Integrative analysis of miRNA-mRNA expression profiles in esophageal fibrosis after ESD

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Abstract. The incidence of esophageal fibrosis and benign esophageal stricture (BES) has increased in recent years due to the curative therapy for early-stage esophageal carcinoma, including partial esophagectomy and esophageal endoscopic submucosal dissection (ESD). The aim of the present study was to identify key genes and associated pathways of esophageal fibrosis after the ESD procedure. During the esophageal ESD procedure, the esophageal tissue in the remaining submucosal layer, referred to as normal esophageal (NE) tissue, was collected, and 1 week thereafter, post-operative esophageal (PE) tissue was obtained. High-throughput sequencing was used to identify dysregulated microRNAs (miRNAs/miRs) between NE and PE tissues. According to the differentially expressed (DE) miRNAs, putative target genes were predicted. Gene Ontology, Kyoto Encyclopedia of Genes and Genomes enrichment analysis and DEmiRNA interaction network analysis were performed. Reverse transcription-quantitative PCR (RT-qPCR) was performed to validate the RNA-sequencing results. A total of 199 miRNAs were determined to be DE between NE and PE tissues. Compared with the expression in the NE group, 83 miRNAs were significantly upregulated, while 116 miRNAs were significantly downregulated. According to these DE miRNAs, forkhead box O1 (FOXO1), paired box 6 (PAX6), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) and adrenoceptor β1 (ADRB1) were DE genes regulated by five DE miRNAs, including miR-223-3p, miR-142-5p, miR-582-5p, miR-21-3p and miR-218-5p. The results suggested that certain pathways were markedly dysregulated, including FOXO, MAPK, AMP-activated protein kinase and signaling pathways regulating the pluripotency of stem cells and proteoglycans in cancer. According to the RT-qPCR results, the expression levels of FOXO1, PAX6, ADRB1, miR-223-3p, miR-582-5p, miR-21-3p and miR-218-5p were consistent with the integrated analysis. In conclusion, FOXO1, PAX6, PIK3CA and ADRB1 may have a role in esophageal fibrosis, regulated by miR-223-3p, miR-142-5p, miR-582-5p, miR-21-3p and miR-218-5p. The present results provided an improved understanding of the changes in the microenvironment during the process of esophageal fibrosis, as well as novel potential targets for the treatment of esophageal fibrosis and BES.

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Introduction

The incidence of benign esophageal stricture (BES) as a result of esophageal fibrosis has increased in recent years and is caused by a variety of esophageal injuries and inflammation, including partial esophagectomy, gastroesophageal reflux, caustic esophageal injury and radiotherapy (1-3). Screening programs of early detection of esophageal carcinoma have been implemented, which have led to earlier diagnosis and improved long-term success in patients with esophageal carcinoma. An increasing number of post-operative esophageal stricture cases require to be treated. The frequency of post-operative esophageal stricture ranges from 10 to 43% after esophagectomy (4,5). In addition, the number of cases of BES after endoscopic submucosal dissection (ESD) has markedly increased over the past decade (4,5). Although ESD is gradually becoming a standard treatment for early esophageal cancer, the post-operative complication of esophageal stricture cannot be ignored. The rate of post-operative esophageal stricture is 88-100% if the lesion measures >3/4 of a circumference (6). In the process of esophageal repair after ESD, fibroblasts produce extracellular matrix (ECM) and transform into myofibroblasts from 1 week after the resection. The latter causes wound contraction, which leads to scar formation and fibrosis. Thus, it is important to clarify the changes in the wound microenvironment ~1 week after ESD (7,8). However, the prevention or treatment of BES remains difficult. The mechanisms of esophageal fibrosis and BES have remained largely elusive. Therefore, investigating the mechanism of esophageal fibrosis after ESD may provide approaches to develop novel treatments.

MicroRNAs (miRNAs/miRs) are a series of small non-coding RNAs with a length of 20-24 nucleotides. It has been reported that miRNAs regulate >60% of protein-coding genes by acting on mRNAs and then inhibiting or reducing their translation (9). Multiple miRNAs and mRNAs interact with each other (10). Of note, dysregulation of certain miRNAs and their target genes may contribute to the process of fibrosis. Identifying novel treatment strategies for BES prevention and treatment is essential. In postoperative epidural fibrosis tissue, mitomycin C (MMC) was able to reduce fibrosis by regulating miR-200b expression and targeting RhoE (11). In a study by Wang *et al* (12), microarray analysis of laminectomy scar tissue indicated that the expression levels of miR-34a, miR-146a and miR-200 were significantly increased, while the levels of miR-16, miR-221 and miR-378a were significantly decreased. Thus, it was speculated that various dysregulated miRNAs and their genes have a role in the process of esophageal fibrosis.

Through performing an integrated analysis, the present study aimed to identify the core miRNAs, differently expressed (DE) genes (DEGs) and their network, as well as to explore pathways associated with the pathogenesis of esophageal fibrosis.

