



MiR-27b-3p exerts tumor suppressor effects in esophageal squamous cell carcinoma by targeting Nrf2

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Abstract

MiR-27b-3p has been reported to function as tumor suppressor in several tumors, including breast cancer and lung cancer. Recently, miR-27b-3p has been identified to be significantly down-regulated in esophageal cancer. However, the clinical significance and biological role of miR-27b-3p in esophageal squamous cell carcinoma (ESCC) still remain unclear. In this study, the expression levels of miR-27b-3p were significantly reduced in ESCC clinical tissues and ESCC cell lines (EC97069 and TE-1). Moreover, down-regulated expression of miR-27b-3p was associated with poor cell differentiation, TNM stage and lymph node metastasis. Specially, overexpression of miR-27b-3p significantly suppressed cell proliferation, migration and invasion in vitro using CCK-8 and transwell assays. Targetscan bioinformatics predictions and luciferase reporter assay confirmed that nuclear factor erythroid 2-related factor 2 (NFE2L2, Nrf2) was a direct target gene of miR-27b-3p. Nrf2 expression was significantly increased in ESCC tissues compared with adjacent tissues. Up-regulated expression of Nrf2 was correlated with TNM stage and lymph node metastasis. Functionally, knockdown of Nrf2 exhibited similar effects to overexpression of miR-27b-3p. Higher expression of ZO-1, E-cadherin and lower expression of N-cadherin, Vimentin and Claudin-1 were observed after miR-27b-3p overexpression of Nrf2 knockdown. Rescue experiments proved that miR-27b-3p suppressed cell proliferation, migration, invasion and epithelial to mesenchymal transition (EMT) via suppression of Nrf2. Taken together, the newly identified miR-27b-3p/Nrf2 axis might represent a new candidate therapeutic target for ESCC treatment.

Keywords Esophageal squamous cell carcinoma · MiR-27b-3p · Nrf2 · Epithelial to mesenchymal transition

Abbreviations

ESCC	Esophageal squamous cell carcinoma
Nrf2	Nuclear factor erythroid 2-related factor 2
EMT	Epithelial to mesenchymal transition
DMEM	Dulbecco's modified eagle's medium
FBS	Fetal bovine serum
NC	Negative control
CCK-8	Cell counting kit-8
PVDF	Polyvinylidene fluoride

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Introduction

Almost half a million (455,800) new cases of esophageal cancer were diagnosed in 2012 globally, resulting in ~400,200 deaths [1]. Esophageal squamous cell carcinoma (ESCC) is the most common form of esophageal cancer in Eastern to Central Asia, South and Africa, accounting for approximately 90% of world's esophageal cancers [2]. There is considerable evidence to support the idea that smoking cigarettes, heavy alcohol consumption, and polycyclic aromatic hydrocarbons play important roles in the pathogenesis of ESCC [3]. Recent molecular biology advances have generated wide interest in the epigenetic alteration of microRNA expression in human ESCC [4]. Further clarification of the molecular mechanisms implicated in ESCC and identification of molecular targets would contribute to improving prevention, early diagnosis, management and prognosis of ESCC [5].

MiRNAs are a classic of evolutionally conserved and single-stranded small non-coding RNAs with length

ranging from 19 to 25 nt, involved in regulating over the half of the human transcriptome controlling diverse biological processes such as proliferation, apoptosis, developmental timing, differentiation, and metabolism [6]. It is well established that the capacity of miRNAs to accelerate mRNA decay and repress translation through binding to the 3'-untranslated region of their target mRNAs allows to produce various biological responses [7]. Over the past two decades, abnormal expression of miRNA occurs frequently in many cases of cancer, including ESCC, with miRNAs function as tumor suppressors or promoters [8]. Since the discovery of miR-27b-3p, its aberrant expression has been observed in multiple cancer types, including cervical cancer [9], gastric cancer [10], breast cancer [11], and lung cancer [12]. Functionally, it was found to impact critical cellular processes and to modulate proliferation, invasion, multi-chemoresistance, apoptosis, and differentiation [10, 13–15]. MiR-27b-3p expression profile has been shown to be promising biomarker for predicting distant metastasis-free survival in triple-negative breast cancer [16]. However, there were no reports about the clinical significance and biological role of miR-27b-3p in ESCC.

As a redox-sensitive transcription factor, nuclear factor erythroid 2-related factor 2 (NFE2L2, Nrf2) is involved in cellular defense against oxidative damage and electrophilic insults [12]. Recently, it has been shown that Nrf2 is frequently amplified and overexpressed in cancer tissues and cells [17]. The role of Nrf2 in the protection of cell stress response and survival was not only found in normal cells but also in cancer cells [18]. Mounting evidence has indicated that Nrf2 is a driving force for cancer progression, tumorigenesis, and chemotherapy resistance [17]. It is capable of heterodimerizing with small MAF proteins to regulate more than 200 genes containing antioxidant response elements (AREs) [19]. The target genes of Nrf2 could regulate the expression of genes involved in redox homeostasis, proliferation, apoptosis, and epithelial-mesenchymal transition [20–22]. Somatic mutations of Nrf2 or Keap1 seems to lead to activation of Nrf2/Keap1 signaling pathway and is connected with chemoradiosensitivity and worse prognosis in ESCC [23]. However, the upstream signaling molecule or molecular mechanisms regulating Nrf2 expression and function remains uncompletely understood in ESCC.

In the current study, we examined the expression levels of miR-27b-3p in clinical 41 paired ESCC tissues and matched normal esophageal tissues and explored the roles of miR-27b-3p in cell proliferation, migration and invasion. Additionally, we investigated the modulation of Nrf2 by miR-27b-3p and identify target motifs and mechanisms of this modulation. This is the first report confirming that miR-27b-3p is involved in the ESCC pathogenesis through direct targeting of Nrf2.

