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Clinical significance of serum miR-223, miR-25 and miR-375 in patients with esophageal squamous cell carcinoma

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Abstract Changes in the expression profiles of microRNAs (miRNAs) have been found in many cancers. The study was aimed to investigate the expression of miR-25, miR-223, and miR-375 in the serum of patients with esophageal squamous cell carcinoma (ESCC) and its effect on survival outcome. We examined the expression levels of miR-25, miR-223, and miR-375 in 20 pairs of ESCC cancer and matched paracancerous tissues, serum samples from 94 healthy volunteers and 194 patients with ESCC using quantitative reverse transcription polymerase chain reaction, and analyzed the relationship between expressions of serum miR-25, miR-223, and miR-375 and ESCC clinicopathological parameters as well as survival. Expressions of miR-25 and miR-223 were significantly increased in ESCC tissues compared with paracancerous tissues ($P = 0.008$ and 0.009, respectively), whereas the expression of miR-375 was significantly decreased in ESCC tissues compared with paracancerous tissues ($P = 0.006$). Expressions of serum miR-25 and miR-223 were significantly higher in ESCC patients than those in healthy controls, and, inversely, expression of serum miR-375 was significantly lower in ESCC patients than those in healthy controls ($P = 0.007$). High expression of serum miR-25 was significantly associated with lymph node metastasis ($P = 0.01$). Survival analysis showed that high expression of serum miR-223 and low expression of serum miR-375 were associated with poor survival in ESCC patients [hazard ratio (HR) = 1.717, 95 % confidence intervals (CI) 1.139–2.588, $P = 0.01$; HR = 1.750, 95 % CI 1.111–2.756, $P = 0.016$, respectively). Furthermore, Patients with high miR-223 and low miR-375 expression had higher risk of death than those with low miR-223 and high miR-375 expression (HR = 3.599, 95 % CI 1.800–7.195, $P = 2.92 \times 10^{-4}$). In conclusion, miR-25, miR-223, and miR-375 were abnormally expressed in ESCC tissues and sera. Serum miR-223 and miR-375 are potential prognostic biomarkers for ESCC.

Keywords Esophageal squamous cell carcinoma · miR-25 · miR-223 · miR-375 · Survival · MicroRNA

Introduction

Esophageal carcinoma is one of the most common malignancies worldwide. Approximately 482,300 newly diagnosed cases of esophageal carcinoma were reported worldwide in 2008, accounting for ~3.8 % of all new cancer cases [1]. In china, esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype, accounting for 90 % of the total esophageal carcinoma [1]. Major therapies for esophageal carcinoma now include surgery, chemotherapy, and radiotherapy. Although survival and functional recovery rates have remarkably improved as a result of the continuous advancement of diagnostic technologies and therapies, the outcome is far from satisfactory with 10–30 % overall 5-year survival rate [2, 3]. Numerous factors influence the prognosis of esophageal carcinoma, such as clinicopathological features [4–6] and abnormal expression of genes and/or proteins [7–10]. However, molecular markers that are of clinical application value are quite limited [10]. Further studies are needed to demonstrate or search for markers that can serve as reliable evidence to determine the efficacy and outcome of esophageal carcinoma treatment.

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MicroRNAs (miRNAs) are a class of small non-coding RNA molecules that can control gene expression post-transcriptionally. These molecules are important in cell growth, proliferation, metabolism, and apoptosis. In recent years, the relationship between miRNAs and human tumors has attracted much attention. The imbalanced expression of several key miRNAs is an important factor in the occurrence and development of cancer [11–13]. Therefore, numerous studies have been using gene chip technology to search for miRNAs with specific expression in cancer tissues by comparing cancer and paracancerous tissues. miRNAs are presumed to regulate nearly one-third of human gene expression [14, 15]. Abnormal patterns of miRNA expression have been observed in lung [16], colorectal [17, 18], esophageal [19, 20], and kidney and bladder [21] cancers. Furthermore, aberrant miRNA expression is closely related to metastasis and prognosis of cancer [22–25]. It has been shown that circulating miRNAs can be steadily detected to suggest the cancer status [25–27]. Thus, circulating miRNAs can be considered as a valuable marker for cancer monitoring. Considering the ease of blood sampling, circulating miRNAs hold better potential for future application as a new cancer biomarker.

