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MicroRNA-556-3p promotes the progression of esophageal cancer via targeting DAB2IP

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Abstract. – OBJECTIVE: To detect the expression of microRNA-556-3p in esophageal cancer (EC) tissues and to elucidate the mechanisms underlying microRNA-556-3p in promoting EC progression.

PATIENTS AND METHODS: QRT-PCR (quantitative Real-Time Polymerase Chain Reaction) was performed to detect microRNA-556-3p expression in 65 cases of EC tissues, 30 cases of normal esophageal tissues and EC cell lines. The overall survival (OS) of EC patients was calculated based on the 10-year follow-up data. For in vitro experiments, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell assay were performed to evaluate the effect of microRNA-556-3p on the proliferative and invasive abilities of EC cells. The effect of microRNA-556-3p on DAB2IP and MAPK pathway was determined by Western blot and qRT-PCR. The binding condition between microRNA-556-3p and DAB2IP was further confirmed by Luciferase reporter gene assay.

RESULTS: MicroRNA-556-3p expression was upregulated in EC tissues than that of paracancerous tissues. EC patients with higher expression of microRNA-556-3p presented a shorter OS than those with lower expression. Moreover, microRNA-556-3p overexpression in EC cells remarkably promoted cell viability. Upregulated microRNA-556-3p in Eca109 and Eca7906 cell lines markedly increased cell proliferation and invasion. The expression level of DAB2IP was negatively regulated by microRNA-556-3p verified by the Luciferase reporter gene assay.

CONCLUSIONS: MicroRNA-556-3p blocked the translation of DAB2IP at mRNA level by directly binding to 3'UTR of DAB2IP, thereafter enhancing the proliferation of Eca109 and Eca7906 cells. MicroRNA-556-3p promoted the occurrence and development of EC. Our study provided a new theoretical basis and therapeutic target for EC treatment.

Key Words:
MicroRNA-556-3p, DAB2IP, Esophageal Cancer, Cell Proliferation, MAPK Pathway.

Introduction

Esophageal cancer (EC) is a common gastrointestinal cancer. Although the morbidity and mortality rates vary greatly among countries, about 300,000 people die from EC each year in the world[1,2]. China is one of the high-incidence areas for EC, with an average death of about 150,000 each year[3]. Tumor metastasis and recurrence are the major causes leading to the poor prognosis of EC. Establishing new therapeutic targets is of great significance for improving the treatment efficacy of EC[4].

MicroRNAs are a series of endogenous, single-stranded, non-coding RNAs discovered in recent years. They are about 18-25 nucleotides in length and widely distributed in eukaryotes[5]. Mature microRNAs are completely or incompletely paired to the 3'-untranslated region (3'UTR) of the target mRNA under the guidance of the RNA-induced silencing complex (RISC). Functionally, microRNA regulates gene expressions and plays a vital role in various diseases[6,7]. The disability gene homolog 2 interacting protein (DAB2IP) is a newly discovered tumor-suppressor gene that can influence cell proliferation, survival and apoptosis by mediating multiple tumor-associated signals[8-10].

Recent studies have shown that microRNAs are involved in the pathogenesis of EC. However, the specific mechanisms of microRNA-556-3p in regulating EC development remain unclear. In-depth studies on exploring the occurrence and progression of EC contribute to improve clinical outcomes of EC.

Patients and Methods

Patients and Sample Collection

A total of 65 EC patients treated in our hospital from July 2012 to July 2017 were enrolled for

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retrospective analysis. The relevant information was shown in Table I. Inclusion criteria were applied for: a) Patients who were pathologically diagnosed as EC for the first time and did not receive any treatment; b) Patients who did not have other major complications and were tolerant to the following treatments. Clinical information of all subjects before and after treatment was collected for further statistical analysis. The study was approved by the Ethics Committee of Jiangsu Province Danyang People's Hospital. All patients and their families received informed consent and signed written informed consent. A total of 30 cases of normal esophageal tissues were collected, placed in a 1.5 mL EP tube and stored at -80°C.

