MicroRNA-34a inhibits esophageal squamous cell carcinoma progression by targeting E2F5

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Summary

**Purpose:** Previous studies have explored the role of microRNA-34a (miR-34a) and E2F transcription family in several tumors, however, the expression level and oncogenesis mechanism of these two factors in esophageal squamous cancer cells (ESCC) remains unclear. Our study aims to explore the inhibitory effect of miR-34a in ESCC as well as its downstream factor E2F5.

**Methods:** We explored the relevant expression level of miR-34a in human tumor tissue as well as in several ESCC cell lines. Through RNA mimic and inhibitor, we examined the specific role of miR-34a in the proliferation, apoptosis and migration of tumor cells, and we further explored the role of downstream factor E2F5 through gain- and loss-of-function analyses.

**Results:** We found that the expression level of miR-34a is significantly downregulated in ESCC tissues as well as in ESCC cell lines, and miR-34a plays an inhibitory role in tumor cell proliferation and migration while it promotes tumor cell apoptosis. We further showed that E2F5 is a direct functional target of miR-34a, as it promotes tumor cell proliferation and migration and inhibits apoptosis.

**Conclusions:** Our results indicate that the intrinsic expression of miR-34a was relatively low in ESCC. The anti-tumor effect of miR-34a is possibly dependent on the regulation of cell-cycle regulator E2F5.

**Key words:** microRNA-34a; esophageal squamous cell carcinoma; E2F5

Introduction

Esophageal carcinoma (EC) ranks the eighth most common cancer and the sixth leading cause of cancer mortality worldwide [1]. In China, the rate of diagnosed EC may be even higher, and over 90% are presented as esophageal squamous cell carcinoma (ESCC) in further pathological diagnosis [2]. Although treatment methods and surgical techniques have been well developed in recent decades, the long-term outcome of these patients has not been significantly improved, with a reported 5-year overall survival rate of only around 10% [3,4]. Therefore, there is a significant need for deepening our understanding in the underline molecular mechanism of ESCC, and further developing novel therapeutic approach to improve patient outcomes. In recent years, targeting therapy for microRNA (miRNA) to treat various human cancers has received widespread attention, as many studies indicate that miRNAs involve in the process of cancer initiation, progression, and resistance to chemotherapy [5,6]. To date, several human endogenous miRNAs have been reported to be dysregulated in EC, including miR-34a, a human endogenous small noncoding RNA which regulates cellular senescence [7,8]. Previous studies suggest that miR-34a is downregulated in EC, which highly associated
with multiple oncological features of tumor cells [9-13]. However, the role of miR-34a in oncogenesis is complicated, and the precise association between miR-34a and biological behavior of ESCC remain unclear. For example, previous studies have suggested that miR-34a might have a direct regulatory effect on various cyclins and cell-cycle pathway proteins, thus affecting tumor cell viability, proliferation, invasion, and chemotherapy resistance [13,14]. E2f family transcription factors might be one of the most important downstream pathways, as the E2F transcription factor 5 (E2F5) plays an oncogenic role in several types of cancer [15]. However, its role in ESCC and the association between it with miR-34a remains poorly characterized.

In this study, we aimed to explore the inhibitory effect of miR-34a in ESCC as well as its downstream factor E2F5. Although both the expression level of miR-34a and E2F transcription factor in gastrointestinal cancer have been multiply studied, currently there have been no studies on the specific roles of the two factors in ESCC, and no research clearly elucidates the correlation between them. By delving into the mechanism of a particular miRNA in ESCC, we hope to provide more evidence on the underlying mechanism of ESCC oncogenesis, and further shield light on the clinical targeting therapy in the near further.

Methods

Cell culture

The cancer and paracancer tissue were isolated from diagnosed ESCC patients in Cancer Hospital of Shantou University Medical College, human ESCC cancer Eca109, KYSE150, EC9706 and TE-1 cell lines as well as HEEC cell line were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The procedures were approved by the Ethics Committee at the Cancer Hospital of Shantou University Medical College, with patient consent obtained before procedure. Cells were cultured in dulbecco’s modified eagle medium (DMEM) medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/L penicillin, and 100 mg/L streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), maintained at 37°C, atmosphere was set at of 95% air and 5% CO₂.

