Original Article
miR-425-5p promoting colorectal carcinoma progression by regulating its target gene PTEN to change activity of the PI3K/AKT/mTOR signaling pathway

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Abstract: This study was designed to explore the role and molecular mechanism of miR-425-5p in colorectal carcinoma (CRC). miR-425-5p and PTEN expression was detected in CRC cells and normal colon epithelial cells. miR-425-5p and PTEN overexpression or inhibitory vectors were transfected into cancer cells, whose proliferation, invasion, and apoptosis were then observed. The expression of autophagy- (LC3B II, p62) and PI3K/AKT/mTOR signaling pathway-related proteins was detected. The correlation of miR-425-5p with PTEN was determined by dual luciferase reporter gene assay (DLRGA). miR-425-5p rose while PTEN reduced in CRC cells. According to cell experiments, inhibiting miR-425-5p or increasing PTEN could inhibit the cell proliferation and invasion, promote their apoptosis, and increase p62 expression, as well as reduce the expression of LC3B II, AKT, p-AKT, mTOR, and p-mTOR. DLRGA confirmed a targeted regulatory relationship between miR-425-5p and PTEN. In conclusion, miR-425-5p can promote CRC progression by regulating its target gene PTEN to change the activity of the PI3K/AKT/mTOR signaling pathway.

Keywords: miR-425-5p, PTEN, cells, CRC

Introduction

Colorectal carcinoma (CRC) is a clinically common malignant tumor of the digestive tract, and also a major cause of death related to cancer [1, 2]. According to statistics, there were more than 1 million patients with CRC worldwide in 2018, and over 550,000 patients with CRC died during the same period [3]. Many methods can be currently selected to treat the disease, such as surgery, chemotherapy, and molecular targeted therapy, but patient prognosis is still unsatisfactory [4, 5]. Therefore, it has been an urgent need to improve the prognosis of CRC in clinical practice.

As a kind of single-chain non-coding RNA, microRNA (miR) promotes the degradation of transcripts via binding to the 3'-untranslated region (UTR) of its target gene mRNA sequences; this has an important impact on cell prolif-

eration, apoptosis, and other biological functions [6]. It is reported that miR regulates one third of human genes and affects their functions and genetic pathways [7]. In cancer, miR plays the role of a tumor suppressor and oncogene by regulating its target genes; thus providing a new perspective for cancer treatment [8]. According to previous studies, miR-425-5p can promote the progression of gastric cancer [9], esophageal squamous cell carcinoma [10], and breast cancer [11], but prevent the progression of prostate cancer [12] and nasopharyngeal carcinoma [13]. Highly expressed in CRC, this miR can be used as a biomarker for the disease, but the mechanism of its action in the disease is still unclear [14]. The prediction by online biological prediction software Targetscan has found a potential target binding site between PTEN and this miR. As reported by a previous study, PTEN affects the biological events of cancer cells through regulating

the activity of the PI3K/AKT/mTOR signaling pathway [15], and this pathway serves as a classical one that mediates important signal transduction in cell biological processes such as growth, proliferation, migration, and survival [16].

Accordingly, we put forward the hypothesis that miR-425-5p can promote CRC progression by regulating its target gene PTEN to change the activity of this pathway. In order to verify the correctness of our hypothesis, we have carried out the following experiments.

Materials and methods

Cell sources and treatment

Cell sources: CRC cells (LOVO, HCT116, SW480, SW620) and normal colon epithelial cells FHC were purchased from ATCC cell bank, America. Cell culture: The five different kinds of cells were respectively put into RPMI1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS) (PBS, Gibco, USA) and 1% penicillin-streptomycin (100X, Solarbio, USA) for culture in an incubator (37°C, 5% CO_2). After adherently growing to 80%-90%, the cells were digested with 0.25% trypsin, and cultured to complete passage. Cell transfection: The cells to be transfected were divided into a miR negative control (miR-NC) group, a miR-425-5p inhibition (miR-425-5p-mimics) group, a PTEN RNA overexpression (si-PTEN) group, and a negative control RNA (si-NC) group, with the corresponding operations carried out based on the instruction of Lipofectamine™ 2000 kit (Invitrogen, USA).