Cell Culture

Human EC cell lines (Eca109, EC9706, TE-10 and TE-11) and human normal esophageal epithelium cell lines (HEEC) were cultured in F12 or RPMI-1640 (Roswell Park Memorial Institute-1640, Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) (HyClone, South Logan, UT, USA). Cells were maintained in a constant temperature incubator at 5% CO_2 and 37°C.

Transfection

Eca109 and Eca7906 cells were collected 1 day before transfection. Cell density was adjusted to 1×10^5 cells/ml and then seeded into 96-well plates (100 μL of cell suspension per well) or 6-well plates (2 mL of cell suspension per well). MicroRNA-inhibitor, microRNA-mimics or control reagents were transfected according to instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells in 96-well plates were used for subsequent MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell assay, and cells in 6-well plates were used for Western blot.

MTT Assays

Eca109 and Eca7906 cells transfected for 0 h, 6 h, 24 h, 48 h, 72 h and 96 h in 96-well plates were collected for the proliferation activity detection, respectively. Briefly, 20 μL of MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and the cells were then incubated for 4 h. After discharging the culture medium, 150 μL of DMSO (dimethyl sulfoxide) was added to each well and cells were shaken for 10 min. The absorbance at 450 nm of each group was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). The growth curve was plotted by calculating the average value of each group. Each group contained 6 replicate wells. The experiment was repeated three times independently.

Cell Invasion Assay

Transwell chambers (Costar, Cambridge, MA, USA) with an 8 μm pore polycarbonate filter were used to assess the invasive properties of tumor cells. Transfected Eca109 and Eca7906 cells (3×10^4 cells) were cultured in 600 μL of RPMI-1640 containing 10% FBS. After incubating at 37°C and 5% CO_2 for 4 h, non-migrated cells on

Table I. Correlation between miR-556-3p expression and clinicopathological characteristics of esophageal cancer.

Clinicopathologic features	Number of cases	Has-miR-556-3p expression		*p*-value
		Low (n=32)	High (n=33)	
Age (years)				0.2669
<60	35	15	20	
≥60	30	17	13	
Gender				0.7018
Female	30	14	16	
Male	35	18	17	
Tumor size				0.4924
<4CM	23	10	13	
≥4CM	42	22	20	
Lung metastasis				0.0186*
Yes	34	12	22	
No	31	20	11	
Lymph node metastasis				0.5402
Absent	30	16	14	
Present	35	16	19	

the upper chamber were removed. Membranes were fixed in methanol and the cell invasion was quantified in five randomly selected fields of each membrane. Each sample was independently assayed in triplicate.

Luciferase Activity Assays

Eca109 and Eca7906 cells in logarithmic growth phase were collected and inoculated in 6-well plates with the cell density of 5×10^4 cells/mL. MicroRNA-inhibitor or microRNA-mimics and wild-type DAB2IP or mutant-type DAB2IP were co-transfected into Eca109 and Eca7906 cells according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After cell transfection for 48 h, cells were harvested and the relative luciferase activity was determined on a single photon detector (Bio-Rad, Hercules, CA, USA) according to the instructions of the Dual-Luciferase Activity Assay Kit. Relative Luciferase activity = firefly Luciferase activity value/Renilla luciferase activity value. The experiment was repeated three times independently.

QRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from each group 24 h after transfection, and complementary Deoxyribose Nucleic Acid (cDNA) product of each sample was obtained by reverse transcription. Totally 2 μL of cDNA was added to the 18 μL of PCR reaction solution (1 μL of 4 μmol/L gene primer, 0.4 μL of red fluorescent dye ROXDye (50×), 6.6 μL of ddH_2O and 10 μL of 2×SYBR Premix Ex Taq) and the amplification was performed on an ABI7900 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). The reaction conditions were as follows: 95°C for 1 min, 95°C for 15 s, 60°C for 30 s and 72°C for 45 s, for a total of 40 cycles. The experiment was repeated three times independently. Primer sequences used in this study were as follows: microRNA-556-3p, F: 5'-GGGGTGTAAACATCCTCGACTG-3', R: 5'-ATTGCGTGTCGTGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Western Blot

