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IPO5 promotes malignant progression of esophageal cancer through activating MMP7

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Abstract. – OBJECTIVE: Previous studies have found that IPO5 is a cancer-promoting gene. However, the role of IPO5 in esophageal cancer has not been reported. This study aims to investigate the expression characteristics of IPO5 in esophageal cancer, and to further analyze its relationship with clinical parameters and prognosis of esophageal cancer.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the expression of matrix metalloproteinase 7 (MMP7) in 45 pairs of tumor tissue specimens and adjacent normal ones collected from esophageal cancer patients. The correlation between IPO5 expression and clinical indicators and prognosis of esophageal cancer patients was analyzed. Meanwhile, IPO5 expression in esophageal cancer cell lines was also detected using qRT-PCR. In addition, the influence of IPO5 on esophageal cancer cell functions was analyzed using cell counting kit-8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assays. Finally, Dual-Luciferase reporter assay and cell reverse experiments were conducted to explore its underlying mechanisms.

RESULTS: In this experiment, qRT-PCR results indicated that IPO5 expression in tumor tissues of esophageal cancer patients was significantly higher than that in adjacent normal ones, and the difference was statistically significant. Compared with esophageal cancer patients with low expression of IPO5, those with high expression of IPO5 had higher pathological stage and lower overall survival rate. Compared with control group, the proliferation ability of esophageal cancer cells in IPO5 knockdown group was significantly decreased. In addition, Western Blot results indicated that the key protein MMP7 was conspicuously elevated in the esophageal cancer cell line after knockdown of IPO5. Dual-Luciferase reporter assay results suggested that IPO5 can specifically bind MMP7. Additionally, the cell reverse experiment demonstrated that MMP7 was responsible for IPO5-regulated malignant progression of esophageal cancer.

CONCLUSIONS: IPO5 expression significantly increased in esophageal cancer tissues, which was associated with pathological staging and poor prognosis of esophageal cancer patients. IPO5 may promote malignant progression of esophageal cancer through the regulation of MMP7.

Key Words:
IPO5, MMP7, Esophageal cancer, Proliferation.

Introduction

Esophageal cancer is one of the most common malignancies of the digestive system in the world. Although its malignancy is not as severe as gastric cancer and liver cancer, according to the National Cancer Center, it ranks fourth among the causes of cancer death in China[1-3]. Esophageal cases in China account for about half of the global cases[4,5]. There are several pathological types of esophageal carcinoma, including adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, small-cell undifferentiated carcinoma, and carcinosarcoma[6,7]. Among them, squamous cell carcinoma (SCC) is the most common subtype in China, accounting for more than 90% of esophageal cancer cases[8,9]. Currently, surgery is the major treatment of esophageal cancer. Statistics have shown that about 90% of esophageal cancer patients are in middle stage or advanced stage when diagnosed, which makes them lose the opportunity of radical operation and thus result in the 5-year survival rate lower than 20%[10,11]. Notably, early-stage esophageal cancer can be radically treated by minimally invasive endoscopic radiofrequency ablation, and its 5-year survival rate is up to 95%. Hence, the early diagnosis and treatment of esophageal can-

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cer are the key to improve the survival rate of patients[11-13]. The early symptoms of esophageal cancer are not evident and specific, and most patients are diagnosed and detected by auxiliary technologies, mainly including X-ray imaging, abscission cytology, and endoscopy[14,15]. These assistive technologies cannot be conventionally applied for high-risk screening due to their complicated clinical operations and low accuracy[15]. In recent years, researches on molecular biological tumor markers have been carried out continuously. Due to the simple operation, high detection efficiency, small trauma to patients, and wide screening range, serum detection of tumor markers is currently considered to have broad prospects in tumor diagnosis[16,17].