Materials and methods

Participants and tissue collection. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University and The First People's Hospital of Changzhou (Third Affiliated Hospital of Soochow University, Suzhou, China; approval no. 2020-100). All participants provided written informed consent. The samples were collected from January 2018 to December 2018. In the normal esophageal (NE) group, the remaining submucosal tissue at the edge of the lesion immediately after the ESD procedure was collected. In the post-operative esophageal (PE) group, the tissue was collected at the edge of the lesion 7 days after ESD. For each group, a total of five cases were enrolled in the study. The inclusion criteria were as follows: i) Patients diagnosed with esophageal high-grade intraepithelial neoplasia or early esophageal carcinoma prior to ESD and by post-operative pathology; and ii) the wound margin and base were both negative. The exclusion criteria were as follows: i) Patients <18 or >80 years of age; and ii) patients with coagulation dysfunction, abdominal aortic aneurysm and those who were not able to tolerate an endoscopic procedure. The characteristics of the participants are displayed in Table I. The tissue was obtained from the patients and immediately stored at -80˚C for further RNA extraction.

Table I. Characteristics of the participants.

Item	NE group (n=5)	PE group (n=5)	P-value
Male sex	3	2	ns
Age (years)	66 (51-76)	66 (57-70)	ns
Postoperative pathology			
High-grade intraepithelial neoplasia	3	4	ns
Early esophageal carcinoma	2	1	ns

Values are expressed as median (range) or n. NE, normal esophageal tissue; PE, esophageal samples taken at 1 week post-operatively; ns, not significant.

Microarray analysis of miRNAs. Total RNA was isolated from the tissue by using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration and purity of total RNA were determined using NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA integrity was accessed by agarose gel electrophoresis. The microarray with the Agilent Human miRNA Microarray kit, Release 21.0, 8x60K (design ID: 070156) and data analysis of the 10 samples from esophageal submucosal tissues were performed by OE Biotechnology Co., Ltd. The miRNA array contained 2,570 probes. miRNA expression was evaluated with an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc.). Total RNA was dephosphorylated, ligated with pCpCy3 and then hybridized to the microarray samples, which were then washed. Using an Agilent Scanner G2505C (Agilent Technologies, Inc.), the array was analyzed.

Data analysis. Raw data and array images were analyzed using Feature Extraction software (version 10.7.1.1; Agilent Technologies, Inc.) and GeneSpring software (version 14.8; Agilent Technologies, Inc.). The genes detected in all samples were further analyzed. DE miRNAs were selected based on fold change and P-value according to Student's t-test. Significant DEGs were considered those exhibiting a fold change ≥2.0 and a P≤0.05. The target genes of these dysregulated miRNAs were predicted with two databases miRDB (http://www.mirdb.org/) and miRWalk (http://mirwalk.umm.uni-heidelberg.de/). Hierarchical clustering was performed to confirm the dysregulated miRNAs among samples between the two groups (NE and PE group).

miRNA-target prediction. In order to identify the key miRNA-mRNA interactions in esophageal fibrosis, target genes of the DE miRNAs were predicted using two databases (miRWalk and miRDB). The intersection of the two databases was considered. Those target genes that were predicted by ≥4 algorithms were included in the study. miRNAs that reduced the target gene expression at the post-transcriptional level and the inversely correlated genes were selected as miRNA targets. The interaction network was built using Cytoscape software 3.7.2 (http://www. cytoscape.org/).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. To elucidate the functional roles of miRNAs and their target genes, GO (molecular function, biological process and cellular component) and KEGG pathway enrichment analyses were performed. A false discovery rate (FDR) <0.05 was considered to indicate a statistically significant difference.

Reverse transcription-quantitative PCR (RT-qPCR) validation of DE miRNAs and DEGs. RT-qPCR analysis was performed to verify the expression of the integrated analysis. Total RNA was isolated from the tissue which was the same as

Table II. Primers used in reverse transcription-quantitative PCR.

Name	Primer sequences (5'-3')
miR-223-3p	F: GCCGAGACCCCAUAAACUG
	R: CAGTGCGTGTCGTGGAGT
miR-142-5p	F: CATAAAGTAGAAAGCACTACT
	R: GCGAGCACAGAATTAATACGAC
miR-582-5p	F: GCGGTTACAGTTGTTCAACC
	R: CTCAACTGGTGTCGTGGA
miR-21-3p	F: ACGTCAACAGCAGTCGATGG
	R: TATGGTTGTTCTGCTCTCTCTGTCTC
miR-218-5p	F: TGCGGCGGCCCCACGCACCAG
	R: CCAGTGCAGGGTCCGAGGT
FOXO1	F: AATCCAGAGGGTGGCAAGAGCG
	R: GGCGAAATGTACTCCAGTTATCAA
PAX6	F: CCCATGCAGATGCAAAAGTC
	R: GCCAGTCTCGTAATACCTGC
PIK3CA	F: CCACGACCATCATCAGGTGAA
	R: CCTCACGGAGATTCTAAAGT
ADRB1	F: GCTGCAGACGCTCACCA
	R: GCGAGGTAGCGGTCCAG
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
β-actin	F: TGGCACCCAGCACAATGAA
	R: CTAAGTCATAGTCCGCCTAGAA

