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Original Article

MiR-590-5p as potential oncogenic microRNA of human colorectal cancer cells by targeting PTEN

Guang-Jun Zhang*, Yuan-Zhou San*, Hai-Qing Zhang, Ji-Fa Zhang, Zhi Yang, Zhen-Feng Yu, Fan-Zhi Kong, Hai-Jun Liu, Xue-Li Zhang

*Department of General Surgery, Fengxian Hospital Affiliated to Southern Medical University, Shanghai, China. *Equal contributors.*

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Abstract: MicroRNA-590-5p (MiR-590-5p) has been proven dysregulated in some human malignancies such as hepatocellular carcinoma and cervical cancer. However, the role and mechanisms of MiR-590-5p involved in the initiation and progression of colorectal cancer (CRC) are far from being fully understood. The objective of this study was to investigate the biological roles of miR-590-5p in CRC development and reveal its underlying molecular mechanism. In this study, we found that miR-590-5p frequently upregulated in CRC tissues and three CRC cell lines. Clinically, CRC patients with higher miR-590-5p predicted worse clinical outcome compared with those with lower miR-590-5p. Knockdown of miR-590-5p inhibited proliferation induced G0/G1 cell cycle arrest and apoptosis of CRC cells. Then, we identified phosphatase and tensin homolog (PTEN) as a direct target of miR-590-5p cells by bioinformatics analysis and confirmed by using luciferase activity assay and Western blot. The patient samples showed that PTEN expression was inversely correlated with the expression of miR-590-5p. Further, we identified that transfection of PTEN siRNA resulted in a recovery on the cell proliferation induced by knockdown of miR-590-5p. Furthermore, results from the western blot analysis demonstrated that knockdown of miR-590-5p significantly decreased the phosphorylation of Akt and mTOR. To sum up, our data suggest that miR-590-5p plays an important oncogenic role in CRC which promotes CRC cells proliferation by targeting PTEN/PI3K/Akt/mTOR pathway.

Keywords: MicroRNA-590-5p, colorectal cancer, PTEN, PI3K/Akt/mTOR pathway

Introduction

Colorectal cancer (CRC) is the third of the most common malignant tumors with the incidence increasing each year [1, 2]. While important efforts in the early detection and prevention of CRC are ongoing, the five year survival rate is only about fifty percent [3]. Therefore, to work out the mechanism of occurrence and progression of CRC and to find effective methods for early diagnosis and treatment of CRC are urgently required for this deadly disease.

MicroRNAs (miRNAs) are a class of endogenous non-coding RNA with 18-22 nt in length, which regulate translation and degraduation of target mRNAs direct interaction with 3'-untranslated region (3'UTR) of the target mRNAs [4]. Numerous researches indicated that miRNAs could contribute to most basic biological processes, such as cell development, apoptosis, invasion and migration [5-7]. And a handful of miRNAs have been involved in the pathogenesis of CRC mainly regulating the expression of oncogenes and tumor suppressors [8, 9]. For example, Yihui Wang et al. demonstrated miR-375 as a tumor growth suppressor in human colorectal cancer, at least, partially through repression of PI3K/Akt pathway [10].

MiR-590 is a small miRNA located on the proximal end of the long arm of human genome chromosome 7, and gives rise to two mature miRNA species, miR-590-3p and the predominant miR-590-5p. A previous study revealed that miR-590-5p was up-regulated in several hepatocellular carcinoma cell lines and promoted proliferation and invasion in human HCCs by directly targeting TGF-βRII [11]. In addition, miR-590-5p is found to be upregulated in the examined renal cell carcinoma (RCC) cell lines, and down-regulation of miR-590-5p resulted in increased PBRM1, inhibited proliferation and invasion of clear cell renal carcinoma (ccRCC) cells [12].

However, the precise molecular mechanisms underlying the role of miR-590-5p in the progression of CRC are not clear. Therefore, we selected miR-590-5p as a subject of our future studies.

In this study, we found miR-590-5p was often upregulated in CRC cell lines and tissues. Mechanistically, our study demonstrated that PTEN is a direct and functional target for miRNA-590-5p in colorectal cancer. The correlation between miR-590-5p and PTEN was further confirmed in CRC clinical samples. Taken together, we propose that miR-590-5p functions as a potential oncogenic microRNA through targeting PTEN/PI3K/Akt/mTOR pathway in human colorectal cancer.