After 48 h of transfection, total protein was extracted and quantified. The proteins were then separated by electrophoresis, transferred to membranes and blocked with 5% skimmed milk. Rabbit anti-human DAB2IP, ERK, and p-ERK antibodies (1:1000) were used to incubate the membranes overnight at 4°C. After incubation with fluorescent secondary antibody (1:1000), signals were acquired using the Odyssey digital imaging system (with GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as an internal reference). The relative expression level of the target protein was expressed as the ratio of the gray value of target protein band to that of the internal reference. The experiment was repeated three times independently.

Statistical Analysis

SPSS 20.0 (Statistical Product and Service Solutions) software (IBM, Armonk, NY, USA) was used for statistical analysis of the results of each experiment. The ***t***-test was used for the comparison between the two groups. SNK method was used for comparison among different groups. Comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

MicroRNA-556-3p Was Overexpressed in EC

MicroRNA-556-3p was overexpressed in EC tissues than that of paracancerous tissues ($p<0.05$, Figure 1A). Survival analysis was conducted based on the 10-year follow-up data of 65 EC patients. The data showed that OS (overall survival) in EC patients with higher expression of microRNA-556-3p was remarkably shorter than those with lower expression (HR=2.551, $p=0.0127$, Figure 1B). MicroRNA-556-3p was also overexpressed in EC patients with pulmonary metastasis compared with those without pulmonary metastasis ($p<0.05$, Figure 1C). ROC curve exhibited that microRNA-556-3p expression was highly sensitive to EC diagnosis (AUC=0.7661, $p<0.05$, Figure 1D). Above results indicated that overexpressed microRNA-556-3p is negatively correlated with EC prognosis.

Overexpressed MicroRNA-556-3p Promoted Proliferation of EC Cells

MicroRNA-556-3p was overexpressed in EC cell lines (Eca109, Eca7906 and KYSE30) compared with those of normal esophageal cell