FOXO1, forkhead box O1; PAX6: paired box 6; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; ADRB1, adrenoceptor β1; F, forward; R, reverse.

the microarray. Total RNA (400 ng) was converted to cDNA using TransScript All-in-One First-Strand cDNA Synthesis SuperMIX (cat. no. AT341-01; Beijing Transgen Biotech Co., Ltd.) following the manufacturer's instructions. The reverse transcription temperature protocols were 42˚C for 15 min, 85˚C for 5 sec and maintained at 4˚C. The final products were stored at -20˚C. qPCR was conducted with Takara SYBR Premix Ex Taq™ (Tli RNaseH Plus; Takara Bio, Inc.) in a 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific, Inc.). The primer sequences are showed in Table II. The reaction procedure consists of 40 cycles: Initial denaturation at 94˚C for 30 sec, followed by 40 cycles of 94˚C for 5 sec and 60˚C for 30 sec. U6 was used as endogenous control for miRNAs and β-actin was for mRNAs. The relative expression values were calculated using the 2-ΔΔCq method (13).

Statistical analysis. Statistical analysis was performed using SPSS 19.0 software (SPSS, Inc.). Values are expressed as the mean ± standard deviation (relative expression of RNAs) or median (range) age. The unpaired Student's t-test was used to compare parameter variables (relative expression of RNAs, age). Fisher's exact test was used to compare categorical variables (male sex, postoperative pathology). P<0.05 was considered to indicate a statistically significant difference.

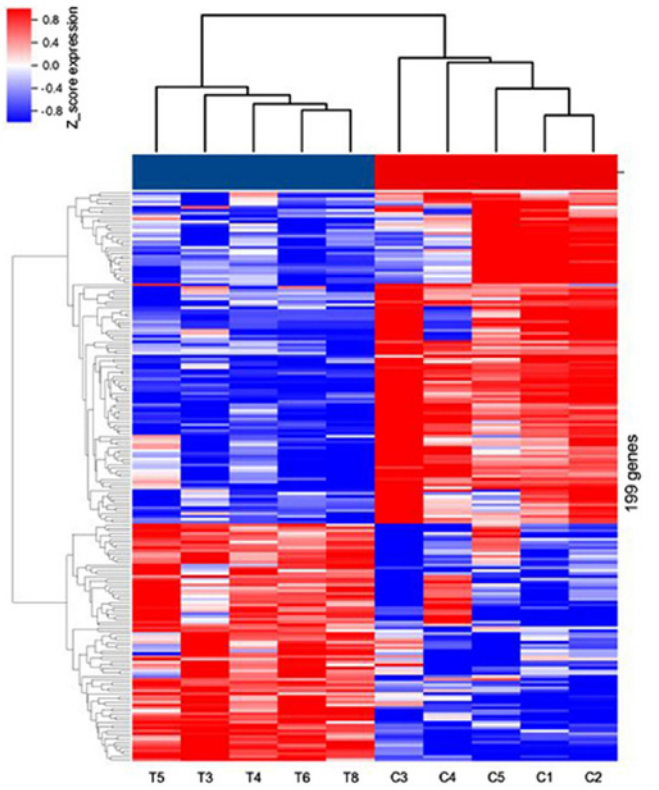

Figure 1. Unsupervised hierarchical clustering identification of two distinct groups (normal esophageal vs. post-operative esophageal group) based on their miRNA expression profile. Sample names are listed at the top. The 199 miRNAs in the heat map were differentially expressed (P<0.05) according to the results of the analysis of target genes. miRNA, microRNA.

Results

Differential expression analysis of genes in esophageal fibrosis. Using miRNA microarray analysis, numerous transcripts were detected in PE and NE tissues. In total, 199 miRNAs were significantly dysregulated with fold change >2.0, P<0.05 and FDR <0.05. Among them, 83 miRNAs were upregulated, while 116 miRNAs were downregulated. The dysregulated miRNAs are displayed in a heat-map (Fig. 1). The top 10 upregulated and downregulated miRNAs are listed in Table III.

GO and KEGG enrichment analyses of dysregulated miRNAs. GO and KEGG pathway enrichment analyses were performed to explore the pathogenesis of esophageal fibrosis. In the GO enrichment analysis, the main affected biological processes included positive regulation of transcription mediated by the RNA polymerase II promoter, negative regulation of transcription mediated by the RNA polymerase II promoter, positive regulation of transcription (DNA template), nervous system development and transcription mediated by the RNA polymerase II promoter. The cellular component terms included cytosol, nucleoplasm, cytoplasm, neuron projection and growth cone. Molecular functions mainly involved protein binding, transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding, protein serine/threonine kinase activity, β-catenin binding and mRNA 3'-untranslated region (UTR) binding (Fig. 2).

