

MiR-455-3p acts as a prognostic marker and inhibits the proliferation and invasion of esophageal squamous cell carcinoma by targeting FAM83F

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Abstract. – **OBJECTIVE:** The aim of the current study was to elucidate the role of miR-455-3p in the pathogenesis of esophageal squamous cell carcinoma (ESCC) and its prognostic value in patients with ESCC.

PATIENTS AND METHODS: Expression levels of miR-455-3p and FAM83F mRNA in ESCC tissues and adjacent normal tissues were detected by quantitative RT-PCR. The X2-test was used to assess miR-455-3p expression on clinicopathological parameters. The association with overall survival of patients was analyzed by Kaplan-Meier survival analysis. Cox's multivariate regression model was performed to identify independent prognostic factors of overall survival. The effect of miR-455-3p on proliferation was evaluated by kit-8 (CCK-8), and cell invasion was evaluated by transwell assays. The molecular target of miR-455-3p was identified using a computer algorithm and confirmed experimentally. Furthermore, the effect of miR-455-3p up-regulation on FAM83F expression was examined by Western blot.

RESULTS: miRNA-455-3p was significantly increased in ESCC tissues and cell lines. Also, miR-455-3p expression was significantly associated with histological grade, lymph nodes metastasis and clinical stage (all $p < 0.05$). The patients with low miR-455-3p expression had shorter survival time than those with high miR-455-3p expression. Furthermore, univariate and multivariate analysis identified low miR-455-3p expression as an unfavorable prognostic factor for overall survival. Moreover, transfection with the miR-455-3p mimic enhanced the cell proliferation and invasion in ESCC cells. Luciferase reporter assays confirmed that miR-455-3p binding to the 3'-UTR regions of FAM83F inhibited the expression of FAM83F in ESCC cells. Western blot confirmed that overexpression of miR-455-3p resulted in down-regulation of FAM83F in ESCC cells.

CONCLUSIONS: Our findings indicate that miR-455-3p plays an anti-oncogenic role in the development of ESCC by downregulation of FAM83F and could be an independent marker for predicting the clinical outcome of ESCC patients.

Key Words:

miRNA-455-3p, Esophageal squamous cell carcinoma, FAM83F, Proliferation, Invasion.

Introduction

Esophageal carcinoma (EC) is one of the most common digestive tract malignancies, and is the sixth most common cause of cancer death worldwide^{1,2}. EC has the two major histological types, including squamous cell carcinoma and adenocarcinoma³. Especially in China, esophageal squamous cell carcinoma (ESCC) is the major histological type⁴. Although significant improvement has been achieved in surgical techniques and adjuvant treatment, the clinical prognosis of ESCC remains very poor due to early lymph node metastasis and invasion of neighboring organs⁵. Recent years, many research focused on the pathogenesis of ESCC. However, the underlying mechanisms of the tumorigenesis and metastasis of ESCC remain largely unclear. Deeper investigation of the molecular mechanism on progression of ESCC was important to explore new biomarkers for the prognosis and treatment of ESCC. MicroRNA (miRNA) is a novel class of short, endogenous, non-coding RNA with 18-25 nucleotides in length⁶. Mature miRNAs negatively regulate their target genes through imperfect com-

plementary sequence pairing to the untranslated region (UTR) of target genes⁷. Growing evidence showed that miRNAs have fundamental importance in biological processes such as cell differentiation, proliferation, and survival^{8,9}. Increasing studies reported that miRNAs are involved in the development and progression of various tumors, including ESCC^{10,11}. Although the regulating mechanism of most of the miRNAs is still not fully clear, the critical role of miRNAs has been shown in various studies. MiR-455-3p is located on chromosome 6. Its role has been studied in several tumors, including breast cancer¹², colon cancer¹³, and glioblastoma¹⁴. However, to our best knowledge, the association between miR-455-3p and ESCC remains unclear. In the present, we firstly determined the expression levels of miR-455-3p in ESCC tissues and cell lines and explore its prognostic value in ESCC patients. Furthermore, we performed *in vitro* assay to study the effect of miR-455-3p in ESCC cell lines. Also, we identified a new oncogene as a direct target of miR-455-3p.

