



miR-488-5p promotes esophageal squamous cell carcinoma progression by suppressing the P53 pathway

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Background: miR-488-3p has been reported to play an important role in cancer progression and metastasis. The protein 53 (P53) gene serves as a mediator and biomarker of esophageal squamous cell carcinoma (ESCC). However, the molecular mechanism underlying miR-488-5p in the pathology of ESCC through the P53 pathway has not been examined.

Methods: The expression levels of miR-488-5p were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Cytological experiments were performed to evaluate the biological functions of miR-488-5p. A bioinformatics analysis was performed to determine the pathways and key miR-488-5p targets associated with ESCC. Correlations between miR-488-5p and P53 signaling pathways were validated by western blotting and the dual luciferase reporter gene system. Finally, the expression level of miR-488-5p was regulated and tumor formation experiments were performed in nude mice.

Results: The qRT-PCR analysis showed that miR-488-5p expression was more upregulated in the KYSE-150 group than the HEEC group. In the KYSE-150 cells, the colony formation assay and flow cytometry analysis indicated that the miR-488-5p inhibitor inhibited cell viability and increased cell apoptosis; however, these effects were recovered by P53 knockdown (KD). In addition, cell invasion and cell migration were inhibited by the miR-488-5p inhibitor, but were also improved by P53 KD. Similarly, the miR-488-5p inhibitor induced the expression of P53 and P21 than normal control (NC) group in which miR-488-5p expression was normal, while P53 KD prevented the effects of the miR-488-5p inhibitor in KYSE-150 cells. Additionally, we found that tumor size was obviously smaller in miR-488-5p overexpression (OE)+ P53 OE mice than miR-488-5p OE mice. Hematoxylin and eosin and immunohistochemistry staining also revealed similar results.

Conclusions: Our results suggest that miR-488-5p promotes ESCC progression by suppressing the P53 pathway. These findings should provide novel ideas for ESCC therapies.

Keywords: KYSE-150; miR-488-5p; esophageal squamous cell carcinoma (ESCC); P53

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Introduction

Esophageal carcinoma, which accounts for 1–2% of all malignant tumors, is one of the most common malignant tumors of the digestive system (1). Among the cases of cancer death worldwide, esophageal carcinoma ranks 6th (2). According to the latest survey data from the International Agency for Research on Cancer, there were 572,000 new esophageal carcinoma cases and 50,800 deaths in 2018 (3). China has high esophageal carcinoma morbidity and mortality rates (4). Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) are the two main pathological types of esophageal carcinoma (5). There are differences between the two pathological types worldwide (6). In China, esophageal carcinoma mainly originates from the esophageal squamous epithelium, and ESCC is the main histological type, while in Western countries, esophageal carcinoma mainly originates from Barrett's esophagus, and EAC is the main histological type (7). The incidence of esophageal carcinoma may be related to genetic factors, smoking, obesity, and chronic gastro esophageal reflux disease (8).

Micro ribonucleic acids (miRNAs) are small, non-coding RNAs that modulate cell development, invasion, migration, proliferation, and apoptosis. Increasing evidences show that abnormal miRNA expression also has such effects on a variety of tumors, such as lung tumors (9), cervical tumors (10), and gastric tumors (11), and numerous studies have proven that miRNAs influence the tumorigenesis. The deregulation of miRNA expression occurs in tumorigenesis, such as esophageal carcinoma; thus, miRNA expression profiles are important in the early diagnosis of esophageal carcinoma. Liu *et al.* found that miRNAs can be used as novel potential biomarkers for ESCC diagnosis (12). The role of miRNAs is similar to that of oncogenes or tumor suppressor genes (13). miR-21 has been reported to induce cell growth, migration, and invasion by targeting phosphatase and tensin homolog (PTEN) and programmed cell death 4 (PDCD4) in ESCC (14,15). In ESCC, more miRNAs have been identified that have a tumor suppressor gene function than an oncogene function (16). The overexpression of miR-382 prevents cell migration and invasion through the mammalian target of rapamycin/eukaryotic translation initiation factor 4E binding protein 1 (mTOR/4E-BP1) signaling pathway in ESCC (16). miR-488-5p, a microRNA, reportedly acts as an inhibitor of androgen receptors in prostate cancer in the earliest research (17). It also has the function

of regulating the bone density of women in desperate situations (18). In recent years, it has been discovered that miR-488-5p plays an important role in tumor progression, such as an oncogene in breast cancer (19) and osteosarcoma (20). However, the association of miR-488-5p with the progression and development of ESCC is still unclear.

Protein 53 (P53) is a tumor suppressor gene containing 11 exons and 10 introns, which can encode proteins containing 393 amino acids (21). Recent studies have shown that P53 is one of the most common genes found in human tumors, and P53 plays an important role in tumor cell apoptosis (22,23). Yao *et al.* found that the expression of P53 significantly increased in ESCC (24). Additionally, Fagundes *et al.* found that the expression of P53 was positively correlated with the severity of esophageal carcinoma (25). However, the mechanism by which P53 effects ESCC progression has not yet been fully elucidated.

In this study, we showed that miR-488-5p was upregulated in ESCC, and that miR-488-5p promoted ESCC progression. Further, we found that P53 was a target gene of miR-488-5p in ESCC. We confirmed that miR-488-5p promoted ESCC progression by suppressing the P53 pathway. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://dx.doi.org/10.21037/jtd-21-1448>).