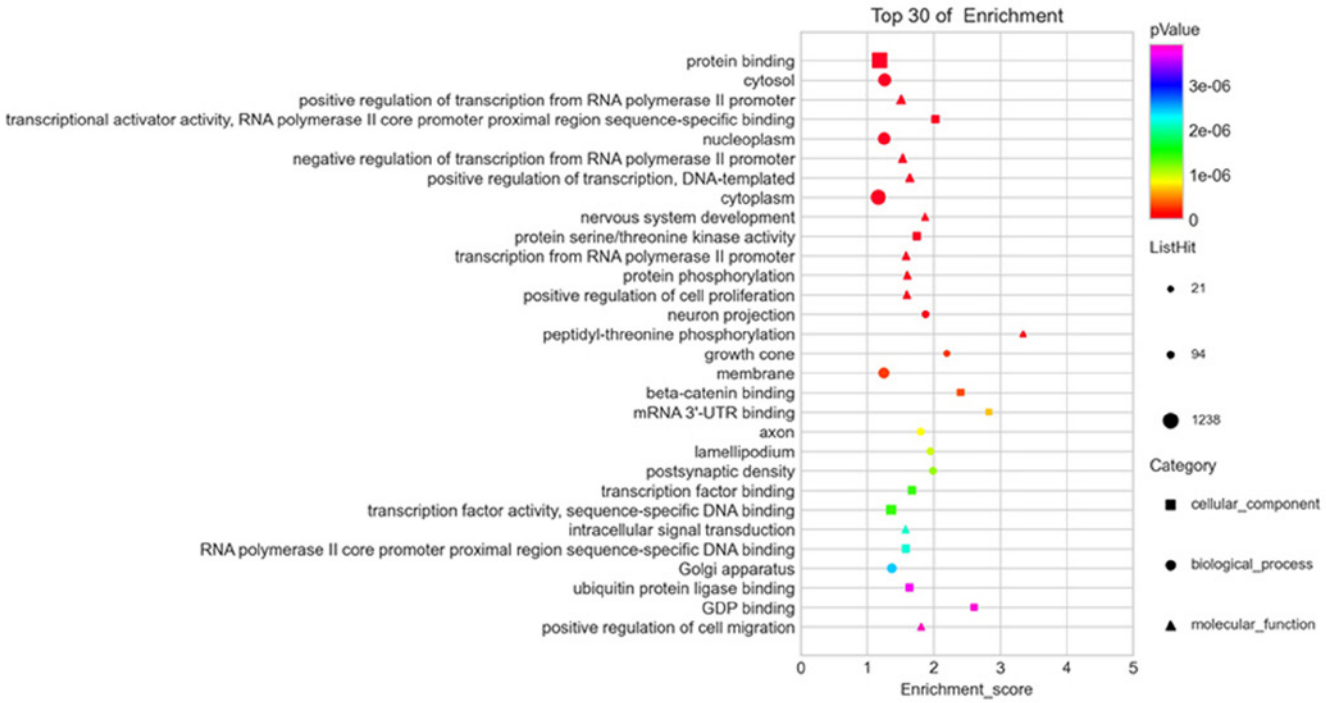

Figure 2. GO analysis of differentially expressed microRNAs. GO analysis according to biological process, molecular function and cellular component, ranked by enrichment score [-log10 (P-value)]. GO, Gene Ontology.

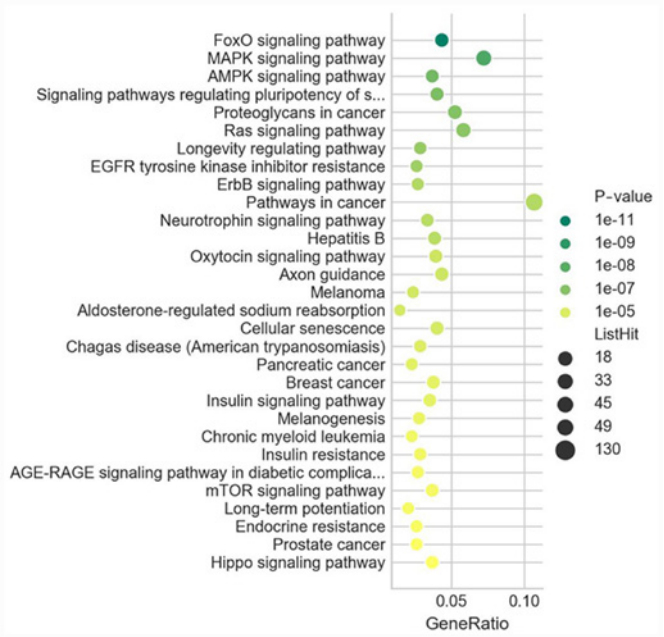

Figure 3. Kyoto Encyclopedia of Genes and Genomes analysis of differentially expressed microRNAs ranked by enrichment score [-log10 (P-value)]. FoxO, Forkhead Box O; AMPK, AMP-activated protein kinase; EGFR, epidermal growth factor receptor; AGE-RAGE, advanced glycation end product-receptor for AGE.

The results of the KEGG pathway enrichment analysis revealed the core pathways involved in esophageal fibrosis, including the forkhead box O (FOXO), MAPK and AMP-activated protein kinase (AMPK) signaling pathways, as well as signaling pathways regulating the pluripotency of stem cells and proteoglycans in cancer (Fig. 3).