Detection methods

PCR-qRT: After the TRIzol kit (Invitrogen, USA) was used to draw total RNA from the cells in each group, its purity, concentration, and integrity were measured by an ultraviolet spectrophotometer and agarose gel electrophoresis. After passing the detection, its reverse transcription and PCR amplification were performed using the reverse transcription kit (one-step method, Beijing TransGen Biotech Co., Ltd.), with U6 used as an internal reference (all primers were designed and synthesized by Jerry Medical Instrument (Shanghai) Co., Ltd.) for miR-425-5p. PCR system was: 10 μL of 2 × Talent qPCR PreMix, each 1.25 μL of upstream and downstream primers, 100 ng of cDNA, and

water that was added to supplement to 20 μL. Conditions for the reaction were pre-denaturation (95°C, 3 min), denaturation (95°C, 5 s), and annealing and extension (60°C, 15 s), cycling for a total of 40 times. With U6 as the internal reference of miR-425-5p, the upstream and downstream primer sequences of miR-425-5p were 5'-GGGGAGTTAGGATTAGG-TC-3' and 5'-TGCGTGTCGTGGAGTC-3', while those of U6 were 5'-ATTGGAACGATACAGAG-AAGATT-3' and 5'-GGAACGCTTCGAATTTG-3'. 2-ΔΔct was used to analyze data [17].

Western blot (WB): The cultured cells were lysed with RIPA buffer (Thermo Scientific, USA), and a BCA protein assay kit (Thermo Scientific, USA) was adopted to test the protein concentration. Subsequently, the protein was electrophoresed to transfer to PVDF membrane, which was then washed with TBST solution, twice. After being placed in 5% skimmed milk powder for 2-hour blocking, the membrane was cleaned with TBST solution, three times, added with primary antibodies [Abcam, USA; LC3B II (1:1000), p62 (1:1000), AKT (1:1000), p-AKT (1:1000), mTOR (1:1000), p-mTOR (1:1000)], and β-actin (1:100), and then reacted at 4°C again overnight in a refrigerator. After washed to remove the primary antibodies, it was added with goat anti-mouse secondary antibody (Abcam, USA; 1:5000) for incubation at 37°C for 1 hour, and finally rinsed with PBS for 5 min, repeated 3 times. Finally, it was developed in a dark room with reference to the ECL kit instructions before remaining liquid on the membrane was removed. The protein bands were scanned to analyze their gray values in Quantity One sortware.

Detection of cell proliferation: The cells were transfected for 24 hours, they were then collected and their concentrations were adjusted to $4*10^4$ cells/well. After that, they were spread on a 96-well plate, and added with 10 μL of CCK solution (Beyotime Biotechnology, Shanghai) at 24, 48, 72, and 96 hours after transfection, respectively, and then cultured at 37°C for 2 hours. Next, optical density values of each well at 490 nm were measured using a microplate reader (Molecular Devices, USA), and the growth curve was plotted.

Detection of cell invasion: The cells were collected with their concentrations adjusted to $4*10^4$ cells/well, and then they were inoculated into the upper chamber. Then, 200 μL of
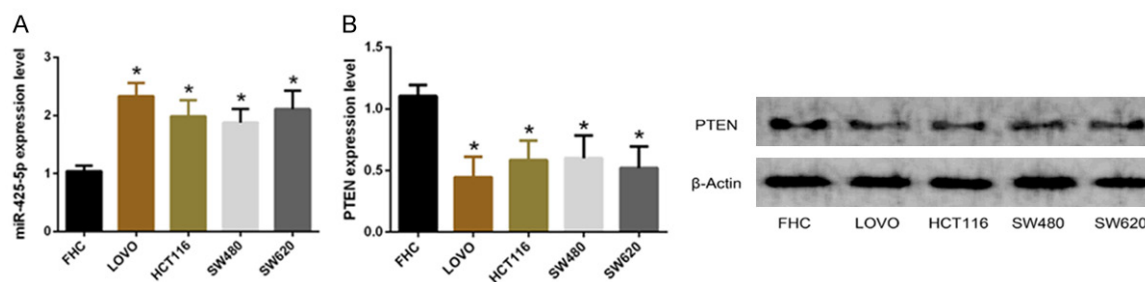
Figure 1. Expression of miR-425-5p and PTEN in CRC cells. A: miR-425-5p showed high expression in LOVO, HCT116, SW480, and SW620 cells. B: PTEN showed low expression in the four cells. Note: *indicates P<0.05.

culture solution was added into this chamber, whereas 500 mL of 20% FBS-containing culture medium was added into the lower one. After the Transwell was cultured at 37°C for 48 hours, the matrix and the cells without penetrating the membrane surface in the upper chamber were wiped off. After being washed with PBS (3 times), the membrane was fixed with paraformaldehyde (10 min), washed with double distilled water (3 times), dried in the air, and then stained with 0.5% crystal violet. Finally, cell invasion was observed under a microscope.