Cell transfection

Following the manufacturer’s protocol, 100 nM of miRNA-34a mimic, miRNA-34a inhibitor or negative control (NC) miRNA-scrambled (Shengong Biological Engineering Technology company, Shanghai, China), and 100 nM of E2F5-specific shRNA or negative control (NC) shRNA (Genepharma, Shanghai, China) were transfected into Eca109 cells using Lipofectamine TM 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The shRNA sequences were as follows: NC shRNA, CATGTCATGTGTCACATCTC; E2F5-shRNA, CAGGAACATCCATGTGCTGTTAT. The miRNA sequences were not provided by the suppliers for commercial secret consideration.

Cell proliferation assay

Cell proliferation was determined by cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). About 5×10³ Eca109 cells were seeded into 96-well plate, added with 100 µL CCK-8 medium, and then incubated for 4 hours at 37°C. The cell absorbance at 450 nm was detected under microplate reader (Bio-Rad, Hercules, CA, USA).

Scrub wound-healing assay

Eca109 cells migration were performed in Scratch wound-healing assays. Cells were firstly cultured in standard medium for 48 h, then a line-shaped wound was made using a 2 mm wide tip. After 24 hours culturing for cell migration, 4% paraformaldehyde (Sigma, St. Louis, MO, USA) were add for fixation, followed with 4’,6-diamidino-2-phenylindole (DAPI) staining. The cell migration was determined under fluorescence microscope (Olympus corporation, Tokyo, Japan), and analyzed using ImageJ 1.8.0.

Transwell assays

ESCC invasion were performed in transwell migration assays. Cells were dissolved and diluted into 1×10⁶ cells/mL using standard serum-free medium, and seed onto the membrane in the upper chamber. After 48 h culturing under standard conditions, the number of migrated cells on the lower chamber was determined under microscope.

Cell apoptosis assay

Flow cytometry was applied to determine the apoptosis of Eca109 cells. After 48 h of cell transfection, cells were digested with 200 µL trypsin without EDTA (ethylenediaminetetraacetic acid) for 1 min. Then cells were centrifuged, and resuspended with 1×Binding Buffer in 1 mL tubes. Each tube was added with 5 µL fluorescein isothiocyanate (FITC) and 10 µL propidium iodide (PI) under dark condition and cultured for 5 min. Afterward, 2 mL phosphate buffered saline (PBS) was added and the flow cytometry was applied.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

RT-qPCR analysis was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was extracted from Eca109 cells in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed using the 260/280 nm absorbance ratio, concentration was quantified using a microplate reader. Then, RT-PCR was performed with 100 ng total RNA. The relative mRNA expression was calculated using the 2-ΔΔCt method. Primer sequences were as follows: miR-34a forward: 5’-CCCAGAACAATAGACACGCTGGA-3’;
miR-34a reverse: 5’-ATCAGCTGGGCACCTAGGACA-3’; E2F5 forward 5’-CCTGTTCCCCCACGTGATG-3’; E2F5 forward 5’-TTTCTG TGGAGTCACTGGAGTCA-3’; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: 5’-TGCACC ACCAACTGCTTAGC-3’; GAPDH reverse: 5’-GGCATGGAC TGTGGTCATGAG-3’.

Western blot analysis

Eca109 cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer for protein extraction (Sigma Aldrich, St. Louis, MO, USA). Total proteins were quantified using bovine serum albumin (BSA) assay kit (P0006, Beyotime, Shanghai, China), then proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). For immunoblots, PVDF membranes carrying the transferred proteins was incubated at 4°C overnight with primary antibodies: E2F5 (1:1000, sc-1082, Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-Cadherin (1:1000, ab15148, Abcam, Cambridge, MA, USA), N-Cadherin (1:1000, ab18203, Abcam, Cambridge, MA, USA), vimentin (1:1000, ab8978, Abcam, Cambridge, MA, USA), GAPDH (1:1,000, ab8245, Abcam, Cambridge, MA, USA). Then PVDF membranes were washed and incubated in a secondary antibody (1:5000, Abcam, Cambridge, MA, USA) for 2 h at room temperature. Finally, the bands were exposed using the BeyoECL kit (Beyotime, Shanghai, China) and Tanon 5200 system.

Luciferase reporter assay

3’-untranslated region (3’-UTR) luciferase reporter assay was measured following the Dual-Luciferase Reporter Assay Kit (Promega Corporation E1910, Madison, WI, USA). The E2F5 mRNA containing the miR-564 binding site was amplified by PCR using the PCR Amplification kit (TaKaRa Biotechnology, Dalian, China). We applied three luciferase reporter recombinant vectors, there were wild-type 3’UTR of E2F5 gene (pGL3-E2F5 wt), Mut E2F5 with mutated miRNA-34a binding sites (pGL3-E2F5 mut), and a wild-type 3’UTR along as control (pGL3-con). The Eca109 cells were seeded in each well of 24-well plates and co-transfected with 3’UTR vectors and hsa-miR-34a mimic (or miRNA mimics control, NC group, (GenePharma Co., Ltd. Shanghai, China) using Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Statistics

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Data were represented as mean ± SD (Standard Deviation). The t-test was used for analyzing measurement data. Comparison between groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). p<0.05 indicated the significant difference.