Recent studies have reported diverse abnormal expression of miR-25 [28, 29], miR-223 [30–32], and miR-375 [17, 19, 33–36] in various types of cancer, including ESCC [29, 32, 36]. MiR-25 and miR-223 function as an oncogene or tumor suppressor in different cancer types [18, 29, 30, 33, 37–39]. MiR-25 represses migration and invasion of ESCC cells by targeting E-cadherin [29]. Overexpression of miR-223 inhibits FBXW7 expression in ESCC cells and then leads to abnormal accumulation of c-Myc and c-Jun proteins [32]. miR-375 acts as a tumor suppressor by targeting several oncogenes in cancer cells [40]. However, numerous previous studies only compared cancer and normal tissues. Since tissue sampling is difficult, especially for patients in advanced stages, which has limited the wide application of miRNA. Furthermore, little data have been reported to date regarding the relationship between circulation miRNAs and prognosis of esophageal cancer patients. Therefore, in the present study, we used quantitative reverse transcription polymerase chain reaction (qRT-PCR) to investigate the expressions of miR-25, miR-223, and miR-375 in ESCC patients. We also investigated the relationship between the expression levels of serum miR-25, miR-223, and miR-375 and the clinical factors as well as analyzed their effects on survival in ESCC patients.

Table 1 Clinicopathological characteristics of ESCC patients

Characteristics	No. of patients	%
Age (years)		
Mean	61.4	
Range	33-81	
Gender		
Female	79	40.7
Male	115	59.3
Smoking		
Never	93	47.9
Current	72	37.1
Former	14	7.2
Unknown	15	7.7
Family history of cancer		
Yes	71	37.1
Esophageal cancer only	52	26.8
No	122	62.9
TNM stage		
Stage I	8	4.1
Stage II	110	56.7
Stage III	57	29.4
Stage IV	7	3.6
Unknown	12	6.2
Lymph node status		
Negative	102	52.6
Positive	81	41.8
Unknown	11	5.7
Tumor size		
Median (cm)	4	
Range (cm)	1–10	
Site of tumor		
Upper esophagus	63	32.5
Middle esophagus	107	55.2
Low esophagus	24	12.4
Differentiation		
Well	8	4.1
Moderate	110	56.7
Poor	70	36.1
Unknown	6	3.1
Pathology type		
Ulcerative type	81	41.8
Medullary type	84	43.3
Fungating type	12	6.2
Constrictive type	4	2.1
Plaque type	4	2.1
Other type	6	3.1

Materials and methods

Patients

With informed consents, we enrolled 20 cases of ESCC patients who underwent surgeries in Zhongshan Hospital between April 2006 and March 2012. Surgical specimens were taken post-operationally for each patient within 30 min, using blades to cut off a piece each of esophageal

cancer and paracancerous tissues (2 cm from the cancer tissue). Fresh specimens obtained by surgical ablation were immediately placed and stored in liquid nitrogen. We collected 2 mL peripheral blood from each of 194 ESCC patients and 98 healthy controls. All patients were pathologically diagnosed as having ESCC using surgical specimens or biopsies. None of the ESCC patients had received any anticancer treatment prior to sampling. Approval was obtained from the Ethics Committee of Zhongshan Hospital prior to specimen collection. Table 1 shows the clinical profiles of patients.

Quantitative real-time PCR

Total RNA was extracted from tissue and serum samples, according to the manual of miRNAVanaTM PARISTM (Ambion, TX, USA). RNA (1 μL) concentration was quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA). RNA was transported and stored in a refrigerator at 80 °C.

TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, CA, USA) was used in reverse transcription. The reaction system was 15 μL, containing 100 ng total RNA, 1.5 μL 10× reverse transcription buffer, 0.15 μL 100 mM deoxyribonucleotide triphosphates, 1.0 μL reverse transcriptase, 0.19 μL RNase inhibitor (20 U/μL), and 3.0 μL specific miRNA primer. Reactions were performed at 16 °C for 30 min, 42 °C for 30 min, and finally at 85 °C for 5 min. The 20 μL reaction mixtures for qRT-PCR included 2 μL cDNA, 10 μL 2 × Universal PCR Master Mix, 1.0 μL TaqMan miRNA assay, and 7 μL nuclease-free water. qRT-PCR was performed on ABI7500 fast real-time PCR system (Applied Biosystems, CA, USA) under the following reaction conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles: at 95 °C for 15 s and at 60 °C for 1 min. Each reaction was repeated thrice. The expression levels of miRNAs in tissue and serum samples were normalized to U6 small nuclear RNA (RNU6B) and miR-16 [41], respectively, and were calculated using the equation $2^{-\Delta\Delta Ct}$. The ΔCt was calculated by subtracting the average Ct value of corresponding reference gene from the average Ct value of the miRNAs of interest. The ΔΔCt was then calculated by subtracting the ΔCt of paracanerous tissue or the average expression of healthy volunteers from ΔCt of ESCC patients. The fold change in gene expression was calculated with the equation $2^{-\Delta\Delta Ct}$. The expression levels of miRNAs were converted into dichotomous variables by splitting the samples into two classes (high and low expression), using the respective mean level expression of miRNA as a cutoff [42].