Methods

Animal models

Twenty female Bagg Albino (BALB)/c nu/nu thymus-deficient nude mice weighing 18 g were obtained from Charles River (Beijing, China). Since gender is not the main factor in the incidence of esophageal cancer (26), we chose female nude mice. All of the mice were disinfected before being reared. Each cage housed 4 mice in a specific-pathogen-free environment. All the mice were kept on a 12-h light-dark cycle. A relative humidity of 50%±10% and a temperature of 22±2 °C were maintained. The mice had free access to food (sterilized special mouse pellet feed) and water, and were regularly subjected to ultraviolet irradiation for disinfection. The KYSE-150 cells [5×10^5 cells in 100 μ L of phosphate-buffered saline (PBS)] (27) were injected subcutaneously into the back of each 4-week-old mouse. The mice were observed every 2 days for at least 8 weeks, and cervical dislocation was then performed to euthanize the mice. Tumor size was assessed based on volume (length

× width × height). The tumor tissue samples obtained from mice were used for the follow-up tests. Experiments were performed under a project license granted by the Institutional Laboratory Animal Care and Use Committee of Hebei Medical University (licenses number: 2018MEC161). All the mice were treated according to national or institutional guidelines for the care and use of animals and the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication, 8th edition, 2011).

Cell culture

Human normal esophageal epithelial cell line (HEEC) and esophageal cancer cell lines (KYSE-150, TE-10, and TE-15) were purchased from American Type Culture Collection (ATCC, USA). The CRISPR/Cas9 system was used to knockout the TP53 gene in the KYSE-150 cells (i.e., KYSE-150 P53^{-/-} cells). The KYSE-150 P53^{-/-} cells were obtained from Synthgene (Nanjing, China). HEEC, KYSE-150, TE-10, TE-15, and KYSE-150 P53^{-/-} cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in an atmosphere containing 5% carbon dioxide (CO₂).

Colony formation assay

The colony formation assay is an effective method for measuring cell viability. The cells in the logarithmic growth phase were inoculated with RPMI-1640 medium at 37 °C with 5% CO₂. When macroscopic colonies appeared in a culture dish, the culture was terminated. The supernatant was discarded and carefully immersed thrice with phosphate-buffered saline (PBS). 5 mL of 4% paraformaldehyde was added to the fixed cells and left for 15 min. The fixative solution was then discarded, an appropriate amount of Giemsa staining solution was added for 10 to 30 min, the staining solution was then slowly washed away with running water, and the plate was left to air dry. The plate was inverted and overlaid using a grid of transparencies. Colonies larger than 10 cells were counted under a microscope (low magnification). Finally, the clone formation rate was calculated.

Cell apoptosis assay

The cells were collected, resuspended, and centrifuged

at 1,000 rpm at room temperature for 5 min. Next, the supernatant was discarded and the sediment was resuspended with 300 μL × binding buffer in each sample, and incubated with 5 μL Annexin V-FITC for 10 min, incubated with 5 μL Propidium iodide (PI) before detection. Finally, the samples were examined using flow cytometer (Beckman Coulter, CA, USA). The tests were repeated 3 times.

Cell invasion assay

Transwell chambers with 24-well plates were used. The Matrigel was prepared in advance, and 100 μL of Matrigel was added to the upper chambers at 37 °C and left overnight to gel. The Matrigel was reconstructed by adding 200 μL of RPMI-1640 medium, and 750 μL of culture medium (with serum) to the lower chamber. Next, the cells were plated at 1×10⁵ cell/mL in the upper chamber of the 24-well plate chamber, and kept at 37 °C for 12–16 h. The cells were then treated with 4% paraformaldehyde for 10 min, and washed thrice with PBS. The cells were treated by GIESMA for 10 min, and then washed 3 times with PBS. Finally, images were captured using a light microscope (Olympus, Tokyo, Japan).

Cell migration assay

A volume of 600 mL of RPMI-1640 medium containing 10% FBS was added to the lower Transwell chamber, and 200 mL of cell suspension was added to the upper chamber and left for 24 hours. Next, the liquid was removed from the upper and lower chambers, and the cells were then treated with 4% paraformaldehyde for 30 min, and washed with PBS. Finally, the cells were treated with 600 μL of 0.1% crystal violet for 10 min and then washed with PBS. The cells were observed under a microscope (Olympus, Tokyo, Japan) and counted.

HE staining

The tumors were separated into NC, miR-488-5p OE, miR-488-5p KD, and miR-488-5p OE + P53 OE groups. The sliced tumors were dewaxed and hydrated, and the slices were then incubated in hematoxylin solution for 5–20 min before being washed with water. Next, the slices were differentiated by differentiation fluid for 30 sec and then soaked in water for 15 min. The slices were then incubated in eosin solution for 10–50 sec. Finally, the slices

were dehydrated, transparentized, sealed, and observed under a microscope (Olympus, Tokyo, Japan).

Immunohistochemistry staining

The sliced tumors were dewaxed, hydrated, and incubated in 3% hydrogen peroxide (H₂O₂) for 10 min to block endogenous peroxidase activity, and then washed with PBS. The slices were incubated with primary antibody (1:100) overnight at 4 °C, and then incubated with secondary antibodies at room temperature for 1–2 h, washed with 1× Tris-buffered saline tween (TBST) and incubated in diaminobenzidine chromogen for 5 min. The nucleus was counterstained with 4',6-diamidino-2-phenylindole (DAPI). The slices were observed under a microscope (Olympus, Tokyo, Japan).