Patients and Methods

Patients and Specimens

119 pairs of ESCC tissues and their corresponding non-tumor adjacent tissues were obtained from patients who underwent surgery at the Three Gorges University People's Hospital and The First People's Hospital of Yichang. All of the specimens were immediately frozen in liquid nitrogen and then stored at -80°C until RNA extraction. None of the patients had received local or systemic therapy prior to surgery. The clinical and pathological characteristics for each patient were also collected. The study was approved by the Research Ethics Committee of Three Gorges University People's Hospital and The First People's Hospital of Yichang. Informed consent was obtained from all of the patients.

Cell Culture and Transfection

Human ESCC cell lines TE1, TE13, EC9706, KYSE30 and a normal human esophageal epithelial cell line (NECC) were purchased from China Academy of Science Cell Library. The above cell lines were maintained in an RPMI-1640 culture medium (Thermo-Fisher Scientific, MA, Waltham, USA) supplemented with 10% fetal bovine serum (FBS) and were kept in a humidified incubator with 5% CO₂ at 37°C. Cells were seeded

into 6 well plates and transfected with miR-455-3p mimics or NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). miR-455-3p mimics was purchased from GenePharma (Pudong, Shanghai, China).

Real-time PCR

Total RNA was extracted from the tissues and cells using a miRcute miRNA isolation kit (Tiangen, Boding, Hebei, China) according to the manufacturer's instructions. The cDNA was synthesized from 5 ng of total RNA by using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). To measure miR-455-3p expression, Real-time RT-PCR was performed using a sequence detector (ABI-Prism). Expression data were uniformly normalized to the internal control U6 and the relative expression levels were evaluated using the $\Delta\Delta C_t$ method. The primers for PCR were designed and purchased from Invitrogen (Carlsbad, CA, USA).

CCK-8 Assay

Cell proliferation was measured using a cell counting kit-8. Cells were seeded into 96-well plates at 2000 cells per well. After incubation for a series of time periods, 10 μ l CCK-8 was added to each well and incubated for 1 h at 37°C. Proliferation rates were determined at 24, 48 and 72 h after transfection.

Transwell Invasion Assays

The invasion assays were carried out using transwell insert chambers (Corning, Haiding, Beijing, China). 2×10^4 cells were seeded into upper chambers pre-coated with matrigel (BD) in serum-free medium in triplicate. Medium with 10% FBS was added to the lower chamber to serve as chemoattractant. After incubating for 48 h, the cells in the lower membrane were stained with 1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and then counted.

Protein isolation and Western blot Analysis

Equal amounts of cell lysates were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk and incubated with anti-FAM83F antibody (Proteintech Group, Wuhan, Hubei, China) or anti-beta-U6 antibody (Sigma-Aldrich, St. Louis, MO, USA). After being washed

extensively, a secondary antibody was then incubated with the membrane for 1.5 h. Signals were detected using the ECL detection reagent.

Dual-Luciferase Reporter Assay

pGL3-FAM83F-3'UTR-MUT or pGL3-FAM83F-3'UTR-WT constructs were transfected into cells together with the miR-455-3p mimics or scramble control. The pRL-TK vector (Promega, Madison, WI, USA) carrying the Renilla luciferase gene was used as an internal control to normalize the transfection efficiency. 24 h after transfection, luciferase activity was measured using dual-luciferase assay (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis was done with SPSS/Win11.0 software (SPSS Inc., Chicago, IL, USA). Most of the data were analyzed using independent two-tailed Student's *t*-test. To compare the association between clinicopathologic variables and miR-455-3p expression, Pearson's χ^2 test were used. Survival was evaluated by Kaplan-Meier survival curves, and the log-rank test was used to evaluate the differences between groups. Survival data were evaluated using univariate and multivariate Cox proportional hazards model. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-455-3p Expression was Downregulated in ESCC Tissues and Cell Lines

To investigate the role of miR-455-3p expression in ESCC progression, we evaluated the

expression levels of miR-455-3p in ESCC tissues and ESCC cells by RT-qPCR. As shown in Figure 1A, the results showed that miR-455-3p was significantly downregulated in ESCC tissues compared with the matched normal tissues ($p < 0.01$). Also, we found that miR-455-3p was dramatically decreased in various ESCC cell lines, including TE1, TE13, EC9706, KYSE30, compared with the normal esophageal epithelial cell line (NECC) (Figure 1B, $p < 0.01$, respectively.).