IQ motif containing GTPase activating protein 1 (IQGAP 1) can adjust the activities of Cdc42 and Rac and combine with actin to participate in various cell processes such as cell adhesion, cell proliferation, cell cycle, and migration. Meanwhile, IpoS can regulate the transcription of IQGAP 1 protein through the Wnt signaling pathway[18,19]. Therefore, we speculated that IpoS may regulate the malignant progression of esophageal cancer cells *via* regulating MMP7[20]. Importin-5 (IPO5) is located on 38q13.11 and has target-controlled relationship with many downstream genes[20,21]. Studies have confirmed that high expression of IPO5 in colorectal cancer and other malignant tumor tissues leads to abnormal regulation of target genes, which provides favorable conditions for the proliferation and differentiation of malignant tumor cells. This study suggested that IPO5 was highly expressed in esophageal cancer cells, and silence of IPO5 can effectively inhibit the malignant progression of esophageal cancer cells, suggesting that IPO5 acted as an oncogene in this cancer.

MMP7 belongs to the matrix metalloproteinase family (MMPs). In normal tissues, it can promote cell proliferation by lysing the normal tissues. However, in tumor tissues, it is secreted and produced by tumor cells and participates in the progression of tumors through multiple pathways[22,23]. Autoantibodies against MMP7 have been found in various malignant tumors[24,25], suggesting that MMP7 may be involved in the occurrence and development of numerous human malignant tumors. Bioinformatics genome prediction suggested that MMP7 may be one of the targeted genes of IPO5. However, IPO5 is not known to be involved in the proliferation and apoptosis of esophageal cancer cells through the targeted regulation of MMP7. Therefore, we detected IPO5 expression in tumor tissue samples and paired adjacent tissues of esophageal cancer patients. The biological function and possible molecular mechanism of IPO5 in esophageal cancer cell lines were further analyzed to provide potential biomarkers for early diagnosis and population screening of esophageal cancer.

Patients and Methods

Esophageal Cancer Patients and Tumor Samples

A total of 45 patients who underwent surgical resection and routine hematoxylin-eosin staining (HE) staining were enrolled. Their matched esophageal cancer tissues and adjacent normal ones were surgically resected. All specimens were frozen and stored in a –80°C refrigerator for subsequent RNA extraction. The investigation was approved by the Ethics Oversight Committee. None of the enrolled patients had chemotherapy before surgery. Patients and their families were fully informed that the specimens would be used for scientific research and signed informed consent. The pathological classification and staging criteria of esophageal cancer were performed according to the international association of cancer (UICC) criteria for esophageal cancer staging.

Cell Lines and Reagents

Four human esophageal cancer cell lines (OE19, OE33, TE-1, and EC-109) and one human normal esophageal epithelial cell line (HEEC) were purchased from American type culture collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Hyclone (South Logan, UT, USA). Cells were cultured with DMEM containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 37°C, 5% CO_2 incubator.

Transfection

The control sequence (sh-NC) and the knockdown IPO5 lentiviral sequence (sh-IPO5) were purchased from GenePharma (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 40%. Lentiviral transfection was performed according to the manufacturer's instructions, and cells were collected 48 h later

for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), Western Blot analysis, and cell function experiments.

Cell Counting Kit-8 (CCK-8) Assay

After 48 h of transfection, cells were collected and plated into 96-well plates at 2000 cells per well. The cells were cultured for 24 h, 48 h, 72 h, and 96 h, respectively, and then CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added. After incubation for 2 h, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

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

To demonstrate the proliferation ability of esophageal cancer cells, the EdU proliferation assay (RiboBio, Guangzhou, China) was performed according to the manufacturer's requirements. After transfection for 24 h, the cells were incubated with 50 μm EdU for 2 h and stained with AdoLo and 4',6-diamidino-2-phenylindole (DAPI). The number of EdU-positive cells was detected by fluorescence microscopy. EdU-positive ratio was calculated as the number of EdU-positive cells (red cells) to the total 4',6-diamidino-2-phenylindole (DAPI) chromogenic cells (blue cells).

QRT-PCR

Total RNA was extracted from the transfected cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), respectively, followed by measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and72°C for 90 s, for a total of 40 cycles. The following primers were used for qRT-PCR reaction: IPO5: forward: 5'-CTGCTGAAGAGGCTAGACAAATG-3', Reverse: 5'-TCTGCCGCAATATCACAAACTT-3'; MMP7: forward: 5'-TGTATGGGGAACTGCTGACA-3', Reverse: 5'-GCGTTCATCCTCATCGAAGT-3'; β-actin: forward: 5'-CAGAGCTCCTCGTCTTGCC-3', Reverse: 5'-GTCGCCACCATGAGAGAC-3'. Data analysis was performed using ABI Step One software (Applied Biosystems, Foster City, CA, USA), and the relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were repeated 3 times.

