 (0.25–3.58)		0.23 (0.09–2.02)		0.75 (0.47–2.27)	
N2	0.31 (0.17–1.15)		0.51 (0.24–0.94)		0.26 (0.13–0.44)		2.59 (0.36–4.86)	
N3	1.08 (0.41–3.54)		0.86 (0.69–2.57)		0.14 (0.05–0.60)		1.26 (0.19–9.66)	
M stage		0.153		0.772		0.237		0.154
M0	0.63 (0.26–2.37)		1.00 (0.36–3.40)		0.31 (0.11–1.92)		2.01 (0.46–4.78)	
M1	1.46 (0.53–11.83)		0.85 (0.70–2.96)		0.09 (0.04–0.65)		0.23 (0.11–5.24)	
TNM stage		0.142		0.196		0.145		0.344
I	1.46 (0.43–9.61)		1.56 (0.52–3.86)		0.76 (0.22–8.59)		3.16 (0.63–5.73)	
II	4.11 (2.23–10.81)		3.36 (2.71–3.58)		1.12 (0.72–1.53)		1.11 (0.25–2.24)	
III	0.48 (0.17–0.75)		0.64 (0.17–1.33)		0.22 (0.08–0.33)		1.73 (0.38–3.25)	
IV	0.85 (0.32–3.51)		0.85 (0.46–2.96)		0.25 (0.05–1.35)		0.74 (0.20–9.13)	

Values are presented as median (interquartile range).

TNM = tumor-node-metastasis.

Although the exact sources of circulating miRNAs are unknown, mature miRNAs are packed into exosomes in cells and then released into the blood through extracellular secretion.²⁷ Therefore, circulating miRNAs can reflect the expression of miRNAs in tumor tissues to a certain degree. The expression patterns of circulating miRNAs are consistent with those of tumor tissue miRNAs in ESCC.^{21,28} Considering that blood samples are more convenient to collect and blood miRNAs are stably detected at reproducible expression levels, circulating miRNAs can be optimal tumor biomarkers.²⁹

miR-18b belongs to the miR-106a-363 cluster located on chromosome X, which encodes six miRNAs (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92-2, and miR-363). Because miR-18b is highly expressed in various tumors, especially in advanced gastric and colorectal cancers, it is mostly regarded as an oncogenic miRNA via participation in the regulation of the cell cycle, proliferation, and migration.³⁰⁻³³ Owing to the paucity of available data on the expression of miR-18b in ESCC, we also analyzed this marker in the present study. The lack of a significant difference in the expression levels of plasma miR-18b between ESCC patients and healthy controls and the absence of a correlation between its expression levels and most clinicopathologic characteristics, except for tumor size, suggests it might not be an efficient candidate biomarker for ESCC.

miR-21, a well-known onco-miRNA, is linked to tumor aggression and carcinogenesis, in part, by preventing apoptosis,³⁴ and is overexpressed in various gastrointestinal cancers.³⁵ In the present study, plasma miR-21 expression was significantly upregulated in ESCC patients compared with healthy controls, which is consistent with the results of previous studies.³⁶⁻³⁸ Although some previous studies have reported that higher blood miR-21 was associated with metastasis,³⁸ the plasma miR-21 level had no relationship with sex, age, tumor location, TNM stage, and other clinicopathologic characteristics in the present study, similar to other existing reports.^{36,39} A recent meta-analysis showed that ESCC patients with high circulating miR-21 levels had significantly shorter overall survival than those with low levels did.¹⁸

The importance of miR-31 in cancers has been suggested; however, its potential roles as a tumor suppressor or oncogenic factor remain controversial. miR-31 is reported to be upregulated in head and neck, lung, and colorectal cancers,⁴⁰⁻⁴² but downregulated in gastric, breast, prostate, and ovarian cancers.⁴³⁻⁴⁶ A study investigating miR-31 in ESCC tissues and sera reported that ESCC patients had a higher expression of miR-31 in serum compared with healthy controls and the level of serum miR-31 was elevated with an increase in the grade of TNM stage, consistent with the results observed in the tissue samples.⁴⁷ However, in the present study, the expression level of plasma miR-31 in ESCC patients was significantly lower than that in healthy controls, and the miR-31 level was not associated with TNM stage and other clinicopathologic characteristics. These inconsistent results of miR-31 might be due to the difference in baseline demographics (sex ratio, age distribution, and TNM stage) of the included patients. Moreover, the functional mechanisms of miRNAs in tumors are extremely complex where one miRNA can regulate several target genes and several miRNAs can regulate one target gene. This complex relationship could lead to varying expression levels of oncogenes and tumor suppressor genes in target genes. Therefore, comprehensive studies on the role of miR-31 in ESCC are needed to establish its potential has oncogenic or tumor suppressor role.