Materials and method

Sample and cell culture

Paired human normal and malignant colorectal tissues were collected from 20 colon cancer patients. None of the patients in the study received any chemotherapy or radiation therapy before surgery. Written informed consent was obtained from all patients. The HT29, SW620 and HCT116 cell lines were from American Type Culture Collection (ATCC). The cell lines were cultivated in DMEM/F12 1:1 modified medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA) under conditions provided by the manufacturer.

RNA extraction and real-time RT-PCR

Total RNA was extracted from cells and tumor tissues by using Trizol reagent (Invitrogen). For miR-590-5p detection, stem-loop RT-PCR assay was performed. U6 snRNA was used as an internal control. For PTEN mRNA detection, M-MLA was used to reverse-transcribe RNA into cDNA, and qRT-PCR was carried out using the SYBR Green qPCR Master Mix on ABI 7300. GADPH was used as an internal control.

miRNA transfection

Shortly before transfection, 1.5×10^5 cells were seeded per well in a six-well plate in 2 ml DMEM/F12 1:1 modified medium containing serum and antibiotics. Before transfection, the cells were incubated under normal growth conditions. Then, miR-590-5p mimic, miR-590-5p inhibitor and miR negative control (NC) (Genepharma, Shanghai, China) were pre-incubated with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with the final concentration of miRNA analogs at 100 nmol l^{-1}.

Cell viability assay

Cells were seeded in the 96 well plates 24 h after transfection at a density of 1500 cells per well. The cell viability assay was performed using Cell Counting Kit-8 (CCK8; Dojindo) according to the manufacturer's protocol. The absorbance at 450 nm was measured. Experiments were performed at three times.

Flow cytometry

For apoptosis assay, the cells were cultured in low-serum medium and collected after 48 h transfection. Cells were subsequently stained with Annexin V-FITC (eBioscience, USA) and PI for 30 min as described by the manufacturer. Apoptosis cells were analyzed by FACS.

Cell cycle analysis

Cells were trypsinized and fixed with 70% ethanol at 4°C overnight before being stained with propidium iodide (PI). DNA contents were detected by LSRII flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed by Flow Jo (Tree Star, Ashland, OR).

Western blotting

After transfection were harvested and lysed in RIPA buffer. Equal amounts as indicated, cells of protein sample was subjected to 10% SDS-PAGE lysis and transferred to PVDF membrane, after blocking in 5% skimmed milk for 30 mins at room temperature, the member was incubated with antibodies against PTEN, Akt, phosphor-Akt (p-Akt), mTOR and phosphor-mTOR (p-mTOR) (Cell Signaling Technology, MA, USA), β-actin (Sigma) for 2 h at room temperature, followed by incubating in with horseradish peroxidase-linked secondary antibody for 1 h at room temperature and visualized with ECL. And the results of Western blots were analyzed using the Image J program.

Luciferase reporter assays

24 h before transfection HCT116 and SW620 cells were seeded into 24 well-plate, then the cells were co-transfected with Renilla luciferase and luciferase reporter plasmids containing miR-590-5p or vector control and wild-

