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MicroRNA-487a promotes proliferation of esophageal cancer cells by inhibiting p62 expression

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Abstract. – OBJECTIVE: MicroRNAs are endogenous, non-coding, small RNAs that can regulate biological processes. Previous studies have found that microRNA-487a serves as an oncogene. However, the role of microRNA-487a in esophageal cancer (EC) has not been reported. The aim of this investigation was to investigate the biological role of microRNA-487a in EC and its underlying mechanism.

PATIENTS AND METHODS: The expression of microRNA-487a in 65 pairs of EC tissues and para-cancerous tissues was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Chi-square test was used to analyze the relationship between microRNA-487a expression with age, sex, clinical stage and distant metastasis of OS patients. Kaplan-Meier survival analysis was conducted to evaluate the correlation between microRNA-487a expression and prognosis of EC patients. Subsequently, microRNA-487a expression in EC cell lines was detected as well. After microRNA-487a knockdown, cell counting kit-8 (CCK-8), EdU and transwell assay were conducted to evaluate the role of microRNA-487a in the biological performances of EC cells, respectively. Meanwhile, the apoptosis of EC cells was determined using flow cytometry. Finally, the interaction between microRNA-487a and p62 was explored by Western blot. Transwell assay was carried out in EC cells co-transfected with p62 overexpression plasmid and si-microRNA-487a.

RESULTS: Compared with para-cancerous tissues, microRNA-487a expression was significantly higher in EC tissues, and the difference was statistically significant. MicroRNA-487a was highly expressed in EC cells as well. Low expression of microRNA-487a was positively correlated with clinical stage, whereas was not correlated with age, sex, lymph node metastasis and distant metastasis of EC patients. Kaplan-Meier survival curves showed that high expression of microRNA-487a was markedly associated with poor prognosis of EC. The knockdown of microRNA-487a significantly inhibited proliferative, migratory and invasive abilities of EC cells but induced cell apoptosis. Western blot results showed that the protein expression of p62 was remarkably upregulated after microRNA-478a knockdown in EC cells. Transwell assay demonstrated that co-transfection with overexpression plasmid of p62 and si-microRNA-487a in EC cells markedly decreased invasive and migratory abilities.

CONCLUSIONS: MicroRNA-487a is highly expressed in EC and is closely correlated with clinical stage and poor prognosis of EC. Our findings confirm that microRNA-487a promotes malignant progression of EC by regulating p62 expression.

Key Words:
MicroRNA-487a, P62, EC, Proliferation.

Introduction

Esophageal cancer (EC) is a common malignant tumor of the digestive tract[1,2]. Previous data have shown that the incidence and mortality of EC in China have not been effectively controlled. Meanwhile, EC incidence and mortality still rank fourth in all malignant tumors[3,4]. In China, esophageal squamous cell carcinoma (ESCC) is the major pathological type of EC, whose incidence shows significant regional differences[3,5]. At present, researches on exploring the pathogenesis of EC have made great progress[6]. Surgical resection is a well-established radical treatment for EC. However, due to the insidious onset and lack of specificity in clinical symptoms, most EC patients are already at the advanced stage and lost the optimal surgical opportunity when first diag-

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nosed[7,8]. Even early-stage EC is fatal to most patients. It is reported that the 5-year survival rate of patients with resectable EC is only about 24%[8,9]. Radiotherapy and chemotherapy can effectively improve the local control rate and long-term survival rate of EC. Meanwhile, they are important auxiliary methods for the clinical treatment of EC. Multidisciplinary comprehensive treatment based on surgery, radiotherapy and chemotherapy is becoming widely applied[7]. However, radiotherapy and chemotherapy have serious toxic and side effects, which may damage normal tissues and cells[10]. Local recurrence and distant metastasis are the main factors leading to poor prognosis in EC patients[11]. Therefore, finding more effective adjuvant treatments and therapeutic targets to inhibit local recurrence and distant metastasis of EC are urgent to be solved in clinical practice.

MicroRNAs are a class of endogenous, single-stranded small RNAs[12,13]. MicroRNAs can regulate the transcription and translation of mRNAs by pairing with 3'UTR of target genes. Moreover, they exert important roles in the differentiation, proliferation and apoptosis of tumor cells[14,15]. With the advent of the post-genome era, the exploration of the biological significance of non-coding sequences has become increasingly prominent. The potential roles of microRNAs in the occurrence and progression of tumor diseases have been well concerned[12]. Accurate prediction of target genes of microRNAs and specific regulation of microRNA expressions may provide new ideas for clinical diagnosis and treatment of malignant tumors[16]. MicroRNA-487a is located at 14q38.12, which has a target-controlled relationship with multiple downstream genes[17,18]. Researchers[17,19] have confirmed that microRNA-487a is highly expressed in malignant tumor tissues and causes abnormal regulation of target genes. Furthermore, it can regulate the proliferation and differentiation of malignant tumor cells.