Results

MiR-34a is downregulated in ESCC tissues and ESCC cell lines

To investigate its possible role in ESCC, we first performed RT-PCR to determine the expression level of miR-34a in 30 paired ESCC and non-tumor tissues. We found that miR-34a was significantly downregulated in ESCC tissues (p<0.001, Figure 1A). Additionally, we examined the expression level of miR-34a in multiple ESCC cell lines (Eca109, KYSE150, EC9706, TE-1) and one normal esophageal epithelium cell line (HEEC). It turned out that miR-34a was markedly decreased in four ESCC cell lines as well (p<0.001) (Figure 1B). The disproportional expression pattern of miR-34a in ESCC and paired adjacent normal tissues suggests that miR-34a might have a regulating role in the initiation and progression of ESCC.

MiR-34a mediates various malignant behaviors in ESCC

To further explore the potential regulating role of miR-34a in ESCC, we performed a series of malignancy-related experiments on Eca109 cell line. Firstly, we applied miR-34a mimics and miR-
34a inhibitor to study the effect of disruption in miR-34a expression on ESCC. The transfection and inhibitor efficiency were evaluated using RT-PCR. The miR-34a expression was observed significantly upregulated (p<0.05) after miR-34a mimics transfection and downregulated (p<0.01) after miR-34a inhibitor administration (Figure 2A). Cell proliferation assays were performed to assess the effect of miR-34a expression on ESCC cell proliferation. After 5 days observation, we clearly identified that the overexpression of miR-34a improved ESCC cell proliferation rate and the administration of miR-34a inhibitor achieved the exact opposite effect (Figure 2B). In the apoptosis assay, we found that miR-34a overexpression facilitated cell death in ESCC cells (p<0.01) and miR-34a downregulation facilitated cell survival (Figure 2C). Wound healing assays were performed to evaluate the migration ability of ESCC cells after transfection with miR-34a mimics or inhibitor. Pictures were taken at 0 h and 8 h. (E): Transwell assay was performed to evaluate the metastasis ability of ESCC cell after transfection with miR-34a mimics or inhibitor.

**Figure 2.** MiR-34a mediates various malignant behaviors in ESCC. (A): The expression level of miR-34a in ESCC cells after 48 h transfection of miR-34a mimics or miR-34a inhibitor (*p<0.05, **p<0.01). (B): CCK8 assay was performed to detect the proliferation rate of ESCC cells after transfection of miR-34a mimics or inhibitor. (C): The apoptosis rate of ESCC cells was detected using flow cytometry 48 h after transfection of miR-34a or inhibitor. (D): Wound healing assay was performed to evaluate the migration ability of ESCC cells after transfection with miR-34a mimics or inhibitor. Pictures were taken at 0 h and 8 h. (E): Transwell assay was performed to evaluate the metastasis ability of ESCC cell after transfection with miR-34a mimics or inhibitor.
MicroRNA-34a inhibits esophageal squamous cell carcinoma

Prolonged cell longevity in ESCC (p<0.001, Figure 2C). Metastasis is a major death cause in ESCC patients. Hence, we performed wound healing assay and transwell assay to evaluate the regulating role of miR-34a in cellular migration and metastasis in ESCC. The wound healing result showed that the overexpression of miR-34a slowed down the migration rate of ESCC cells (p<0.05) while the down-regulation of miR-34a improved the migration rate (p<0.01, Figure 2D). The transwell assay indicated that miR-34a was an inhibitory factor in ESCC metastasis, as overexpression of miR-34a decreased the invasion rate of ESCC cells (p<0.05) while the downregulation of miR-34a improved the invasion rate (p<0.001, Figure 2E).