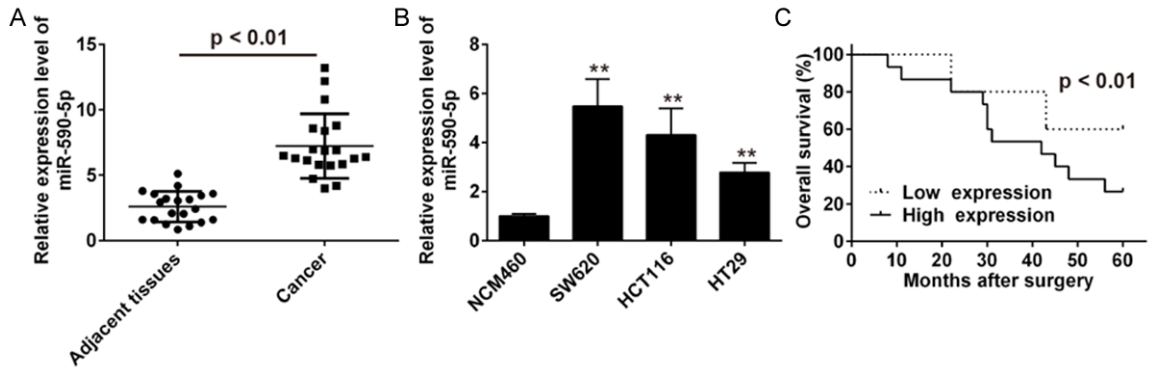

Figure 1. Upregulation of miR-590-5p expression in human colorectal cancer (CRC) cell lines and tissues compared with the corresponding controls. A. The expression levels of miR-590-5p were examined in CRC tissues with 20 pairs of CRC tissues and normal tissue. Transcript levels were normalized to U6 expression and data are shown as the relative levels compared with corresponding normal colorectal specimen. B. qRT-PCR for miR-590-5p was performed using three CRC cell lines and one immortalised normal colon mucosal epithelial cell line (NCM460). The mean and standard deviation of miR-590-5p expression levels relative to the miR-590-5p expression level of NCM460 are shown. C. Kaplan-Meier overall survival curve based on miR-590-5p expression. Data (A and B) are shown as mean ± SEM of three independent experiments. **P<0.01.

type or mutated target gene 3'UTR with Lipofectamine 2000. 24 h after transfection, luciferase activities were measured using dual-luciferase reporter assay system (Promega). The Renilla activity was used as an internal control. Each transfection was performed in triplicate.

Small interfering RNA (siRNA)

The small interference RNA (siRNA) targeting human PTEN and the negative control siRNA were purchased from Sigma (Louis, MO, USA). Cells were seeded (2×10^5 cells/well) in six-well plates. After incubation for 24 hours, cells were transfected with miR-590-5p inhibitor or siRNA against PTEN using Lipofectamine 2000 transfection reagent according to the manufacturer's instruction.

Statistical analysis

All values were expressed as mean ± SD and processed by GraphPad Prism 5.0 software. Differences among the groups were assessed by Student's t-test, and they were considered statistical significance if P<0.05.

Results

Upregulation of miR-590-5p in CRC tissues and cell lines

To evaluate the expression of miR-590-5p in clinical specimens, qRT-PCR was used to detect between 20 pairs of CRC tissues and normal tissue. As shown in **Figure 1A**, miR-590-5p expression was significantly upregulated in CRC tissues compared to the normal tissue in all the detected specimens. It implied that miR-590-5p might be involved in the progression of CRC. In parallel, miR-590-5p was expressed at higher levels in three CRC cell lines than that in normal CRC line (NCM460) (**Figure 1B**, P<0.01).

To investigate whether miR-590-5p expression is associated with clinical outcome in CRC patients, CRC patients were divided into two different groups according to the median miR-590-5p level. MiR-590-5p expression above or below the median was considered as high or low expression. As indicated in **Figure 1C**, CRC patients with higher expression of miR-590-5p predicted worse overall survival (OS) compared with those with lower miR-590-5p expression. Therefore, we concluded that altered expression of miR-590-5p might be associated with the progression of CRC.

Effects of miR-590-5p in SW620 and HCT116 in vitro

Given its increased expression in CRC cells, we next investigated whether miR-590-5p could regulate CRC cell growth, cell cycle and apoptosis in vitro. To do this, we transfected CRC cells with chemically synthesized miR-590-5p inhibitors and its negative control (NC) into SW620 and HCT116 cells. qRT-PCR analy-

