Exosome-mediated miR-25/miR-203 as a potential biomarker for esophageal squamous cell carcinoma: improving early diagnosis and revealing malignancy

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Background: Esophageal squamous cell carcinoma (ESCC) is the leading cause of cancer death in men and women worldwide. The poor prognosis and rapid increase in ESCC incidence highlight the need to promote early detection and prediction. Identifying key molecular targets involved in ESCC monitoring and progression is critical for ESCC patients.

Methods: This study examined miR-25/miR-203 as a biomarker for ESCC patients. Real-time quantitative polymerase chain reaction (PCR) was used to detect miR-25/miR-203 expression levels in tissues and serum exosomes, and MiR-25/miR-203 upregulation was confirmed in ESCC.

Results: We found that the miR-25/miR-203 ratio in cancer tissues from 36 ESCC patients was significantly enhanced compared with that in adjacent tissues. Moreover, the serum level of miR-25/miR-203 in 57 ESCC patients was higher than that in 31 healthy volunteers. Intriguingly, in 38 ESCC patients, the level of miR-25/miR-203 decreased significantly after surgery. Using ROC curve statistical analysis, we found that each group of miR-25/miR-203 had obvious sensitivity and high specificity. The miR-25/miR-203 relationship with the clinicopathological features of ESCC patients was also analyzed. MiR-25/miR-203 was significantly associated with the ESCC TNM-stage and lymph node metastasis, which predicts the prognosis of ESCC and reflects tumor progression.

Conclusions: This study highlights the feasibility of using exosome-mediated miR-25/miR-203 as a vital noninvasive biomarker for the detection and treatment monitoring of ESCC.

Keywords: Biomarkers; microRNAs; exosomes; serum; esophageal squamous cell carcinoma (ESCC)

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies worldwide (1), and its incidence has increased six-fold over the past four decades (2,3). Despite the rapid development of conventional surgical resection, chemotherapy, radiotherapy, and other esophageal cancer treatment strategies, the 5-year survival rate is still less than 20% (4,5). Therefore, it is imperative to

identify a novel noninvasive biomarker for ESCC diagnosis and treatment monitoring with high stability and specificity.

The exosome is a nanosized vesicular body secreted by cells, which encloses biologically active substances and participates in many important physiological and pathological processes (6,7). The exosomal membrane protects the exosome contents from degradation by enzymes in the extracellular environment (8). MicroRNAs are a class of noncoding small ribonucleic acids (RNAs) that are ubiquitous in organisms. Growing evidence has indicated that they have important regulatory effects on a variety of biological activities, such as growth, development, cell proliferation, differentiation, and metabolism (9-11). Numerous studies have demonstrated that the dysregulated expression of several key miRNAs is closely associated with cancer malignancies as well as the prognosis and prediction of response to treatment in different human cancers (12-14). MiR-203 has been shown to be abnormally expressed in different cancers and to promote abnormal cancer proliferation, invasion, and metastasis (15,16). MiR-203 is also hypermethylated in esophageal cancer. Hypermethylation is an important modification that causes the expression of miR-203 to decrease, which promotes the development and progression of ESCC. Furthermore, when miR-203 is upregulated over 4.5-fold, it is significantly associated with lymph node metastasis (17). MiR-25 belongs to the miR-106b-25 cluster, which is related to hepatocellular tumors (18). Kim *et al.* (19) also found that, in humans, miR-25 is significantly upregulated in gastric cancer tissues and adjacent tissues compared with normal tissues. The high expression of miR-25 may be a high-risk-related factor for gastric cancer tissue penetrating the serosa, lymph nodes, and distant metastasis. It is known that microRNAs are stable in body fluids and are not susceptible to environmental factors (16). Considering the desire for a novel, noninvasive, convenient, clinical tool, serum miR-25/miR-203 holds great potential for future application as a new biomarker for ESCC.

The present study aimed to characterize miR-25/miR-203 in peripheral serum, with a view to developing the diagnostics and monitoring of ESCC. We present the following article in accordance with the STARD reporting checklist (available at https://dx.doi.org/10.21037/tcr-21-1123).

Methods

Patients

In 2017, ESCC patients undergoing thoracoscopic surgery combined with laparoscopic surgery at the Department of Thoracic Surgery, Fujian Medical University Union Hospital were selected for this study. Surgical specimens were collected straight away post-operation from each patient, by using scalpels to cut off a sample of the cancer tissues as well as adjacent tissues. All of the specimens were immediately stored in liquid nitrogen. We collected preoperative peripheral blood from 57 ESCC patients and postoperative peripheral blood from 38 ESCC patients and used peripheral blood from 31 healthy volunteers as a control. Clinical staging was determined according to the American Joint Committee on Cancer (AJCC) 8th edition of ESCC TNM-Staging. The study was approved by the Ethics Committee of Fujian Medical University Union Hospital. Written informed consent was obtained from all participants. The registration number is 2020KY099. The study is in accordance with the Helsinki Declaration (as revised in 2013).