P62/IGF2BP2 (insulin-like growth factor 2 mRNA binding protein 2) is a tumor-associated antigen protein, which is found in cDNA library screening for serum samples of hepatocellular carcinoma patients[20,21]. The expression of p62 gene is also regulated by growth and development. P62 can only found in the fetal stage. However, it has not been found in the liver tissue of healthy adults, showing characteristics of fetal protein[22]. Interestingly, during the transition from chronic hepatitis or cirrhosis to hepatocellular carcinoma, the presence of p62 antibody in serum samples has already been found. However, p62 antibody cannot be found under the same detection in patients with early-stage of chronic hepatitis or cirrhosis[23]. Current researches have shown the presence of p62 antibody in various malignancies. This suggests that p62 may be involved in the development of many human malignant tumors[24]. Based on the above characteristics, the aim of this study was to explore whether p62 could serve as a serological marker for early diagnosis of EC. We also explored the mechanism of microRNA-487 in regulating the development of EC.

EC tissues and para-cancerous tissues were first collected, and the expression level of microRNA-487a was detected. The diagnostic value of microRNA-487a in EC patients was evaluated. Subsequently, the biological functions of microRNA-487a in EC cell lines were determined. Our work might provide a new basis for the EC treatment.

Patients and Methods

Patients and Esophageal Cancer Samples

65 EC patients who underwent surgical resection in our hospital were enrolled. All patients were diagnosed as EC and did not receive preoperative chemotherapy. EC tissues and para-cancerous tissues were harvested and preserved in -80°C. Informed consent was obtained from enrolled patients and their families before sample collection. This study was approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University.

Cell Lines

Four human EC cell lines (OE19, OE33, TE-1 and EC-109) and one human esophageal epithelial cell line (HEEC) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and maintained in a 5% CO_2 incubator at 37°C. Culture medium was replaced every 2-3 days. Cell passage was performed until 90% of confluence.

Cell Transfection

Two small interference plasmids of microRNA-478a were constructed, namely si-microRNA-487a-1 and si-microRNA-487a-2. Meanwhile, overexpression plasmid of p62 was constructed as

well. For cell transfection, the cells were first seeded into 6-well plates. When the density of cells reached 70%, cell transfection was performed according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Cell Proliferation Assay

48 h after transfection, EC cells were seeded into 96-well plates with 2000 cells per well. After culturing for 6 h, 24 h, 48 h, and 72 h, respectively, cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) reagent was added to each well. After 2 hours of incubation in the dark, the optical density (OD) value of each well at the wavelength of 490 nm was measured using a microplate reader.

Flow Cytometry Analysis

The transfected EC cells were stained with Annexin V-FITC/PI following the manufacturer's recommendations (Sigma-Aldrich, St. Louis, MO, USA). The apoptotic rate of cells was determined by flow cytometry. Cells were distinguished from living cells, necrotic cells and apoptotic cells. Finally, the apoptotic rate was calculated in each sample.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

24 h after transfection, EC cells were incubated with 50 μm EdU for 2 h, followed by staining with AdoLo and 4',6-diamidino-2-phenylindole (DAPI) according to manufacturer's recommendations (R&D Systems, Minneapolis, MN, USA). EdU-positive cells were observed under a fluorescence microscope. The positive rate was calculated as the number of EdU-positive cells to the number of total DAPI-stained cells.

Transwell Assay

48 h after transfection, EC cells were digested and re-suspended in serum-free medium. Cell density was adjusted to 2.0×10^5/mL. Transwell chamber containing Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) or not was placed in 24-well plates. 200 μL of cell suspension containing 1.0×10^5 cells was added to the upper chamber. Meanwhile, 500 μL of medium containing 20% FBS was added to the lower chamber. 48 hours later, the cells were fixed with 4% paraformaldehyde for 15 min until chamber removal. Subsequently, they were stained with 0.2% crystal violet for 20 min. The inner layer cells were carefully removed. 5 fields were randomly selected for each sample. Penetrating cells were captured, and the number of cells was calculated.

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

Total RNA was first extracted from transfected cells, followed by reverse transcription. QRT-PCR was performed to determine the relative mRNA levels of microRNA-487a and p62. The primers used in the work were as follows: microRNA-487a: forward, 5'-CGCTGGCAATCATACAGGGACAT-3', reverse, 5'-GTGCAGGGTCCGAGGGT-3'; U6: forward, 5'-CTCGCTTCGGCAGCACA-3', reverse, 5'-AACGCTTCACGAATTTGCGT-3'; P62: forward, 5'-CCAGAGAGTTCCAGCACAGA-3', reverse, 5'-CCGACTCCATCTGTTCCTCA-3'; β-actin: forward, 5'-CAGAGCTCCTCGTCTTGCC-3', reverse, 5'-GTCGCCACCATGAGAGAC-3'. The relative expression level of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method and analyzed by ABI Step One software (Applied Biosystems, Foster City, CA, USA). Each sample was detected in triplicate.

Western Blot

Total protein in EC cells and tissues was extracted. The concentration of total protein was determined using bicinchoninic acid (BCA) protein determination kit (Peirce, Rockford, IL, USA). The extracted protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Western blot was conducted according to the standard procedures. Primary and secondary antibodies were provided by Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analyses. Data were expressed as mean ± standard deviation. *t*-test was used to compare the difference between the two groups. One-way ANOVA was applied to compare the differences among different groups, followed by Post-hoc test. The correlation between microRNA-487a expression and clinical indexes of EC patients was analyzed by chi-square test. Kaplan-Meier method was used to calculate the survival time of EC patients, and the difference between different curves was compared by Log-rank test. $p<0.05$ was considered statistically significant.