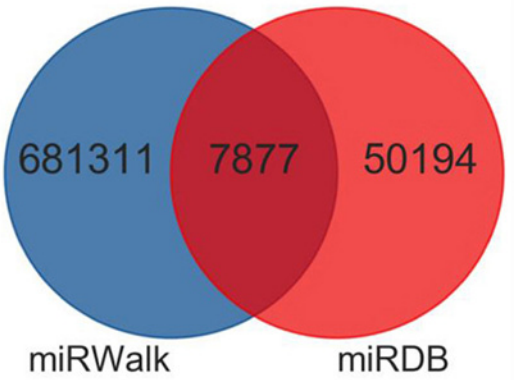

Figure 4. miRNA-target prediction using two databases (miRWalk and miRDB). Blue and red colors represent target genes predicted by the databases miRWalk and miRDB, respectively. miR/miRNA, microRNA.

Target genes predicted by DE miRNAs and miRNA-mRNA interaction network. A total of 7,878 miRNA-mRNA interaction pairs were obtained, of which 2,745 miRNA-mRNA pairs were upregulated and 5,133 miRNA-mRNA pairs downregulated (Fig. 4). According to these pairs, the miRNA-mRNA interaction network was built. The network consisted of the top 10 related dysregulated miRNAs (miR-218-5p, miR-345-5p, miR-424-3p, miR-1246, miR-142-5p, miR-21-3p, miR-223-3p, miR-223-5p, miR-582-5p and miR-605-5p) and 143 predicted DEGs (Fig. 5). In total, five miRNAs (miR-223-3p, miR-142-5p, miR-582-5p, miR-21-3p and miR-218-5p) were selected as core DE miRNAs that interacted with the majority of DEGs according to the interaction network analysis, and the GO and KEGG analyses. According to the DE miRNAs obtained, four predicted DEGs [FOXO1, paired box 6 (PAX6), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic

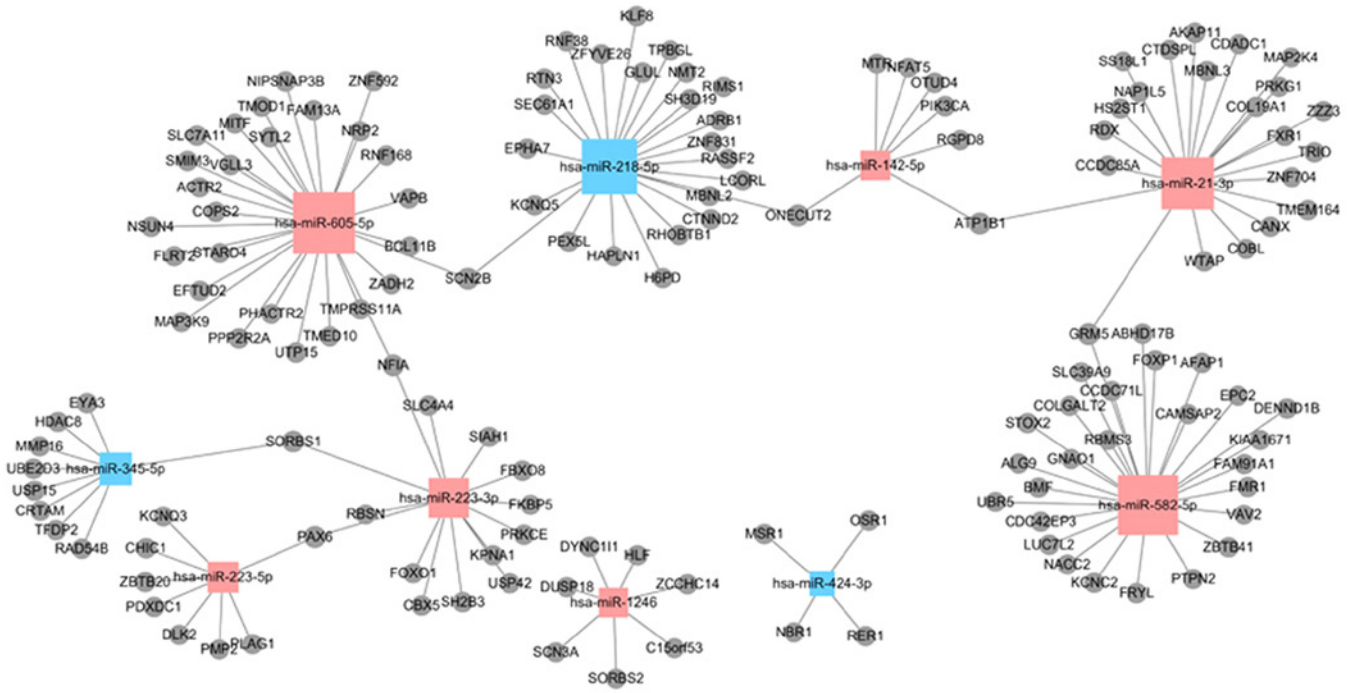

Figure 5. Esophageal fibrosis-specific differentially expressed miR-mRNA regulatory network. Red represents upregulation and blue represents downregulation. miR, microRNA; hsa, *Homo sapiens*.)