Western blot

Protein from the tumors or cells was isolated using cell lysis buffer (Thermo Fisher, MA, USA). Next, the protein was separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membranes. Next, the membranes were incubated with 0.5% bovine serum albumin for 1 h at room temperature and then washed in PBS. The membranes were then incubated with primary antibodies (1:1,000) at 4 °C overnight. They were then washed and incubated in secondary antibody and kept at room temperature for 1–2 h. Finally, the bands were evaluated using scanning densitometry through enhanced chemiluminescence (ECL; Thermo Fisher, MA, USA). Actin served as the internal control.

qRT-PCR

RNAs from the cell cultures were isolated using TRIzol reagent (Invitrogen, Shanghai, China) in accordance with the manufacturer's instructions. Reverse transcription of the extracted RNA that was converted into complementary deoxyribonucleic acid (cDNA) was conducted using the HiScript[®] III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). MiR-488-5p expression was analyzed on a reverse transcription polymerase chain reaction (RT-PCR) detection system (Analytic, Jena, Germany) and quantified using the $\Delta\Delta$ CT method with the following reverse transcription primers: 5'-GTCGTATCCAGTGCA

GGGTCCGAGGTATTTCGCACTGGATACGACGACC AA-3'; FP: 5'-GCGATTGAAAGGCTATTTTC-3'; and RP: 5'-AGTGCAGGGTCCGAGGTATT-3'.

Luciferase reporter assay

The putative binding sites of miR-488-5p on the P53 3'-untranslated region (UTR) were predicted by TargetScan7.2. pMIR-P53 3'-UTR-WT, and pMIR-P53 3'-UTR-Mut luciferase reporter plasmids were purchased from Genechem (Shanghai, China). The sequences of TP53 (position 784–804) that bind to miR-488-5p were partly mutated and inserted into the reporter plasmid to identify the binding specificity. The miR-488 mimic and its control were transfected into cells. After 48 h of transfection, the relative luciferase activities of the cells were measured using the Dual-Glo Luciferase Assay System (Promega, Shanghai, China) in accordance with the manufacturer's introductions. The Renilla signal was used to normalize luciferase activity.

Statistical analysis

The data are presented as mean \pm standard deviation. The statistical analyses included a 1-way analysis of variance followed by Bonferroni's multiple comparison test (for comparisons of more than two groups) or the Student's *t*-test (for comparisons of two groups) (GraphPad Prism). A *P* value <0.05 was considered statistically significant.

Results

miR-488-5p promotes the development of ESCC in vitro

As *Figure 1A* shows, miR-488-5p expression was significantly more upregulated in KYSE-150 group than the HEEC group. The KYSE-150 cells were transfected with the miR-488-5p inhibitor, and the transfection efficiency was confirmed by the quantitative RT-PCR assay (see *Figure 1B*). First, the colony formation assays and cell apoptosis detection results indicated that compared to the KYSE-150+NC group, the miR-488-5p inhibitor group showed decreased cell viability and increased cell apoptosis (see *Figure 1C,1D*). Additionally, the miR-488-5p inhibitor group showed less cell migration than the KYSE-150+NC group (see *Figure 1E*). In addition, cell invasion was also inhibited by the miR-488-5p inhibitor (see *Figure 1F*). These results indicate that miR-488-5p could promote ESCC progression in KYSE-150 cells.