Results

MicroRNA-487a was Highly Expressed in EC

We first examined the expression of microRNA-487a in 65 pairs of EC tissues and para-cancerous tissues by qRT-PCR. The results showed that compared with para-cancerous tissues, microRNA-487a expression was significantly higher in EC tissues, and the difference was statistically significant (Figure 1A, 1B). Similarly, compared with human normal esophageal epithelial cells (HEEC), microRNA-487a was highly expressed in EC cells, and the difference was statistically significant (Figure 1C). Among EC cell lines, TE-1 and EC-109 cells expressed the highest level of microRNA-487a, which were selected for the following experiments.

MicroRNA-487a Expression was Correlated with Clinical Stage and Overall Survival of EC Patients

Based on microRNA-487a expression in EC tissues, the enrolled patients were divided into two groups, including high-level microRNA-487a group and low-level microRNA-487a group. Chi-square test was used to analyze the relationship between microRNA-487a expression with age, sex, clinical stage, lymph node metastasis and distant metastasis of EC patients. Low expression of microRNA-487a was positively correlated with clinical stage, whereas was not correlated with age, sex, lymph node metastasis and distant metastasis of EC patients (Table I). To explore the relationship between microRNA-487a expression and the prognosis of EC patients, we collected relevant follow-up data. Kaplan-Meier survival curves showed that high expression of microRNA-487a was markedly associated with poor prognosis of EC. The lower expression level of microRNA-487a, the better prognosis of EC ($p<0.05$, Figure 1D). This suggested that microRNA-487a might be a new biological indicator for predicting the prognosis of EC.

Knockdown of MicroRNA-487a Inhibited Proliferative Ability of EC Cells

To explore the effect of microRNA-487a on the proliferative ability of EC cells, we first suc-

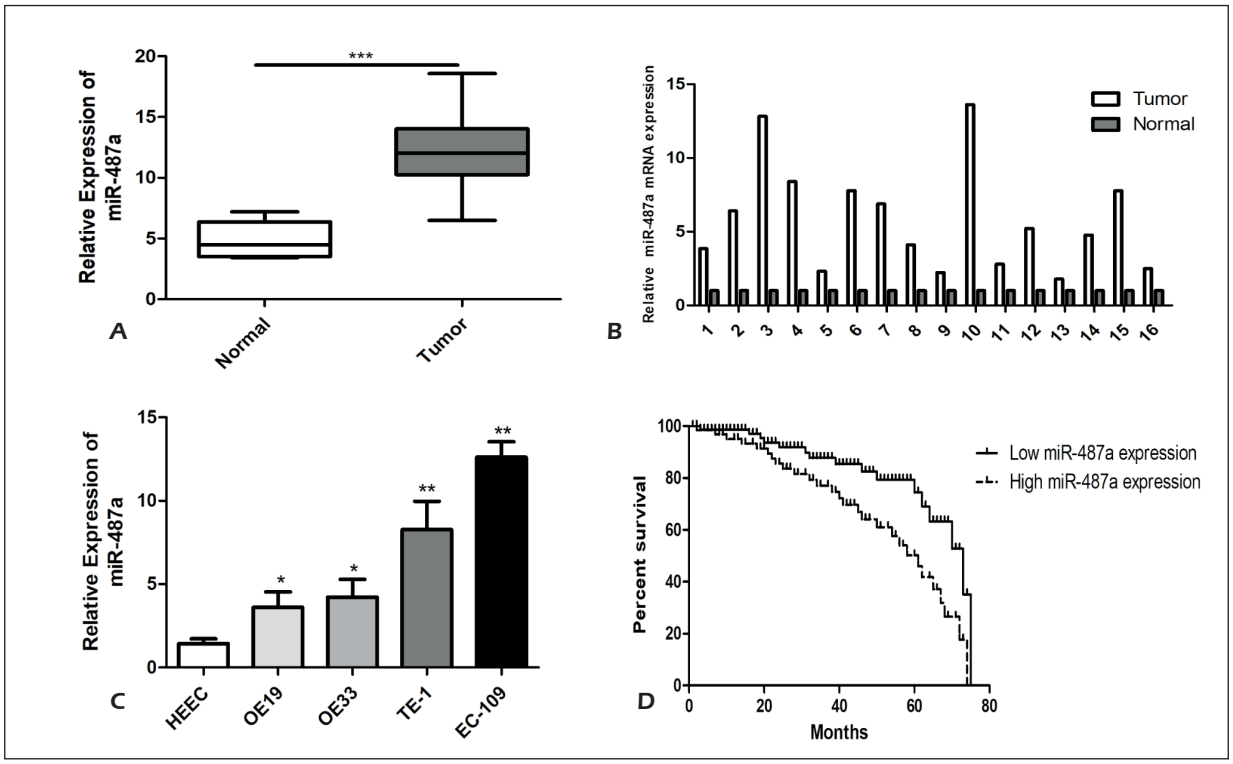

Figure 1. MicroRNA-487a was highly expressed in EC tissues and cell lines. **A-B**, MicroRNA-487a expression in EC tissues and para-cancerous tissues detected by qRT-PCR. **C**, MicroRNA-487a expression in EC cell lines. **D**, Survival curves of microRNA-487a expression in EC patients. Data were expressed as mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of miR-487a expression with clinicopathologic characteristics of esophageal cancer.