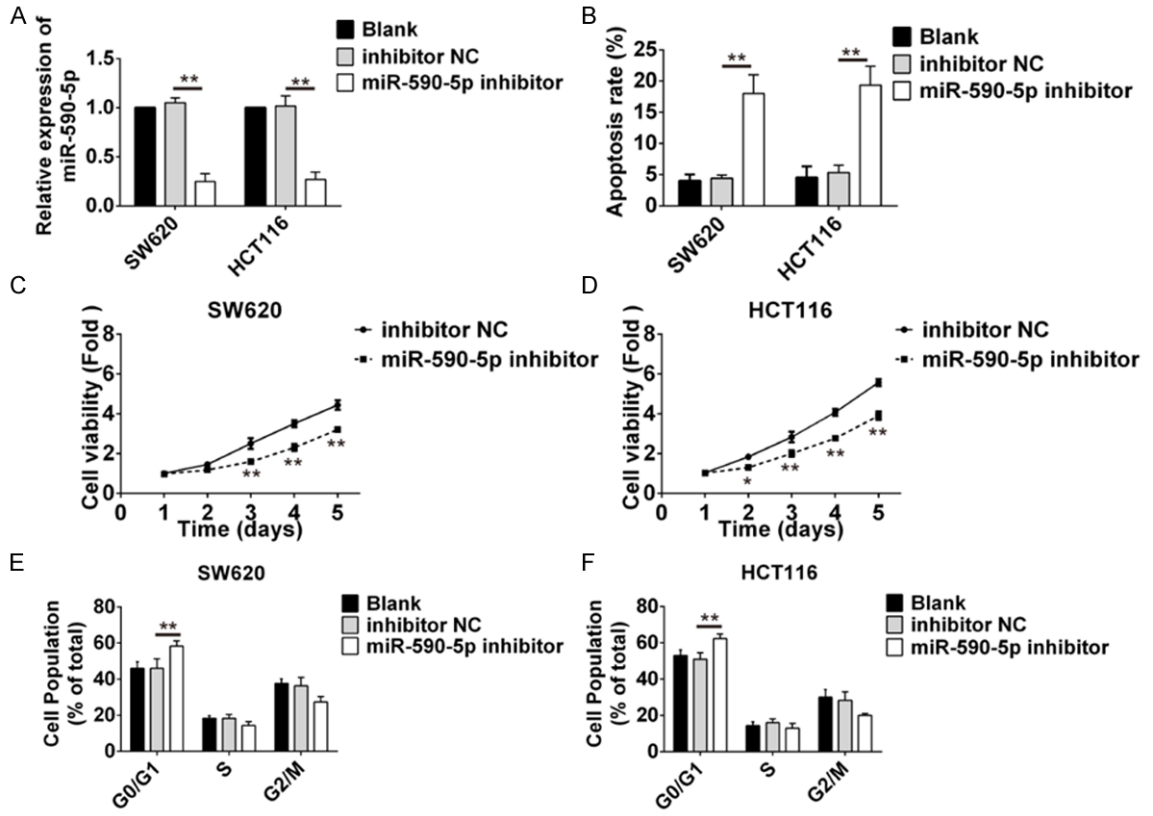

Figure 2. Effect of miR-590-5p on proliferation of CRC cells in vitro. A. MiR-590-5p inhibitors decreased miR-590-5p expression compared to NC, as evaluated by qRT-PCR. B. Annexin V-FITC/PI-stained cells transfected with miR-590-5p showed a higher rate of apoptosis, as evaluated by flow cytometry. C, D. Cell proliferation, determined by CCK-8 assay, revealed that knockdown of miR-590-5p significantly inhibited SW620 and HCT116 cells proliferation. E, F. The results showed that knockdown of miR-590-5p caused cell cycle arrest in the G0/G1 phase in SW620 and HCT116 cells, as evaluated by flow cytometry. Data are shown as mean ± SEM of three independent experiments. *P<0.05, **P<0.01 compared with NC.

sis showed that miR-590-5p expression was effectively reduced by miR-590-5p inhibitors (**Figure 2A**). Knockdown of miR-590-5p promoted apoptosis (**Figure 2B**) and inhibited proliferation (**Figure 2C**, **2D**) in SW620 and HCT116 cells. Further, we asked whether miR-590-5p knockdown affected cell-cycle regulation. As indicated in **Figure 2E** and **2F**, we found that most of miR-590-5p inhibitors transfected cells were arrested in the G0/G1 phase of the cell cycle. All these results suggested that miR-590-5p executed an oncogenic effect on CRC cells.

PTEN is a direct target of miR-590-5p in CRC cells

Using algorithms for target gene prediction, TargetScan [13] and miRanda [14], a negative regulator for PI3K activity [15], PTEN was identified as one of the potential targets of miR-590-5p. The predicted binding of miR-590-5p with PTEN 3' UTR was illustrated in **Figure 3A**. To experimentally confirm that PTEN is a direct target gene of miR-590-5p, the dual-luciferase activity assay was performed. Results showed that miR-590-5p significantly suppressed the luciferase activity of the wildtype (WT) 3'-UTR of PTEN, without effect on its mutant (Mut) (**Figure 3B**). In addition, western blot analysis showed that miR-590-5p overexpression markedly decreased the protein level of PTEN, whereas miR-590-5p inhibition increased the protein expression of PTEN (**Figure 3C**). All these results indicate that PTEN is a direct target of miR-590-5p.