Statistical analysis

Data analysis was performed with SPSS 17.0 (SPSS Inc, Chicago, USA). $P < 0.05$ was considered statistically significant. Rank-sum test was used to compare the differences in the expression of serum miR-25, miR-223, and miR-375 between ESCC patients and healthy controls. χ^2-test was used to determine the relationship between the expression of these serum miRNAs and the ESCC clinicopathological parameters. Receiver-operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to assess the predictive power of individual miRNAs. Kaplan–Meier method and log-rank test were used to investigate the association between the expression of the three serum miRNAs and the survival in ESCC patients. Cox regression model was used to test the independence of risk factors. The end point was overall survival from the time of initial histological diagnosis.

Results

MiR-25, 223, and 375 in primary ESCC tissues

Differential expression of miR-25, miR-223, and miR-375 in cancer and paracancerous tissues were statistically significant ($P = 0.008$, 0.009 and 0.006, respectively) (Fig. 1). The expression levels of miR-25 and miR-223 were increased, whereas the expression level of miR-375 was decreased in ESCC tissues (fold change = 2.66, 3.42 and 2.71, respectively).

Expression of serum miR-25, 223, and 375 in ESCC patients

We hypothesized that the expression levels of miR-25, miR-223, and miR-375 in primary ESCC tissues would influence serum levels of miR-25, miR-223, and miR-375 in ESCC patients. We first compared the expression levels of miR-25, miR-223, and miR-375 in 20 pairs of ESCC tissues and sera. There were statistically significant correlations between tissue and serum levels of miR-25, miR-223, and miR-375 (correlation coefficients ranged from 0.579 to 0.663, all $P < 0.01$) (Fig. S1). We further investigated the serum miRNA expression in 194 ESCC patients and 98 healthy controls. We found statistically significant differences between ESCC patients and healthy controls in expression levels of miR-25, miR-223, and miR-375 ($P = 0.009$, 0.001 and 0.007, respectively) (Fig. 1).

Relationship between expression of serum miR-25, miR-223, and miR-375 and clinicopathological characteristics in ESCC patients

We also analyzed the relationship between serum miR-25, miR-223, and miR-375 expression and clinicopathological