Parameters	Number of cases	miR-487a expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.407
<60	27	17	10	
≥60	38	20	18	
Gender				0.694
Male	32	19	13	
Female	33	18	15	
T stage				0.040
T1-T2	37	17	20	
T3-T4	28	20	8	
Lymph node metastasis				0.251
No	40	25	15	
Yes	25	12	13	
Distance metastasis				0.631
No	55	32	23	
Yes	10	5	5	

cessfully constructed si-microRNA-487a. Transfection efficacy was verified by qRT-PCR (Figure 2A, 2B). EdU assay was performed to detect the proliferative abilities of EC cells transfected with si-NC or si-microRNA-487a. The data showed that the proliferative rate of EC cells transfected with si-microRNA-487a was markedly decreased compared with those transfected with controls (Figure 2C, 2D). Similar results were obtained in the CCK-8 assay, as microRNA-487a knockdown significantly decreased the proliferative ability of EC cells (Figure 2E, 2F).

Knockdown of MicroRNA-487a Induced Apoptosis in EC Cells

To explore the effect of microRNA-487a on apoptotic rate, flow cytometry was used to detect the apoptosis of EC cells transfected with si-microRNA-487a or si-NC. Staining of Annexin V-FITC/PI showed the apoptotic rate in the si-microRNA-487a group was remarkably higher than that of the si-NC group (Figure 3A, 3B).

Knockdown of MicroRNA-487a Inhibited Migratory and Invasive Abilities of EC Cells

Transwell assay was conducted to explore the effects of microRNA-487a on the migratory and invasive abilities of EC cells. The number of penetrating TE-1 cells was significantly decreased after microRNA-478a knockdown, suggesting a reduction in migratory and invasive abilities (Figure 3C, 3D). EC-109 cells showed the same results as those in TE-1 cells (Figure 3E, 3F).

P62 Modulated MicroRNA-487a Expression in EC Cells

To further explore the mechanism of microRNA-487a in promoting the malignant progression of EC, a possible relationship was found between p62 and microRNA-487a through bioinformatics analysis. Subsequently, qRT-PCR and Western blot were conducted to determine p62 expression in EC tissues and para-cancerous tissues. The results showed that P62 was highly expressed in EC tissues (Figure 4A). Meanwhile, it was highly expressed in EC cells than HEEC cells as well (Figure 4B). Additionally, 16/65 pairs of EC tissues were selected to determine the expressions of microRNA-487a and p62. The data revealed a negative correlation between microRNA-487a and p62 in EC tissues (Figure 4C). At the cellular level, p62 was significantly upregulated after microRNA-487a knockdown in TE-1 and EC-109 cells (Figure 4D).

To further explore the interaction between microRNA-487a and p62 in EC cells, we overexpressed p62 in cells transfected with si-microRNA-487a. The transfection efficacy of overexpression plasmid of p62 was verified by Western blot (Figure 5A, 5B). Transwell assay demonstrated that the invasive and migratory abilities of EC cells co-transfected with overexpression plasmid of p62 and si-microRNA-487a were markedly decreased (Figure 5C, 5D).