Materials and methods

Clinical specimens

A total of 41 paired tumor tissues and matched adjacent normal tissues were obtained from ESCC patients who underwent esophagus resection between June 2015 and September 2017 at Xinjiang Medical University Affiliated Tumor Hospital (Xinjiang, China). All samples were histologically examined to confirm as to esophageal hyperplasia, dysplasia or non-malignant tissues by two experienced pathologists, which next were immediately snap-frozen in liquid nitrogen and stored at -80°C for subsequent analysis. All ESCC patients had not received any other treatment and signed the written informed consent before esophagus resection. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Xinjiang Medical University Affiliated Tumor Hospital (Xinjiang, China).

Cell lines and culture conditions

Human ESCC cell lines (TE-1, EC9706 and KYSE-150) and the normal human esophageal epithelial cell line HET-1A were purchased from the American Type Culture Collection and cultured in DMEM with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% CO_2 .

Cell transfection

The miR-27b-3p mimics, miR-27b-3p inhibitor and their corresponding negative control (mimics NC and inhibitor NC), as well as two Nrf2-specific small interfering RNAs (si-Nrf2-1: 5'-GGUGUCUAGUUACAUAUA-3' and si-Nrf2-2: 5'-UAAGCUAGUGGCUCAGAAU-3') and negative control siRNA (si-NC: 5'-UUCUCCGAACGUGUCACGUTT-3') were all synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China). The full-length sequences of Nrf2 was amplified by PCR and sub-cloned into the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) for the overexpression of Nrf2. The empty pcDNA3.1 vector was used as negative control. Transfection of the aforementioned plasmids into EC9706 or TE-1 cells was performed as demanded with the use of Lipofectamine 2000 kit (Invitrogen) under the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR)

According to the corresponding instructions provided by manufactures, isolation of miRNA from tissues and cells was

conducted using the mirVANA RNA isolation kit (Ambion; Thermo Fisher Scientific, Inc.) and reverse transcription was performed using miScript reverse transcription kit (Qiagen, Inc.). Quantitation detection of miR-27b-3p was performed on an Applied Biosystems Prism 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the SYBR Green master mix (Takara Biotechnology Co., Ltd., Dalian, China). For Nrf2 mRNA analysis, total RNA was obtained from tissues using TRIzol reagent (Invitrogen) and cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen). RT-qPCR was conducted using SYBR green master mix (Takara Biotechnology Co., Ltd.). The primers for PCR analysis were as follows: miR-27b-3p forward: 5'-CGGCGGTTACAGTGGCTAA-3' and reverse: 5'-GTGCAGGGTCCGAGGT-3'; U6 forward: 5'-CTCGCTTCGGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'; Nrf2 forward: 5'-CCTTGTACA ACTCAGCATC-3' and reverse: 5'-GGCTTCTTAGTATCT TTCTT-3'; GAPDH forward: 5'-GAAGGTGAAGGTCGG AGTC-3' and reverse: 5'-GAAGATGGTGATGGGATT TC-3'. Comparative quantification was determined using $2^{-\Delta\Delta Cq}$ method with U6 or GAPDH as the internal control. All PCR assay was performed in triplicate.

Cell proliferation assay

The proliferation of EC9706 or TE-1 cells was assessed using a cell counting kit-8 (CCK-8) assay. In brief, 4×10^3 cells were seeded into each well of 96-well plates and cultured for 0, 24, 48 and 72 h, respectively. At each time point, 10 μ l CCK-8 (Dojindo, Tokyo, Japan) was added to each well. Following 2 h incubation, the optical values at a wavelength of 450 nm were measured using a microplate reader (BioTek, Winooski, VT, USA). All experiments were performed in triplicate.

Transwell assay

The migration and invasion ability of cells was evaluated using Transwell chambers (BD Biosciences, San Jose, CA, USA) coated with or without Matrigel, respectively. Briefly, approximately 1×10^5 EC9706 or TE-1 cells were harvested and seeded in the upper chamber containing serum-free DMEM medium. Meanwhile, medium containing 10% FBS was added to the lower chamber. After culturing for 24 h, migrated or invasive cells attached to the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 20 min at room temperature. The number of migrated or invasive cells was counted in five randomly selected fields (magnification, $\times 200$) under a microscope. All experiments were performed in triplicate.

Luciferase reporter assay

The putative binding sites of miR-27b-3p on the NFE2L2 (Nrf2) were predicted by TargetScan7.1 (https://www.targetscan.org/vert_71/). The full-length 3'-UTR of the human Nrf2 gene was amplified by PCR using human genomic DNA as a template and inserted into the pmiRGLO vector (Promega Corporation, Madison, WI, USA) to generate wild type (WT) Nrf2. Meanwhile, the mutant (MUT) sequences of Nrf2 3'UTR that contained mutations in the conserved miR-27b-3p binding site were produced through site-directed mutagenesis by Genescript (Nanjing, China), which were then integrated into pmiRGLO vector to generate MUT Nrf2. For luciferase reporter assay, 10 nM WT Nrf2 or MUT Nrf2 were co-transfected in EC9706 cells together with 10 nM miR-27b-3p mimics and mimics NC, as well as TE-1 cells together with 10 nM miR-27b-3p inhibitor and inhibitor NC using Lipofectamine 2000 kit (Invitrogen). After 48 h, the relative luciferase activities were determined using a Dual-Luciferase Reporter Assay system (Promega Corporation). All experiments were performed in triplicate.

Western blotting

Total cell proteins were extracted using RIPA lysis buffer and protein concentration was determined using a Bicinchoninic Acid Protein Assay kit both provided by Beyotime Institute of Biotechnology (Jiangsu, China). Equal amounts of protein samples (30 μ g) were subjected to separation on 10% SDS-PAGE gels and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk for 2 h at room temperature. Subsequently, membranes were incubation with the primary antibodies against Nrf2, ZO-1, E-cadherin, N-cadherin, Vimentin, Claudin-1 and GAPDH overnight at 4 °C, followed by the incubation with the HRP-linked secondary antibodies at room temperature for 2 h. Finally, protein bands were captured through an enhanced chemiluminescence system (Beyotime Institute of Biotechnology).