PTEN is involved in the role of miR-590-5p in the regulation of CRC cell activity

To further evaluate the relationship between miR-590-5p and PTEN in CRC, the expression of PTEN was detected between 20 pairs of CRC tissues and normal tissue using qRT-

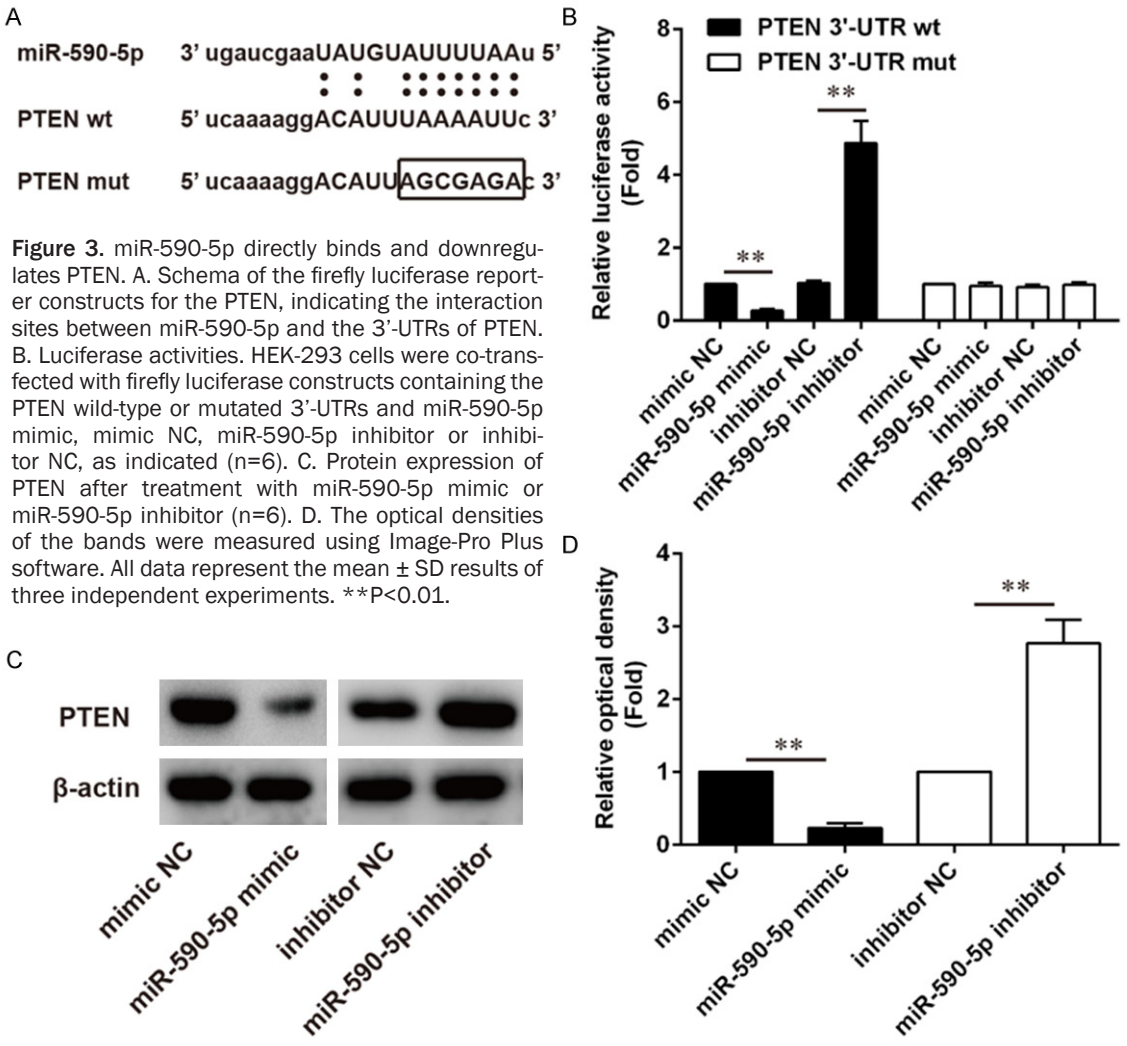

Figure 3. miR-590-5p directly binds and downregulates PTEN. A. Schema of the firefly luciferase reporter constructs for the PTEN, indicating the interaction sites between miR-590-5p and the 3'-UTRs of PTEN. B. Luciferase activities. HEK-293 cells were co-transfected with firefly luciferase constructs containing the PTEN wild-type or mutated 3'-UTRs and miR-590-5p mimic, mimic NC, miR-590-5p inhibitor or inhibitor NC, as indicated (n=6). C. Protein expression of PTEN after treatment with miR-590-5p mimic or miR-590-5p inhibitor (n=6). D. The optical densities of the bands were measured using Image-Pro Plus software. All data represent the mean ± SD results of three independent experiments. **P<0.01.