Western Blot Assay

The transfected cells were collected after 72 h of culture and the proteins were extracted for quantitative detection. Proteins were extracted according to the protein extraction step and protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Total protein was separated using dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland), and blocked with 5% skim milk powder for 1 h at room temperature. Membranes were incubated with primary antibodies against MMP7 (1:500) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2000) overnight at 4°C. On the next day, the membranes were rinsed 3 times with Tris-Buffered Saline and Tween-20 (TBST) and incubated with secondary antibody (1:1 000) for 1 h at room temperature. After that, the protein samples on the membrane were finally developed and analyzed with enhanced chemiluminescence (ECL) kit. All experiments were repeated 3 times.

Dual-Luciferase Reporter Assay

Based on the predicted binding sequences in the seed region of MMP7 and IPO5, wild-type and mutant-type IPO5 plasmids were constructed. HEK293T cells were seeded in 24-well plates and co-transfected with MMP7 mimic/NC and pMIR Luciferase reporter plasmids using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. After 48 h of transfection, relative Luciferase activity was calculated using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 (SPSS; IBM, Armonk, NY, USA) statistical software. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Differences between groups were analyzed by paired sample *t*-test. The relationship between IPO5 and clinicopathological features was tested by χ^2-test. Survival analysis was performed by Kaplan-Meier method. $p<0.05$ was considered statistically significant.

Results

IPO5 Was Highly Expressed in Esophageal Cancer Tissues and Cell Lines

The expression of IPO5 in 45 pairs of esophageal cancer tissues and their corresponding adjacent ones, as well as in esophageal cancer cell lines was detected by qRT-PCR. The results indicated that compared with adjacent non-tumor tissues, the expression level of IPO5 in tumor tissues was significantly upregulated, and the difference was statistically significant (Figure 1A). Meanwhile, compared with human normal esophageal epithelial cells (HEEC), IPO5 was highly expressed in esophageal cancer cell lines (Figure 1B), especially in EC-109 and OE33 cells, which were therefore selected for subsequent experiments.

IPO5 Expression Was Correlated with Clinical Stage and Overall Survival in Esophageal Cancer Patients

The above tissue samples were divided into high expression and low expression group according to the median mRNA expression of IPO5. Subsequently, Chi-square test was performed to analyze the interaction between IPO5 expression and clinical indicators of esophageal cancer patients, such as age, gender, pathological stage, lymph node or distant metastasis. As shown in Table I, high expression of IPO5 was positively correlated with the pathological stage, but it was not correlated with other indicators. In addition, Kaplan-Meier survival curves were plotted to analyze the relationship between the expression of IPO5 and the prognosis of esophageal cancer patients. As a result, high expression of IPO5 was found to be conspicuously associated with the poor prognosis of esophageal cancer ($p<0.05$; Figure 1C). It is suggested that IPO5 might be a new biological indicator for predicting the prognosis of esophageal cancer.

Knockdown of IPO5 Inhibited Esophageal Cancer Cell Proliferation

To explore the influence of IPO5 on the proliferation ability of esophageal cancer cells, IPOT5 expression was silenced in EC-109 and OE33 cells (Figure 1D and 1E). Subsequently, CCK-8 (Figure 2A) and EdU (Figure 2B) assay were performed to examine the cell proliferation ability. Knockdown of IPO5 markedly decreased proliferation ability in esophageal cancer.