Statistical analysis

All data are presented as the mean \pm standard deviation (SD) from three independent experiments. SPSS 21.0 statistical software (IBM Corp., Armonk, NY, USA) was applied to analyze the statistics. The Chi-squared test was employed to examine possible correlations between the clinicopathologic parameters and miR-27b-3p or Nrf2 expression. The Student's t-test was applied for comparisons of differences between two groups, while one-way analysis of variance with Tukey's post hoc test was conducted for multi-group differences. The $p < 0.05$ was defined to be statistically significant.

Results

Reduced miR-27b-3p in ESCC tissues and cell lines

Using RT-qPCR, we determine the expression level of miR-27b-3p in clinical 41 paired ESCC tissues and matched adjacent tissues. As shown in Fig. 1a, miR-27b-3p expression was significantly decreased in ESCC tissues compared with adjacent tissues. According to the medium value of

miR-27b-3p in ESCC tissues, patients were classified as exhibiting low ($n = 22$) and high ($n = 19$) miR-27b-3p expression levels. Statistical analysis (Table 1) reveals that lower miR-27b-3p was remarkably correlated with poor cell differentiation ($p = 0.0067$), TNM stage ($p = 0.0240$) and lymph node metastasis ($p = 0.0013$). In addition, the expression of miR-27b-3p was significantly down-regulated in three ESCC cell lines compared with HET-1A, a normal esophageal epithelial cell line (Fig. 1b). These results

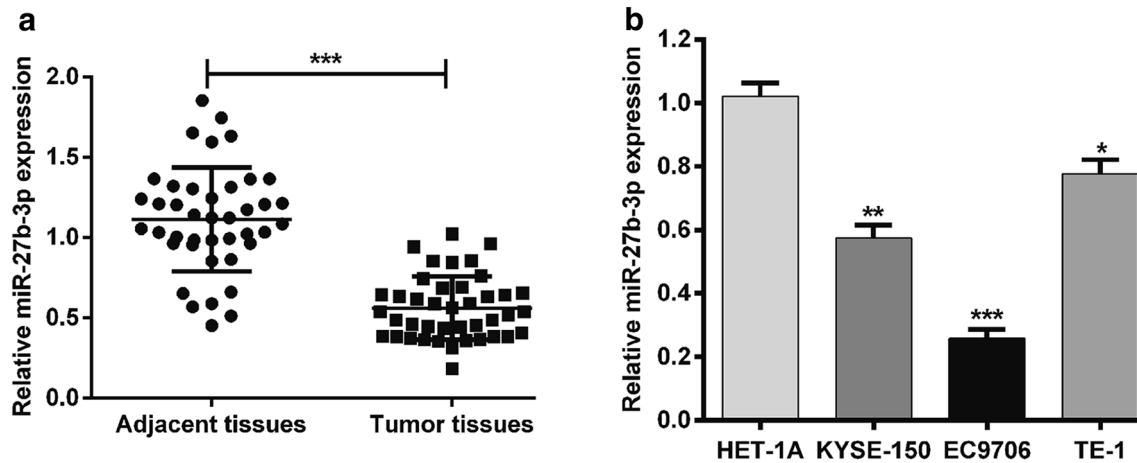


Fig. 1 Expression levels of miR-27b-3p in ESCC tissues and cell lines. **a** Expression of miR-27b-3p in 41 paired ESCC tissues and adjacent non-tumor tissues were examined by reverse transcription-quantitative PCR. *** $p < 0.001$, compared with adjacent tissues;

b Expression of miR-27b-3p in three ESCC cell lines and normal human esophageal epithelial cell line HET-1A. Data were presented as the mean \pm SD of triplicate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with HET-1A

Table 1 Relationships between miR-27b-3p expression and clinical-pathologic parameters in ESCC patients

Characteristics	Cases ($n = 41$)	MiR-27b-3p expression		p value (Chi-square test)
		Low ($n = 22$)	High ($n = 19$)	
Gender				0.7894
Male	29	16	13	
Female	12	6	6	
Age (year)				0.9842
< 50	17	9	8	
≥ 50	24	13	11	
Cell differentiation				0.0067*
Well/moderate	16	12	4	
Poor	25	10	15	
TNM stage				0.0240*
I-II	14	6	8	
III-IV	27	16	11	
Lymph node metastasis				0.0013*
Negative	18	13	5	
Positive	23	9	14	

Bold represents significant differences

ESCC esophageal squamous cell carcinoma

* $p < 0.05$

revealed that miR-27b-3p may be an important regulator in ESCC carcinogenesis.

MiR-27b-3p inhibited cell proliferation, migration and invasion in ESCC cells

Among the investigated three ESCC cells, EC9706 exhibited the lowest miR-27b-3p expression, while TE-1 had higher miR-27b-3p expression. We next selected EC9706 and TE-1 cells for gain-of-function and loss-of-function assays, respectively, to investigate the biological function of miR-27b-3p in ESCC cells. At first, RT-qPCR demonstrated that

miR-27b-3p mimics transfection significantly elevated miR-27b-3p expression in EC9706 cells, while miR-27b-3p inhibitor transfected remarkably reduced miR-27b-3p expression in TE-1 cells (Fig. 2a). The data from CCK-8 assay (Fig. 2b) showed the proliferation rate of EC9706 cells was markedly decreased by transfection of miR-27b-3p mimics compared with the negative control, while that of cells in miR-27b-3p inhibitor group was increased in TE-1 cells. In addition, in vitro migration assay (Fig. 2c) demonstrated that miR-27b-3p overexpression significantly attenuated the migration ability of EC9706 cells and miR-27b-3p knockdown promoted TE-1 cell migration ability. Similar trends were