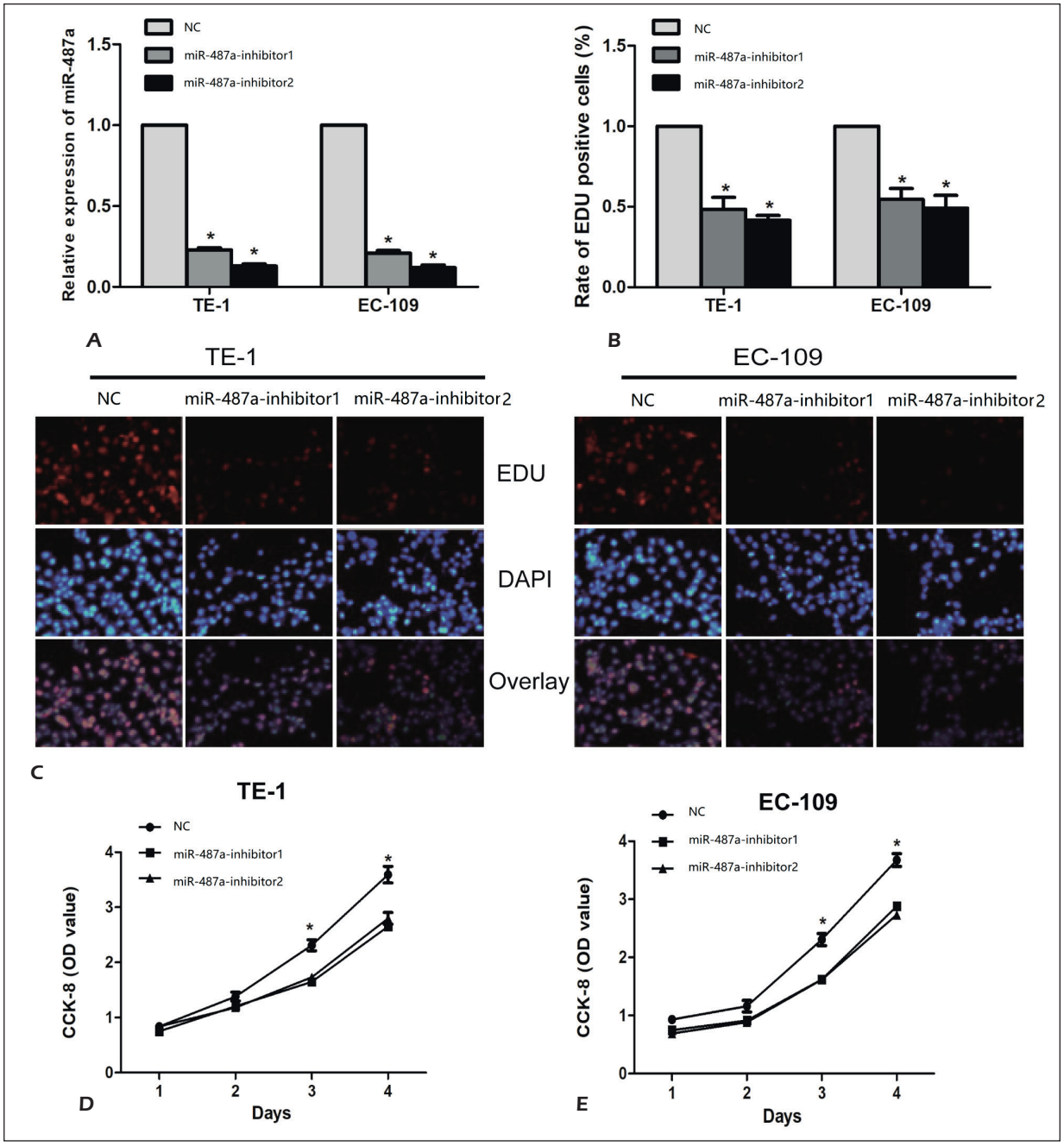

Figure 2. Knockdown of microRNA-487a inhibited cell proliferation. **A**, Transfection efficacy of si-microRNA-487a. **B**-**C**, Proliferative rate of TE-1 and EC-109 cells detected by EdU assay after transfection of si-microRNA-487a. **D**-**E**, Proliferative rate of TE-1 and EC-109 cells detected by CCK-8 assay after transfection of si-microRNA-487a. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Discussion

Tumor is a complex disease with multi-gene and multi-stage development. It activates one or several proto-oncogenes and silences tumor-suppressor genes, eventually escaping the regulatory mechanisms in the body. Tumor cells proliferate independently and finally present malignant phenotypes, such as invasive and infiltrative characteristics[25]. EC is a common malignancy of the digestive tract in the world, with more than 300,000 new cases every year[2-4]. As a high-risk country, nearly half of the new cases are found in China. Meanwhile, there are 150,000 death cases of EC