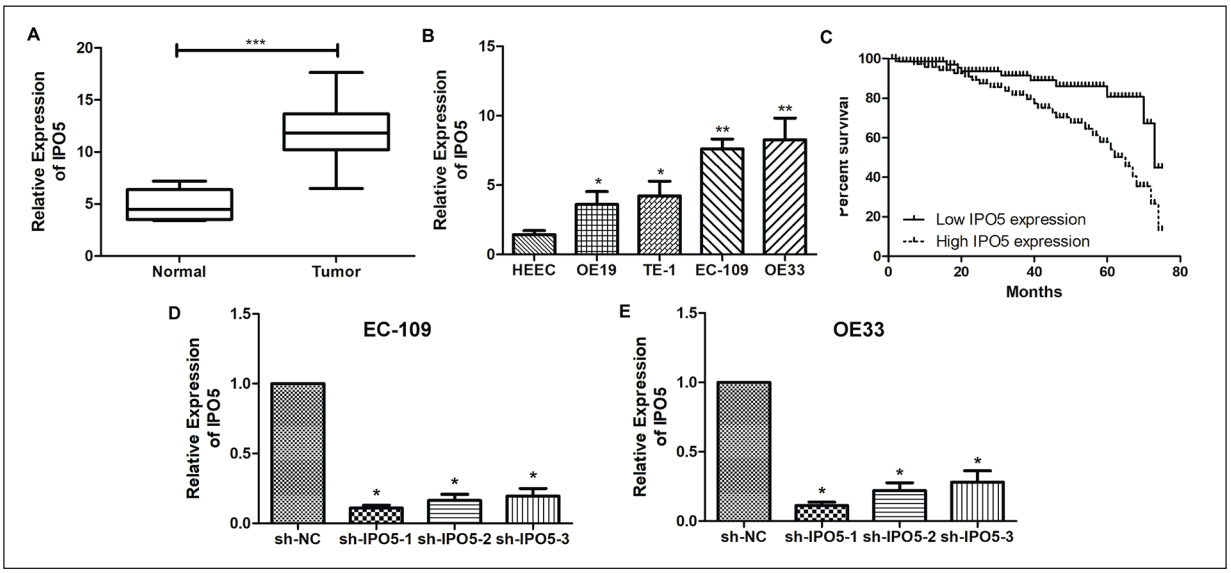

Figure 1. IPO5 was highly expressed in esophageal cancer tissues and cell lines. **A,** QRT-PCR was used to detect the difference in expression of IPO5 in esophageal cancer tumor tissues and adjacent normal tissues; **B,** QRT-PCR was used to detect the expression level of IPO5 in esophageal cancer cell lines; **C,** Kaplan-Meier survival curves of esophageal cancer patients based on IPO5 expression. The prognosis of esophageal cancer patients with high expression of IPO5 was significantly worse than those with low expression; **D,** QRT-PCR verified the transfection efficacy of sh-IPO5 in EC-109 cell line; **E,** QRT-PCR verified the transfection efficacy of sh-IPO5 in the OE33 cell line. Data are mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of IPO5 expression with clinicopathologic characteristics of esophageal cancer.

Parameters	No. of cases	IPO5 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.259
< 60	17	12	5	
≥ 60	28	15	13	
Gender				0.626
Male	22	14	8	
Female	23	13	10	
T stage				0.003
T1-T2	27	21	6	
T3-T4	18	6	12	
Lymph node metastasis				0.197
No	30	20	10	
Yes	15	7	8	
Distance metastasis				0.464
No	35	22	13	
Yes	10	5	5	