PCR. The mRNA levels of PTEN in colorectal cancer tissues were significantly decreased when compared with normal colorectal samples (**Figure 4A**). Moreover, the expression levels of PTEN in tumor tissues inversely correlated with the miR-590-5p levels (Spearman R^2: 0.4331, P=0.0016; **Figure 4B**). Importantly, transfection of PTEN siRNA could recover the inhibition of CRC cell proliferation induced by miR-590-5p inhibitor (**Figure 4C**, **4D**). Taken together, these data suggest that miR-590-5p may promote CRC cell proliferation by regulating the expression of PTEN.

Knockdown of miR-590-5p inhibited PI3K/Akt/mTOR activation in CRC cells

The PI3K/Akt/mTOR signaling pathway is known to be directly associated with cell growth, proliferation and survival in many cancers including colorectal cancer. As a major downstream factor of PTEN, PI3K/Akt could be negatively regulated by PTEN [16]. Our finding that PTEN was identified as a target of miR-590-5p encouraged us to investigate whether miR-590-5p activates PI3K/Akt/mTOR signaling. Therefore, we checked the expression of Akt, phosphorylated Akt, mTOR and phosphorylated mTOR using Western Blot. As expected, the level of phosphorylated Akt and phosphorylated mTOR was markedly decreased after miR-590-5p inhibitor treatment in both SW620 and HCT116 cells (**Figure 5A**, **5B**). Accordingly, the results implied that miR-590-5p may act as an oncogene in the progress of CRC via the PI3 K/Akt/mTOR signaling pathway.

Discussion

In the present study, we found that miR-590-5p was frequently upregulated in colorectal