Serum exosome isolation

Serum exosomes were isolated using the modified polyethylene glycol (PEG) separation method. An appropriate amount of polyethylene glycol 6000 (average molecular mass 6000) was dissolved in pure water and sodium chloride was added to make sodium chloride solution 1 M, thereby obtaining a storage solution of a 2× concentration. The serum was then mixed with one volume of the above PEG solution and incubated at 4 ℃ overnight. It was then spun in a refrigerated centrifuge at the highest speed for one hour, and the pellet was collected and resuspended in PBS.

RNA extraction and reverse transcription

Total RNA extraction with TRIzol was performed as follows: (I) the homogenized sample was placed at room temperature for 5 min, and 0.2 times the volume of chloroform was added. The samples were then spun for 15 s, mixed well, placed at 15–30 ℃ for 2–3 min, and then centrifuged at 4 ℃ and 13,000 rpm. Centrifugation resulted in a lower red phenol-chloroform phase, an intermediate phase, and an upper colorless aqueous phase. (II) The upper aqueous phase was carefully transferred to a new centrifuge tube, one volume of isopropanol was added, and the mixture was gently inverted. After incubation at room temperature for 10 min, the sample was centrifuged at 13,000 rpm for 15 min at 4 ℃, and the supernatant was discarded. (III) The

pellet was mixed with 70% alcohol and inverted several times to wash off the salt contained in the precipitate. If the precipitate is larger, the pellet was broken up. The sample was then centrifuged again at 13,000 rpm for 10 min at 4 ℃, the supernatant was discarded, and the procedure was repeated one more time. (IV) The sample was placed on the ultraclean workbench, and the tube cover was opened, allowing the pellet to dry at room temperature, and then the appropriate amount of RNase-free water was added to dissolve and mix up the pellet, and this was immediately followed by centrifugation. (V) A total of 1.2 µL was used to analyze the RNA concentration and purity. Reverse transcription was performed as follows. (I) Two microliters of RNA template was pre-denatured at 70 ℃ for 10 min. (II) The sample was incubated in an ice bath for 5 min. (III) The transcription system was prepared in the following order: 25 mmol/L MgCl$_2$ (4 µL) → reverse transcription buffer (2 µL) → 10 mmol/L dNTP (2 µL) →RNase (0.5 µL) → template (2 µL) → random primer or specific downstream primer (1 µL) → DEPC water (8.5 µL). (IV) The mixture was incubated for 15 min at room temperature. (V) Finally, the mixture was incubated at 42 ℃ for 1 h.

Quantitative real-time PCR

The Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA, USA) was used to carry out qRT-PCR with the SYBR PremixExX Taq kit (Takara, Shiga, Japan). Primer sequences for qRT-PCR were obtained from the online database miRbase (http://www.miRbase.org/). *Table 1* lists the primer sequences. The ΔΔCt was calculated by subtracting the average Ct value of the corresponding reference gene from the average Ct value of the miRNAs of interest. The ΔCt was then calculated by subtracting the ΔCt of precancerous tissue or the average expression in healthy volunteers from the 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 cut-off point.

Data statistics

All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, USA) using the mean ± standard deviation. P<0.05 was considered statistically significant. The two groups were compared with the use of one-way analysis of variance; ROC curves and the area under the ROC curve (AUC) were used to assess the specificity and sensitivity of each miRNA for the diagnosis of ESCC. The rank-sum test was used to compare differences in serum miR-25 and miR-203 expression between ESCC patients and healthy controls. The chi-square test was used to determine the relationship between the expression of these serum miRNAs and the clinical-pathological parameters of ESCC.

Results

The up-regulation of serum exosome miR-25/miR-203 in primary ESCC

To verify the expression of miR-25/miR-203 in the ESCC serum samples, we analyzed the levels of miR-25/miR-203 in the serum of 57 ESCC patients and 31 healthy volunteers. We found that the miR-25/miR-203 ratio in ESCC was significantly higher than that in the control group, as shown by the ROC curve (AUC =0.821, sensitivity =71.9%, specificity =96.6%, *Figure 1A,1B*). We also collected 36 ESCC tumors and adjacent tissues and confirmed that the expression level of miR-25/miR-203 in tumor tissues was higher than that in adjacent tissues, as shown by the ROC curve (AUC =0.924, sensitivity =91.7%, specificity =80.6%), as shown in *Figure 1C,1D*. To determine whether miR-25/miR-203 has value in monitoring the effect of ESCC tumor resection and recurrence. we analyzed the expression levels of serum miR-25/miR-203 in the preoperative and postoperative serum of 38 ESCC patients and showed that the expression level of miR-25/miR-203 was significantly reduced after surgery, as indicated by the ROC curve (AUC =0.831, sensitivity =97.4%, specificity =65.8%), as shown in *Figure 1E,1F*.