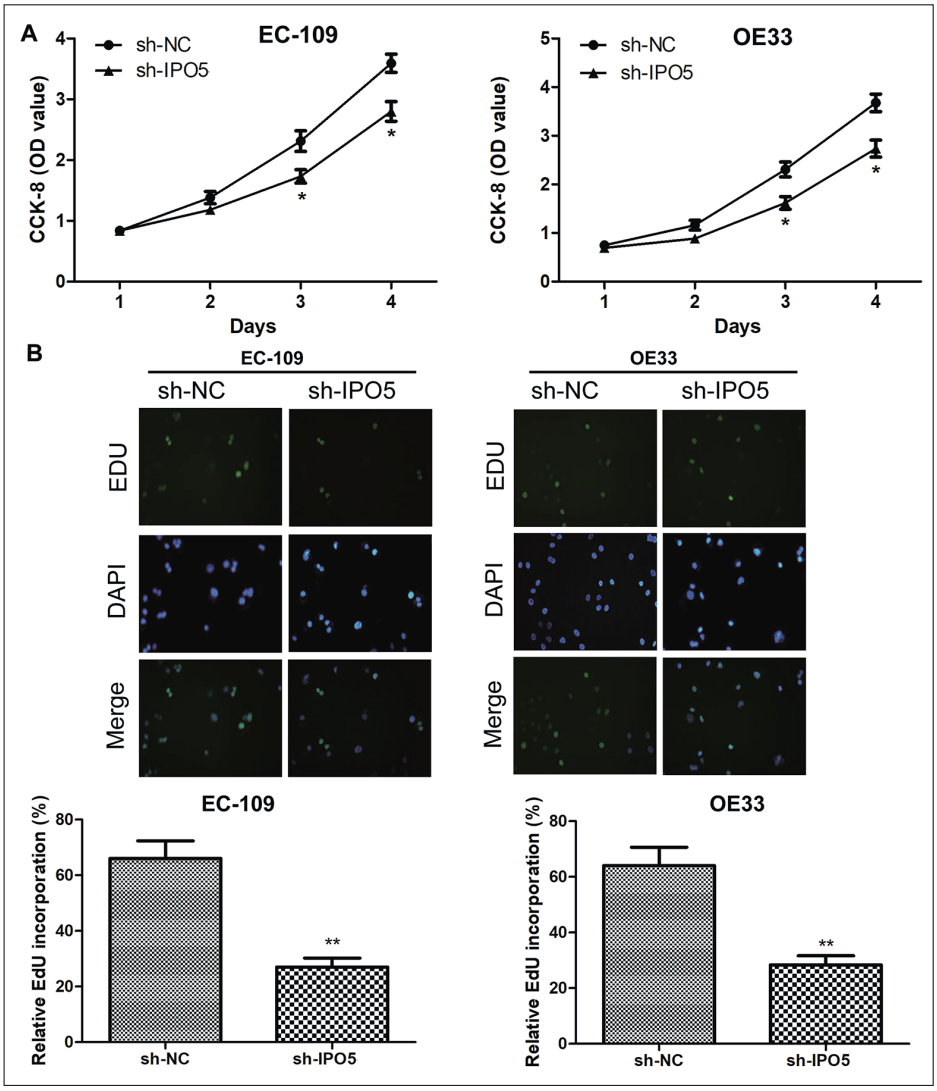

Figure 2. Knockdown of IPO5 inhibited esophageal cancer cell proliferation. **A,** CCK-8 assay was used to detect the effect of IPO5 knockdown on the proliferation of esophageal cancer cells EC-109 and OE33; **B,** EdU assay examined the effect of IPO5 knockdown on proliferation of esophageal cancer cells EC-109 and OE33 (Magnification: 40×). Data are mean ± SD, **p*<0.05, ***p*<0.01.

Knockdown of IPO5 Decreased MMP7 Level

To further explore how IPO5 promoted the malignant progression of esophageal cancer, bioinformatics analysis was applied, and MMP7 was predicted to be the downstream gene binding IPO5. Western Blot and qRT-PCR results indicated that the expression level of MMP7 was significantly reduced in EC-109 and OE33 cells with IPO5 knockdown (Figure 3A, 3B). Moreover, Dual-Luciferase reporter assay verified that overexpression of MMP7 significantly attenuated Luciferase activity in wild-type IPO5 vector, while the mutant-type one was not affected, further demonstrating that IPO5 can be targeted by MMP7 (Figure 3C). At the same time, qRT-PCR revealed that the expressions of IPO5 and MMP7 in esophageal cancer tissues were positively correlated (Figure 3D).

IPO5 Regulated Proliferation of Esophageal Cancer Cells Through MMP7

To further explore the interaction between IPO5 and MMP7 in esophageal cancer cell lines, we co-transfected MMP7 overexpression plasmid and sh-IPO5 in EC-109 and OE33 cells. Transfection efficiency was determined by qRT-PCR and Western Blot (Figure 4A, B). Interestingly, EdU assay revealed that overexpression of MMP7 in EC-109 and OE33 cells with IPO5 knockdown could partially restore the weakened proliferation ability of esophageal cancer cells induced by knockdown of IPO5 (Figure 4C).