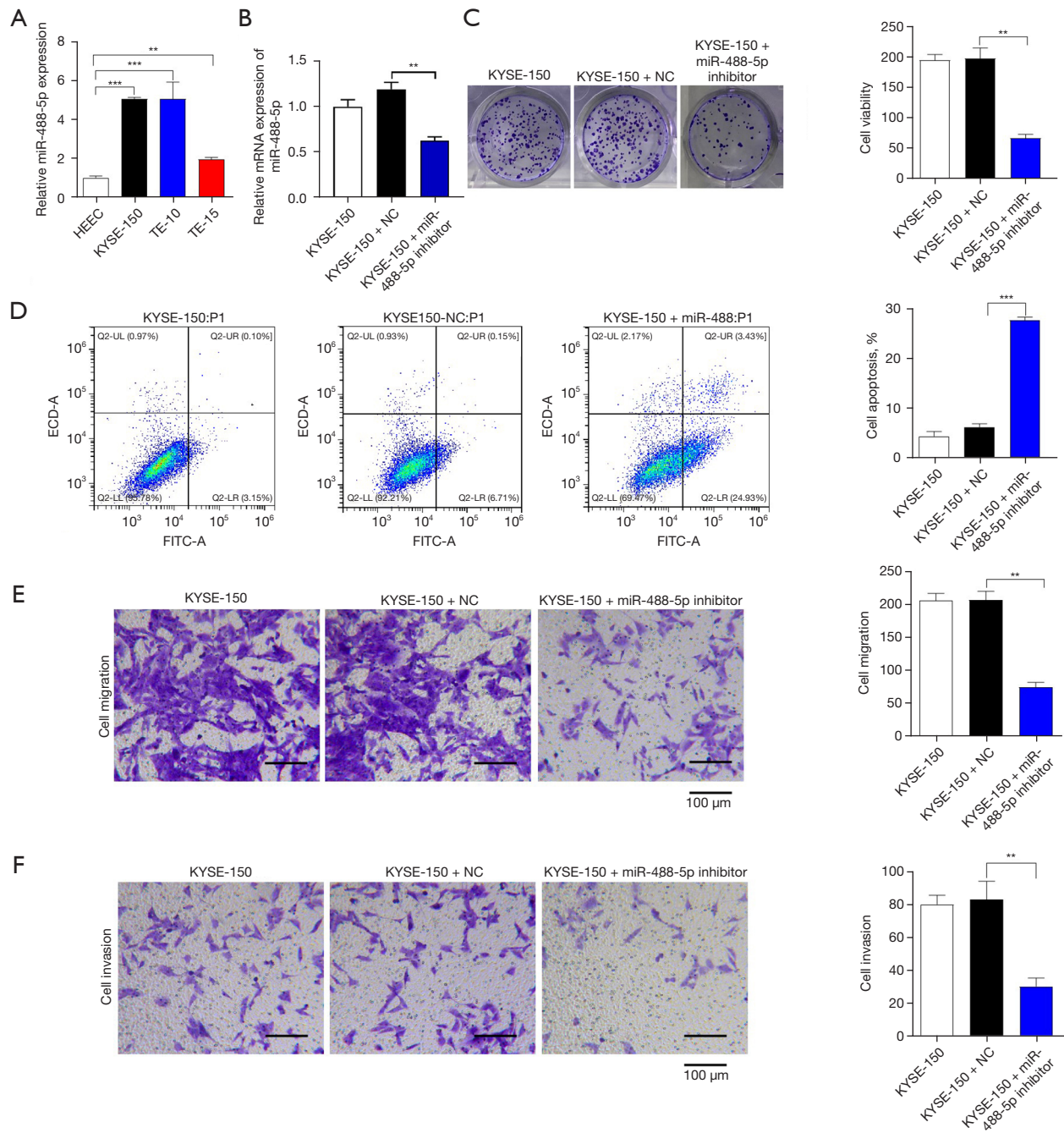


Figure 1 miR-488-5p promotes the development of ESCC *in vitro*. KYSE-150 cells were treated with the miR-488-5p inhibitor for 48 h. (A) Relative values of miR-488-5p mRNA levels in the HEEC, KYSE-150, TE-10, and TE-15 groups. (B) The transfection efficiency of the miR-488-5p inhibitor was confirmed by a qRT-PCR assay. (C) A colony formation assay showed cell viability. (D) A flow cytometry assay detected cell apoptosis. (E,F) A Transwell assay assessed cell migration and cell invasion. Cells were stained with Giemsa stain and were observed under light microscopy ($\times 400$ magnification). Values are mean \pm standard error, compared to the KYSE-150 group, $**P < 0.01$, $***P < 0.001$, $n = 3$ per group.