Relationship Between miR-455-3p Expression and ESCC Patients' Clinicopathologic Variables

The relationship between miR-455-3p expression level and clinicopathological characteristics was shown in Table I. Statistically significant correlations could be found between miR-455-3p expression and histological grade, lymph nodes metastasis and clinical stage (all $p < 0.05$). However, there was no significant correlation between miR-455-3p expression and other clinicopathologic features such as age, gender, and tumor size ($p > 0.05$). Taken together, our data revealed a relationship between the expression of miR-455-3p and ESCC development.

The Association Between miR-455-3p Expression and Overall Survival of Patients with ESCC

Kaplan-Meier analysis and log-rank test were used to evaluate the effects of miR-455-3p expression and the clinicopathological features on overall survival of ESCC patients. As shown in Figure 1C, the data revealed the patients with low miR-455-3p expression had shorter survival time

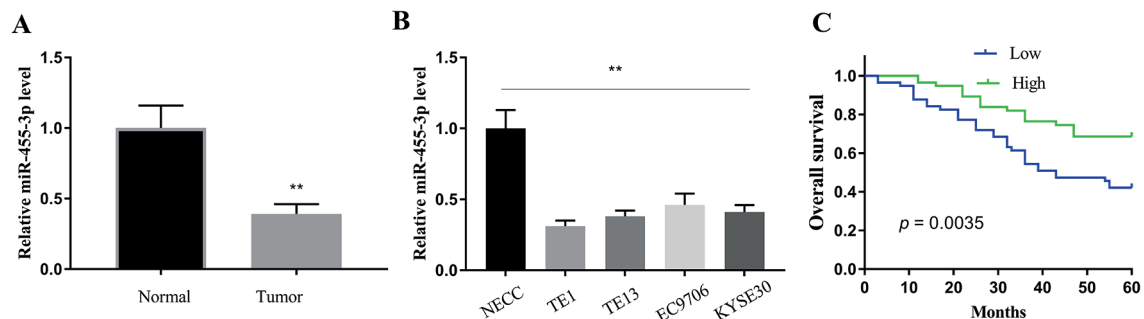


Figure 1. Increase expression of miR-455-3p in ESCC is associated with poor prognosis. (A) Expression of miR-455-3p in ESCC tissue samples and matched non-tumor tissue samples were detected by qRT-PCR. (B) Expression of miR-455-3p in TE1, TE13, EC9706, KYSE30 and NECC that were detected by qRT-PCR. (C) Kaplan-Meier curves for survival time in patients with ESCC divided according to miR-455-3p expression. * $p < 0.05$, ** $p < 0.01$.