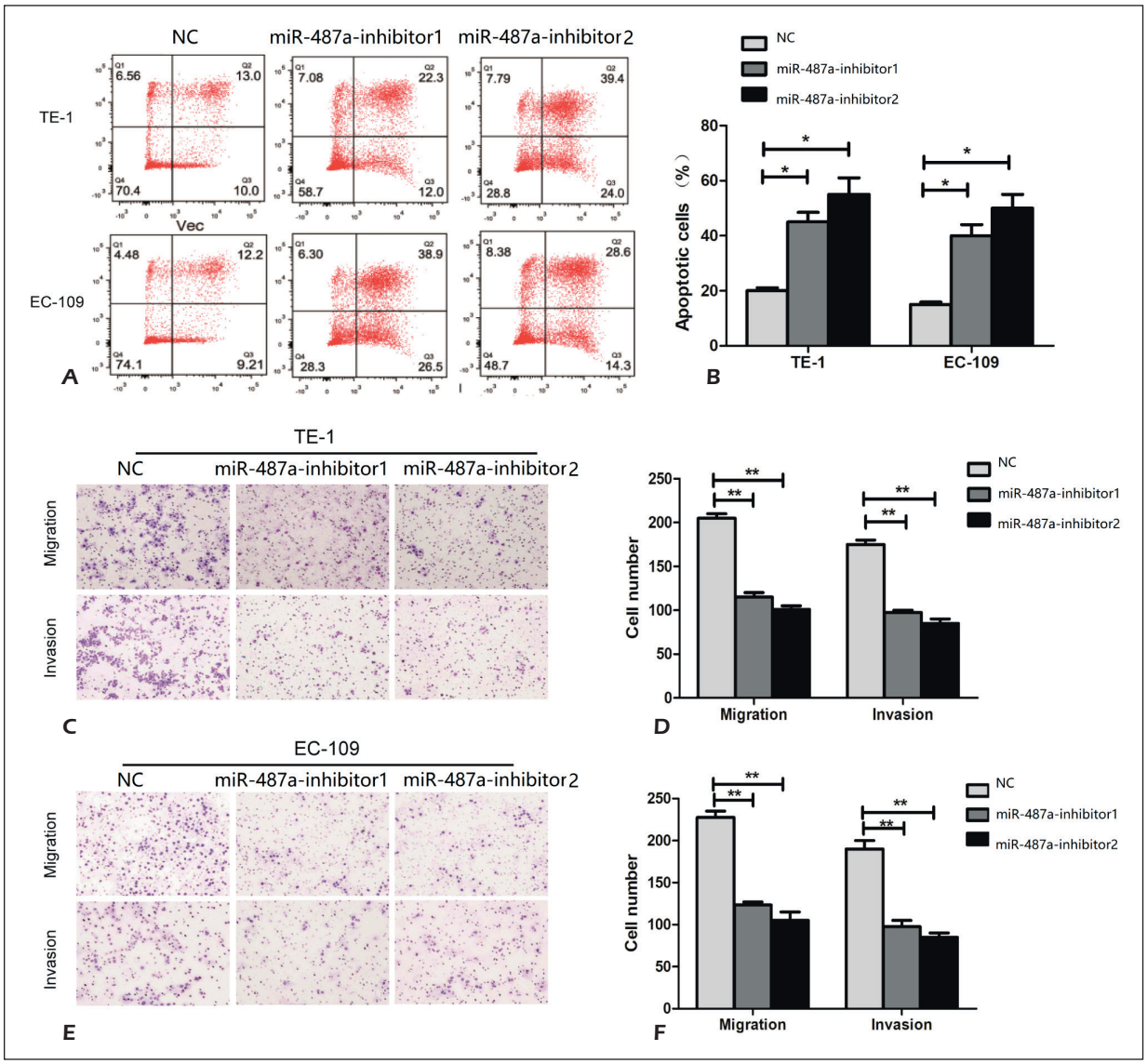

Figure 3. Knockdown of microRNA-487a inhibited cell migration and invasion. **A-B**, Apoptotic rate of TE-1 and EC-109 cells after transfection of si-microRNA-487a. **C-D**, Invasive and migratory abilities in TE-1 cells after transfection of si-microRNA-487a. E, F, Invasive and migratory abilities in EC-109 cells after transfection of si-microRNA-487a. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

in our country[3-5]. Metastasis and recurrence are the main factors for poor prognosis of EC. So far, the 5-year recurrence rate of EC patients after radical resection is still as high as over 40%[11]. However, the specific pathogenesis of EC is still not comprehensively understood. The incidence and mortality of EC remain high in part of the regions. Furthermore, the clinical outcomes of EC patients are far away from satisfying[6-8]. In recent years, the biological roles of microRNAs in regulating the differentiation, proliferation, cell cycle and apoptosis of tumor cells have been widely studied[12,13]. With the development of molecular biology, more and more microRNAs have been discovered. In addition, their biological functions in tumors have been gradually clarified.

Insidious onset of EC makes most EC patients lose the best opportunity for surgery. Therapeutic efficacy of the combined treatment is not very effective. Therefore, finding new treatments is the key to improve the overall survival of EC patients[7-10]. Proliferation, invasion and migration are the most significant biological characteristics of malignant tumors. They are also the fundamental