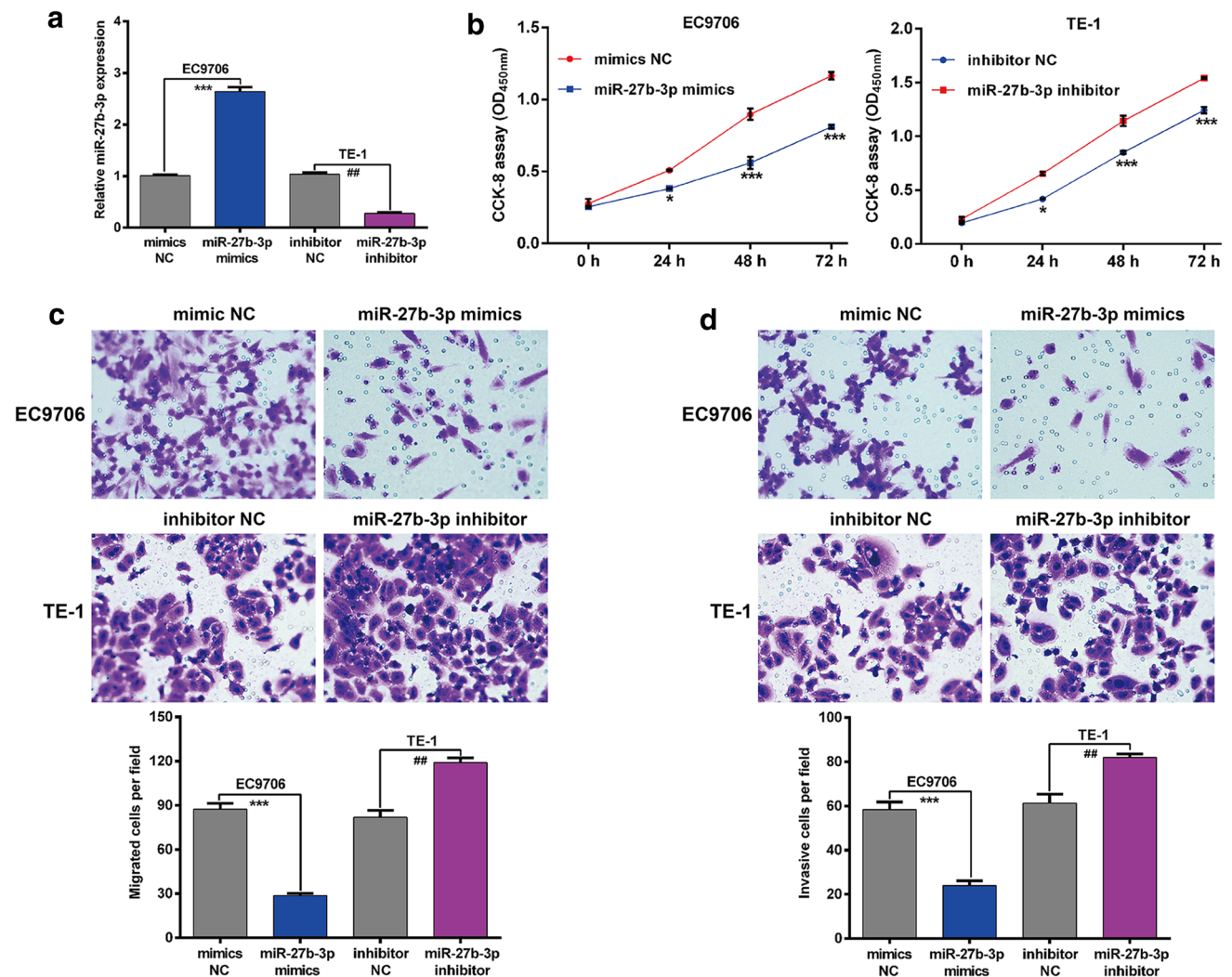


Fig. 2 Effects of miR-27b-3p expression on ESCC cell proliferation, migration and invasion. EC9706 cells were transfected with miR-27b-3p mimics or mimics NC, while TE-1 cells were transfected with miR-27b-3p inhibitor or inhibitor NC. **a** Expression of miR-27b-3p was determined using reverse transcription-quantitative PCR in EC9706 and TE-1 cells. **b** Cell proliferation was measured by a Cell Counting Kit-8 assay. **c** Transwell no matrigel-coated assay performed to determine the migration ability of EC9706 and TE-1

cells. **d** Transwell assay with matrigel-coated was performed to determine the invasion ability of EC9706 and TE-1 cells. Representative images (upper panel; Magnification, ×200) and quantification (below panel) for EC9706 and TE-1 were shown. Data were presented as the mean ± SD of triplicate experiments. **p* < 0.05, ****p* < 0.001, compared with mimics NC; ##*p* < 0.05, ###*p* < 0.001, compared with inhibitor NC

also obtained from the in vitro invasion assay in EC9706 and TE-1 cells (Fig. 2d).

Nrf2 was a direct target of miR-27b-3p

Next, the target genes of miR-27b-3p were searched using the website TargetScan. The predicted results revealed one potential miR-27b-3p binding site in the 3'UTR of Nrf2 mRNA (Fig. 3a). Subsequently, luciferase reporter assay with plasmids containing WT Nrf2 or MUT Nrf2 was employed to validate the specific and direct binding between miR-27b-3p and Nrf2 mRNA. As shown in Fig. 3b, overexpression of miR-27b-3p dramatically reduced the luciferase reporter activity of WT but not MUT Nrf2 in EC9706 cells. On contrary, miR-27b-3p inhibitor transfection significantly increased the luciferase activity of the WT 3'UTR of Nrf2, without effect on the MUT Nrf2 in TE-1 cells (Fig. 3c). Furthermore, western blotting analysis was used to observe the expression of Nrf2 regulated by miR-27b-3p. The results (Fig. 3d) demonstrated that miR-27b-3p overexpression reduced the protein expression of Nrf2 in EC9706 cells, while knockdown elevated the protein expression of Nrf2

in TE-1 cells. In addition, we determined the mRNA expression of Nrf2 in clinical 41 paired ESCC tissues and matched adjacent tissues. The results showed that Nrf2 mRNA expression was higher in ESCC tissues than that in adjacent tissues (Fig. 3e). The ESCC patients were divided into high and low Nrf2 expression group based on the median value of the relative Nrf2 mRNA expression. The association between Nrf2 expression and clinical-pathologic parameters was presented in Table 2. The high Nrf2 expression was identified to be associated with TNM stage ($p=0.0142$) and lymph node metastasis ($p=0.0032$).