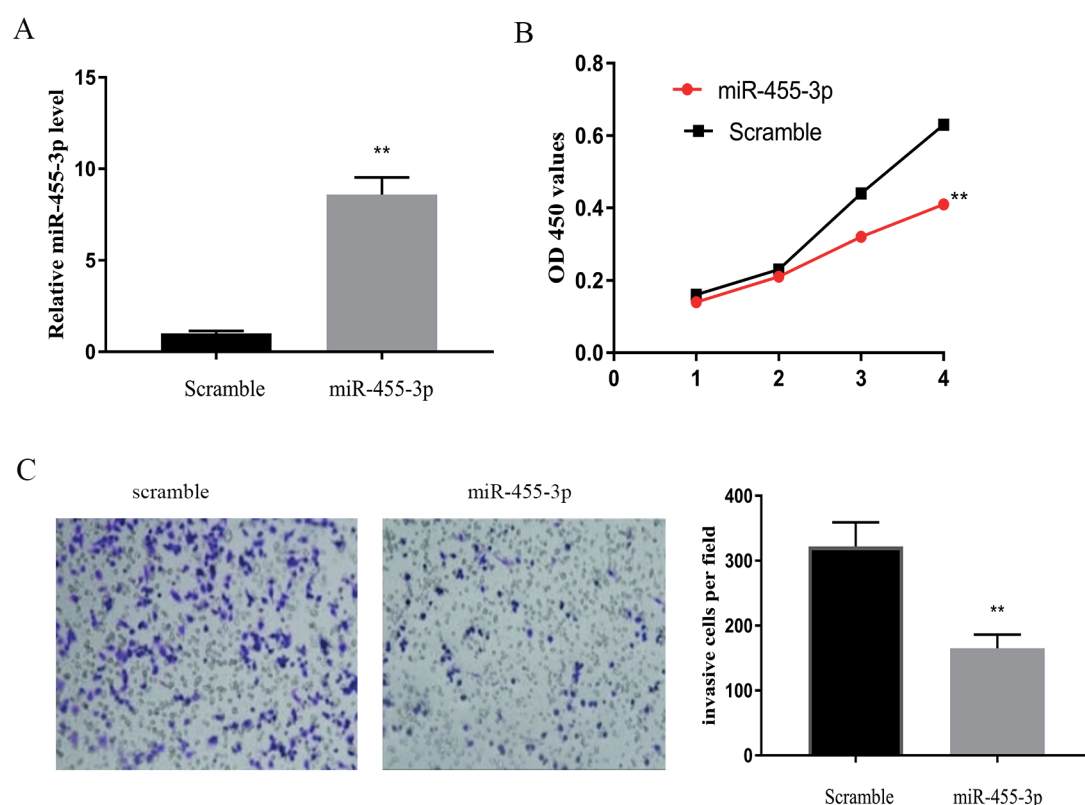


Figure 2. Over-expression of miR-455-3p promotes ESCC cell proliferation and invasion. **(A)** Over-expression of miR-455-3p in TE1 cells were detected by qRT-PCR analysis. **(B)** The cell proliferation ability of the different cells *in vitro* was detected by CCK-8 assay. **(C)** Transwell invasion assays of the TE1 cell lines. Data are presented as the mean $\pm$ SD of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

Table I. Association of miR-455-3p expression with clinicopathological variables in ESCC patients.

		miR-455-3p expression			p
Parameters	Group	Total	Low	High	
Gender	Male	46	21	25	0.697
	Female	73	36	37	
Age (years)	< 60	56	25	31	0.503
	≥ 60	63	32	31	
Tumor size (cm)	< 4	80	41	39	0.295
	≥ 4	39	16	23	
Histological grade	G1	83	32	51	0.002
	G2+G3	36	25	11	
Lymph nodes metastasis	Absence	77	30	47	0.008
	Presence	42	27	15	
Clinical stage	I-II	71	27	44	0.009
	III-IV	48	30	18	

than those with high miR-455-3p expression ($p < 0.001$). Furthermore, univariate analysis of overall survival showed that histological grade ($p = 0.007$), lymph nodes metastasis ($p = 0.002$), clinical stage ($p = 0.004$) and miR-455-3p expression

($p = 0.001$) were prognostic indicators (Table II). More importantly, multivariate analysis confirmed that miR-455-3p expression was a poor independent prognostic factor for overall survival in patients with ESCC ($p = 0.003$, Table II).