miR-375 was first identified in the murine pancreatic MIN 6 β -cell line, and several studies have shown that it functions as a tumor suppressor gene in various cancers.⁴⁸⁻⁵⁰ miR-375 has also been reported to play a tumor suppressive role in ESCC by inhibiting cell proliferation, colony formation, and metastasis *in vitro* and *in vivo*.⁵¹ In the present study, the significantly lower expression levels of plasma miR-375 in ESCC patients than in healthy controls were consistent with the results of previous studies, which also showed a similar miR-375 expression tendency in plasma and primary ESCC tissue samples in most cases.^{21,36} Similarly to our results, there was no significant correlation between the level of plasma miR-375 and clinicopathologic characteristics in these studies.^{21,36} A recent meta-analysis demonstrated that the downregulation of miR-375 was significantly correlated with poor overall survival in patients with head and neck cancer or ESCC.²²

Our study has some notable limitations that should be addressed in future studies. First, because we focused on the level of plasma miRNAs at the time of diagnosis, we did not collect long-term follow-up data, such as therapeutic response and survival. Second, the number of cases included in the present study was relatively small, as this was a single institution-based study. Therefore, a larger sample size is required to validate the role of miRNAs as biomarkers for ESCC, as shown in the present study. Third, profiles of circulating miRNAs could differ according to the specimens analyzed, such as plasma versus serum, and the RT-qPCR test methods (TaqMAN probe method versus SYBR green method). Some meta-analyses have suggested that plasma-based specimens have a higher accuracy than

serum-based specimens,^{52,53} whereas others showed the opposite tendency.^{54,55} Generally, the TaqMAN probe method might have a higher specificity than the SYBR green method, which may, in turn, have a higher sensitivity than the TaqMAN probe method. A recent meta-analysis of miRNAs in ESCC showed that both plasma and serum could be recommended as clinical specimens and there was no statistically significant difference between miRNA detection methods.¹⁶ Finally, miRNAs detected in patients with ESCC are not organ-specific. Even though we excluded concurrent malignancies via endoscopy, laboratory tests, and imaging studies using chest and abdominal CT and/or positron-emission tomography with CT, there is still a possibility that undetected malignancies present in other organs were a source of plasma miRNAs.⁵⁶ Therefore, plasma miRNAs cannot substitute endoscopy with forceps biopsy for ESCC diagnosis. However, they may be a good diagnostic method in population with a high risk of ESCC. Nevertheless, our results regarding plasma miRNAs should be validated in other studies prior to clinical application.

In conclusion, we identified a higher expression of plasma miR-21 and lower expression of plasma miR-31 and miR-375 in ESCC patients than in healthy controls. The validation of these findings in a large-scale case-control study could lead to these plasma miRNAs serving as potential non-invasive biomarkers for the detection of ESCC. The additional role of these miRNAs as predictive biomarkers for prognosis and response to chemotherapy should be explored in large, prospective, long-term follow-up studies.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021;71(3):209-49.
[PUBMED](#) | [CROSSREF](#)
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;136(5):E359-86.
[PUBMED](#) | [CROSSREF](#)
3. Jung HK. Epidemiology of and risk factors for esophageal cancer in Korea. *Korean J Helicobacter Up Gastrointest Res* 2019;19(3):145-8.
[CROSSREF](#)
4. Pennathur A, Farkas A, Krasinskas AM, Ferson PF, Gooding WE, Gibson MK, et al. Esophagectomy for T1 esophageal cancer: outcomes in 100 patients and implications for endoscopic therapy. *Ann Thorac Surg* 2009;87(4):1048-54.
[PUBMED](#) | [CROSSREF](#)
5. Kim GH. Diagnosis and clinical management of esophageal squamous dysplasia. *Korean J Helicobacter Up Gastrointest Res* 2021;21(1):4-9.
[CROSSREF](#)
6. Chiam KH, Shin SH, Choi KC, Leiria F, Militz M, Singh R. Current status of mucosal imaging with narrow-band imaging in the esophagus. *Gut Liver* 2021;15(4):492-9.
[PUBMED](#) | [CROSSREF](#)
7. Lee MW, Kim GH, Jeon HK, Park SJ. Clinical application of circulating tumor cells in gastric cancer. *Gut Liver* 2019;13(4):394-401.
[PUBMED](#) | [CROSSREF](#)
8. Mroczko B, Kozłowski M, Groblewska M, Łukaszewicz M, Nikliński J, Jelski W, et al. The diagnostic value of the measurement of matrix metalloproteinase 9 (MMP-9), squamous cell cancer antigen (SCC) and carcinoembryonic antigen (CEA) in the sera of esophageal cancer patients. *Clin Chim Acta* 2008;389(1-2):61-6.
[PUBMED](#) | [CROSSREF](#)
9. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;120(1):15-20.
[PUBMED](#) | [CROSSREF](#)

10. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008;9(2):102-14.
[PUBMED](#) | [CROSSREF](#)
11. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem* 2010;285(23):17442-52.
[PUBMED](#) | [CROSSREF](#)
12. Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol* 2009;19(2):43-51.
[PUBMED](#) | [CROSSREF](#)
13. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008;105(30):10513-8.
[PUBMED](#) | [CROSSREF](#)
14. Kong YW, Ferland-McCollough D, Jackson TJ, Bushell M. microRNAs in cancer management. *Lancet Oncol* 2012;13(6):e249-58.
[PUBMED](#) | [CROSSREF](#)
15. He Y, Lin J, Kong D, Huang M, Xu C, Kim TK, et al. Current state of circulating microRNAs as cancer biomarkers. *Clin Chem* 2015;61(9):1138-55.
[PUBMED](#) | [CROSSREF](#)
16. Liu F, Tian T, Xia LL, Ding Y, Cormier RT, He Y. Circulating miRNAs as novel potential biomarkers for esophageal squamous cell carcinoma diagnosis: a meta-analysis update. *Dis Esophagus* 2017;30(2):1-9.
[PUBMED](#) | [CROSSREF](#)
17. Wan J, Wu W, Che Y, Kang N, Zhang R. Insights into the potential use of microRNAs as a novel class of biomarkers in esophageal cancer. *Dis Esophagus* 2016;29(5):412-20.
[PUBMED](#) | [CROSSREF](#)
18. Gao S, Zhao ZY, Zhang ZY, Zhang Y, Wu R. Prognostic value of microRNAs in esophageal carcinoma: a meta-analysis. *Clin Transl Gastroenterol* 2018;9(11):203.
[PUBMED](#) | [CROSSREF](#)
19. Hirajima S, Komatsu S, Ichikawa D, Takeshita H, Konishi H, Shiozaki A, et al. Clinical impact of circulating miR-18a in plasma of patients with oesophageal squamous cell carcinoma. *Br J Cancer* 2013;108(9):1822-9.
[PUBMED](#) | [CROSSREF](#)
20. Wang LL, Li HX, Yang YY, Su YL, Lian JS, Li T, et al. MiR-31 is a potential biomarker for diagnosis of head and neck squamous cell carcinoma. *Int J Clin Exp Pathol* 2018.11(9):4339-45.
[PUBMED](#)
21. Wu C, Li M, Hu C, Duan H. Clinical significance of serum miR-223, miR-25 and miR-375 in patients with esophageal squamous cell carcinoma. *Mol Biol Rep* 2014;41(3):1257-66.
[PUBMED](#) | [CROSSREF](#)
22. Wang P, Xu L, Li L, Ren S, Tang J, Zhang M, et al. The microRNA-375 as a potentially promising biomarker to predict the prognosis of patients with head and neck or esophageal squamous cell carcinoma: a meta-analysis. *Eur Arch Otorhinolaryngol* 2019;276(4):957-68.
[PUBMED](#) | [CROSSREF](#)
23. Amin MB, Edge SB, Greene FL, Byrd DR, Brookland RK, Washington MK, et al. *AJCC Cancer Staging Manual*. 8th ed. New York, NY, USA: Springer; 2017.
24. Choi MK, Kim GH, I H, Park SJ, Lee MW, Lee BE, et al. Circulating tumor cells detected using fluid-assisted separation technique in esophageal squamous cell carcinoma. *J Gastroenterol Hepatol* 2019;34(3):552-60.
[PUBMED](#) | [CROSSREF](#)
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 2001;25(4):402-8.
[PUBMED](#) | [CROSSREF](#)
26. Yao C, Liu HN, Wu H, Chen YJ, Li Y, Fang Y, et al. Diagnostic and prognostic value of circulating microRNAs for esophageal squamous cell carcinoma: a systematic review and meta-analysis. *J Cancer* 2018;9(16):2876-84.
[PUBMED](#) | [CROSSREF](#)
27. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;9(6):654-9.
[PUBMED](#) | [CROSSREF](#)
28. Zhou X, Wen W, Zhu J, Huang Z, Zhang L, Zhang H, et al. A six-microRNA signature in plasma was identified as a potential biomarker in diagnosis of esophageal squamous cell carcinoma. *Oncotarget* 2017;8(21):34468-80.
[PUBMED](#) | [CROSSREF](#)

29. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 2008;18(10):997-1006.
[PUBMED](#) | [CROSSREF](#)
30. Egeland NG, Jonsdottir K, Aure MR, Sahlberg K, Kristensen VN, Cronin-Fenton D, et al. MiR-18a and miR-18b are expressed in the stroma of oestrogen receptor alpha negative breast cancers. *BMC Cancer* 2020;20(1):377.
[PUBMED](#) | [CROSSREF](#)
31. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, et al. A microRNA polycistron as a potential human oncogene. *Nature* 2005;435(7043):828-33.
[PUBMED](#) | [CROSSREF](#)
32. Han X, Zhang Y, Wang D, Fu X, Li M, Wang A. Upregulation of microRNA-18b induces phosphatase and tensin homolog to accelerate the migration and invasion abilities of ovarian cancer. *Oncol Lett* 2017;14(5):5631-7.
[PUBMED](#) | [CROSSREF](#)
33. Zhu L, Xu X, Tang Y, Zhu X. TRIP6 functions as a potential oncogene and facilitated proliferation and metastasis of gastric cancer. *Biologics* 2019;13:101-10.
[PUBMED](#) | [CROSSREF](#)
34. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* 2005;65(14):6029-33.
[PUBMED](#) | [CROSSREF](#)
35. Guraya S. Prognostic significance of circulating microRNA-21 expression in esophageal, pancreatic and colorectal cancers; a systematic review and meta-analysis. *Int J Surg* 2018;60:41-7.
[PUBMED](#) | [CROSSREF](#)
36. Komatsu S, Ichikawa D, Takeshita H, Tsujiura M, Morimura R, Nagata H, et al. Circulating microRNAs in plasma of patients with oesophageal squamous cell carcinoma. *Br J Cancer* 2011;105(1):104-11.
[PUBMED](#) | [CROSSREF](#)
37. Kurashige J, Kamohara H, Watanabe M, Tanaka Y, Kinoshita K, Saito S, et al. Serum microRNA-21 is a novel biomarker in patients with esophageal squamous cell carcinoma. *J Surg Oncol* 2012;106(2):188-92.
[PUBMED](#) | [CROSSREF](#)
38. Cai EH, Gao YX, Wei ZZ, Chen WY, Yu P, Li K. Serum miR-21 expression in human esophageal squamous cell carcinomas. *Asian Pac J Cancer Prev* 2012;13(4):1563-7.
[PUBMED](#) | [CROSSREF](#)
39. Dong Y, Wu WK, Wu CW, Sung JJ, Yu J, Ng SS. MicroRNA dysregulation in colorectal cancer: a clinical perspective. *Br J Cancer* 2011;104(6):893-8.
[PUBMED](#) | [CROSSREF](#)
40. Lajer CB, Nielsen FC, Friis-Hansen L, Norrild B, Borup R, Garnæs E, et al. Different miRNA signatures of oral and pharyngeal squamous cell carcinomas: a prospective translational study. *Br J Cancer* 2011;104(5):830-40.
[PUBMED](#) | [CROSSREF](#)
41. Liu X, Sempere LF, Ouyang H, Memoli VA, Andrew AS, Luo Y, et al. MicroRNA-31 functions as an oncogenic microRNA in mouse and human lung cancer cells by repressing specific tumor suppressors. *J Clin Invest* 2010;120(4):1298-309.
[PUBMED](#) | [CROSSREF](#)
42. Slaby O, Svoboda M, Fabian P, Smerdova T, Knoflickova D, Bednarikova M, et al. Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. *Oncology* 2007;72(5-6):397-402.
[PUBMED](#) | [CROSSREF](#)
43. Zhang Y, Guo J, Li D, Xiao B, Miao Y, Jiang Z, et al. Down-regulation of miR-31 expression in gastric cancer tissues and its clinical significance. *Med Oncol* 2010;27(3):685-9.
[PUBMED](#) | [CROSSREF](#)
44. Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, et al. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA* 2008;14(11):2348-60.
[PUBMED](#) | [CROSSREF](#)
45. Schaefer A, Jung M, Mollenkopf HJ, Wagner I, Stephan C, Jentzmik F, et al. Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. *Int J Cancer* 2010;126(5):1166-76.
[PUBMED](#) | [CROSSREF](#)
46. Creighton CJ, Fountain MD, Yu Z, Nagaraja AK, Zhu H, Khan M, et al. Molecular profiling uncovers a p53-associated role for microRNA-31 in inhibiting the proliferation of serous ovarian carcinomas and other cancers. *Cancer Res* 2010;70(5):1906-15.
[PUBMED](#) | [CROSSREF](#)