Detection of cell apoptosis: A suspension with $1*10^6$ cells/mL was prepared, and fixed with 70% ethanol solution (4°C, 30 min). Next, the cells were placed in Annexin V-FITC/7-AAD mixed solution for incubation in a dark room for 5 min. The FC500MCL flow cytometer system (BD, USA) was used for detection.

Detection of target genes: miR-425-5p downstream target genes were predicted using the online biological prediction website Targetscan. The PTEN-3'UTR wild type (Wt), PTEN-3'UTR mutant (Mut), miR-424-mimics, and miR-NC were all transferred into HEK-293T cells using the Lipofectamine™ 2000 kit again. Forty-eight hours later, luciferase activity in the cells was detected using a dual luciferase reporter gene assay (DLRGA) kit (Promega, USA).

Statistical processing

In this study, GraphPad Prism 7 (GraphPad Software, San Diego, USA) was used for data analysis and figure illustrations. Measurement data were expressed by mean ± standard deviation (Mean ± SD). The comparison between two groups was analyzed by independent samples t test. The comparison between multiple groups was first analyzed by one-way analysis

of variance (ANOVA), and pairwise comparison was then analyzed by LSD-t test. The comparison of expression between multiple time points was first analyzed by repeated measures ANOVA, and then tested by Bonferroni. When P<0.05, there was a statistically significant difference.

Results

Expression of miR-425-5p and PTEN in CRC cells

PCR-qRT and WB were used for detecting miR-425-5p and PTEN expression in CRC cells (LOVO, HCT116, SW480, SW620) and normal colon epithelial cells (FHC). *miR-425-5p* showed high expression (P<0.05), while PTEN showed low expression in the above four CRC cells (P<0.05). Additionally, *miR-425-5p* had the highest expression in LOVO and SW620 cells, which were therefore chosen for subsequent cell experiments. See **Figure 1**.