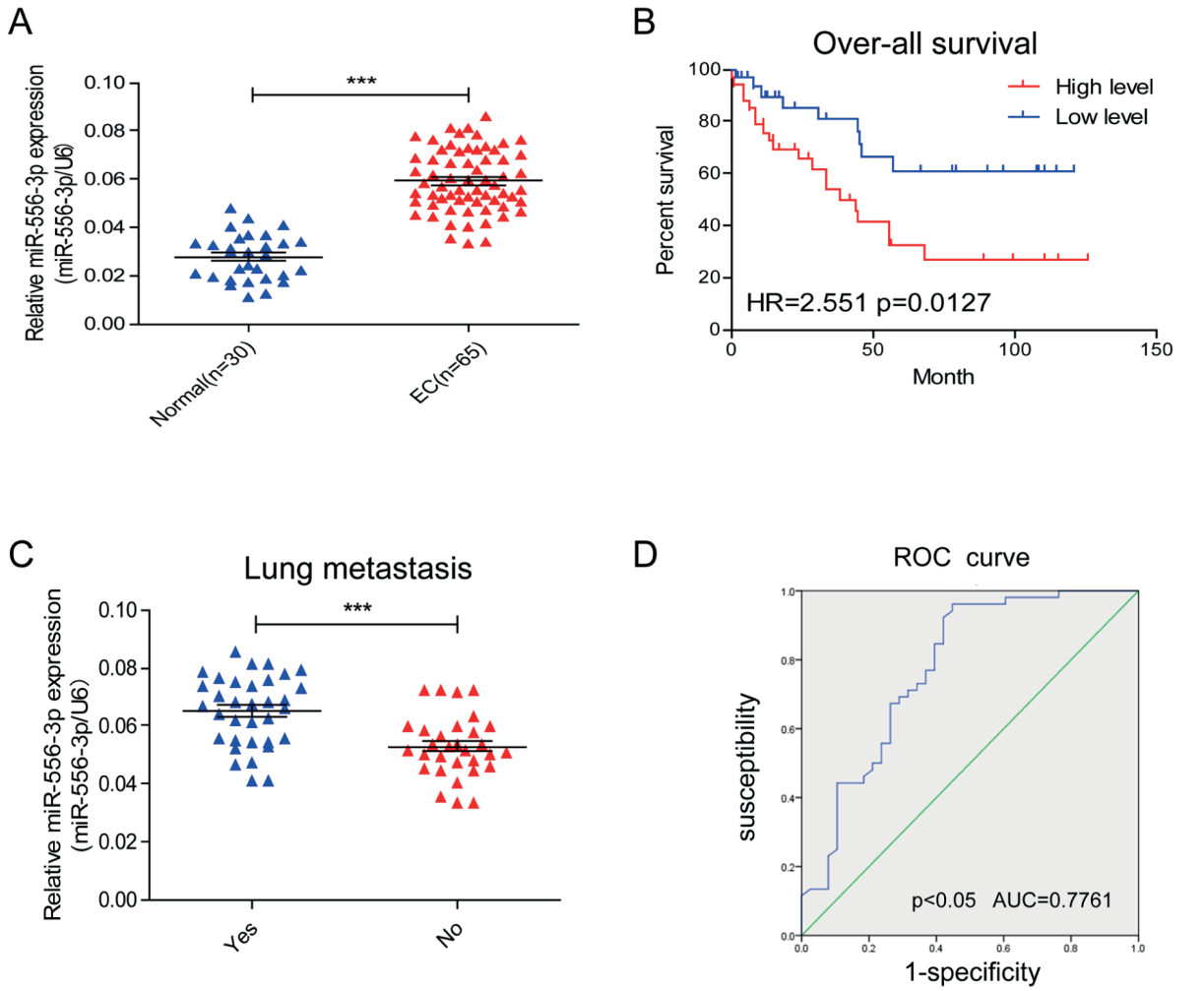

Figure 1. MicroRNA-556-3p was overexpressed in EC. ***A,*** The expression of microRNA-556-3p in 65 EC patients was significantly higher than that of 30 controls. ***B,*** The overall survival of EC patients with higher expression of microRNA-556-3p was significantly lower than those with lower expression. ***C,*** MicroRNA-556-3p expression in EC patients with pulmonary metastasis was significantly higher than those without pulmonary metastasis. ***D,*** ROC curve between microRNA-556-3p expression and the diagnostic sensitivity of EC.

lines (HEEC and TE-4) (p<0.05, Figure 2A). Transfection efficacies of microRNA-337-3p mimics and inhibitors were verified by qRT-PCR (Figure 2B and 2C). MTT assay showed that the proliferative activity of Eca109 and Eca7906 cells was markedly increased after microRNA-337-3p overexpression (Figure 2D-2F). Besides, cell invasion assay also indicated that overexpressed microRNA-337-3p in Eca109 and Eca7906 cells remarkably enhanced proliferative and invasive activities (Figure 2E-2G). The above data indicated that overexpression of microRNA-556-3p promotes the proliferation and invasion of EC cells.

MicroRNA-556-3p Participated in the Development of EC by Regulating DAB2IP and MAPK Pathways

Bioinformatics analysis predicted the binding site for microRNA-556-3p and DAB2IP (Figure 3A). The results of Luciferase reporter assay showed that microRNA-556-3p significantly inhibited the Luciferase activity in Eca109 cells transfected with wild-type DAB2IP, suggesting that microRNA-556-3p may interfere with the mRNA expression of DAB2IP (Figure 3B). Western blot results exhibited that expression levels of DAB2IP, ERK and p-ERK in Eca109 and Eca7906 cells were decreased after microRNA-337-3p ove-

rexpression (Figure 3C-3F). The above results demonstrated that microRNA-556-3p participates in EC development by targeting DAB2IP and activating the MAPK pathway.