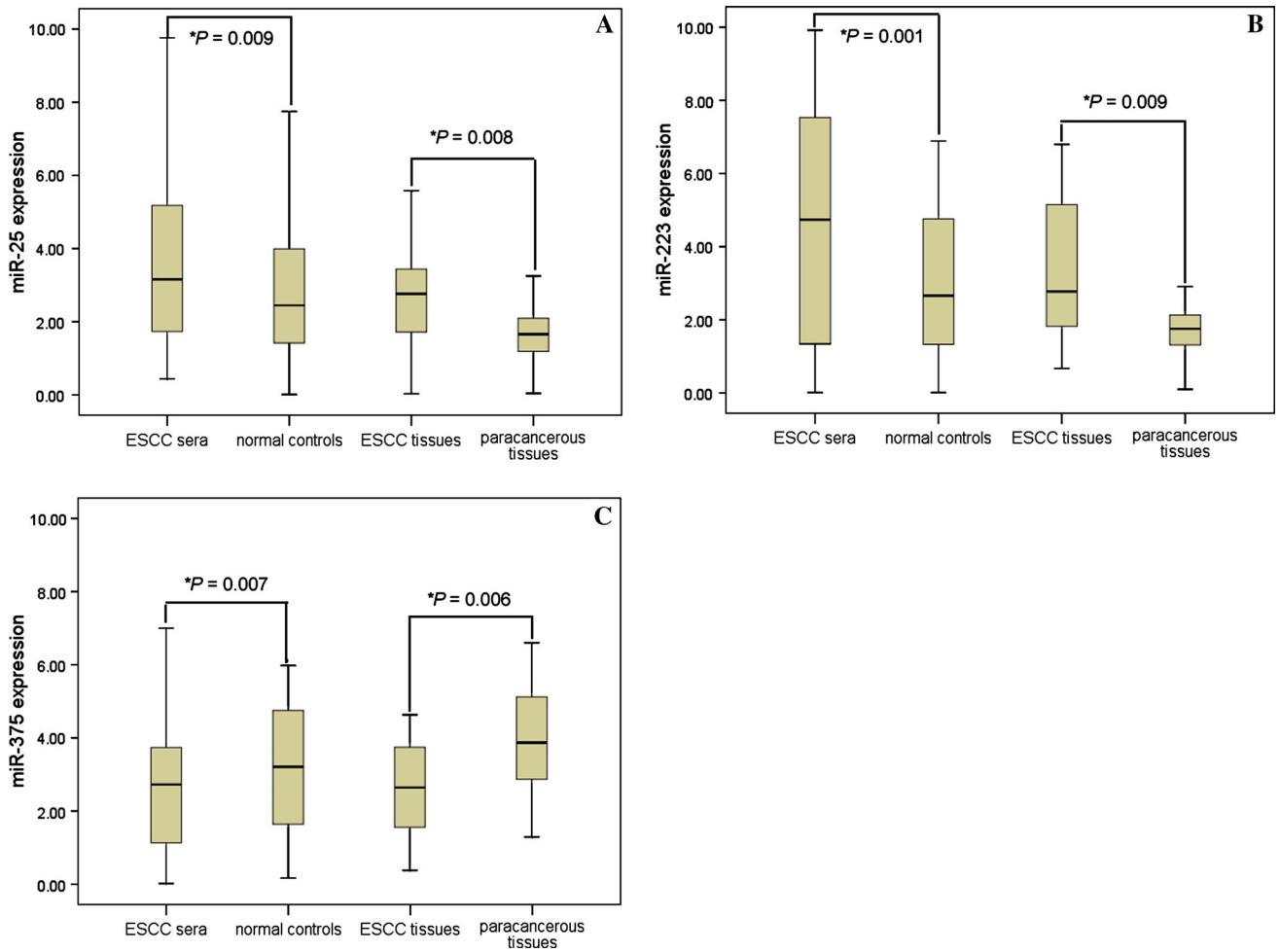

Fig. 1 Differential expression of miR-25, miR-223 and miR-375 in serum samples of 194 ESCC cases and 94 healthy controls as well as in 20 paired ESCC cancer and matched paracancerous tissues. **a** miR-25, **b** miR-223, **c** miR-375

features in ESCC patients in order to better understand their potential roles in the development and progression of ESCC. High expression of serum miR-25 was significantly associated with lymph node metastasis ($P = 0.01$). The AUC based on serum miR-25 was 0.593 (Fig. 2a). No other significant differences were found between the expression levels of serum miR-25, miR-223 and miR-375, and clinicopathological characteristics (all $P > 0.05$) (Table 2).

Survival analysis

The median survival for the whole group was 31.3 months. The overall survival rates at 1, 3, and 5 years were 85.8, 42.5, and 29.4 %, respectively. Patients with upregulated miR-223 expression had significantly shorter median survival time (MST) than those with low miR-223 expression (26.3 vs. 36.9 months; $P = 0.007$, log-rank test; Fig. 3a). By contrast, MST was significantly longer for those with upregulated miR-375 expression than those with low miR-375 expression (39.3 vs. 27.8 months; $P = 0.016$, log-rank test) (Fig. 3b). The AUC based on serum miR-223 and miR-375 expression was 0.734 and 0.720, respectively (Fig. 2b, c). No significant association was observed between miR-25 expression and survival ($P = 0.525$). Multifactor Cox analysis showed that high serum miR-223 expression and low serum miR-375 expression were independent predictors of survival in ESCC patients [Hazard ratio (HR) = 1.717, 95 % confidence intervals (CI) 1.139–2.588, $P = 0.010$; HR = 1.750, 95 % CI 1.111–2.756, $P = 0.016$, respectively) (Table 3).