Knockdown of Nrf2 imitated the suppressive effects of miR-27b-3p on ESCC cells

Because of Nrf2 was negatively regulated by miR-27b-3p, we thus speculated that Nrf2 might be an oncogene in ESCC cells. To validate this, we performed loss-of-function assay in EC-9706 cells by transfection with si-Nrf2-1 or si-Nrf2-2. As demonstrated in Fig. 4a, si-Nrf2-1 or si-Nrf2-2 transfection obviously down-regulated the protein expression of Nrf2 in EC9706 cells. Consistent with

Fig. 3 Nrf2 was a direct target gene of miR-27b-3p. **a** Human Nrf2 3'UTR fragments containing a wild-type or MUT miR-27b-3p target site were cloned downstream of the luciferase reporter gene. **b, c** EC9706 cells were co-transfected with WT or MUT Nrf2 and miR-27b-3p mimics or mimics NC, while TE-1 cells were co-transfected with WT or MUT Nrf2 and miR-27b-3p inhibitor or inhibitor NC. After 48-h transfection, dual-luciferase activity was detected. Data were presented as the mean \pm SD of triplicate experiments. $**p < 0.01$, compared with mimics NC; $##p < 0.01$, compared with inhibitor NC; **d** Western blotting was performed to detect the protein expression of Nrf2 in EC9706 cells transfected with miR-27b-3p mimics or mimics NC, as well as TE-1 cells transfected with miR-27b-3p inhibitor or inhibitor NC. **e** In total, 41 cases of patients with ESCC were analyzed to determine the mRNA expression of Nrf2 in tumor tissues and adjacent non-tumor tissues. $***p < 0.001$, compared with adjacent tissues

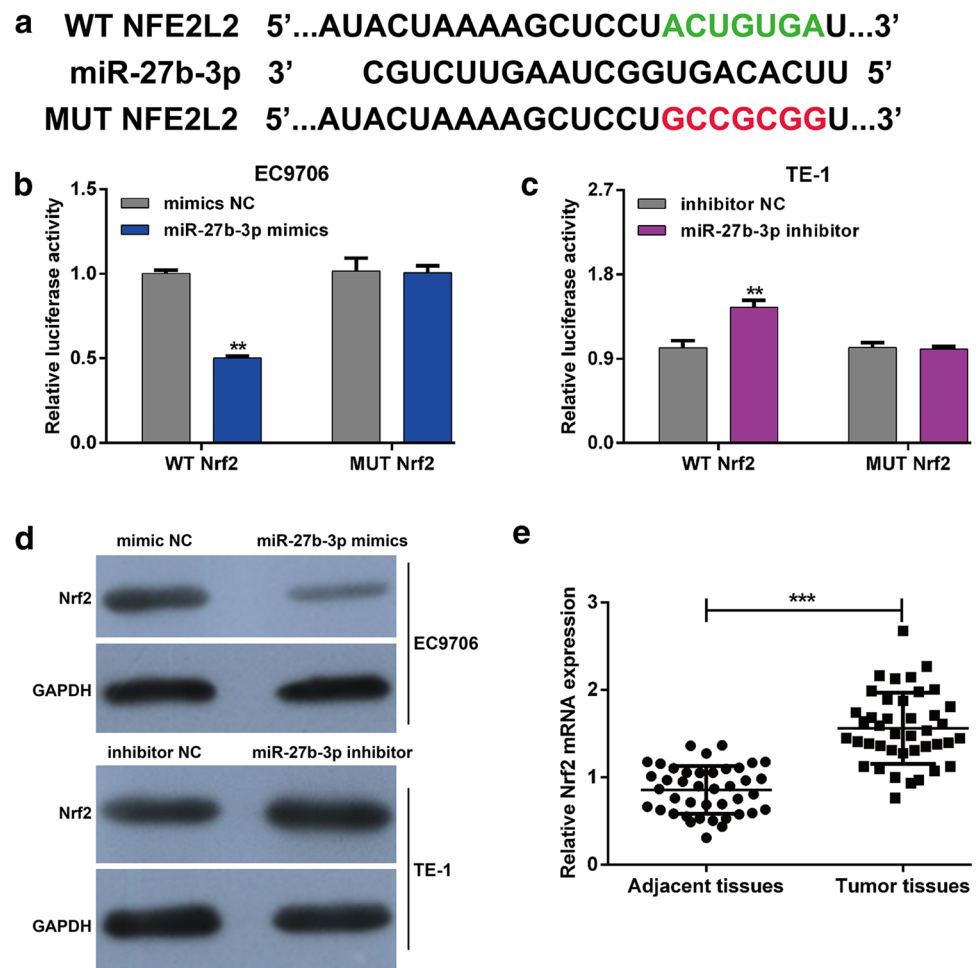


Table 2 Relationships between Nrf2 expression and clinical-pathologic parameters in ESCC patients

Characteristics	Cases (<i>n</i> = 41)	Nrf2 expression		<i>p</i> value (Chi-square test)
		Low (<i>n</i> = 16)	High (<i>n</i> = 25)	
Gender				0.5543
Male	29	11	18	
Female	12	5	7	
Age (year)				0.6532
< 50	17	5	12	
≥ 50	24	11	13	
Cell differentiation				0.0649
Well/moderate	16	6	10	
Poor	25	10	15	
TNM stage				0.0142*
I-II	14	7	7	
III-IV	27	9	18	
Lymph node metastasis				0.0032*
Negative	18	10	8	
Positive	23	6	17	