The clinical significance of miR-25/miR-203 in patients with primary ESCC

To confirm the clinical significance of serum miR-25/miR-203 upregulation in ESCC, we analyzed the clinical data of 57 cases of ESCC (*Table 1*). Pearson's χ^2 analysis of miR-25/miR-203 expression and clinicopathological features indicated that miR-25/miR-203 upregulation was associated with ESCC clinical stages. Our analysis showed that miR-25/miR-203 overexpression was associated with T stage (P=0.043) and TNM stage (P=0.013) but was not related to age, gender, location, smoking, tumor differentiation or hemangioma. It would therefore appear that miR-25/

Table 1 Clinicopathological characteristics of 57 ESCC patients with respect to miR-25/miR-203 expression

Characteristic	MiR-25/miR-203, n (%)		P value
	Low expression (n=16)	High expression (n=41)	
Health *vs.* cancer			0.0001*
Health	30 (96.78)	1 (3.22)	
Cancer	16 (28.07)	41 (71.93)	
Age (years)			>0.05#
<60	8 (14.04)	19 (33.33)	
≥60	8 (14.04)	22 (38.60)	
Gender			p>0.05#
Female	2 (3.51)	8 (14.04)	
Male	14 (24.56)	33 (57.89)	
TNM stage			0.013*
I	8 (14.04)	6 (10.53)	
II	6 (10.53)	19 (33.33)	
III	2 (3.51)	16 (28.07)	
T classification			0.043*
T1	9 (15. 79)	9 (15. 79)	
T2	1 (1.75)	5 (8.77)	
T3	6 (10.53)	27 (47.37)	
N classification			>0.05#
N0	13 (22.81)	19 (33.33)	
N1	3 (5.26)	12 (21.05)	
N2	0	7 (12.28)	
N3	0	3 (5.26)	
Vascular tumor thrombus			>0.05#
Yes	3 (5.26)	14 (24.56)	
No	13 (22.81)	27 (47.37)	
Location			>0.05#
Up/middle	10 (17.54)	27 (47.37)	
Lower	6 (10.53)	14 (24.56)	
Differentiation			>0.05#
Highly	5 (8.77)	12 (21.05)	
Moderate	9 (15.79)	22 (38.60)	
Low	2 (3.51)	7 (12.28)	
Smoking			>0.05#
Yes	13 (22.81)	24 (42.11)	
No	3 (5.26)	17 (29.82)	