To perform a comprehensive analysis of the two markers, we assigned ESCC patients to three groups: group one, patients with low miR-223 and high miR-375 expression; group two, patients with high miR-223 or low miR-375 expression; group three, patients with high miR-223 and low miR-375 expression. Patients in group one had longer MST than those in group two or three ($P = 0.002$) (Fig. 3c). In the multivariate Cox-regression model,

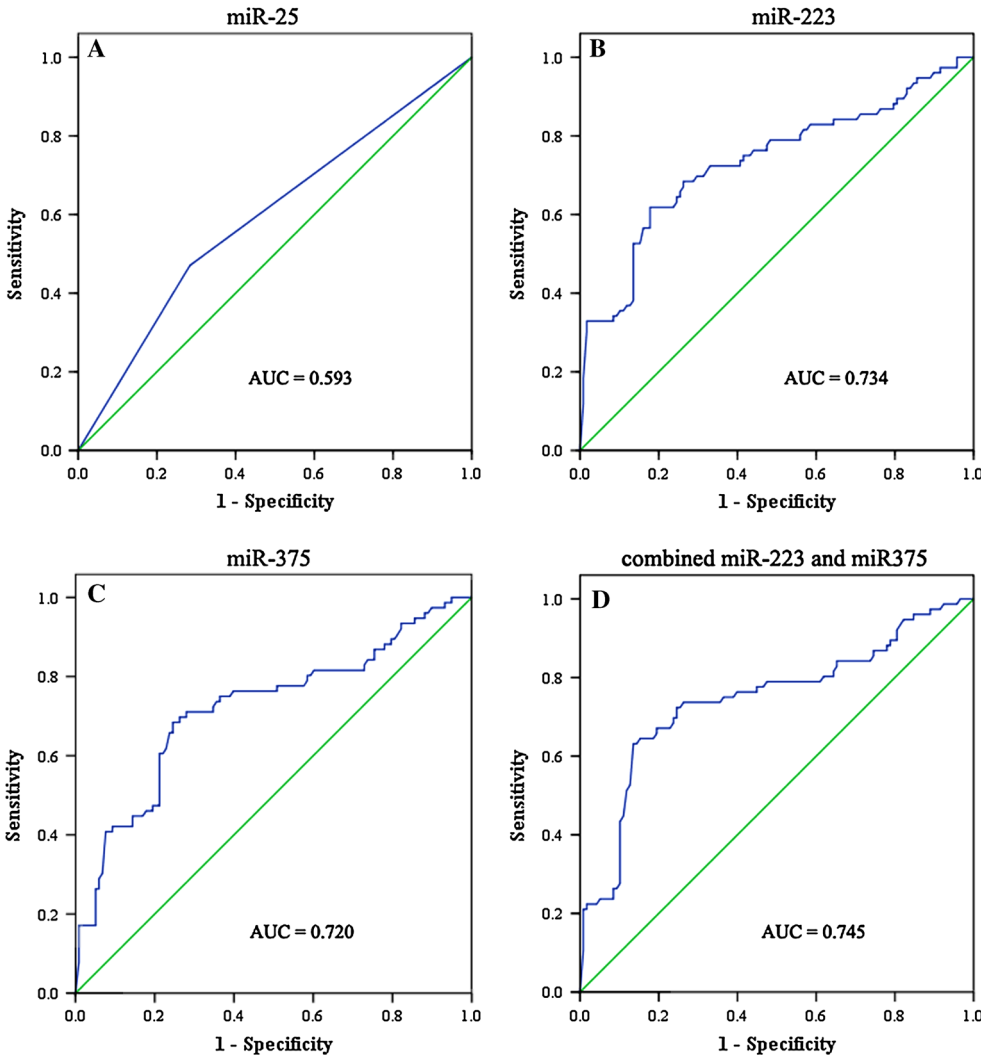

Fig. 2 ROC analyses of miR-25, miR-223 and miR-375 based on serum expression. **a** Serum miR-25 yielded AUC of 0.593 (95 % CI 0.511–0.676) with 47.1 % sensitivity and 71.6 % specificity in discriminating ESCC patients with lymph node metastasis. **b** ROC curve for 5-year survival for serum miR-223. c ROC curve for 5-year survival for serum miR-375. **d** ROC curve for 5-year survival for combined miR-223 and miR-375

patients in group two had a cancer-related death risk 2.527 times higher (95 % CI 1.268–5.039, $P = 0.008$), and those in group three had a cancer-related death risk 3.599 times higher (95 % CI 1.800–7.195, $P = 2.92 \times 10^{-4}$) than those in group one. The AUC based on combined miR-223 and miR-375 was 0.745 (Fig. 2d).