Bold represents significant differences

ESCC esophageal squamous cell carcinoma

**p* < 0.05

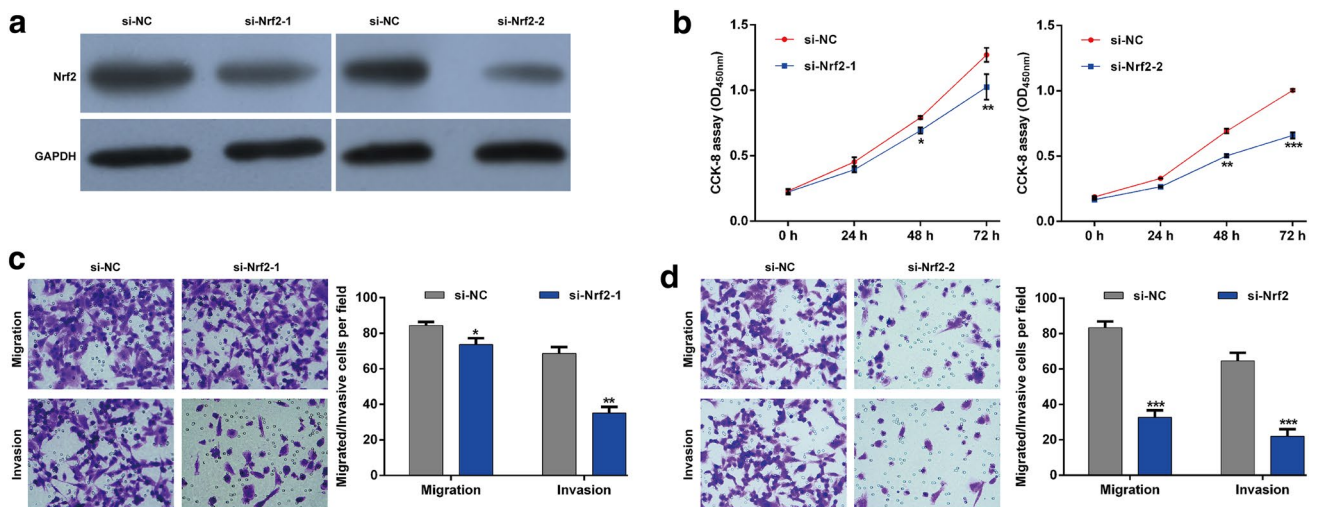


Fig. 4 Effects of Nrf2 knockdown on ESCC cell proliferation, migration and invasion. EC9706 cells were transfected with si-Nrf2-1, si-Nrf2-2 or si-NC. **a** The protein expression of Nrf2 was detected by western blotting. **b** Cell proliferation was measured by a Cell Count-

ing Kit-8 assay. **c, d** Transwell assays were performed to determine the migration and invasion ability of EC9706 cells. Data were presented as the mean \pm SD of triplicate experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, compared with si-NC

miR-27b-3p overexpression, we found knockdown of Nrf2 by si-Nrf2-1 or si-Nrf2-2 transfection significantly suppressed the cell proliferation rate (Fig. 4b), migration and invasion capacities (Fig. 4c, d) in EC9706 cells.

Restoration of Nrf2 reversed the suppressive role of miR-27b-3p on ESCC cells

To further confirm the association between miR-27b-3p and

Nrf2 in ESCC cell function, rescue experiments were performed in EC9706 cells by co-transfection with miR-27b-3p mimics and Nrf2 overexpression plasmid. Western blotting assay indicated that the down-regulation of Nrf2 by miR-27b-3p mimics transfection alone was restored by co-transfection with miR-27b-3p mimics and Nrf2 overexpression plasmid (Fig. 5a). Then, we conducted a CCK-8 assay to test cell proliferation. We discovered that the proliferation of EC9706 cells diminished as a result of miR-27b-3p overexpression, and such diminishment could be restored by the co-transfection of Nrf2 overexpression plasmid (Fig. 5b). Additionally, Nrf2 overexpression also abolished impaired cell migration and invasion ability of EC9706 cells induced by miR-27b-3p overexpression (Fig. 5c). The above data collectively demonstrated that miR-27b-3p suppressed ESCC proliferation, migration and invasion by targeting Nrf2.

MiR-27b-3p inhibited EMT by targeting Nrf2 in ESCC cells

We further assessed whether miR-27b-3p-induced ESCC cell migration and invasion by targeting Nrf2 was related to EMT. To confirm this, we detected the protein expression

of EMT related markers, including ZO-1, E-cadherin, N-cadherin, Vimentin and Claudin-1 in EC9706 cells. As depicted in Fig. 6a, we found that the expression of ZO-1 and E-cadherin in si-Nrf2 group was up-regulated compared to the si-NC group, while the N-cadherin, Vimentin and Claudin-1 showed an opposite situation. Interestingly, miR-27b-3p overexpression increased ZO-1 and E-cadherin, and decreased the N-cadherin, Vimentin and Claudin-1 levels, which was notably reversed by restoration of Nrf2 (Fig. 6b). Therefore, these data indicated that miR-27b-3p inhibited the EMT through targeting Nrf2 in ESCC cells.