47. Zhang T, Wang Q, Zhao D, Cui Y, Cao B, Guo L, et al. The oncogenetic role of microRNA-31 as a potential biomarker in oesophageal squamous cell carcinoma. *Clin Sci (Lond)* 2011;121(10):437-47.
[PUBMED](#) | [CROSSREF](#)
48. Yan JW, Lin JS, He XX. The emerging role of miR-375 in cancer. *Int J Cancer* 2014;135(5):1011-8.
[PUBMED](#) | [CROSSREF](#)
49. Avissar M, Christensen BC, Kelsey KT, Marsit CJ. MicroRNA expression ratio is predictive of head and neck squamous cell carcinoma. *Clin Cancer Res* 2009;15(8):2850-5.
[PUBMED](#) | [CROSSREF](#)
50. Mathé EA, Nguyen GH, Bowman ED, Zhao Y, Budhu A, Schetter AJ, et al. MicroRNA expression in squamous cell carcinoma and adenocarcinoma of the esophagus: associations with survival. *Clin Cancer Res* 2009;15(19):6192-200.
[PUBMED](#) | [CROSSREF](#)
51. Kong KL, Kwong DL, Chan TH, Law SY, Chen L, Li Y, et al. MicroRNA-375 inhibits tumour growth and metastasis in oesophageal squamous cell carcinoma through repressing insulin-like growth factor 1 receptor. *Gut* 2012;61(1):33-42.
[PUBMED](#) | [CROSSREF](#)
52. Liu L, Wang S, Cao X, Liu J. Diagnostic value of circulating microRNAs for gastric cancer in Asian populations: a meta-analysis. *Tumour Biol* 2014;35(12):11995-2004.
[PUBMED](#) | [CROSSREF](#)
53. Liu L, Wang S, Cao X, Liu J. Analysis of circulating microRNA biomarkers for breast cancer detection: a meta-analysis. *Tumour Biol* 2014;35(12):12245-53.
[PUBMED](#) | [CROSSREF](#)
54. Xin H, Li X, Yang B, Zhang L, Han Z, Han C. Blood-based multiple-microRNA assay displays a better diagnostic performance than single-microRNA assay in the diagnosis of breast tumor. *Tumour Biol* 2014;35(12):12635-43.
[PUBMED](#) | [CROSSREF](#)
55. Wu K, Li L, Li S. Circulating microRNA-21 as a biomarker for the detection of various carcinomas: an updated meta-analysis based on 36 studies. *Tumour Biol* 2015;36(3):1973-81.
[PUBMED](#) | [CROSSREF](#)
56. Lee HJ, Kim GH, Park SJ, Kwon CH, Lee MW, Lee BE, et al. Clinical significance of TWIST-positive circulating tumor cells in patients with esophageal squamous cell carcinoma. *Gut Liver* 2021;15(4):553-61.
[PUBMED](#) | [CROSSREF](#)