*, P value was determined using Pearson's chi-square test; #, no statistical.
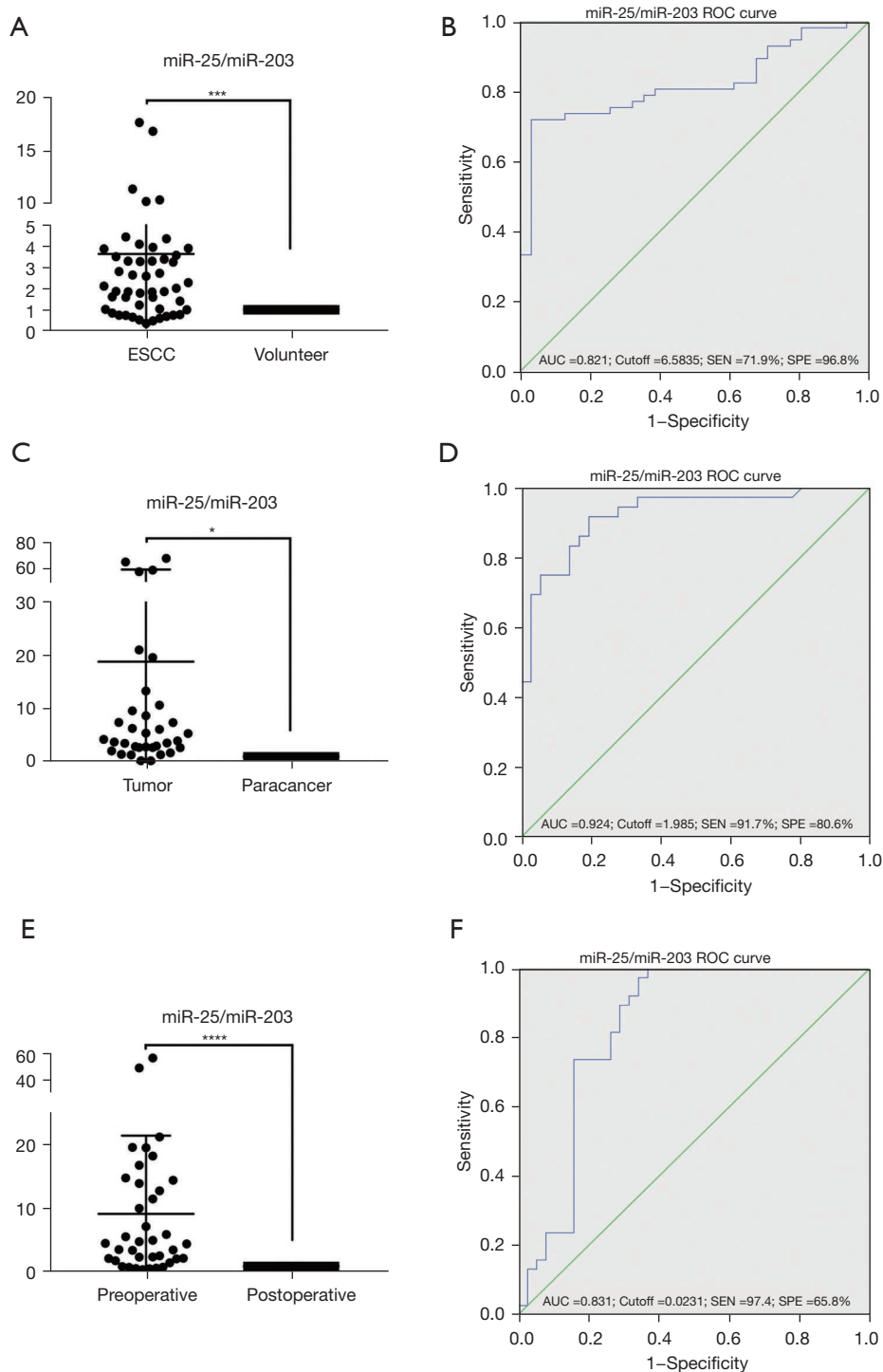
Figure 1 The up-regulation of exosome miR-25/miR-203 in primary ESCC. (A,B) The level of miR-25/miR-203 in serum was higher than that in the healthy volunteer group (P<0.05): ROC curve (AUC =0.821, sensitivity =71.8%, specificity =96.6%); (C,D) we also found the level of miR-25/miR-203 in tumor was higher than that in non-tumors tissue tissues (P<0.05): ROC curve (AUC =0.924, sensitivity =91.7%, specificity =80.6%); intriguingly, (E,F) the level of miR-25/miR-203 decreased significantly after surgery (P<0.05): ROC curve (AUC =0.831, sensitivity =97.4%, specificity =65.8%). *P<0.05, ***P<0.001, ****P<0.0001.

miR-203 could be used as a potential biomarker test for the diagnosis and treatment of ESCC.

Discussion

In recent decades, although therapeutic strategies and biological targets for ESCC have made great progress, the prognosis of this malignant cancer is still poor. Therefore, researchers are increasingly shifting their focus to the relationship between miRNAs and cancers. It is critical to identify specific and sensitive biomarkers for early detection, prognosis and treatment response and as potential targets for prevention and therapy (20).

The pathogenesis of ESCC is complex, and miRNAs may affect its occurrence and progression through various pathways. Studies have found that microRNA expression is significantly different between ESCC and paracancerous tissue. For example, microRNA-10a inhibits cell proliferation and metastasis of ESCC by targeting Tiam1, suggesting that microRNAs play an important role in ESCC progression (21). Research aimed at the characterization of circulating miRNA complexes in human serum revealed that circulating miRNAs not only copurified with the Ago2 ribonucleoprotein complex but also cofractionated with vesicles such as exosomes (22). Furthermore, the biological properties of the exosomal membrane allows microRNAs in exosomes to resist enzymatic degradation in the extracellular environment (8). Therefore, an exosomal miRNA with dysregulated expression was also detected in peripheral circulation, and it can be used as a biomarker for ESCC diagnosis (23).