Table II. Univariate and multivariate analysis of overall survival in patients with ESCC

Variable	Univariate analysis		Multivariate analysis	
	HR p		HR p	
Gender	1.347	0.317	-	-
Age (years)	1.183	0.474	-	-
Tumor size (cm)	1.537	0.219	-	-
Histological grade	3.784	0.007	3.249	0.011
Lymph nodes metastasis	4.567	0.002	3.982	0.006
Clinical stage	3.884	0.004	3.283	0.007
miR-455-3p expression	4.873	0.001	4.238	0.003

Upregulating miR-455-3p Inhibited Proliferation and Invasion in ESCC

To investigate the cellular functions of miR-455-3p in prostate cancer, we performed overexpression experiments in TE1 cells. As shown in Figure 2A, the increased levels of miR-455-3p can be detected in the TE1 cells after transfection (Figure 2A). CCK-8 assay showed miR-455-3p could suppress cell growth compared with the control miRNA (Figure 2B). We next determined whether miR-455-3p could affect invasion of TE1 cells. As shown in Figure 2C, we discovered that ectopic expression of miR-455-3p mimics inhibited the invasion of TE1 cells. Taken together, our results suggested that miR-455-3p could suppress cell proliferation and invasion in ESCC *in vitro*.

FAM83F was a Direct Target of miR-455-3p in ESCC Cells

Using the bioinformatics software TargetScan for target gene prediction, FAM83F was identified as a potential target of miR-455-3p (Figure 3A). To confirm whether FAM83F was a direct target gene of miR-455-3p, we performed luciferase reporters. The results indicated that wild type 3'-untranslated region resulted in a significant reduction in luciferase activity, whereas mutations of the key-binding region showed no variation compared with control (Figure 3B and 3C). Furthermore, Western blot analysis and PCR showed overexpression of miR-455-3p decreased FAM83F expression in both TE1 and TE13 cells (Figure 3D and 3E). These data suggested that FAM83F was a downstream target of miR-455-3p.

Discussion

Previous studies have shown that miR-455-3p served as oncogene or anti-oncogene in dif-

ferent types of tumors. For instance, Li et al¹² reported that over-expression of miR-455-3p enhanced cell proliferative, invasive and migration abilities in triple negative breast cancer cell lines by targeting tumor suppressor EI24, suggesting that miR-455-3p served as a tumor promoter in this disease. On the contrary, Zheng et al¹³ found that overexpression of miR-455-3p induces apoptosis in colon cancer cells by regulating the expression of Bcl-2, Bax, and caspase-3, suggesting its suppressive role in colon cancer. Unfortunately, the potential role of miR-455-3p in other tumors remains largely unknown. In the present study, we firstly determined the expression levels of miR-455-3p in ESCC tissues and cell lines using RT-PCR. Our results revealed that miR-455-3p was significantly down-regulated in both ESCC tissues and cell lines. Then, we found that miR-455-3p expression was significantly associated with histological grade, lymph nodes metastasis and clinical stage. Those results indicated that low miR-455-3p expression may be associated with advanced tumors. Furthermore, we compared the overall survival in patients with ESCC and found that patients with low miR-455-3p expression had shorter survival time than those with high miR-455-3p expression. Also, by univariate and multivariate analysis, we identified that low miR-455-3p expression as an unfavorable prognostic factor for overall survival. The above results highlighted the important clinical significance of miR-455-3p in patients with ESCC. To explore the role of miR-455-3p in tumor cells proliferation and invasion, we performed CCK-8 and transwell assays. We found that over-expression of miR-455-3p enhanced the cell proliferation and invasion in ESCC cells, suggesting that miR-455-3p upregulation might play an important role in promo-