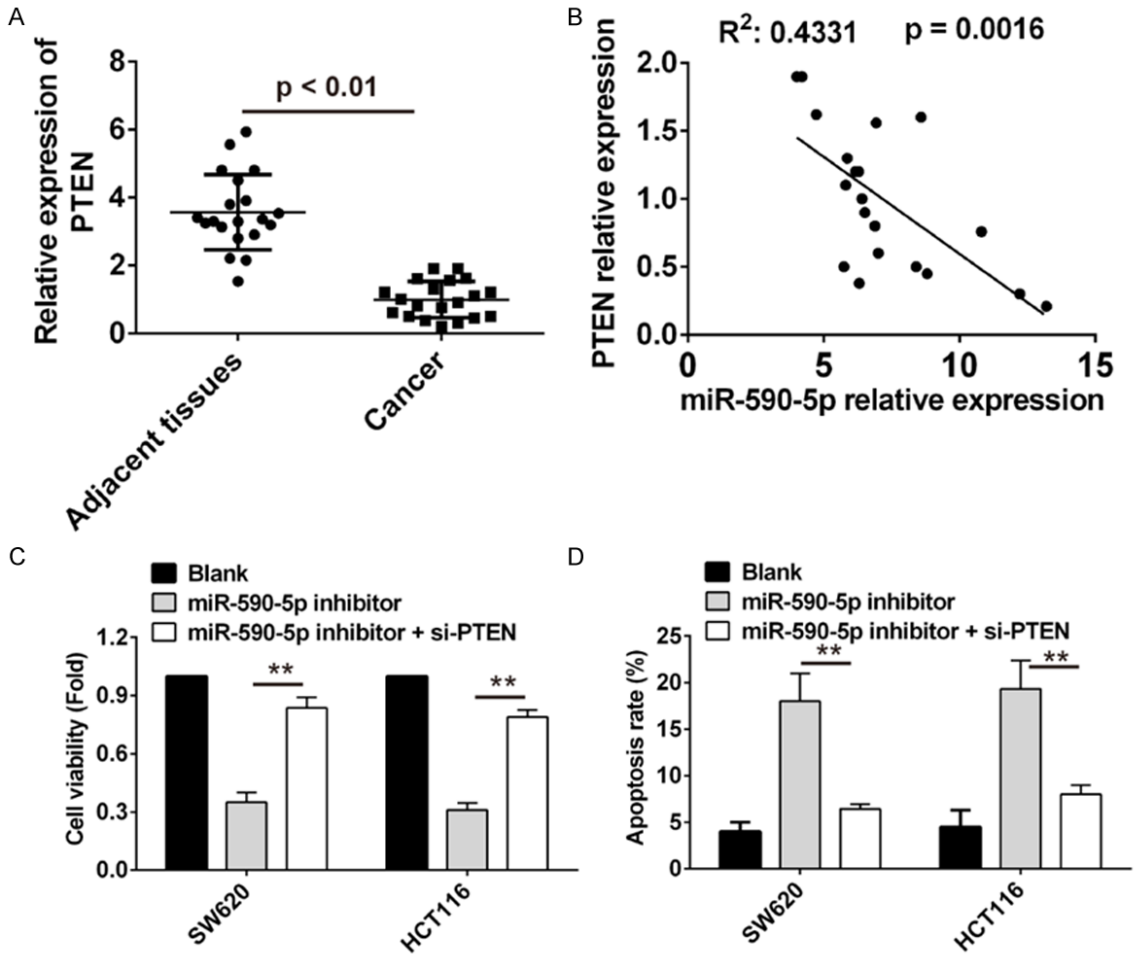

Figure 4. PTEN is involved in the role of miR-590-5p in regulation of CRC cell activity. A. The expression of PTEN was detected in 20 pairs of CRC tumors and adjacent normal tissues by qPCR. B. Pearson's correlation was performed to analyze the correlations between miR-590-5p and PTEN in CRC tissues (R^2=0.4331; P=0.0016). C. The cell viability of SW620 and HCT116 cells after transfection with miR-590-5p inhibitor and si-PTEN. D. Flow cytometry analysis of SW620 and HCT116 cells after transfection with miR-590-5p inhibitor and si-PTEN. All values are the mean ± SD of triplicate measurements, and experiments were repeated 3 times with similar results. **P<0.01 vs miR-590-5p inhibitor group.

cancer patient samples and CRC cell lines. Subsequent analysis indicated that miR-590-5p was significantly correlated with colorectal cancer patient overall survival. Functional analyses showed that knockdown of miR-590-5p promoted apoptosis, inhibited proliferation and induced cell cycle arrest in G0/G1 in vitro. We then identified PTEN as a direct target of miR-590-5p. In CRC samples, the expression levels of miR-590-5p were inversely correlated with PTEN expression, and transfection of PTEN siRNA could recover the inhibition of CRC cell proliferation induced by miR-590-5p inhibitor. More importantly, we proved that miR-590-5p may promote cell growth through the PTEN/PI3K/Akt/mTOR signaling pathway.

Recently, increasing evidence has associated dysregulated expression patterns of miRNAs to various types of human cancers, including CRC. Zhao L et al. found that miR-138-5p was frequently downregulated in CRC tissues and was associated with advanced clinical stage, lymph node metastasis and poor overall survival, which suggested that miR-138-5p was a tumor suppressor in CRC [17]. A study performed by Liu C et al. showed that miR-410 was upregulated in CRC tissues and cell lines, and may function as an oncogenic miR by suppressing the basal level of apoptosis [18]. Recent study showed that miR-590-5p can inhibit the metastasis of breast cancer, which indicated that miR-590-5p may act as a poten-