miR-425-5p as an oncogene

miR-425-5p-inhibitor and miR-NC were transfected into LOVO and SW620 cells to know the role of miR-425-5p in CRC cells. *miR-425-5p* expression in the cells transfected with miR-425-5p-inhibitor reduced (P<0.05). According to the detection by the microplate reader, miR-425-5p-inhibitor could inhibit LOVO and SW620 cells from proliferation. According to Transwell assay, miR-425-5p-inhibitor reduced the number of the cells penetrating the membrane. According to the flow cytometry, the apoptosis of the cells greatly rose after transfection with miR-425-5p-inhibitor. According to the detection of autophagy-related proteins, LC3B II reduced but p62 rose in the cells after transfection with miR-425-5p-inhibitor. See **Figure 2**.
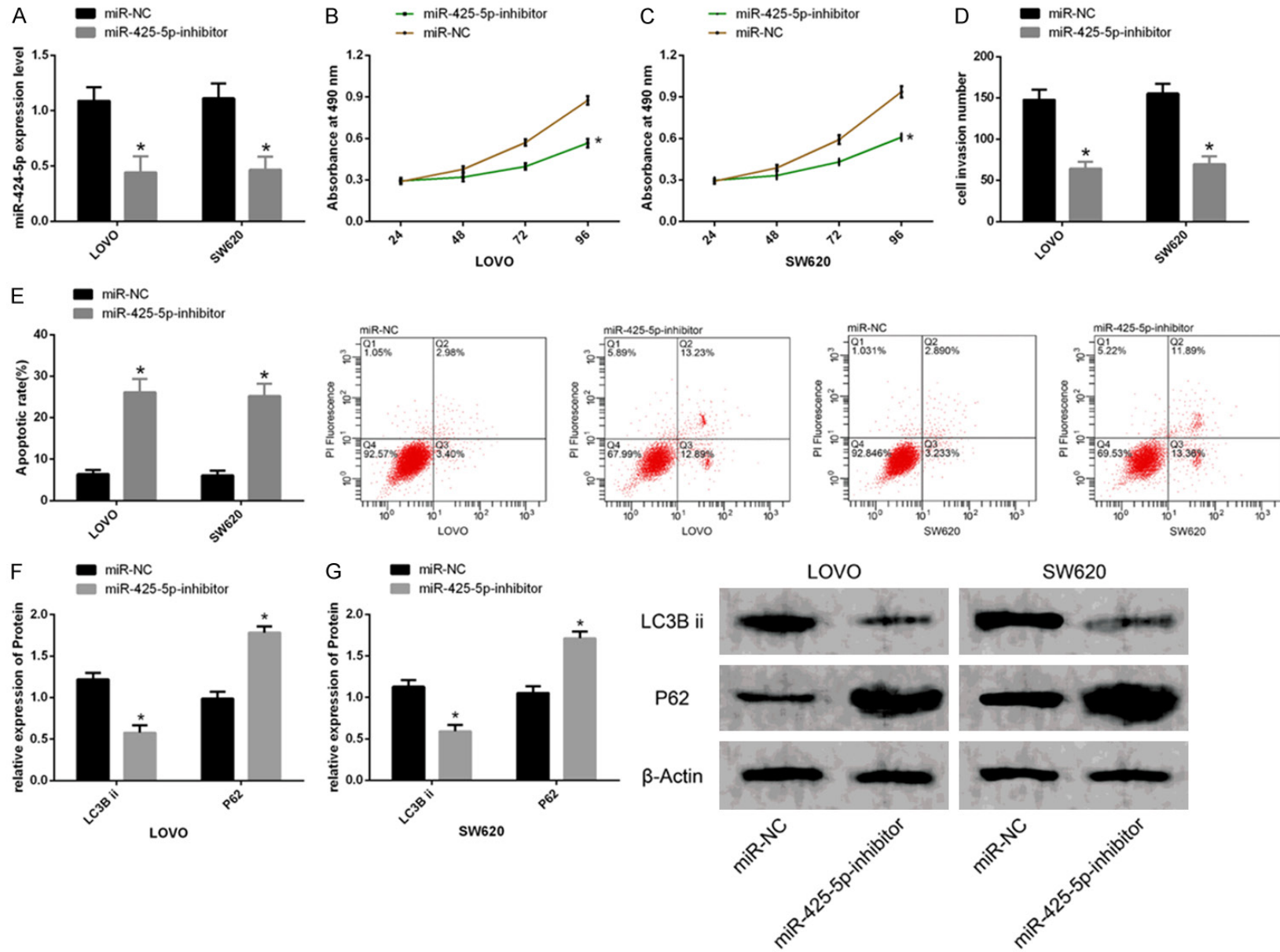
Figure 2. Effects of miR-425-5p changes on the biological function of CRC cells. A: miR-425-5p expression decreased in LOVO and SW620 cells transfected with miR-425-5p-inhibitor. B, C: The proliferation of the cells transfected with miR-425-5p-inhibitor was inhibited. D: The invasion of the cells transfected with miR-425-5p-inhibitor was inhibited. E: The apoptotic rate of the cells transfected with miR-425-5p-inhibitor significantly increased. F, G: LC3B II expression decreased while p62 expression increased in the cells transfected with miR-425-5p-inhibitor. Note: LC3B ii was LC3B II. *indicates P<0.05.
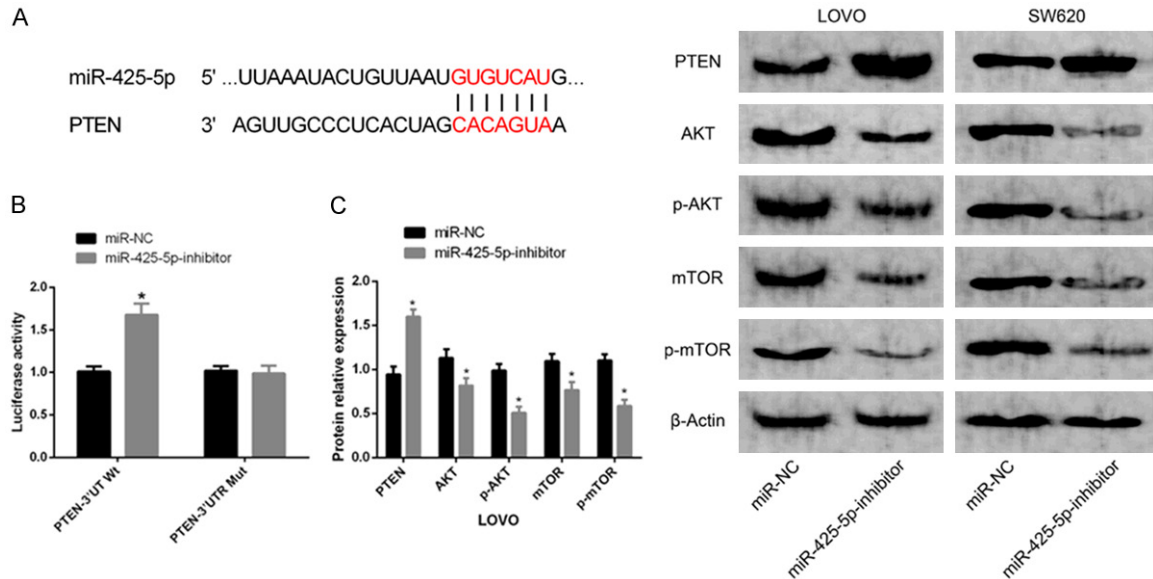
Figure 3. Relationship between miR-425-5p and PTEN. A: There was a targeted binding site between miR-425-5p and PTEN. B: The transfection of miR-425-5p-inhibitor could increase PTEN-3'UT Wt luciferase activity in the cells, but PTEN-3'UTR Mut luciferase activity had no change. C: PTEN expression increased, while the expression of AKT, p-AKT, mTOR, and p-mTOR decreased in the cells transfected with miR-425-5p-inhibitor. Note: *indicates P<0.05.