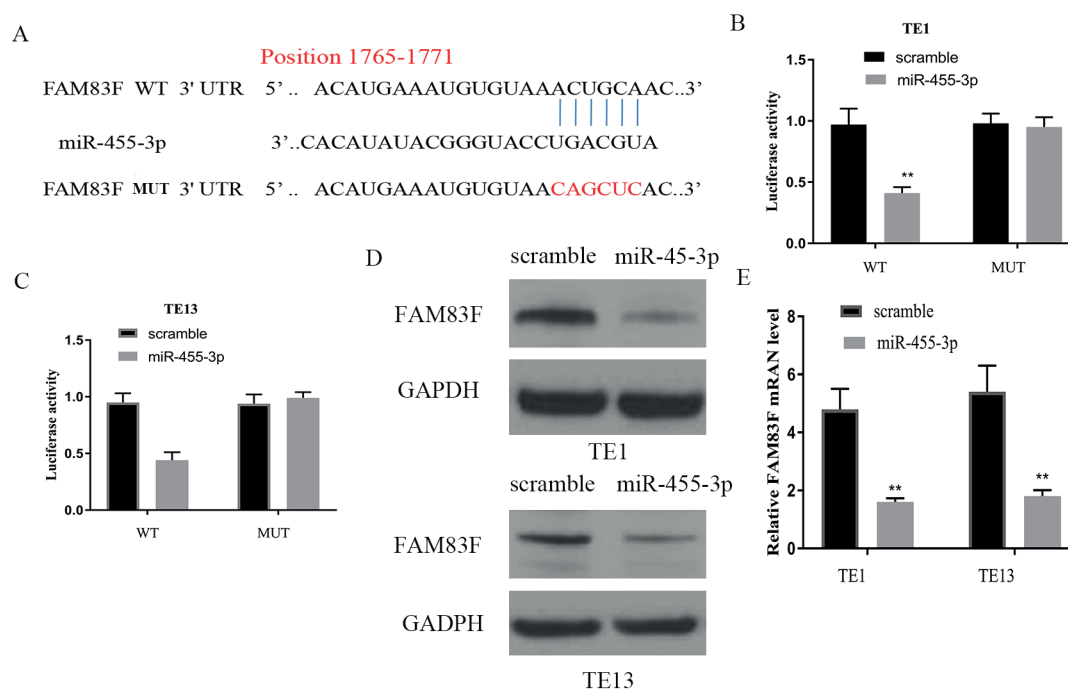


Figure 3. miR-455-3p targeted FAM83F by binding to its 3'-UTR. **(A)** The predicted binding sites for miR-455-3p in the 3'UTR of FAM83F and the mutations in the binding sites are shown. **(B, C)** Luciferase activity assay shown that miR-455-3p suppressed Wt FAM83F 3'-UTR luciferase activity, while it had no effect on Mut FAM83F 3'-UTR luciferase activity compared to control in TE1 and TE13 cells. **(D)** The protein expression of FAM83F in both TE1 and TE13 cell was measured by Western blot. **(E)** The mRNA expression of FAM83F in both TE1 and TE13 cell was measured by qRT-PCR. * $p < 0.05$; ** $p < 0.01$.

ting carcinogenesis and progression of ESCC. To further study the potential mechanism how miR-455-3p regulated the cells behavior, we searched the potential target gene of miR-455-3p, and our attention focused on FAM83F. FAM83 family was recently identified as a novel oncogene family which was reported to play an important role in progression and development of tumors¹⁵⁻¹⁷. Cipriano et al¹⁸ reported that knockdown of FAM83 family members in human breast cancer cells suppresses tumorigenicity. Previous findings by Lee et al¹⁵ showed that FAM83 family members could affect the tumor cells behavior by regulating EGFR/RAS signaling. More importantly, Mao et al¹⁹ identified the role of FAM83F in promoting ESCC cell proliferation, migration and invasion. They also found that miR-143 acts as a tumor suppressor by directly targeting FAM83F in ESCC. Those results indicated FAM83F served as oncogene and was regulated by miRNAs. Thus, we searched the TargetScan and found that FAM83F may be a target gene of miR-455-3p. Then, luciferase reporter assay was performed to confirm the potential target of miR-455-3p.

The results showed that miR-455-3p directly targeted the FAM83F.

Conclusions

We firstly found frequent downregulation of miR-455-3p in ESCC. Upregulated levels of miR-455-3p led to decreased cellular proliferation and invasion. We also revealed that MiR-455-3p expression in ESCC patients could act as a prognostic biomarker. Mechanism research showed that miR-455-3p exerted its role by targeting FAM83F. Therefore, miR-455-3p could be regarded as a novel therapeutic target for ESCC.

Conflict of interest

The authors declare no conflicts of interest.

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