Discussion

Globally, EC is one of the most common malignancies and its incidence has been increasing each year[11]. China is one of the countries with high incidence of EC. The mortality rate of EC ranks the second to digestive tract malignancy in China[12,13]. Although many oncogenes, tumor-suppressor genes and tumor-related signaling pathways have been discovered in recent years, the pathogenesis of EC remains unclear[14]. So far, effective prognostic indicators of EC are still lacking. The exploration of novel prognostic factors and therapeutic targets for the individua-

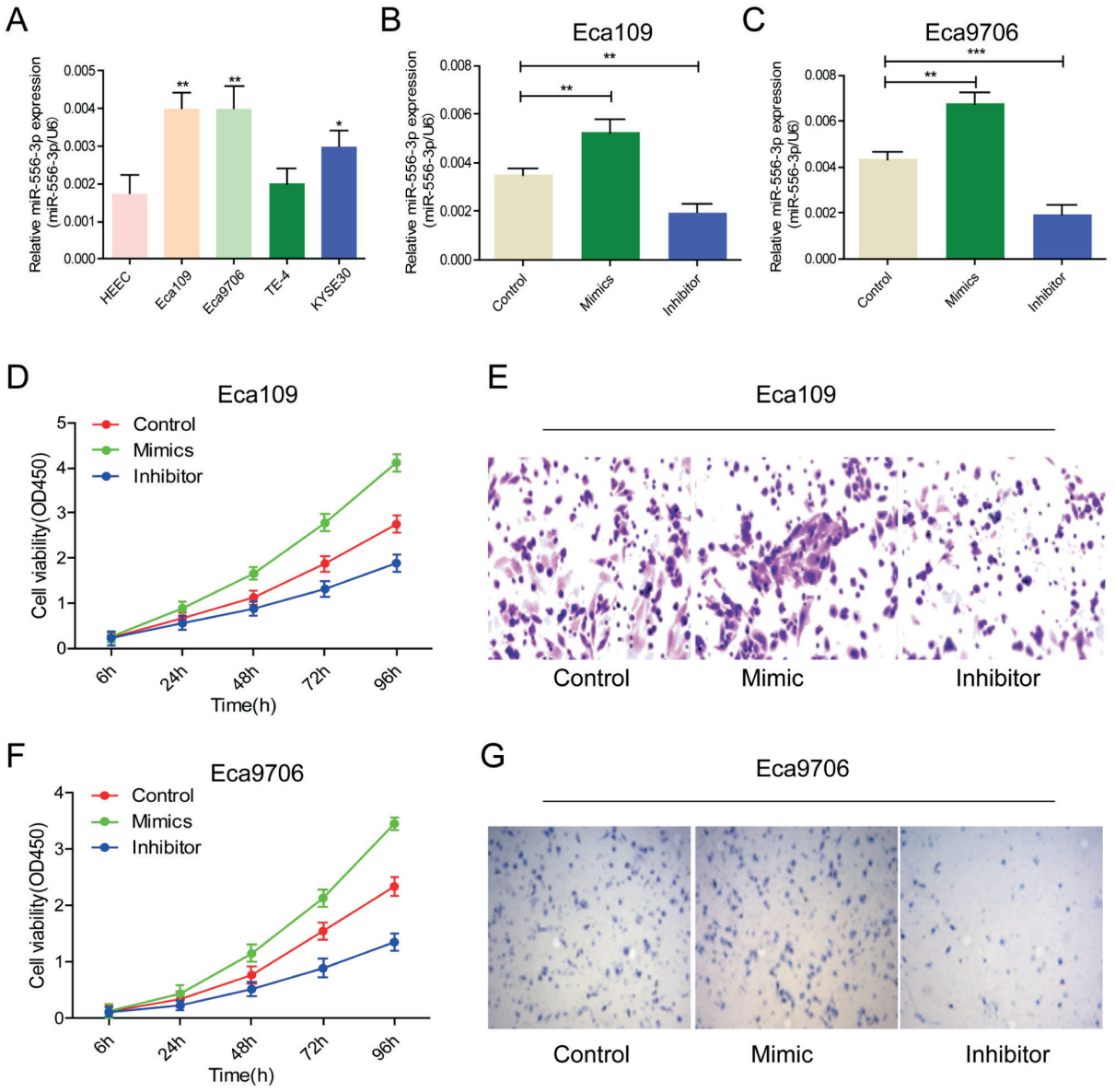

Figure 2. Overexpression of microRNA-556-3p promoted proliferation of EC cells. ***A,*** The expression level of microRNA-556-3p in normal esophageal cell line HEEC and EC cell lines Eca109, Eca7906, TE-4, KYSE30. ***B, C,*** Transfection efficacy of microRNA-556-3p mimics and inhibitor in Eca109 and Eca7906 cells. ***D, F,*** Changes in cell viability after overexpression or knockdown of microRNA-556-3p in Eca109 and Eca7906 cells. ***E-G,*** Changes in cell invasion after microRNA-556-3p overexpression or knockdown in Eca109 and Eca7906 cells.

lized treatment of EC is of clinical significance. It is generally believed that key molecules related to the occurrence and progression of EC could be served as novel prognostic and therapeutic targets of EC[15-17].

MicroRNAs are a class of endogenous, gene-encoded, single-stranded RNAs with 18-24 nucleotides in length. MicroRNAs exert their functions by complete or incomplete complementary pairing with the 3′UTR of target genes at post-transcriptional level[18,19]. The expression levels of microRNAs are considered as reference frame for the diagnosis of various diseases, especially tumors. Li et al[20] have demonstrated some differentially expressed microRNAs in EC cells. Identification and analysis of the certain microRNAs contribute to accurate diagnosis of EC[21]. MicroRNA-556-3p is reported to be overexpressed in endometriosis. The specific role of microRNA-556-3p in tumors, however, has not yet been reported. DAB2IP is proved to be an important prognostic indicator of colorectal cancer and gallbladder cancer. The overexpression of DAB2IP exerts a crucial role in the regulation of tumors.