Relationship between miR-425-5p and PTEN

miR-425-5p downstream target genes were predicted through Targetscan to know the molecular mechanism of *miR-425-5p* in CRC. There was a targeted binding site between miR-425-5p and PTEN. According to the DL-RGA, the transfection of miR-425-5p-inhibitor could increase PTEN-3'UT Wt luciferase activity in the cells (P<0.05), without effect on PTEN-3'UTR Mut luciferase activity (P>0.05). According to WB, PTEN expression increased, while the expression of AKT, p-AKT, mTOR, and p-mTOR decreased in the cells transfected with miR-425-5p-inhibitor (P<0.05). See **Figure 3**.

PTEN as a tumor suppressor gene

Next, we investigated whether the transfection of sh-PTEN and sh-NC into LOVO and SW620 cells would affect their biological functions. The results showed that PTEN in the cells transfected with sh-PTEN increased (P<0.05). According to the detection of cell biological

functions, the proliferation and invasion of those cells transfected with sh-PTEN were inhibited, but their apoptotic rate rose. The effects of sh-PTEN were consistent with those of miR-425-5p-inhibitor. WB showed that p62 expression increased while the expression of LC3B II, AKT, p-AKT, mTOR, and p-mTOR decreased in the cells (P<0.05). See **Figure 4**.