MiR-34a directly targets the 3'-UTR of E2F5 in ESCC

To better understand the regulating mechanism of miR-34a in ESCC, we resorted to TargetScan online platform to identify the potential downstream gene of miR-34a. E2F transcription factor 5 protein (E2F5) was identified as an interesting candidate since it has been reported to be upregulated in various cancers [16-19]. To determine whether miRNA-34a is capable of regulating E2F5 expression in ESCC cells, RT-PCR was used to examine the expression change in E2F5 after miR-34a disruption. The results demonstrated that transfection of miR-34a mimics significantly decreased E2F5 mRNA level (p<0.05), while the administration of miRNA-34a inhibitor significantly upregulated E2F5 mRNA level (p<0.01) (Figure 3A). Additionally, western blot analysis also showed similar results as transfection of miRNA-34a mimics and miRNA-34a inhibitor into ESCC cells could significantly decrease and increase E2F5 protein level, respectively (Figure 3B). Furthermore, we explored TCGA database and identified that E2F5 mRNA level of E2F5 in ESCC}

![Figure 3. MiR-34a directly targets the 3'-UTR of E2F5 in ESCC. (A): RT-PCR was performed to detect the mRNA level of E2F5 in ESCC cells transfected with miR-34 mimics or inhibitor. (B): WB was performed to detect the protein level of E2F5 in ESCC cells transfected with miR-34 mimics or inhibitor. (C): The mRNA expression level of E2F5 in ESCC according to TCGA database. (D): The E2F5 gene 3'UTR included 7 sequential pairing bases with the 5' of miR-34a, indicating that E2F5 may be a potential target of miR-34a. Dual-luciferase assays were used to detect the luciferase activity in Eca109 cells transfected with pGL3-con, pGL3-E2F5 wt or pGL3-E2F5 mut in the presence of miR-34a mimics. (E): The correlation between the expression of miR-34a and E2F5 mRNA (*p<0.05, **p<0.01, ***p<0.001).]
Figure 4. E2F5 enhances multiple malignant characters in ESCC and facilitate ESCC metastasis via regulating EMT process. (A): The expression level of E2F5 was detected in sh-E2F5 cell line using RT-PCR and WB. (B): Apoptosis rate was detected in sh-E2F5 cells with or without addition of miR-34a inhibitor using flow cytometry. (C): CCK-8 assay was performed to detect the proliferation rate of sh-E2F5 cells with or without addition of miR-34a inhibitor. (D): Wound healing assay was performed to evaluate the migration ability of sh-E2F5 cells with or without addition of miR-34a inhibitor. (E): Transwell assay was performed to evaluate the metastasis ability of sh-E2F5 cells with or without addition of miR-34a inhibitor. (F): Western blot detection of several EMT-related proteins (*p<0.05, **p<0.01).
was significantly upregulated in ESCC tumor tissues which is consistent with the downregulation of miR-34a in ESCC (p<0.001, Figure 3C). Next, we continued to explore whether miR-34a inhibits E2F5 mRNA expression by directly binding to the specific site in the 3'UTR region of E2F5 gene. Two luciferase reporter recombinant vectors containing either wild-type 3'UTR of E2F5 gene (pGL3-E2F5 wt) or a Mut E2F5 with mutated miRNA-34a binding sites (pGL3-E2F5 mut) were constructed. The experiment result indicated that miR-34a mimics significantly decreased the luciferase activity of wt E2F5 reporter, while showed no effect on the luciferase activity of mut E2F5 reporter (p<0.01, Figure 3D). What's more, we found a negative association between miR-34a levels and E2F5 mRNA levels in ESCC (Figure 3E). Collectively, we confirmed E2F5 as a direct target of miR-34a in ESCC.

**E2F5 enhances multiple malignant characters in ESCC and facilitate ESCC metastasis via regulating EMT process**

The results above have demonstrated that E2F5 is a direct target of miR-34a in ESCC. Hence, we moved on to investigate whether the change in E2F5 expression level could affect the malignant characters in ESCC. We constructed stably E2F5-knockdown cell line using short-harpin RNA and RT-PCR was preformed to evaluate the knockdown efficiency (p<0.01, Figure 4A). Western blot assay was also performed with a similar result (Figure 4A). The apoptosis assay result demonstrated that E2F5 acted as an anti-apoptosis factor in ESCC while the extra administration of miR-34a counteracted the E2F5-knockdown effect (p<0.01, Figure 4B). In the proliferation assay, the knockdown of E2F5 greatly weakened the proliferative ability of ESCC cells while the extra administration of miR-34a pulled the proliferation curve back to normal. These results showed that miR-34a mediates ESCC cell behaviors by targeting gene E2F5. In the wound healing and tranwell assay, the knockdown of E2F5 substantially harmed cellular migration ability in ESCC and the extra addition of miR-34a inhibitor rescued the E2F5-deficiency effect (Figure 4C and D). The epithelial-mesenchymal transition (EMT) is crucial process in cancer metastasis [20]. Considering the impact of miR-34a in the metastatic process in ESCC, we continued to explore the potential relationship between miR-34a and the EMT process. The western blot results demonstrated big changes in the expression of EMT-related genes after miR-34a-knockdown. The administration of miR-34a inhibitor decreased the expression level of E-cadherin while increased the expression level of N-cadherin and Vemintin (Figure 4F). With the extra knockdown of E2F5, the expression level of E-cadherin, N-cadherin and Vemintin level restored to relatively normal (Figure 4F). Above all, these results revealed miR-34a inhibit the metastasis process in ESCC via regulating the EMT process.