In this study, our results were consistent with the results of previous microRNA chips showing that miR-203 was upregulated, and miR-25 was downregulated, in ESCC. MiR-25 and miR-203, which are important members of the microRNA family, are specifically expressed in epithelial tissues, and their expression levels are significantly different in cancer. They can also be used as oncogenes, and, as cancer suppressor genes, they interact with a variety of microRNA target genes to participate in different stages of cancer development. The results showed that miR-203 and miR-25 may have certain diagnostic value in ESCC, and the combination of the two may have better diagnostic performance.

The previous studies have investigated the role of miR-203 in cancer (17,24-29). Cui *et al.* (17) found that miR-203 has good diagnostic and prognostic value in patients at the early stage of ESCC. Guo *et al. in vitro* studies

showed that knocking out microRNA-203 reduces the cisplatin chemosensitivity of osteosarcoma cell by targeting RUNX2 (26). miR-203 induces independent growth of MET characteristics and growth factors by inhibiting SNAI2 in prostate cancer (27). In addition, miR-203 also inhibits the growth of bladder cancer cells by targeting the Twist family bHLH transcription factor-1 (28). MiR-203 regulates the proliferation and apoptosis of ovarian cancer cells by targeting the expression of SOCS3, and affects proliferation through the JAK-STAT pathway (29). We found that serum miR-203 was significantly higher in ESCC patients with lymph node metastasis than those without lymph node metastasis. Moreover, the serum miR-203 level was significantly lower than that before surgery. We further determined that there was a significant association between preoperative serum miR-203 levels and the pathological stage of ESCC. Guo *et al.* (30) showed that miR-25 was upregulated in ESCC tissues. Komatsu *et al.* (31) showed that serum miR-25 diagnosed ESCC with high sensitivity and specificity, which is consistent with our results, and its carcinogenesis was associated with the negative regulation of tumor suppressor genes. Preoperative patients and patients with recurrent squamous cell carcinoma also showed high miR-25 expression, and the concentration in plasma of patients after surgery was significantly reduced. These observations indicate that plasma miR-25 is beneficial for the early diagnosis of ESCC and the dynamic monitoring of recurrence of ESCC patients. When multiple serum miRNAs are combined, there is higher sensitivity and specificity for cancer diagnosis (32).

At least from a clinical practice perspective, the strength of our current research is the use of serum rather than tissue biomarkers. The extraction of serum test biomarkers is a more feasible noninvasive method that can be used for ESCC diagnostics, real-time monitoring, and assessment of treatment outcomes. The heterogeneity of individual cancers is an important issue in the study of biomarkers using ESCC tissue, which may affect the reproducibility of research results and, thus, affect the transformation of reagents into clinical practice (33). Using blood or serum-derived biomarkers are considered strategies to overcome this particular problem. After proper validation, such biomarkers may be more robust and, therefore, easier to translate into clinically useful blood tests. Furthermore, the two selected miRNAs with a similar dysregulation status may also exist in other tumors (12-15,18). Thus, if the selected miR-25/miR-203 combination is a regulatory

miRNA that controls the driver genes of ESCC, then studies of these miRNA combinations can improve the discriminability of ESCC with other cancer types (34,35). However, focusing on discoveries of a single miRNA able to target multiple driver genes could be a barrier to research progress. The results of this study are similar and suggest that using a single miRNA for the diagnosis of esophageal cancer can be limiting, and the combined detection of multiple miRNAs can significantly improve diagnostic efficiency.

Inevitably, there were limitations to this study. First, because the collection of specimens occurred in the past year or two, long-term follow-up data are not available yet. Therefore, there are no complete survival data, which makes it impossible to assess the prognostic value of miR-25/miR-203 in ESCC. Moreover, our study only investigated ESCC, and no other cancer types, such as adenocarcinoma. Although it seems reasonable to expect that miR-25/miR-203 may play a role in ESCC diagnostics, real-time monitoring, and assessment of treatment outcomes, this possibility still needs investigation. However, the results of this study demonstrate significant potential for the development of exosome-mediated miR-25/miR-203 for ESCC diagnosis and therapeutic evaluation, and so doing more work to confirm this would be justifiable.

Conclusions

In this study, the expression level of miR-25/miR-203 in ESCC tissues and blood samples was detected. The results showed that the target microRNA was differentially expressed in ESCC and showed a significant change trend after surgery. However, there are still some shortcomings in our study, the number of cases included in our study is very limited, and a complete prognosis is not yet available. But the clinical significance of miR-25/miR-203 in ESCC deserves further study. Taken together, through the integration of miR-25/miR-203, our results strongly demonstrated that exosome-mediated miR-25/miR-203 levels may have a better potential value in cancer diagnosis and monitoring in ESCC patients.

Acknowledgments

Footnote

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