Discussion

Increasing evidence reveals that some miRNAs including miR-145, miR-133a, miR-133b, miR-21, and miR-25 are dysregulated in ESCC [24–26]. In this study, we identified that miR-27b-3p is another miRNA which is downregulated in ESCC tissues compared with matched adjacent tissues. Besides, we also found that miR-27b-3p expression is lower in three ESCC cell lines than in normal esophageal epithelial cell line HET-1A. Notably, the reduction in miR-27b-3p is

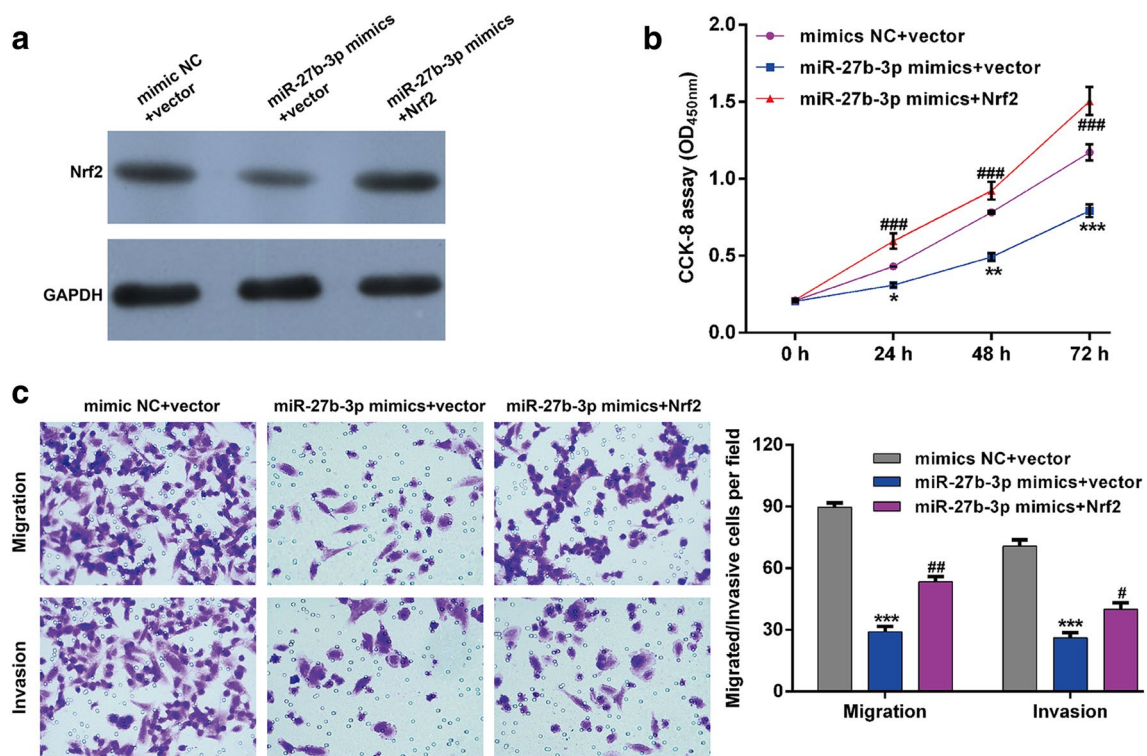


Fig. 5 Restoration of Nrf2 reversed the suppressive role of miR-27b-3p on ESCC cells. EC9706 cells were co-transfected with miR-27b-3p mimics and Nrf2 overexpression plasmid. **a** The protein expression of Nrf2 was detected by western blotting. **b** Cell proliferation was measured by a Cell Counting Kit-8 assay. **c** Transwell

assays were performed to determine the migration and invasion ability of EC9706 cells. Data were presented as the mean \pm SD of triplicate experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, compared with mimics NC + vector; # p < 0.05, ### p < 0.01, #### p < 0.001, compared with miR-27b-3p mimics + vector