Discussion

The pathogenesis of esophageal cancer remains unknown. With the progression of cancer research, no or little gene mutations were found in certain cancers to date. Moreover, miRNAs play vital roles in post-transcriptional regulation in many biological processes. Therefore, researchers are increasingly shifting their focus on the relationship between miRNAs and tumors. In the present study, we used qRT-PCR to determine the expressions of serum miR-25, miR-223, and miR-375 in ESCC patients and healthy controls. We found that serum miR-223, miR-25 and miR-375 expression are potential biomarkers for the prognosis of ESCC patients.

miR-25 and miR-223 are upregulated in many types of cancer [29, 30, 37, 43, 44], promoting cancer cell proliferation, migration and invasion [29, 30, 37, 45]. However, miR-25 may act as tumor suppressor, and inhibits proliferation of colon cancer [18] and anaplastic thyroid carcinoma cells [38]. These results indicates that miRNAs plays distinct roles under different cellular. miR-375 is frequently downregulated and functions as a tumor suppressor gene in various cancers [17, 33–36, 40]. Furthermore, the expression levels of miR-223 and miR-375 were not affected by neo-adjuvant chemoradiation therapy [46]. In the current study, we found that abnormal expression of serum miR-25, miR-223, and miR-375 also exists in ESCC patients. The sources of serum miRNAs are relatively unknown. Serum miRNAs can be substances that have been released into the blood as a result of tumor cell

Table 2 Association between serum expression of miR-25, miR-223 and miR-375, and the clinicopathological features

Characteristics	miR-25			miR-223			miR-375		
	Low	High	*P* value	Low	High	*P* value	Low	High	*P* value
Age (years)									
>60	43	63	0.354	46	60	0.61	39	67	0.159
≤60	30	58		35	53		24	64	
Gender									
Female	27	52	0.411	32	47	0.77	22	57	0.254
Male	46	69		49	66		41	74	
Smoking									
Never	30	63	0.437	37	56	0.468	28	65	0.861
Current	28	43		31	41		24	48	
Former	7	8		8	6		5	9	
Family history of cancer									
Yes	26	46	0.737	30	42	0.985	23	49	0.904
No	47	75		51	71		40	82	
TNM stage									
Stage I	5	3	0.519	3	5	0.35	3	5	0.453
Stage II	40	70		48	62		32	78	
Stage III	21	36		21	36		19	38	
Stage IV	3	4		5	2		4	3	
Lymph node status									
Negative	48	54	0.01	47	50	0.289	32	70	0.647
Positive	23	58		31	55		28	53	
Tumor size (cm)									
>4	28	44	0.639	28	44	0.305	22	50	0.913
≤4	26	48		35	39		22	52	
Site of tumor									
Upper esophagus	21	42	0.547	27	36	0.976	20	43	0.987
Middle esophagus	41	66		44	63		35	72	
Low esophagus	11	13		10	14		8	16	
Differentiation									
Well	3	5	0.76	3	5	0.799	4	4	0.516
Moderate	38	72		49	61		35	75	
Poor	28	42		28	42		21	49	
Pathology type									
Ulcerative type	33	48	0.525	32	49	0.731	30	51	0.53
Medullary type	32	52		37	47		28	56	
Fungating type	5	7		4	8		2	10	
Constrictive type	0	4		2	2		0	4	
Plaque type	2	2		1	3		1	3	
Other	1	5		4	2		2	4	

apoptosis or disintegration [47]. Valadi et al. [48] showed that mature miRNAs are packaged into exosomes in cells by lipids or lipoproteins and enter the blood through extracellular secretion. Therefore, serum miRNA may also be partly expressed from the active secretion of tissue cells. Our results show that the expression trend of serum miRNA is consistent with that in tumor tissues, suggesting that serum miRNA expression reflect the expression in tumor tissues to a certain extent. Serum samples are convenient to collect, and serum miRNAs are stable enough to serve as optimal tumor biomarkers [49].