Discussion

In recent years, the incidence of malignant tumor has shown an overall growth trend glob-

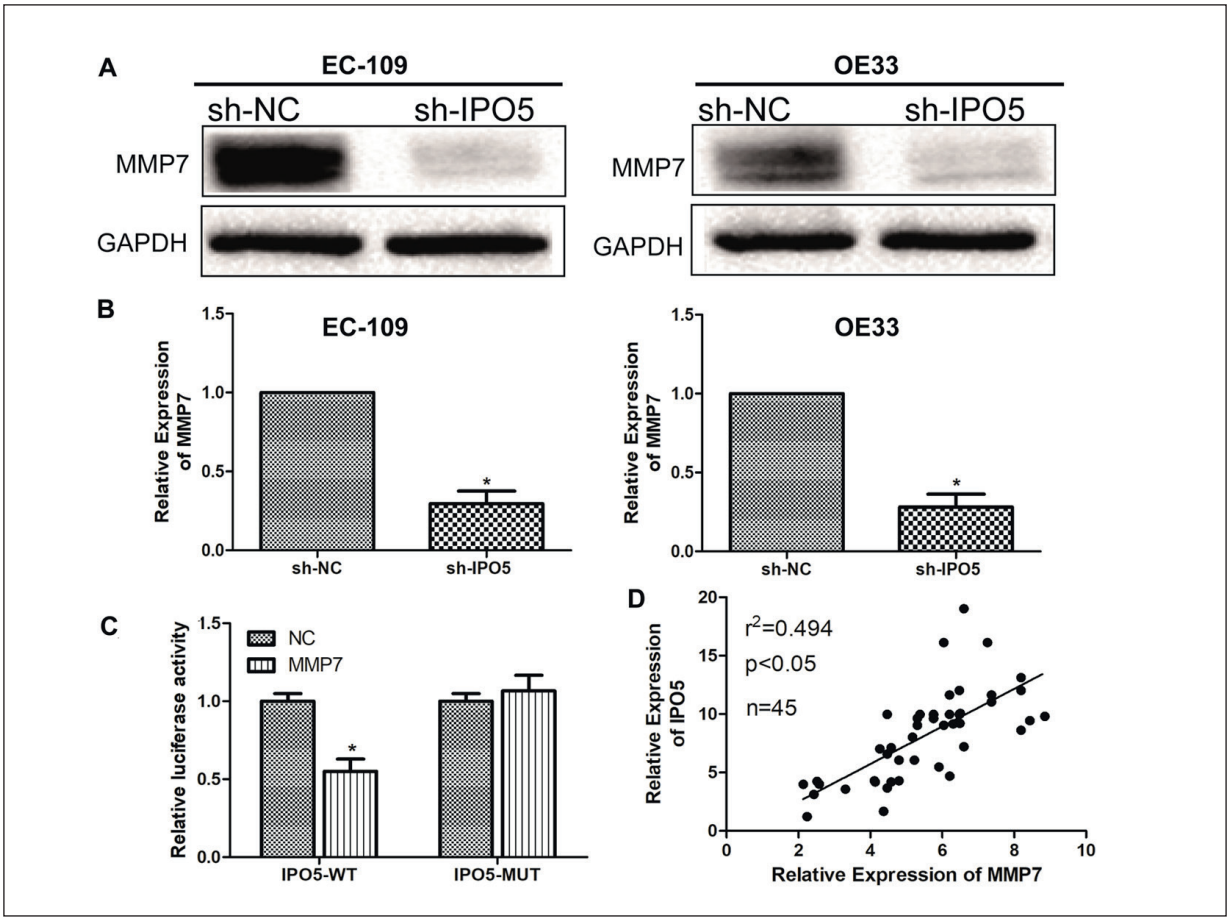

Figure 3. IPO5 regulated the expression of MMP7 in esophageal cancer tissues and cell lines. **A,** Western Blotting verified the protein expression level of MMP7 after interference with IPO5 in EC-109 and OE33 cell lines; **B,** QRT-PCR verified mRNA expression level of MMP7 after interference with IPO5 in EC-109 and OE33 cell lines; **C,** Dual-Luciferase reporter assay validated the direct targeting of IPO5 and MMP7. **D,** Significant positive correlation between the expression levels of IPO5 and MMP7 in esophageal cancer tissues. Data are mean ± SD, *p<0.05.