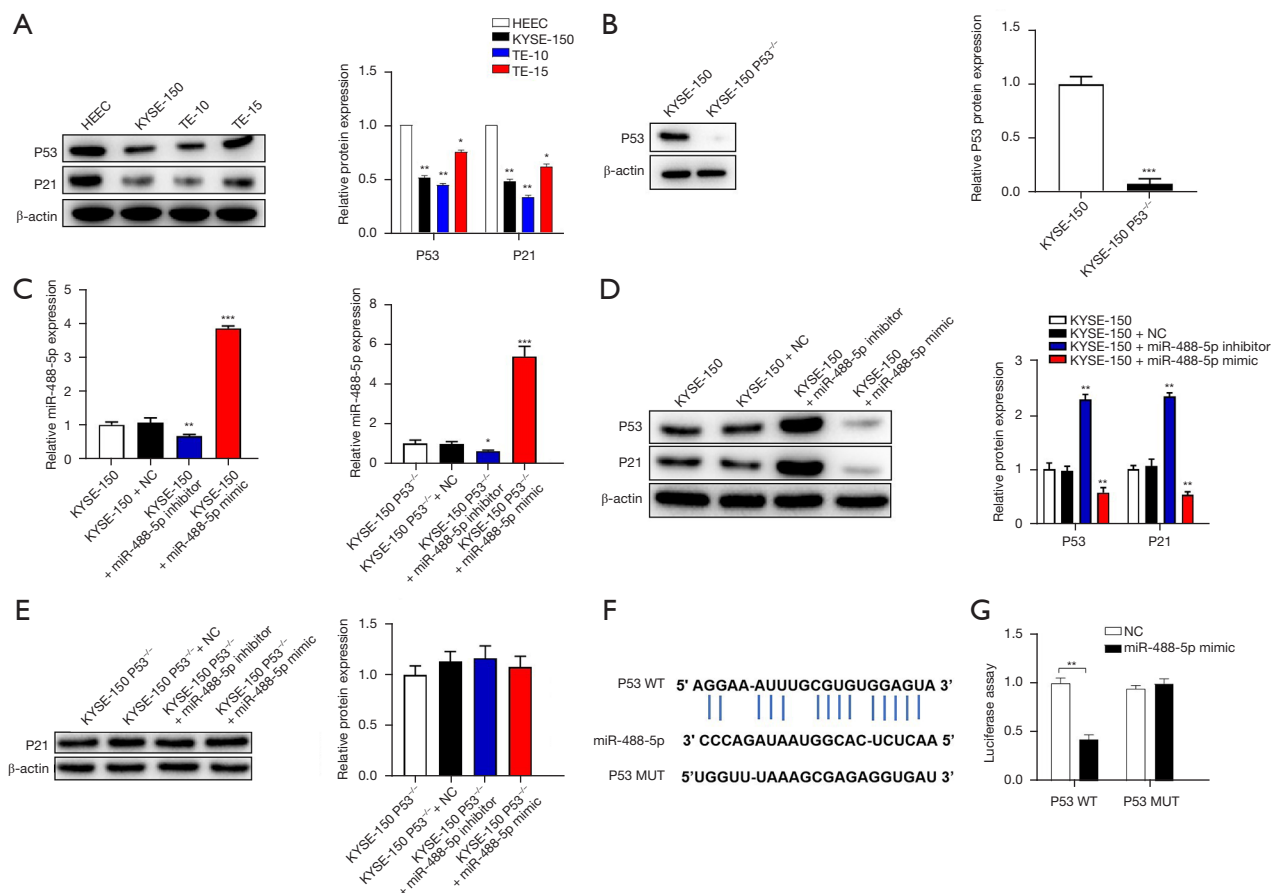


Figure 2 P53 is a target gene of miR-488-5p. KYSE-150 cells were treated with the miR-488-5p inhibitor for 48 h. (A) A Western blot assay detected P53 and P21 protein expression in the HEEC, KYSE-150, TE-10 and TE-15 groups. (B) A Western blot assay detected P53 protein expression in the KYSE-150 P53^{-/-} cells. The KYSE-150 cells and KYSE-150 P53^{-/-} cells were treated with the miR-488-5p inhibitor or the mimic for 48 h. (C) The transfection efficiency of the miR-488-5p inhibitor or the mimic in the KYSE-150 cells or KYSE-150 P53^{-/-} cells was confirmed by a qRT-PCR assay. (D) A Western blot assay detected P53 and P21 protein expression in the KYSE-150 cells. (E) A Western blot assay detected P21 protein expression in the KYSE-150 P53^{-/-} cells. (F,G) A luciferase reporter assay was performed at 48 h after transfection KYSE-150 with luciferase reporter plasmid containing the wild type or mutant form of P53 3'-UTR along with the control mimic or the miR-488-5p mimic. Cells were stained with Giemsa stain and were observed under light microscopy (×400 magnification). Values are mean ± standard error, compared to the HEEC, KYSE-150, or KYSE-150 P53^{-/-} groups. *, P<0.05; **, P<0.01; ***, P<0.001. n=3 per group.

P53 is a target gene of miR-488-5p

We then explored the relationship between miR-488-5p and P53 in ESCC. The Western blot showed that the levels of P53 and P21 protein were more downregulated in KYSE-150 group than the HEEC group (see *Figure 2A*). We generated TP53 knockout cells in KYSE-150. The Western blot assay confirmed that the protein expression of P53 was markedly decreased in KYSE-150 P53^{-/-} cells (see *Figure 2B*). We then treated KYSE-150 cells or KYSE-150

P53^{-/-} cells with the miR-488-5p inhibitor and miR-488-5p mimic for 48 h. The RT-qPCR assay showed that miR-488-5p inhibitor suppressed miR-488-5p expression, while the miR-488-5p mimic significantly upregulated miR-488-5p in the KYSE-150 and KYSE-150 P53^{-/-} cells (see *Figure 2C*). The MiR-488-5p inhibitor obviously increased P53 and P21 expression, while the miR-488-5p mimic decreased P53 and P21 expression (see *Figure 2D*). However, the miR-488-5p inhibitor and mimic had no function on P21 expression in KYSE-150 P53^{-/-} cells (see *Figure 2E*). Next, the online

TargetScan7.2 database was used to predict the target of miR-488-5p. We found that P53 might be a potential target of miR-488-5p; a promising binding site between miR-488-5p and P53 is shown in *Figure 2F*. To confirm whether miR-488-5p affected P53 expression, the luciferase reporter assay was performed. The luciferase activity was significantly more decreased following co-transfection with pMIR-P53 3'-UTR-WT and miR-488-5p than co-transfection with pMIR-P53 3'-UTR-Mut and miR-488-5p. These results indicate that miR-488-5p can specifically bind to the 3'-UTR of P53, and negatively regulate the P53 expression (see *Figure 2G*).

miR-488-5p facilitates ESCC by P53 inhibition in vitro

To confirm whether miR-488-5p promotes ESCC via the P53 pathway, we treated KYSE-150 cells with miR-488-5p mimic or miR-488-5p inhibitor for 48 h. Colony formation assay demonstrated that compared to the miR-488-5p mimic, miR-488-5p inhibitor decreased cell viability in KYSE-150 cells (see *Figure 3A*). Similarly, apoptosis detection suggested that cell apoptosis was upregulated by miR-488-5p inhibitor compared to miR-488-5p mimic in KYSE-150 cells (see *Figure 3B*). In addition, we also found that cell migration (see *Figure 3C*) and cell invasion (see *Figure 3D*) were also more inhibited by miR-488-5p inhibitor than the miR-488-5p mimic in KYSE-150 cells. As a comparison, KYSE-150 P53^{-/-} cells were also treated with the miR-488-5p mimic or miR-488-5p inhibitor for 48 h. There were no significant differences in cell viability (see *Figure 3E*), apoptosis (see *Figure 3F*), cell migration (see *Figure 3G*), and invasion (see *Figure 3H*) between the P53 deletion KYSE-150 cells treated with the miR-488-5p inhibitor and the miR-488-5p mimic. These results confirmed that P53 is an important target for miR-488-5p in aggravating the development of ESCC.