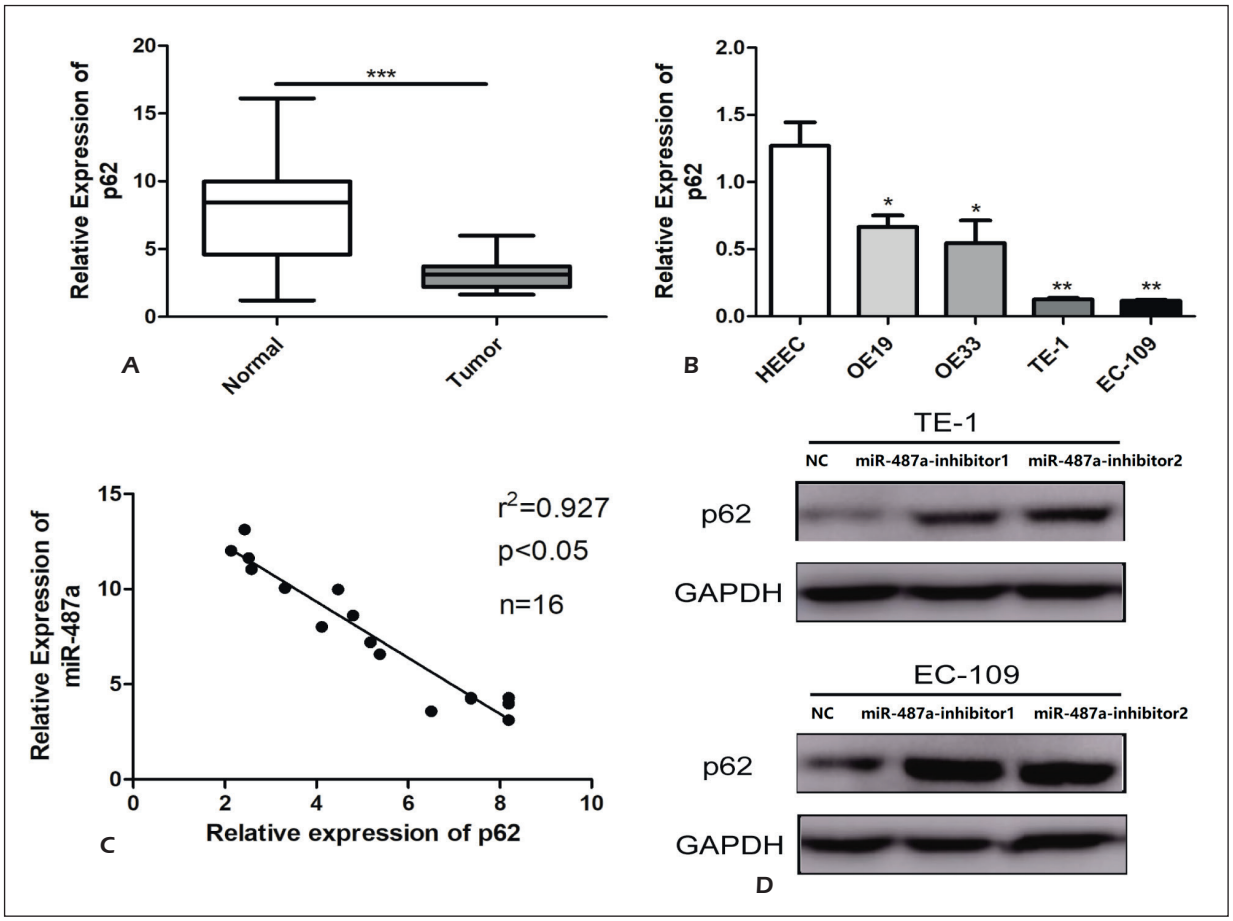

Figure 4. MicroRNA-487a regulated p62 expression in EC. **A**, P62 expression in EC tissues and para-cancerous tissues. **B**, P62 expression in EC cells. **C**, MicroRNA-487a expression was negatively correlated with p62 expression in EC cells. **D**, P62 expression in TE-1 and EC-109 cells after transfection of si-microRNA-487a. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

causes of their difficult treatment. Therefore, the inhibition of proliferation, invasion and migration of malignant tumors is of great significance in controlling the progression of malignant tumors and improving survival rate[10,11]. Previous works have demonstrated that tumor proliferation and invasion are complex processes involving multiple factors. The regulation of microRNAs on genes related to the proliferation and invasion have been extensively studied.

MicroRNAs are differentially expressed in normal cells and malignant tumor cells. MicroRNAs, which are located in tumor genomic instability regions and tumor-associated genomic regions, are abnormally expressed in tumors[12-14]. Several researches have shown that each microRNA has multiple target genes, forming a complex network regulation system. MicroRNAs are involved in the development and progression of tumors, serving as oncogenes or tumor-suppressor genes. They provide a new strategic technology platform for gene therapy of malignant tumors[15]. By analyzing serum samples of EC patients and healthy people, some microRNAs are found to be dysregulated in EC patients. This indicates their potential role in the progression of EC[16]. For example, miR-21 is highly expressed in serum samples of EC patients, which is correlated with tumor stage, differentiation and lymph node metastasis. Meanwhile, it is believed as an oncogene in EC[26]. Other microRNAs are also crucial in the EC development, such as miR-16, miR-25, miR-92c, miR-155, miR-208, miR-214, miR-223, miR-296, etc. Besides, higher serum levels of let-7c, miR-145, miR-200c, and miR-223 are observed in EC patients as well[27-31].

MicroRNA-487a is one of the newly discovered microRNAs in recent years. It serves as a