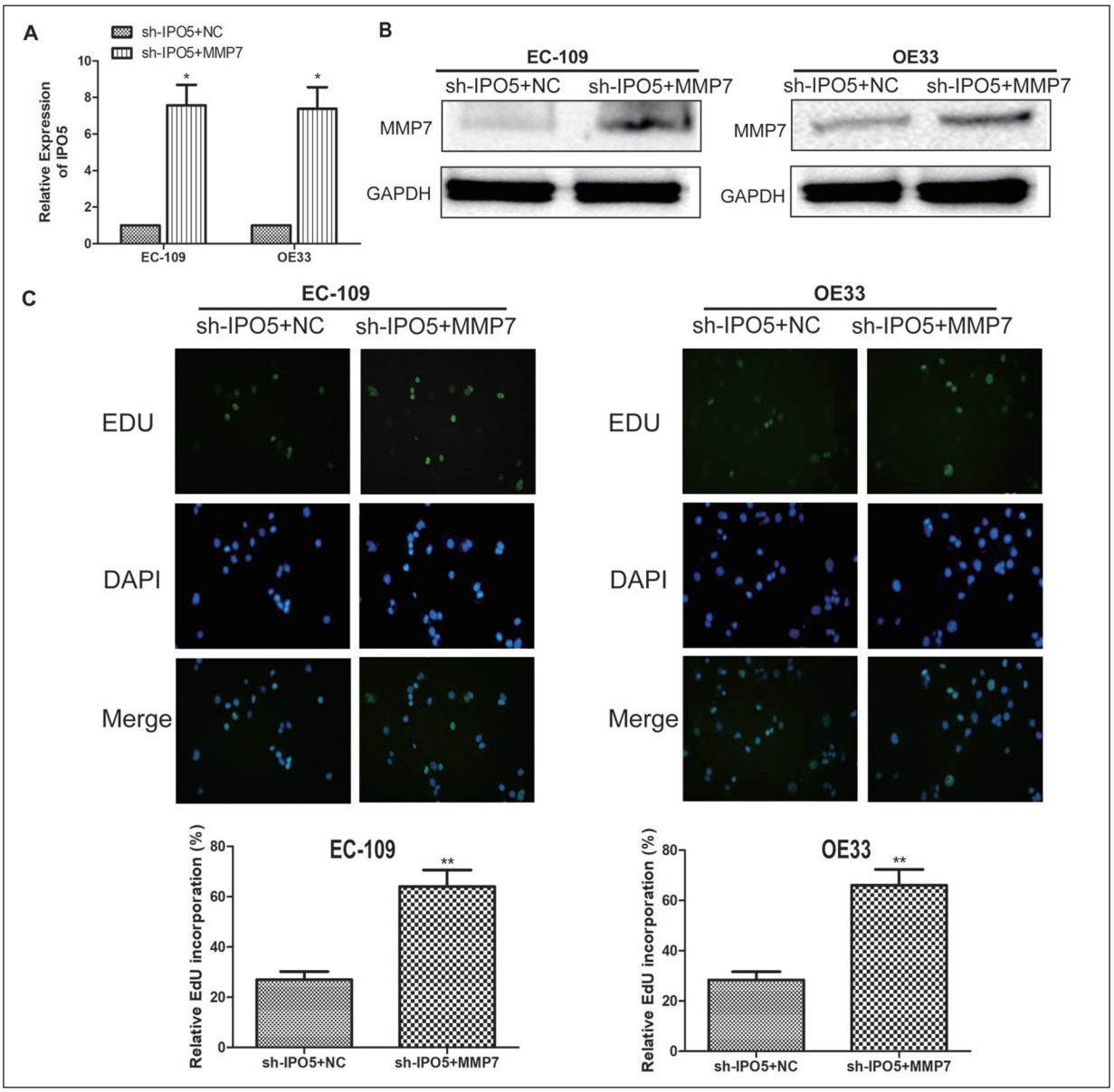

Figure 4. IPO5 regulated proliferation in esophageal cancer cells through regulating MMP7s. **A,** MRNA expression levels of MMP7 in esophageal cancer cell lines co-transfected with sh-IPO5 and MMP7 overexpressing plasmid were detected by qRT-PCR; **B,** Western blotting was used to detect the protein expression level of MMP7 in esophageal cancer cell lines co-transfected with sh-IPO5 and MMP7 overexpressing plasmid; **C,** EdU assay was used to detect the effects of IPO5 and MMP7 on the proliferation of esophageal cancer cells (magnification: 40×). Data are mean ± SD, **p*<0.05, ***p*<0.01.

ally, which is a serious threat to human health and social development[1,2]. Esophageal cancer is one of the common malignant gastrointestinal tumors in human beings, and China is a high incidence area of esophageal cancer, which seriously affects people's life and health[1-4]. At present, the treatment of esophageal cancer relies on combined treatment of surgical treatment, radiotherapy, and chemotherapy. Nevertheless, the prognosis of esophageal cancer is still poor, and the survival rate is low. Therefore, it is necessary to improve the conventional therapies and find new therapeutic targets to improve the existing treatment scheme[7,8]. Since the onset of esophageal cancer is insidious, most patients are already in the advanced stage

when they are diagnosed[5-10]. Cell proliferation is the most significant biological characteristic of malignant tumors and the fundamental reason for poor therapeutic efficacy of malignant tumors[9-11]. Therefore, effective inhibition of tumor cell proliferation is of great significance to control the progress of malignant tumors and improve the survival rate[11,12]. Malignant progression of tumor is a complex process involving multiple factors, and the regulation of gene transcription on proliferation-related genes is a hotspot of current research[13-17].