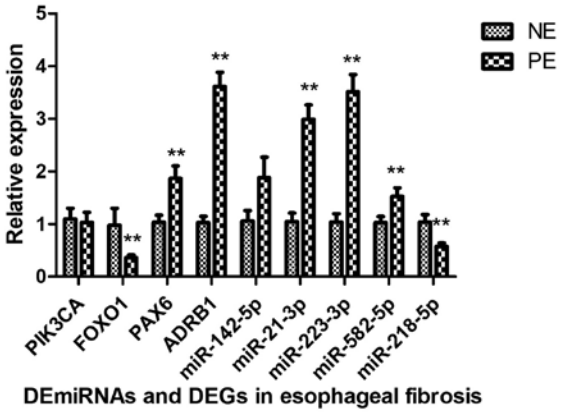

Figure 6. Reverse transcription-quantitative PCR results of DE micRNAs and target DE genes in esophageal fibrosis. DE, differentially expressed; miRNA/miR, microRNA; NE, normal esophageal tissue; PE, esophageal samples taken at 1 week post-operatively; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; FOXO1, forkhead box O1; ADRB1, adrenoceptor β1; PAX6, paired box 6. (**P<0.05 vs. NE group).

subunit alpha (PIK3CA) and adrenoceptor β1 (ADRB1)] were selected, which were also involved in the process of fibrosis.

Verification of expression changes in miRNAs and mRNAs. To confirm whether the genes were consistent with the integrated analysis, five DE miRNAs (miR-223-3p, miR-142-5p, miR-582-5p, miR-21-3p and miR-218-5p) and four targets (FOXO1, PAX6, PIK3CA and ADRB1) were selected. The results revealed that FOXO1 and miR-218-5p were downregulated, while miR-223-3p, miR-582-5p, miR-21-3p, PAX6 and ADRB1 were upregulated. However, the expression of PIK3CA and miR-142-5p was inconsistent with the results of the integrated analysis (Fig. 6).

Table III. Top 10 overexpressed and top 10 downregulated miRNAs in esophageal samples taken at 1 week post-operatively vs. normal esophageal samples.

Probe ID	P-value	FC	log2FC	Direction of regulation
hsa-miR-223-3p	2.75×10^{-5}	64.46	6.01	Up
hsa-miR-3663-5p	1.94×10^{-5}	48.76	5.61	Up
hsa-miR-223-5p	1.84×10^{-4}	36.43	5.19	Up
hsa-miR-142-5p	4.40×10^{-4}	35.13	5.13	Up
hsa-miR-31-3p	0.022	30.50	4.93	Up
hsa-miR-605-5p	2.38×10^{-4}	27.57	4.79	Up
hsa-miR-4451	2.81×10^{-6}	24.63	4.62	Up
hsa-miR-1246	1.56×10^{-7}	16.45	4.04	Up
hsa-miR-582-5p	9.05×10^{-3}	13.71	3.78	Up
hsa-miR-21-3p	4.53×10^{-4}	13.17	3.72	Up
hsa-miR-4647	7.84×10^{-5}	-58.61	-5.87	Down
hsa-miR-6129	3.89×10^{-6}	-46.88	-5.55	Down
hsa-miR-4522	4.10×10^{-5}	-43.70	-5.45	Down
hsa-miR-1273c	3.93×10^{-7}	-40.00	-5.32	Down
hsa-miR-4446-3p	9.54×10^{-7}	-38.85	-5.28	Down
hsa-miR-218-5p	5.03×10^{-3}	-36.99	-5.21	Down
hsa-miR-5699-5p	1.62×10^{-4}	-34.97	-5.13	Down
hsa-miR-424-3p	4.28×10^{-7}	-33.47	-5.06	Down
hsa-miR-345-5p	6.97×10^{-6}	-31.75	-4.99	Down
hsa-miR-6857-5p	9.26×10^{-7}	-26.64	-4.748	Down

Hsa, *Homo sapiens*; miRNA/miR, microRNA; FC, fold change.

Discussion

Studies have indicated that miRNAs have a vital role in the process of fibrosis, including myocardial, pulmonary and liver fibrosis (10-12). However, to the best of our knowledge, no previous studies have reported on the function of miRNAs in the pathobiology of esophageal fibrosis and subsequent BES. In the clinic, the prevention or treatment of BES remain challenging and identifying novel strategies for the prevention or treatment of esophageal fibrosis is of great importance. In the present study, miRNA microarray analysis was performed for evaluating DE miRNA expression associated with esophageal fibrosis. Compared with those in the NE control group, 83 overexpressed miRNAs and 116 downregulated miRNAs were identified in the patients 1 week after the esophageal ESD procedure. According to the results of GO and KEGG analyses, as well as the interaction network, four DEGs (FOXO1, PAX6, PIK3CA and ADRB1) and five DE miRNAs (miR-223-3p, miR-142-5p, miR-582-5p, miR-21-3p and miR-218-5p) were associated with esophageal fibrosis.