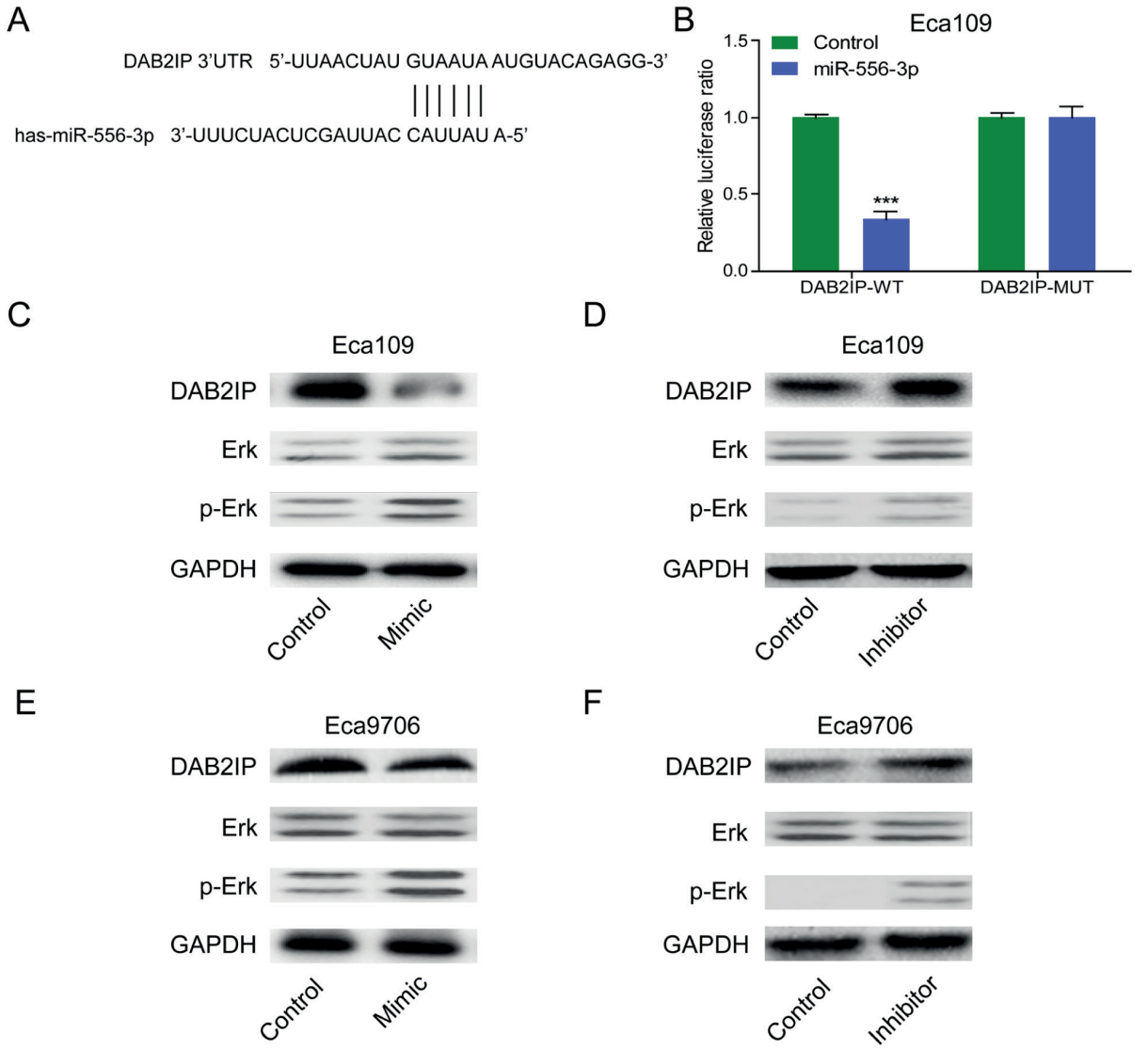

Figure 3. MicroRNA-556-3p participated in the development of EC by regulating DAB2IP and MAPK pathways. ***A,*** Bioinformatics analysis predicted the binding sites for microRNA-556-3p and DAB2IP. ***B,*** Luciferase reporter gene assay was performed in the Eca109 cells. ***C, D,*** Protein expressions of DAB2IP, ERK, and p-ERK in EC cells after overexpression or knockdown of microRNA-556-3p. ***E, F,*** Protein expressions of DAB2IP, ERK, and p-ERK in EC cells after overexpression or knockdown of microRNA-556-3p.

Dysregulated DAB2IP may lead to abnormal cell proliferation, eventually resulting in the persistent deterioration of tumors[22].

Our work revealed that microRNA-556-3p was overexpressed in EC tissues than that of paracancerous tissues. MicroRNA-556-3p overexpression remarkably promoted the proliferative and invasive capacities of EC cells. Through bioinformatics software, such as Targetscan, miRWalk and miRDB, we predicted that microRNA-556-3p is bound to the 3'UTR of DAB2IP. Therefore, we suggested that microRNA-556-3p might promote the proliferation of EC cells ***via*** blocking the mRNA translation of DAB2IP, which was further confirmed by Luciferase activity assay. To explore the effect of microRNA-556-3p on MAPK pathway, EC cells were transfected with microRNA-556-3p mimics or inhibitors, respectively. Upregulation of microRNA-556-3p effectively inhibited protein expressions of DAB2IP, ERK and p-ERK. The above results suggested that microRNA-556-3p directly inhibits the expression level of DAB2IP at the post-transcriptional level, thereby promoting the proliferation of EC cells.

In summary, microRNA-556-3p blocked the mRNA translation by binding to 3'UTR of DAB2IP, thereby promoting the proliferation of Eca109 and Eca7906 cells. Our research showed that microRNA-556-3p promotes the occurrence and development of EC, which provides a new theoretical basis for the gene therapy of EC.

Conclusions

We found that microRNA-556-3p inhibits the mRNA translation of DAB2IP by targeting the 3'UTR of DAB2IP. MicroRNA-556-3p stimulates the occurrence and development of EC, which provides a new theoretical basis and therapeutic target for EC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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