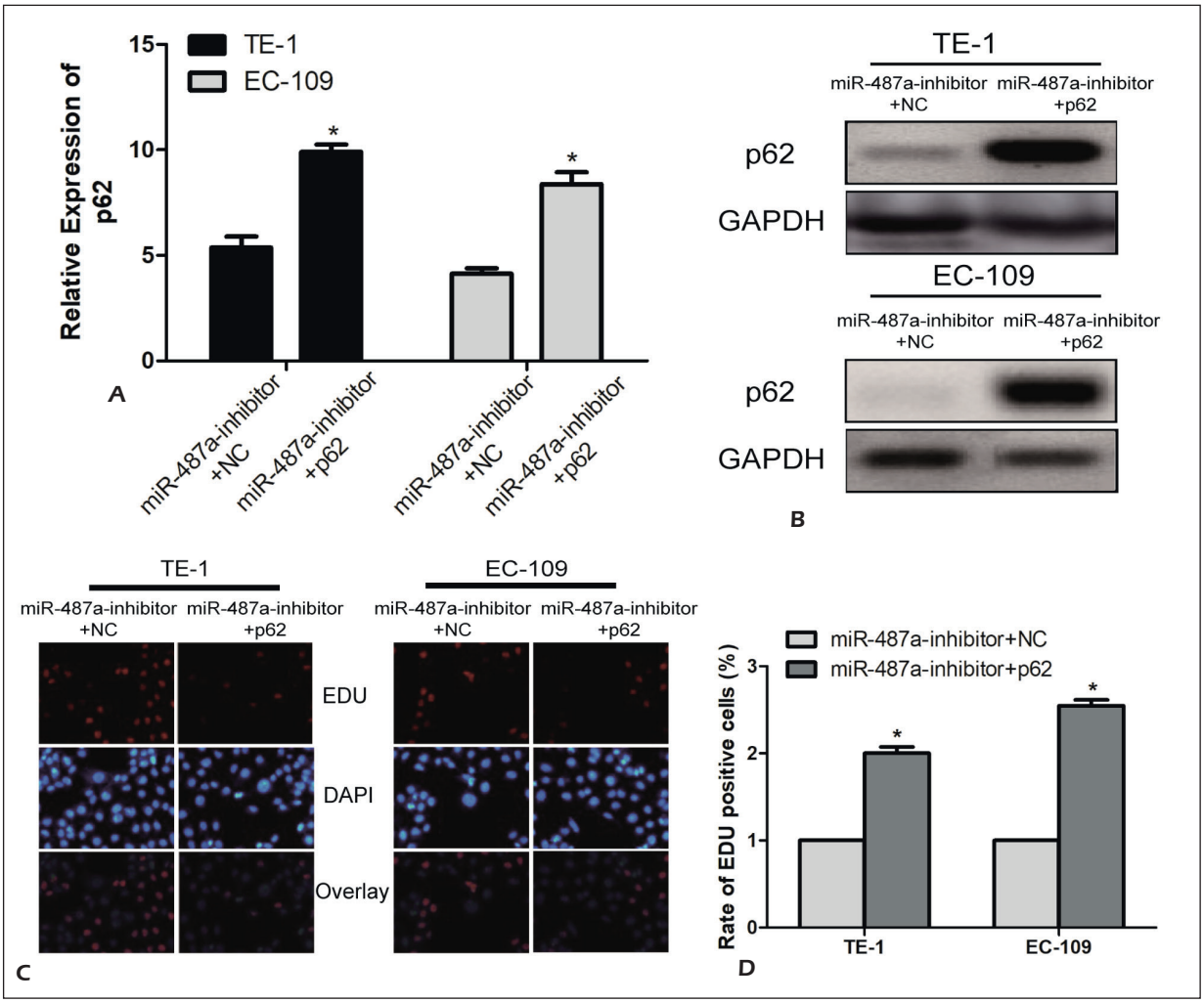

Figure 5. MicroRNA-487a regulated EC by p62. **A**, P62 expression in EC cells co-transfected with overexpression plasmid of p62 and si-microRNA-487a by qRT-PCR. **B**, P62 expression in EC cells co-transfected with overexpression plasmid of p62 and si-microRNA-487a by Western blot. **C-D**, Proliferation abilities in EC cells after co-transfection with overexpression plasmid of p62 and si-microRNA-487a. Data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

tumor-suppressor gene by targeting downstream gene expressions[17]. A large number of studies have confirmed that highly expressed microRNA-487a in various malignant tumors regulates the proliferation and differentiation of tumor cells[17]. The specific role of microRNA-487a in EC, however, is rarely reported. In this investigation, we selected two EC cell lines (TE-1 and EC-109) as research objects. The influences of lowly expressed microRNA-487a on the proliferation, apoptosis, invasion and migration of EC cells were accessed. Firstly, we collected 65 pairs of EC tissues and para-cancerous tissues and detected microRNA-487a expression. The results showed that microRNA-487a was lowly expressed in EC tissues, and was negatively correlated with clinical progression, pathological grade and poor prognosis of EC patients. Therefore, we considered that microRNA-487a could inhibit the EC development. Afterward, small interference plasmid of microRNA-487a was conducted. The subsequent results indicated that the knockdown of microRNA-487a markedly inhibited the proliferative, migratory and invasive abilities of EC cells.

Apoptosis is a progress of orderly and autonomous cell death under gene control, thereby maintaining the stability of the body environment[32]. Unlike the passive process of cell necrosis, apoptosis is an active process, and is closely related to the activation and expressions of a

series of genes[32]. Studies have indicated that the occurrence and development of malignant tumors are associated with the proliferation and apoptosis of tumor cells. Apoptosis has a negative regulation effect, which can effectively inhibit tumor growth. Therefore, how to induce the apoptosis of tumor cells has become a new direction for tumor treatment[32,33]. In our study, flow cytometry showed that downregulation of microRNA-487a induced apoptosis of TE-1 and EC-109 cells, suggesting that targeting microRNA-487a expression might be a new approach for the treatment of EC.

The expression of p62 is regulated by growth and development. It is only found in the fetal stage, but not in the liver of adults. Therefore, p62 exerts the characteristics of tumor-like fetal protein[20,21]. During the transition from chronic hepatitis and cirrhosis to hepatocellular carcinoma, p62 is found in serum samples of affected patients. However, it cannot be detected before the onset of cancer[22,23]. Previous studies have demonstrated that P62 exists in various malignancies. As a cytoplasmic protein, p62 can bind to microRNA-487a[24]. Through rescue experiments, we found the interaction between p62 and microRNA-487a in EC. Further understanding of the biological function of p62 gene and its role in tumor development will be more conducive to the diagnosis, treatment and prognosis of tumors. This undoubtedly brings good news to EC patients and their families.

To verify whether microRNA-487a regulated the occurrence and progression of EC by targeting p62, we first detected p62 expression after microRNA-487a knockdown in EC cells. Subsequently, we found that the p62 overexpression markedly altered the proliferative ability of EC cells. These results indicated that microRNA-487a regulated EC development by altering p62 expression.

Conclusions

MicroRNA-487a was highly expressed in EC, which was closely correlated with clinical stage and poor prognosis of EC. Our findings showed that microRNA-487a promotes malignant progression of EC by regulating p62 expression.

Conflict of Interests

The authors declare that they have no conflict of interest.

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