Regarding the collected tissue, the process of esophageal fibrosis after ESD, in which the mucosa and majority of the submucosal layer were dissected, were evaluated (6). The majority of fibroblasts and fibrocytes are located in the connective tissue (submucosal or serosa layer) of the esophagus. Thus, after ESD, the surrounding fibroblasts are activated and participate in the process of esophageal fibrosis mainly in the remaining submucosal layer (7). In addition, in the process of esophageal repair after ESD, fibroblasts produce ECM and transform into myofibroblasts from 1 week after the resection. Thus, the remaining submucosal tissue after the ESD procedure was harvested immediately and 7 days thereafter.

FOXO1, a potential target gene of miR-223-3P, which usually has a key role in cell function, is a member of the FOX transcription factor family and was reported to participate in cell metabolism, proliferation, migration, apoptosis and angiogenesis progression (14). In a study by Han *et al* (15), miR-223-3p was indicated to be highly expressed in neuroblastoma cell lines as compared with its expression in normal cell lines, and it was able to bind to the 3'-UTR of FOXO1, thus resulting in a reduction in FOXO1 expression. The results revealed that miR-223-3p has an oncogenic role by promoting cell proliferation and invasion, and inhibiting apoptosis via targeting FOXO1. In other studies, miR-223 was also dysregulated in skeletal muscle and cardiac fibroblasts and acted as a fibrogenic factor (16,17). In the present study, miR-223-3p and miR-223-5p were highly expressed and may serve as therapeutic targets. However, whether they are associated only with inflammation or with the process of esophageal fibrosis was not confirmed. The precise role of miR-223 requires further investigation.

PAX6 is a member of the PAX family of transcription factors and has been indicated to have essential roles in multiple organs and cell lines, including the eyes, central nervous system, pancreatic islet cells and olfactory system. It has been reported that PAX6 acted as an anti-fibrotic agent for cardiac fibroblast differentiation and cystic fibrosis and repressed the expression of the pro-fibrotic factor TGF-β1 (18). Interference against PAX6 also inhibited the activation and proliferation of hepatic stellate cells (HSCs). Thus, PAX6 upregulation may induce HSC activation and proliferation, resulting in liver fibrosis (19). Based on these results, future studies should explore the fibrogenic effect of PAX6 on esophageal fibrosis.

ADRB1 is expressed in the apical membrane of human bronchial ciliated epithelia and participates in the process of pulmonary cystic fibrosis (20). Kiriazis *et al* (21) investigated the effect of ADRB1 on cardiac hypertrophic growth and fibrosis. The results also revealed the key role of β-adrenoceptor signaling and that ADRB1 may be an alternative therapeutic target for cardiac hypertrophy and fibrosis. The study by Ito *et al* (22) suggested that DRB1 has an important role in myocyte apoptosis and fibrosis in cardiac muscle and skeletal muscle. In the present study, ADRB1 was one of the target mRNAs of miR-218-5p, which may also be a regulator of the process of fibrosis.

Steiner *et al* (23) indicated that PIK3CA mutation leads to abnormal scarring involving the PI3K-Akt-mTOR pathway. Yang *et al* (24) reported that PIK3CA regulated myocardial fibrosis and oxidative stress via the PI3K/Akt signaling pathway. Wegner *et al* (25) indicated that PIK3CA and TGF-β1 contributed to prostatic stromal hypertrophy and collagen accumulation. However, in the present study, the RT-qPCR results for PIK3CA revealed no significant difference between the two groups, whilst PIK3CA was negatively correlated with miR-142-5p, which was predicted with two databases miRDB and miRWalk. In other studies, PIK3CA was also negatively regulated by miR-142-5p in multiple tumors (26,27). It may be speculated that this inconsistency may result from the small size of the samples and their heterogeneity. Thus, whether PIK3CA regulates esophageal fibroblast activation requires further investigation.

In cardiac fibrosis after myocardial infarction, miR-142 contributed to cardiac fibroblast activation by targeting the adenomatous polyposis coli and the subsequent Wnt signaling pathway (28). In idiopathic pulmonary fibrosis, miR-142-3p had a protective role against the pro-fibrotic effect of TGF-β1 (29). In the process of non-small cell lung cancer, upregulated miR-142-5p suppressed cell proliferation by regulating PIK3CA (27). Together with the present results, the above suggested that miR-142 may have key roles in inflammation and fibrosis by regulating the expression of PIK3CA. However, the RT-qPCR results for miR-142-5p indicated no significant difference. In the microarray results, the miR-142 expression in the majority of patients with PE was higher than that in the NE group. However, there was excessive heterogeneity among different samples. In addition, a previous study suggested that miR-142-5p regulates PIK3CA and has a role in fibrosis (27). Thus, whether miR-142-5p and PIK3CA are involved in the process of esophageal fibrosis requires further evaluation.

miR-582-5p has also been revealed to be closely associated with inflammatory and immune response signaling in previous studies. It was reported to have a key role in the development of chronic inflammation of the airway and subsequent airway remodeling (30). In the cirrhotic liver, hepatic cytochrome P4503A (CYP3A) activity was determined to be decreased and miR-582-5p was highly expressed, which suggested that miR-582-5p expression may regulate hepatic CYP3A activity and liver cirrhosis (31).