IPO5 is one of the newly discovered genes regulating tumor occurrence and development in recent years, which can serve as a cancer-promoting gene through targeted regulation of downstream genes[18,19]. IPO5 is highly expressed in various malignant tumor tissues, which provides favorable conditions for the proliferation and differentiation of malignant tumor cells to some extent. However, the effect of IPO5 on esophageal cancer is rarely reported[20,21]. On the basis of previous researches, esophageal cancer cell lines, including EC-109 and OE33 were selected in this study. First, the expression of IPO5 was found to be upregulated in 45 pairs of esophageal cancer tissues compared to that of adjacent tissues. Meanwhile, IPO5 level was positively correlated with the pathological stage and poor prognosis of esophageal cancer, suggesting that IPO5 may play a pro-cancer role in esophageal cancer. In addition, CCK-8 and EdU assay revealed that knockdown of IPO5 could significantly inhibit the proliferation of esophageal cancer cells.

Autoantibodies of MMP7 can be found in various malignant tumors[22-25], and MMP7 is also a cytoplasmic protein binding the mRNA encoding IPO5. We next analyzed the involvement of MMP7 in IPO5-regulated development of esophageal cancer. In esophageal cancer cells, MMP7 level was positively regulated by IPO5. Besides, MMP7 was highly expressed in esophageal cancer tissues as well. Through bioinformatics prediction and Dual-Luciferase reporter assay, we further proved that MMP7 was the downstream gene binding IPO5. Notably, overexpression of MMP7 partially reversed the regulatory effects of silenced IPO5 on proliferation of esophageal cancer cells. Our findings undoubtedly provide a new direction in the treatment of esophageal cancer. IPO5/MMP7 axis may be utilized as effective markers for screening and treating esophageal cancer.

Conclusions

We first confirmed that the expression of IPO5 in esophageal cancer tissues was significantly upregulated, which was remarkably correlated with pathological staging and poor prognosis of esophageal cancer patients. In addition, IPO5 may promote the proliferation ability of esophageal cancer *via* regulating MMP7.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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