**Discussion**

Esophageal cancer (EC) is a highly fatal malignancy, the incidence of which has been climbing up gradually in western countries in recent decades [21,22]. In China, the incidence of esophageal cancers including esophageal adenocarcinoma (ESAC) and ESCC were the fifth highest [23]. In cancer-related deaths, metastasis remains the number one cause [24]. At first diagnosis, nearly 50% esophageal cancer patients are diagnosed with metastasis to distant lymph nodes or organs [2]. The prognosis of metastatic EC is rather poor with the five-year survival rate being less than 5% [25]. Hence, to improve the prognosis of esophageal cancer patients, inhibiting tumor progression and metastasis is holding great significance. Multiple miRNAs have been reported to participate in the occurrence and development of various cancers and showed differential expression [26]. Furthermore, several miRNAs were identified playing fundamental roles in ESCC. Has-miR-100-5p and has-miR-133b were reported associated with better prognosis in ESCC patients [27]. MiRNA-200c improves radiosensitivity of esophageal cancer by affecting cell cycle arrest and targeting P21 [28]. MiR-502 activates esophageal cancer cell proliferation by promoting AKT phosphorylation [29]. However, the possible role of miRNA in the metastasis of esophageal cancer is lack of study.

MiR-34a was reported to be downregulated in various cancers. A previous study demonstrated that miR-34a was significantly decreased in gastric cancer cells and suppressed EGFR-dependent MMP-7 activation [30]. To study the possible role of miR-34a in ESCC, we first determined the expression level of miR-34a was significantly downregulated in ESCC. Furthermore, miR-34a is also decreased in ESCC cell lines compared with normal esophageal epithelium cell line while showing no difference among different ESCC cell lines. Next, we performed a series of gain and loss function experiments and identified that miR-34a acts as an inhibitive factor in ESCC cell proliferation and a pro-apoptosis factor as well. According to the results of wound healing and transwell assay, miR-34a exhibits anti-metastasis function in ESCC. Normally, miRNAs function via binding with the 3'UTR region of their target genes. In our study, we identified E2F5 as the potential downstream tar-
get of miR-34a. The miR-34a/E2F5 pathway has been identified and reported in gastric cancer for regulating the chemosensitivity [15]. Luciferase reporter assay was performed to confirm the physical binding between miR-34a and E2F5 in ESCC. Previous studies have revealed that E2F5 was upregulated in various types of malignancies including breast, epithelial ovarian, hepatocellular carcinoma and prostate cancer and closely related to tumor progression and prognosis [16-19]. However, the role of E2F5 in ESCC remains unkown. According to the TCGA database, E2F5 was significantly up-regulated in ESCC, indicating being an oncogene in ESCC. Hence, E2F5-knockdown ESCC cell line was constructed to study the its biological function in ESCC. As expected, E2F5 performed the exact opposite function as miR-34a, acting as pro-proliferation, anti-apoptosis, pro-metastasis factor in ESCC. In addition, rescue experiment was also performed using miR-34a inhibitor which counteracted all the effect induced by E2F5 knockdown, confirming E2F5 being the direct target of miR-34a. Epithelium-mesenchymal transition (EMT) is a key process in ESCC metastasis [31-33]. We detected the expression change of several EMT-related proteins (E-cadherin, N-cadherin, Vemitin) using western blot technique after the administration of miR-34a inhibitor. The result showed that miR-34a effectively inhibited the EMT process in ESCC, suggesting miR-34a/E2F5 signaling pathway regulates metastasis in ESCC via controlling EMT process.

Conclusions

In conclusion, the present study demonstrated that miR-34a is greatly downregulated in both ESCC tissues and cell lines and acts as a tumor suppressor especially in ESCC metastasis. Furthermore, we identified E2F5 as the target gene of miR-34 for its regulating function in ESCC. The miR-34a/E2F5 signaling pathway inhibits tumor metastasis in ESCC via affecting EMT process. Collectively, miR-34a presents as a promising therapeutic target in ESCC.

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Conflict of interests

The authors declare no conflict of interests.

References