miR-488-5p promotes ESCC progression in vivo

In addition, we also established mice model to determine the effects of miR-488-5p on ESCC *in vivo*. We found that the miR-488-5p OE-induced ESCC tumor size was reduced by P53 OE, and ESCC tumor size was more diminished in the miR-488-5p knockdown (KD) group than the NC group (see *Figure 4A,4B*). Hematoxylin and eosin (HE) staining revealed similar results (see *Figure 4C*). The immunohistochemical expressions of P53 and P21 were decreased after being treated with the miR-488-5p

mimic and increased after being treated with the miR-488-5p inhibitor. The overexpression of P53 reversed the decreased P53 and P21 expression trend caused by miR-488-5p overload. The proliferation of tumors also exhibited the same behavior when assessed using Ki67 immunohistochemical detection (see *Figure 4D*). The above *in vivo* experiment results indicated that miR-488-5p promoted ESCC progression by suppressing P53 and P21 expression.

Discussion

In recent years, miRNAs have been the subject of extensive research worldwide (28,29). More and more evidence has shown that miRNAs are related to a number of types of tumor progression (30). The discovery of miRNA has generated novel ideas for therapeutic targets of ESCC. In the present study, we found that miR-488-5p expression was significantly upregulated in ESCC cells, indicating the potential role of miR-488-5p in ESCC. Thus, we aimed to explore the role and molecular mechanism of miR-488-5p in ESCC.

Previous studies have suggested that the abnormal expression of miR-488-5p is involved in the development of various diseases. For example, miR-488-5p is considered a novel prognostic biomarker for breast cancer (19). Zhou *et al.* found that transfection of a microRNA-488-5p inhibitor resulted in an increase in apoptosis and a decrease in proliferation in osteosarcoma (20). Arnold *et al.* revealed that miR-488-5p suppressed melanoma development (31). It can be seen that miR-488-5p is expressed both as an oncogene and also as a tumor suppressor gene. It is not uncommon for the same miRNA molecule to exhibit different functions under different circumstances. For example, different studies believe that miR-23b-3p has diametrically opposite effects on breast cancer. Pellegrino *et al.* (32) found that miR-23b-3p regulates focal adhesion, cell spreading, cell-cell junctions and the formation of lamellipodia in breast cancer, and exerts a tumor suppressor effect. Jin *et al.* (33) believed that miR-23b (by comparing the nucleic acid sequence with the database here is indeed miR-23b-3p) and miR-27b gene knockdown can significantly inhibit the growth of breast cancer, by knocking down miR-23b and miR-27b can enhance the expression of Nischarin, so miR-23b-3p is considered to be an oncogene. It can be seen that the same miRNA molecule may have different functions in different situations, and even in the same disease, there