Rescue experiment

After miR-425-5p-inhibitor+si-PTEN was transfected into LOVO and SW620 cells, changes in their biological functions were detected. The proliferation, invasion, and apoptosis of those transfected with miR-425-5p-inhibitor+si-PTEN were not different from those of the cells transfected with miR-NC; while their proliferation and invasion rose and their apoptotic rate reduced compared with those of the cells transfected with miR-425-5p-inhibitor. According to the WB, the transfection of miR-425-5p-inhibitor+si-PTEN did not rise or reduce the
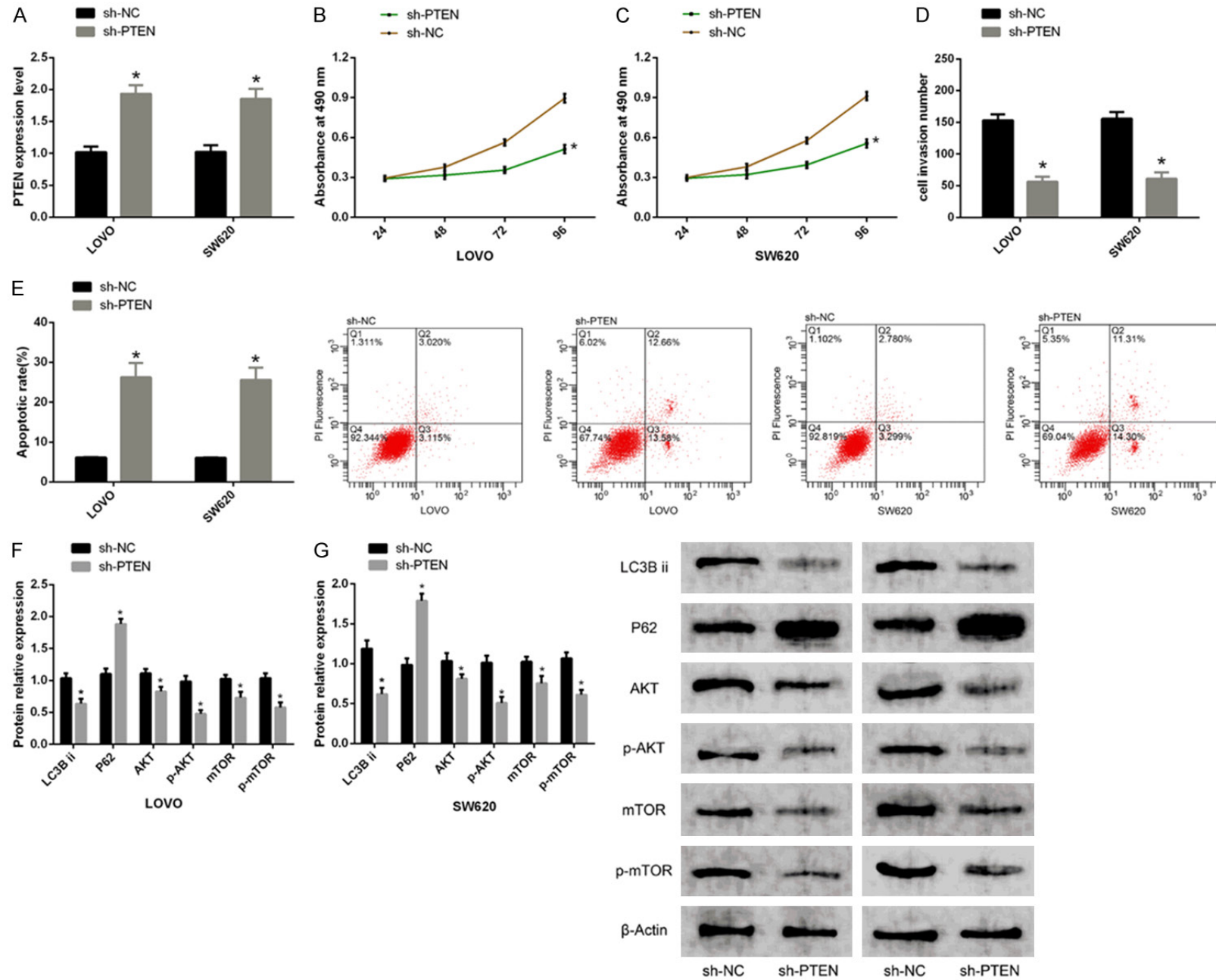
Figure 4. Effects of PTEN changes on the biological function of CRC cells. A: PTEN expression in the cells transfected with sh-PTEN increased. B, C: The proliferation of the cells transfected with sh-PTEN was inhibited. D: The invasion of the cells transfected with sh-PTEN was inhibited. E: The apoptotic rate of the cells transfected with sh-PTEN increased. F, G: p62 expression increased while the expression of LC3B II, AKT, p-AKT, mTOR, and p-mTOR decreased in the cells transfected with sh-PTEN. Note: LC3B ii was LC3B II. *indicates P<0.05.

expression of LC3B II, p62, AKT, p-AKT, mTOR, and p-mTOR in the cells (P>0.05). See **Figure 5**.