Abnormal miRNA expression is associated with tumor formation and development and affects patient prognosis. Dysregulated expressions of miR-223, miR-25 and miR-

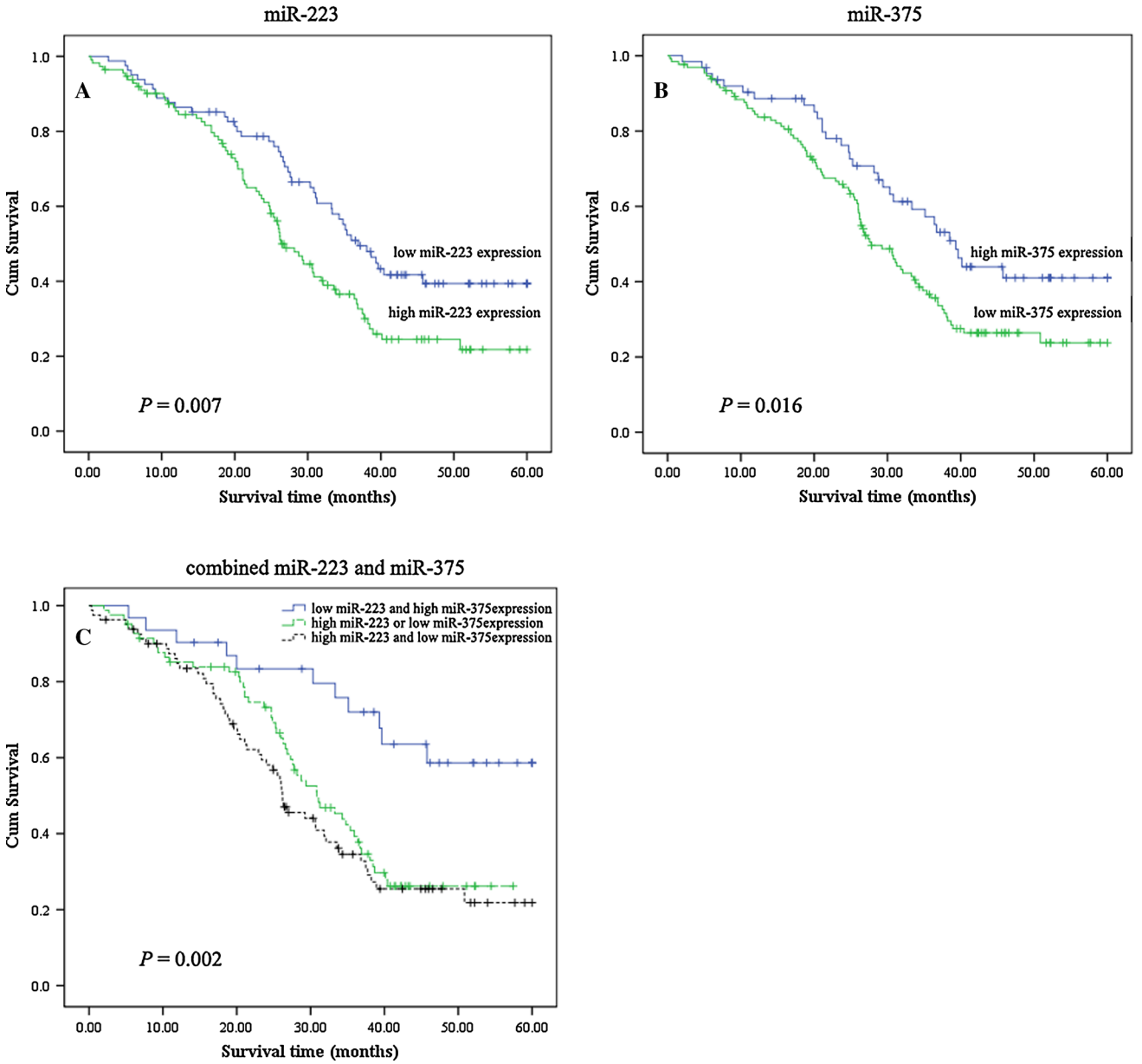

Fig. 3 Kaplan–Meier survival curves based on expression levels of serum miR-223 and miR-375 in ESCC patients. **a** miR-223. **b** miR-375. **c** Combined analysis of the expression levels of serum miR-233 and miR-375

375 were reported to be associated with poor prognosis in some human cancers [23, 29, 30, 32, 46, 50, 51]. Previous study showed that miR-223 expression in ESCC tissue was inversely associated with the survival, which was correlated to the suppression of the FBXW7 gene functions by high miR-223 expression [32]. Li et al. [30] found that overexpression of miR-223 stimulated nonmetastatic gastric cancer cells migration and invasion and was associated with poor metastasis-free survival. Recent studies showed that low miR-375 expression was associated with worse survival in patients with head and neck squamous carcinoma [23], Barrett's esophagus and adenocarcinoma [46] or ESCC [36]. MiR-25 can suppress p57 and CDH1 expression, and thereby promote cancer cell migration and invasion [28, 29]. Overexpression of miR-25 in ESCC tissue was associated with a high risk of metastasis [30]. In the present study, we found that abnormal expression of miR-223, miR-25 and miRNA-375 in the serum of ESCC patients are significantly associated with poor prognosis, which were consistent with aforementioned studies. High expression of serum miR-223 and low expression of serum miRNA-375 are the independent markers to predict poor prognosis of ESCC patients. In addition, we combined low expression of serum miR-223 with high expression of