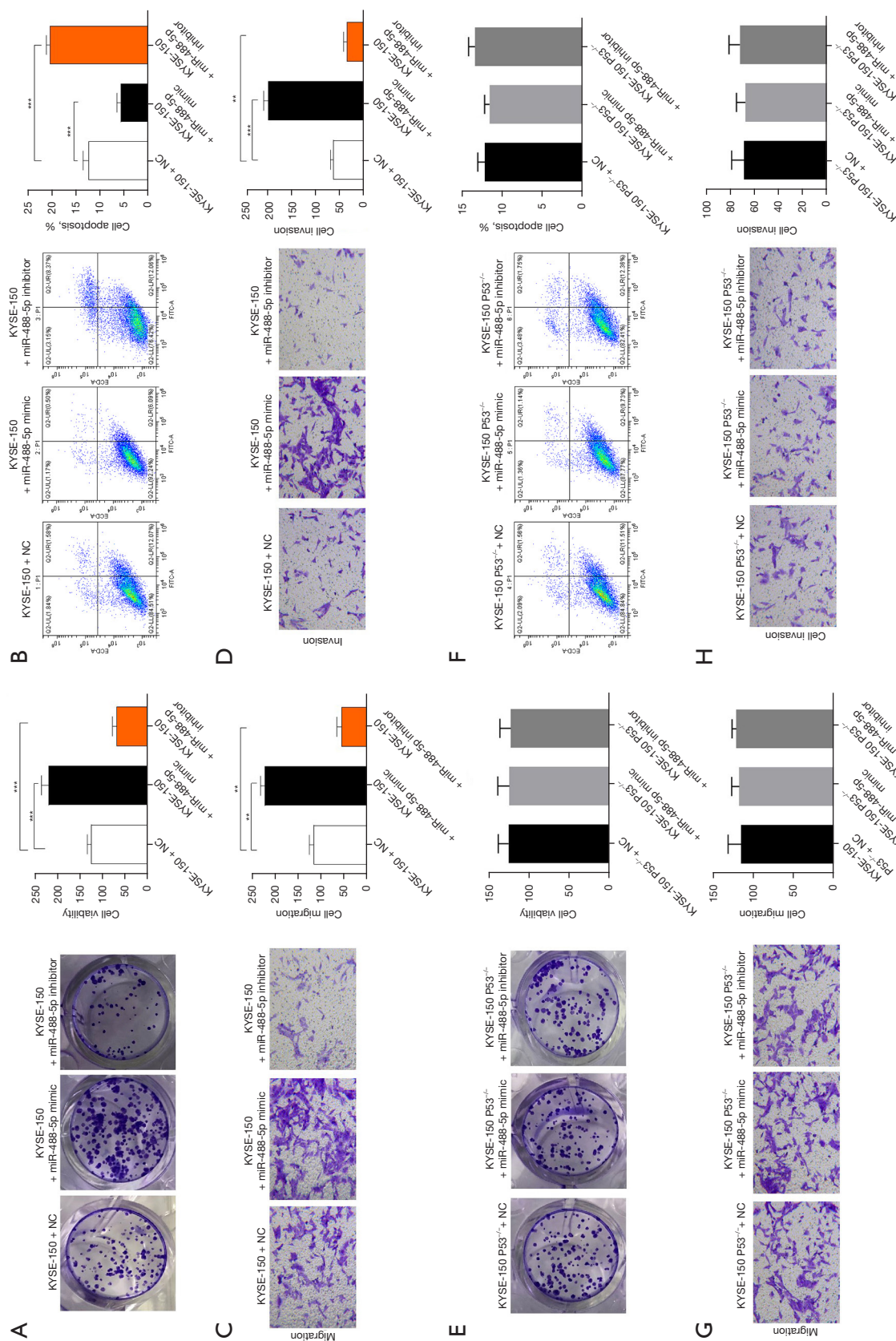


Figure 3 miR-488-5p facilitates ESCC by P53 inhibition *in vitro*. The KYSE-150 cells were treated with the miR-488-5p mimic or the miR-488-5p inhibitor for 48 h, and the KYSE-150 P53^{-/-} cells were treated with the miR-488-5p mimic or the miR-488-5p inhibitor for 48 h. (A,E) A Transwell assay assessed cell migration and cell invasion. Cells were stained with Giemsa stain and were observed under light microscopy (×400 magnification). Values are mean ± standard error, compared to the KYSE-150 + NC group (A,B,E,F) or the KYSE-150 P53^{-/-} + NC group (C,D,G,H). **, P<0.01; ***, P<0.001. n=3 per group.