Discussion

The development and progression of CRC is a multi-step process, during which an imbalance between oncogenes and tumor suppressor genes is considered to be a major factor leading to CRC progression [18]. miRs play the role of both oncogenes and tumor suppressor genes in a variety of tumors, so they are considered to be important molecules to promote the production of new anti-cancer drugs [19]. Our study explored the effects and mechanism of action of miR-425-5p in CRC. This miR rose in the four CRC cells purchased from ATCC, which is similar to previous research results [14]. Then, we inhibited miR-425-5p in the CRC cells, and found that this treatment inhibited the cells to proliferate and invade and promoted their apoptosis; LC3B II expression decreased while p62 expression increased in the cells. The increase of LC3B II expression and the decrease of p62 expression indicate the enhancement of cell autophagy [20]. This suggests that miR-425-5p functions as a cancer gene in CRC, so it can be used as a therapeutic target for the disease. However, the pathway of its action remains unclear.

A targeted binding site between miR-425-5p and PTEN was found through Targetscan. Considered as a tumor suppressor gene, PTEN inhibits the activation of the PI3K/AKT signaling pathway to inhibit cancers [21]. In this study, we overexpressed PTEN in CRC cells and observed the effects on their biological functions. The results showed that their proliferation and invasion were inhibited, but their apoptotic rate increased; LC3B II expression decreased and p62 expression increased in the cells. These findings demonstrate that PTEN has potential as a therapeutic target for CRC. According to previous studies, PTEN can be specifically regulated by a variety of miRs. For example, it can be specifically regulated by miR-494, thus promoting liver cancer cells to proliferate, migrate, and invade, and improving

drug resistance to sorafenib [22]. It can also be specifically regulated by miR-32 to induce the three malignant behaviors of liver cancer cells [23]. Moreover, it can be specifically inhibited by miR-106a, making it play the role of an oncogene in non-small cell lung cancer [24]. These findings reveal that miRs may exert many great functions in tumor development through regulating PTEN. According to the DLRGA, miR-425-5p-inhibitor transfection could increase PTEN-3'UTR Wt luciferase activity, without effect on PTEN-3'UTR Mut luciferase activity in the cells. Besides, miR-425-5p-inhibitor transfection upregulated PTEN expression in the cells, which indicates that PTEN can be negatively regulated by miR-425-5p. According to the rescue experiment, PTEN inhibition could reverse the changes in biological functions of CRC cells caused by miR-425-5p inhibition. These findings show that miR-425-5p can target PTEN to promote CRC progression. Playing an indispensable role in many cellular processes, PI3K/AKT/mTOR signaling pathway is usually active in tumors (including CRC) [25, 26]. PTEN is a well-known antagonist of PI3K signaling transduction [27]. Therefore, we detected marker proteins of this pathway in the cells after treatment with miR-425-5p inhibition and PTEN overexpression. The transfection of both miR-425-5p-inhibitor and sh-PTEN could reduce the expression of pathway-related proteins (AKT, p-AKT, mTOR, and p-mTOR), while miR-425-5p-inhibitor+si-PTEN transfection did not affect the expression of these proteins. Accordingly, the hypothesis is correct that miR-425-5p can promote CRC progression by regulating its target gene PTEN to change the activity of this pathway.

The above research has confirmed that miR-425-5p can promote CRC progression by regulating PTEN to change the activity of this pathway. However, there are some limitations in this study. First, tumor-forming experiments in nude mice were not carried out, so we cannot know whether miR-425-5p and PTEN affect tumor growth in mice. Secondly, the clinical roles of miR-425-5p and PTEN were not explored.