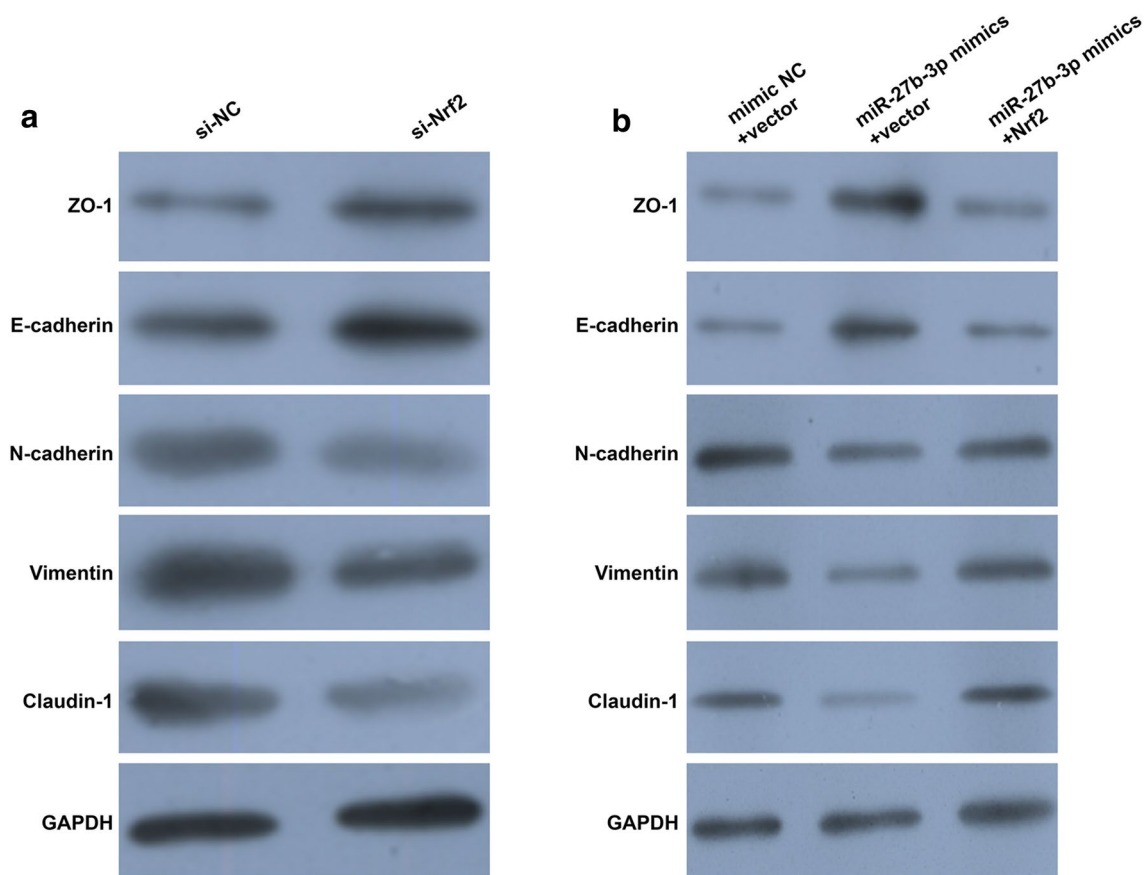


Fig. 6 MiR-27b-3p regulated EMT through Nrf2. Western blot analysis results of the protein levels of ZO-1, E-cadherin, N-cadherin, Vimentin and Claudin-1 under Nrf2 knockdown (a) or the co-transfection of miR-27b-3p mimics and Nrf2, mimics NC, and empty vector as control (b)

consistent with previous studies [10, 11, 27], which have reported reduced miR-27b-3p expression profiles in gastric cancer [10], breast cancer [27], and lung cancer [11]. In a further, the correlation of miR-27b-3p levels with poor cell differentiation, TNM stage and lymph node metastasis were highly significant. Similarly, triple-negative breast cancer studies revealed that higher expression of miR-27b-3p is linked to metastasis in patients [16]. Our study indicates that miR-27b-3p can be used as a potential diagnostic biomarker for ESCC.

To determine whether miR-27b-3p implicated in the pathogenesis of ESCC, we choose EC9706 for gain-of-function experiments and TE-1 for loss-of-function experiments. As a result, knockdown of miR-27b-3p promoted some cellular processes like cell proliferation, migration and invasion, while miR-27b-3p overexpression has opposite effects on these malignant biological behaviors. Recent studies using gastric cancer specimens and cell lines by Tao et al. [10] indicated the role of miR-27b-3p in the suppression of cell proliferation. Ectopic expression of miR-27b-3p in Bads-200 cells significantly reduces cell proliferation, colony formation, and reverses multi-chemoresistance [27].

Also miR-27b-3p is able to suppress cells viability and survival of NCI-H446 and A549 cells in vitro [11]. In addition, miR-27b-3p attenuates the malignant phenotype in colorectal cancer cells by directly interacting with Rab3D [16].

It is well established that complex biological procedures conferred by a given miRNA are majorly dependent on the target of this miRNA [28]. Then, we used website TargetScan to identify potential target genes and binding sites of miR-27b-3p. Among these targets, Nrf2, which mutation has been shown to confer tumorigenesis potential and chemoradiation therapy in advanced ESCC, was selected for further investigation. Luciferase reporter assay revealed that miR-27b-3p binds selectively to the 3'UTR of Nrf2 mRNA and reduces its translation. Furthermore, Nrf2 knockdown imitated the effects of miR-27b-3p on proliferation, migration and invasion, while restoration of Nrf2 rescued them. Notably, Nrf2 overexpression did not completely abrogate the suppressive role of miR-27b-3p on ESCC cell migration and invasion. On the one hand, Nrf2 overexpression might activate other molecules associated with cell mobility and other targets of miR-27b-3p may function with Nrf2 to suppress the function role of

Nrf2 overexpression on cell mobility. On the other hand, cell status might be not good enough in miR-27b-3p mimics + Nrf2 group when performing transwell well assays. Anyway, compared with miR-27b-3p mimics + vector group, we confirmed Nrf2 overexpression caused significantly elevated migration and invasion. These results suggest that miR-27b-3p function as a tumor suppressor in ESCC might through targeting Nrf2.

It has been shown that cell proliferation pathways converge on redox-dependent signaling processes [29]. Fan et al. [29] revealed that the Nrf2-Keap1 pathway represent a critical switch for glioma cells proliferation. The growth of pancreatic cancer cells can be enhanced by soluble factors from stellate cells, the mechanism is associated with metabolic reprogramming and ROS detoxification that activated by Nrf2 [30]. Besides, ERK/Nrf2 signaling pathway is responsible for inhibition of proliferation in chrysin-treated glioblastoma cells [31]. It is clear from Zhang et al. [32] that Nrf2 induces hepatocellular carcinoma cells proliferation at least partially by upregulating Bcl-xL and MMP-9 expression. In this study, the involvement of the above-described signaling pathways may play major roles during miR-27b-3p/Nrf2 axis mediated ESCC cells proliferation. Interestingly, an emerging data has revealed the essential role of the EMT program in the acquisition of migratory and invasive capabilities in epithelial cells [33]. In the current study, increased expression of ZO-1 and E-cadherin, and decreased expression of the N-cadherin, Vimentin and Claudin-1 were observed in EC9706 cells with miR-27b-3p overexpression and Nrf2 silencing. The effects of miR-27b-3p on these EMT biomarkers can be reversed by restoration of Nrf2. Our results suggest that miR-27b-3p could inhibit cell migration and invasion by regulating EMT program through targeting Nrf2.

In summary, this was the first study to systemically determine the tumor suppressor role of miR-27b-3p in ESCC. Our findings revealed that miR-27b-3p was downregulated in ESCC tissues and cell lines, and the expression of miR-27b-3p was correlated with poor cell differentiation, TNM stage and lymph node metastasis. We also concluded that miR-27b-3p inhibited proliferation, invasion and migration of ESCC cells through downregulation of Nrf2.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate This study was conducted in accordance with the Declaration of Helsinki and approved by the

Ethics Committee of Xinjiang Medical University Affiliated Tumor Hospital (Xinjiang, China).

Ethical standards This study was approved by the Committee on the Ethics of Ethics Committee of Xinjiang Medical University Affiliated Tumor Hospital (Approval number: XMU3109). All of the experiments were performed in accordance with the Declaration of Helsinki.

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