Table 3 Univariate and multivariate Cox regression analysis of overall survival

Features	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*P* value	HR (95 % CI)	*P* value
Tumor features				
Age (years), >60 vs. ≤60	1.344 (0.936–1.931)	0.108		
Gender, male vs. female	1.194 (0.830–1.716)	0.339		
Smoking, never vs. current/former	0.948 (0.779–1.153)	0.593		
Family history of cancer, yes vs. no	1.276 (0.883–1.842)	0.194		
Tumor size (cm), >4 vs. ≤4	1.512 (0.982–2.326)	0.060		
Site of tumor	1.349 (1.039–1.751)	0.025	1.464 (1.098–1.951)	0.009
Tumor differentiation	1.800 (1.277–2.537)	0.001	1.478 (1.013–2.158)	0.043
Pathology type	1.029 (0.875–1.211)	0.726		
TNM stage	1.577 (1.195–2.083)	0.001	1.712 (1.134–2.586)	0.011
LNM, positive vs negative	1.558 (1.072–2.268)	0.020	1.111 (0.644–1.915)	0.706
Expression of miRNAs				
miR-25 (high vs. low)	1.127 (0.778 to 1.637)	0.526		
miR-223 (high vs. low)	1.672 (1.148 to 2.434)	0.007	1.717 (1.139 to 2.588)	0.010
miR-375 (high vs. low)	0.605 (0.401 to 0.913)	0.017	1.750 (1.111 to 2.756)	0.016
Combined miR-223 and miR-375	1.557 (1.201 to 2.017)	0.001	1.732 (1.298 to 2.310)	1.89×10^{-4}

LNM lymph node metastasis

serum miRNA-375 for further analysis, and found that ESCC patients without these two risk factors have longer survival. Therefore, serum miR-223 and miR-375 expression may be used as biomarkers to predict survival in ESCC patients. However, studies with adverse conclusions also exist. Karakatsanis et al. [22] revealed that miR-223 expression in liver cancer tissues is not significantly associated with clinical profiles (including metastasis) or survival of patients. Yao et al. [50] suggested that diffuse large B cell lymphoma patients with high miR-223 expression had significantly longer survival time than those with low miR-223 expression. Stamatopoulos et al. [51] reported that reduced miR-223 expression predicted poor prognosis in patients with chronic lymphocytic leukemia. Zhang et al. [52] found that gastric cancer patients with high miR-375 expression had higher risk of recurrence and shorter survival time than those with low expression of miR-375. These inconsistent results may be due to the disparity of tumor tissue sources. Furthermore, mechanisms by which miRNAs function in tumors are extremely complex. One miRNA can control several target genes, whereas several miRNAs can control one target gene. Members of one miRNA cluster can possibly work on target genes. This complex relationship can result in the differential expression between oncogenes and anti-oncogenes in target genes, which eventually affect prognosis. Abnormal expression of miR-223 and miR-375 are associated with the clinicopathological features such as cancer staging and distal metastasis [19, 21]. However, our findings showed no significant association between abnormal expression of serum miR-223 and miRNA-375 and clinicopathological features of ESCC patients. This result may have been caused by different cancers or different mechanisms by which miR-223 and miRNA-375 function.

In summary, we found that ESCC prognosis is associated with the abnormal expression of serum miR-223, miR-25 and miR-375, which could be potential new biomarkers for outcome prediction for ESCC patients. Our findings have provided new insights into studies on the pathogenesis of ESCC and the determination of its prognosis. Suppressed proliferation and induced apoptosis via regulated expression of miRNAs can offer new alternatives in tumor treatment. However, reports on the interaction between miR-223, miR-25 and miR-375 and their corresponding target genes are limited. Functional analysis of these miRNAs will help further reveal the mechanism in the tumorigenesis of ESCC.

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Conflict of interest The authors declare that they have no conflict of interest.

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