High expression of miR-21 has been determined in multiple fibrotic tissues. As reported by Yang *et al* (32), miR-21 contributed to the development of hepatic fibrosis via the TGF-β/Smad7 signaling pathway. miR-21 also regulated myofibroblast transformation and myocardial fibrosis. TGF-β promoted cardiac fibroblast to myofibroblast transformation and myocardial fibrosis by upregulating miR-21, which in turn sponged the target mRNA Jagged1 (33). Furthermore, miR-21 was also reported to be a fibrosis-associated miRNA that promoted inflammation in ligamentum flavum tissue by activating IL-6 expression, leading to ligamentum flavum fibrosis and hypertrophy (34). In the present study, the expression level of miR-21-3p was significantly increased in the PE group, which is consistent with other studies (32-34).

miR-218-5p was reported to be significantly downregulated in smokers with chronic obstructive pulmonary disease compared with its expression in never-smokers. It was mostly highly expressed in the bronchial airway epithelium and was associated with airway obstruction (35). Li *et al* (36) and Brkić *et al* (37) indicated that miR-218-5p regulated the proliferation, migration and EMT of human glioma cells. Pterygium is a chronic ocular inflammatory disease that may result in various ocular surface symptoms. Upregulated miR-218-5p significantly suppressed the expression of epidermal growth factor receptor via the PI3K/Akt/mTOR pathway in epithelial cells affected by pterygium (38). As the results of the present study, miR-218-5p was downregulated in the PE group, suggesting that its overexpression may counteract the fibrotic process.

According to the KEGG enrichment analysis, the PIK3CA, FOXO, MAPK and AMPK pathway was among the top signaling pathways. These pathways have all been reported as key signaling pathways in the activation of fibrosis, which supports the present results (23-25,15,39,40).

In the clinic, certain drugs may be used to prevent or treat esophageal stricture, including topical use of corticosteroids and mitomycin C (MMC) (41,42). In a previous study by our group, topical injection of MMC was able to release the post-ESD esophageal stricture (42). However, the mechanisms of esophageal fibrosis have remained elusive, to the best of our knowledge. Only MMC was reported to reduce epidural fibrosis by regulating miR-200b expression and the target gene RhoE (11). In a study by Wang *et al* (12), after MMC treatment of laminectomy scar tissue, the expression levels of miR-34a, miR-146a and miR-200 were significantly increased, while the levels of miR-16, miR-221 and miR-378a were significantly decreased. However, whether these genes are differentially expressed during the process of esophageal fibrosis remains unclear. Thus, future studies should aim to validate the associations of the aforementioned drugs and these DE miRNAs and DE mRNAs.

The present study has several limitations. First, the post-ESD esophageal stricture model is not suitable for other types of esophageal fibrosis such as esophagectomy, as the mucosa and majority of submucosal layer were dissected during the ESD procedure, but the lamina propria and serosa remained intact. However, to the best of our knowledge, there is no animal model or study referring to the mechanism of esophageal stricture resulting from esophagectomy. However, all BES types are the result of esophageal fibrosis due to myofibroblast contraction and substantial deposition of ECM components. Thus, it may be partially confirmed which factors determine the process of esophageal fibrosis. In addition, only five cases were recruited in each group. Furthermore, the tissue was collected by endoscopic biopsy, which features broad heterogeneity. Thus, further studies with larger sample sizes are required.

In conclusion, using high-throughput sequencing, DE miRNAs and their DEGs were detected in the process of esophageal fibrosis. According to the RT-qPCR results, the expression levels of FOXO1, PAX6, ADRB1, miR-223-3p, miR-582-5p, miR-21-3p and miR-218-5p were consistent with the integrated analysis, but PIK3CA and miR-142-5p could not be confirmed. These reasons may include the large heterogeneity of patients and the false positive chip results. Furthermore, miR-223-3p may participate in esophageal fibrosis by targeting FOXO1 and PAX6. miR-218-5p also has a promoting role by targeting ADRB1. miR-142-5p may facilitate fibrosis by regulating PIK3CA. In addition, miR-582-5p and miR-21-3p also had roles in esophageal fibrosis. Further studies are required to functionally characterize the role of these candidate RNAs. The current results may enhance the understanding of the microenvironmental changes in the process of esophageal fibrosis and may provide targets for novel treatment strategies for esophageal fibrosis and BES.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus (accession no. GSE169354) repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169354).

Authors' contributions

CX conceived and designed the study, authenticated the raw data and performed manuscript review. YZ developed the methodology, performed the experiments, analyzed the data and wrote the manuscript. Both authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University and The First People's Hospital of Changzhou (Third Affiliated

Hospital of Soochow University, Suzhou, China; approval no. 2020-100). All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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