Exploration of the mechanism of action of miR-425-5p in CRC
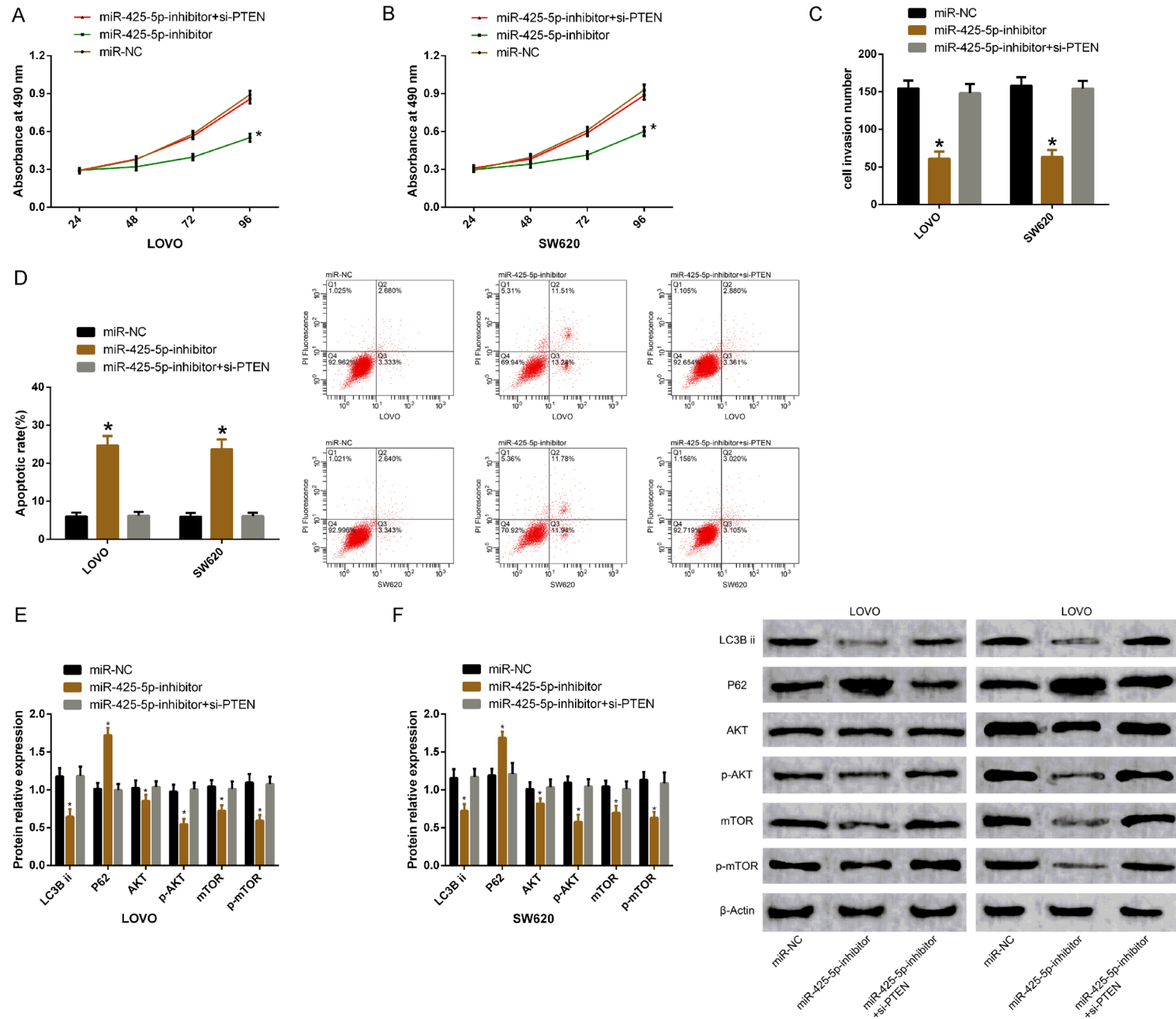
Figure 5. Rescue experiment. A, B: The proliferation of the cells transfected with miR-425-5p-inhibitor+si-PTEN was not different from that of the cells transfected with miR-NC, but that increased compared with that of the cells transfected with miR-425-5p-inhibitor. C: The invasion of the cells transfected with miR-425-5p-inhibitor+si-PTEN was not different from that of the cells transfected with miR-NC, but that increased compared with that of the cells transfected with miR-425-5p-inhibitor. D: The apoptotic rate of the cells transfected with miR-425-5p-inhibitor+si-PTEN was not different from that of the cells transfected with miR-NC, but that decreased compared with that of the cells transfected with miR-425-5p-inhibitor. E, F: The transfection of miR-425-5p-inhibitor+si-PTEN did not increase or decrease the expression of LC3B II, p62, AKT, p-AKT, mTOR, and p-mTOR in LOVO and SW620 cells. Note: LC3B ii was LC3B II. *indicates $P<0.05$.

Therefore, we will conduct more studies to supplement this research.

In summary, we have demonstrated that miR-425-5p can promote CRC progression by regulating its target gene PTEN to change the activity of this PI3K/AKT/mTOR signaling pathway. Thus, miR-425-5p can provide a new therapeutic strategy for the disease.

Disclosure of conflict of interest

None.

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