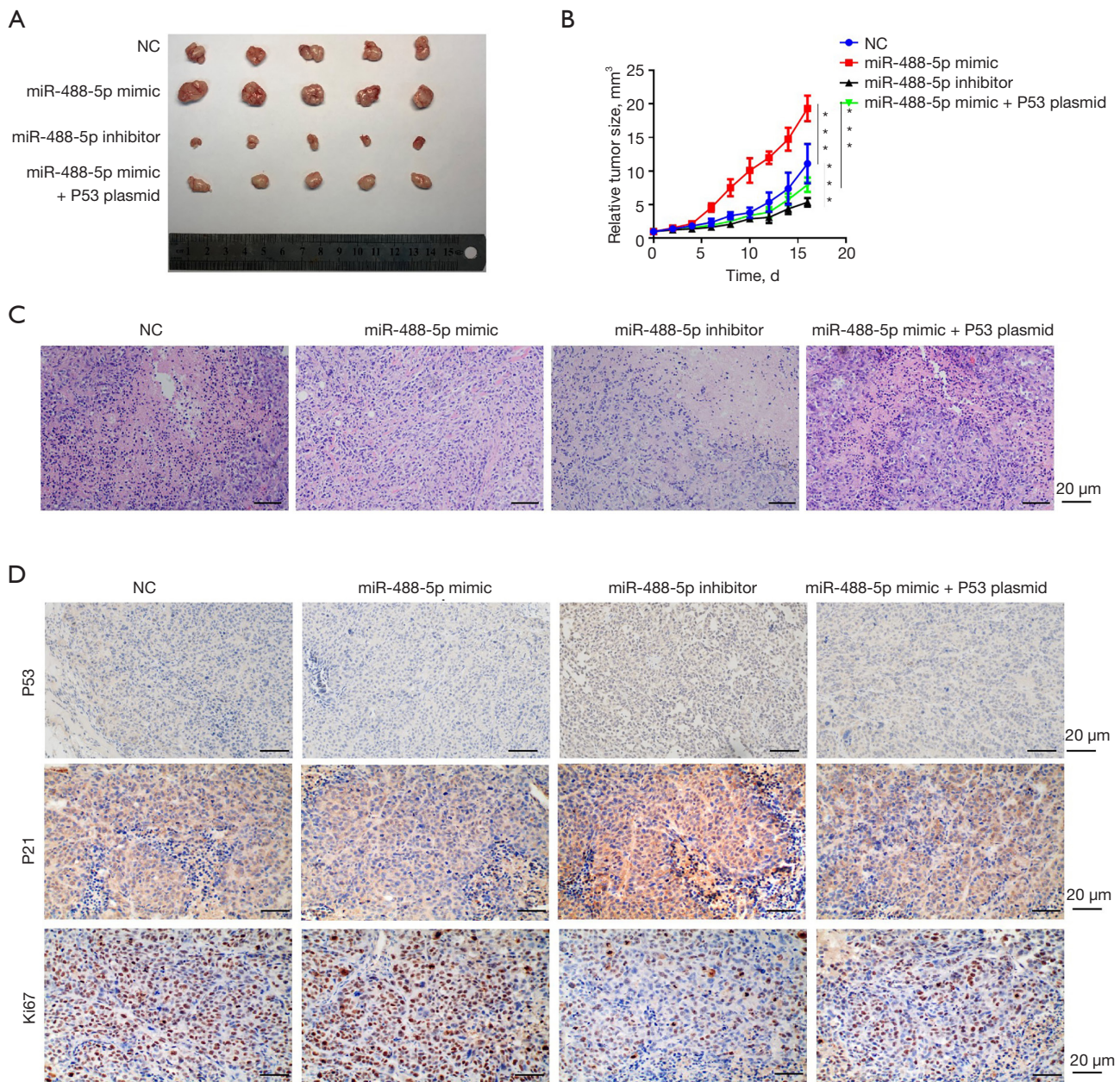


Figure 4 miR-488-5p promotes ESCC *in vivo*. Tumors were separated in the NC, miR-488-5p OE, miR-488-5p KD, and miR-488-5p OE + P53 OE mice models. (A) Representative images of tumors from the NC, miR-488-5p OE, miR-488-5p KD and miR-488-5p OE + P53 OE groups. (B) Relative tumor size of tumor. (C) Representative images of HE staining of tumors. Bar =20 μ m. (D) Representative images of immunohistochemical staining for P53, P21 and Ki67 (brown color) in tumors. Bar =20 μ m. Values are mean \pm standard error, compared to the NC group. ***, $P < 0.001$. $n = 5$ per group.

may be diametrically opposite manifestations. The specific reasons are still unclear. Additionally, Huang *et al.* found that miR-488-5p inhibited mycobacterium tuberculosis associated macrophage polarization (34). However, no

research had previously been conducted on whether miR-488-5p regulated ESCC progression. Our research studied the pathological function of ESCC using KYSE-150 cells. We found that cell viability, invasion, and migration

were significantly decreased by the miR-488-5p inhibitor compared to NC in KYSE-150 cells, while cell apoptosis was upregulated. These results indicate that miR-488-5p appears to be an oncogene in ESCC.

MiRNA is a key regulator of transcriptional and post-transcriptional gene silencing in tumor development (28,35). Evidence has shown that miRNAs play biological roles by directly binding to the 3'-UTR of mRNAs and thereby suppressing mRNA level. For example, Wang *et al.* showed that miR-488-3p promoted osteoblast function by targeting the Dickkopf WNT signaling pathway inhibitor 1 (Dkk1) (36). MiR-488-3p was shown to regulate the production of proinflammatory cytokines in acute gouty arthritis by targeting the 3'-UTR of interleukin-1 β (IL-1 β) (37). In the present study, we performed a bioinformatics analysis to clarify the mechanism underlying miR-488-5p, and predicted that P53 was a possible target of miR-488-5p. P53 has been reported to inhibit DNA damage and chromosomal aberrations, and thus prevent the transmission of these aberrations to daughter cells (38). P53 is one of the most important tumor suppressor genes and is frequently mutated in human cancers (39). For example, Xu *et al.* suggested that the P53 signaling pathway suppressed ESCC cell growth and promoted apoptosis (40). P21 has been shown to be a downstream transactivation target of P53 (41). Further, the level of P21 has been shown to be positively correlated with P53 in ESCC (42). Tang *et al.* clarified that the upregulation of P53 and P21 increased the survival of patients with ESCC (43). Our research indicated that P53 and P21 levels were decreased in ESCC cell lines.

In addition, miR-488-5p negatively regulated P53 and P21 expression in KYSE-150 cells. Interestingly, miR-488-5p had no effect on P21 expression in KYSE-150 P53^{-/-} cells. We performed a luciferase assay and confirmed that miR-488-5p negatively regulated P53 expression directly by binding to the 3'-UTR of P53 mRNA. In addition, the promotion effects of the miR-488-5p mimic on cell viability, cell invasion, and cell migration were markedly more inhibited in KYSE-150 P53^{-/-} cells than KYSE-150 cells, and the miR-488-5p inhibitor also produced similar results. We also explored the mechanism of miR-488-5p *in vivo*, and found miR-488-5p OE-induced ESCC tumor size was reduced by P53 OE, and ESCC tumor size was also significantly diminished in the miR-488-5p knockdown (KD) group than the NC group. HE staining showed similar results. The immunohistochemical expression of

P53 and P21 decreased with miR-488-5p OE treatment, which were prevented by P53 OE.

It should be noted that this study had some limitations. For example, drug resistance is the most common challenge that arises in ESCC treatment (44); however, the question of whether miR-488-5p plays a role in promoting drug sensitivity has not yet been investigated. We will explore the function of miR-488-5p in relation to the overexpression and inhibition of miR-488-5p in drug-sensitive ESCC cells in the future.

In conclusion, miR-488-5p promoted ESCC progression by mediating P53 expression. Further analyses of the mechanism of miR-488-5p in ESCC could lead to novel ideas being generated for ESCC treatments.

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Ethical Statement: The authors are accountable for all aspects of the work, including ensuring that any questions related to the accuracy or integrity of any part of the work have been appropriately investigated and resolved. Experiments were performed under a project license granted by the Institutional Laboratory Animal Care and Use Committee of Hebei Medical University (license number: 2018MEC161). All mice were treated according to national or institutional guidelines for the care and use of animals and the Guide for the Care and Use of Laboratory Animals published by the United States

National Institutes of Health (NIH publication, 8th edition, 2011).

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