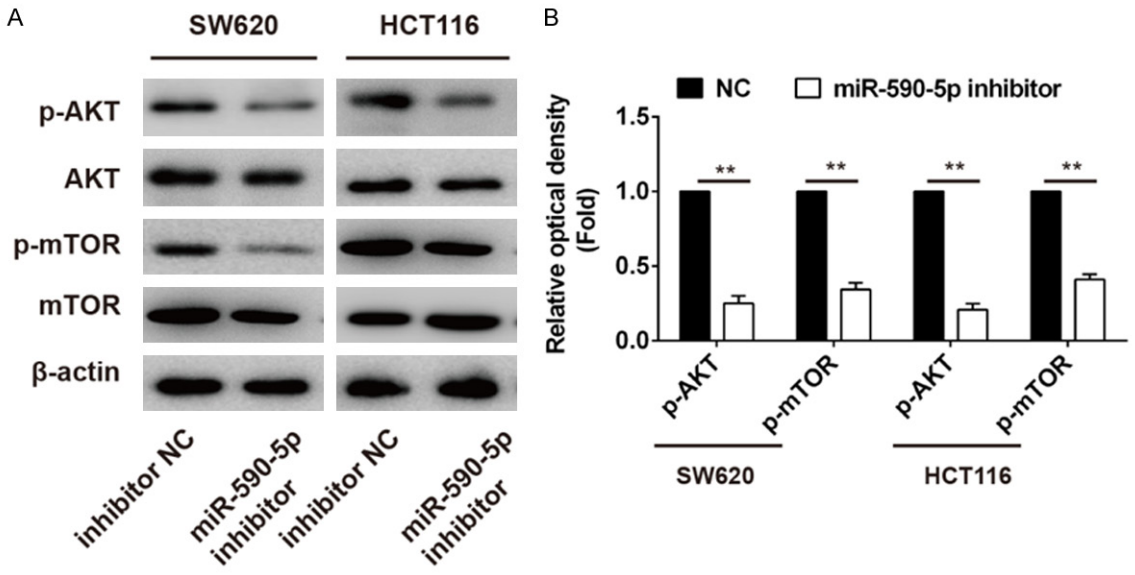

Figure 5. Knockdown of miR-590-5p suppresses PI3K/Akt/mTOR pathway. A. The expression of p-Akt, Akt, p-mTOR, and mTOR proteins was detected by western blotting and representative blots are shown, β-actin was used as loading control. B. The optical densities of the bands were measured using Image-Pro Plus software. All experiments were repeated at least three times. **P<0.01 vs control group.

tial tumor suppressor gene [19]. However, limited studies have pay attention on the biological roles of miR-590-5p on colorectal cancer. In this study, our data showed that miR-590-5p expression is upregulated in CRC. We subsequently showed that knockdown of miR-590-5p significantly promoted apoptosis, inhibited proliferation and induced cell cycle arrest in G0/G1 in vitro. We further demonstrated that miR-590-5p overexpression was correlated with poor overall survival. These data suggest that miR-590-5p may function as a tumor oncogene in CRC.

MiRNAs enforce their function via degradation or translational inhibition of their target mRNAs at the posttranscriptional level [20]. PTEN, a tumor suppressor gene, is involved in cancer cell proliferation, migration, invasion and apoptosis through several signal pathways [21]. Recently, a variety of studies have demonstrated that loss or down-regulation of PTEN plays an important role in development and progression of malignancies including CRC [22-25]. Yazdani et al. found that down-regulated expression of the PTEN protein probably contributed to growth, invasion, and metastasis of colorectal carcinoma [26]. However, the relationship between miR-590-5p and PTEN has never been reported before. In this study, PTEN was identified as a target of miR-590-5p based on ioinformatic analyses and validated by a dual luciferase reporter assay. Further RT-PCR analysis confirmed that the level of PTEN mRNA expression was inversely correlated with miR-590-5p expression in CRC samples and also that overexpression of PTEN could attenuate the oncogenic role of miR-590-5p. Together, these data suggested that miR-590-5p might promote CRC cell proliferation through regulating PTEN.

The PI3K/Akt/mTOR pathway is an intracellular signaling pathway, which regulates several customary cellular functions such as cell proliferation, cell metabolism, angiogenesis, cell cycle progression apoptosis and autophagy [27, 28]. Seveasl studies demonstrated that the PI3K/Akt/mTOR pathway involved in the progression of CRC [29-31]. For example, Zhang X et al. demonstrate that miR-218 suppresses the proliferation, migration and invasion of colon cancer cells by targeting the PI3K/Akt/mTOR signaling pathway and MMP9 [32]. Moreover, PTEN, a target of miR-590-5p, could regulate the activation of PI3K/Akt/mTOR signaling pathway. In the present study, we found that knockdown of miR-590-5p regulated the total and phosphorylated protein levels of Akt and its down-

stream target, mTOR protein in CRC cells, indicating that miR-590-5p may be an important regulator of PI3K/Akt/mTOR signaling pathway. All these results indicated that miR-590-5p promoted CRC cell growth by PI3K/Akt/mTOR signaling pathway activity.

In summary, our present study demonstrates miR-590-5p as a potential oncogenic microRNA in human colorectal cancer by directly targeting PTEN, and further activated the PI3K/Akt/mTOR pathway. The ability of miR-590-5p to promote CRC cell growth may provide us a novel perspective on patient treatment.

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Disclosure of conflict of interest

None.

Address correspondence to: Xue-Li Zhang and Hai-Jun Liu, Department of General Surgery, Fengxian Hospital Affiliated to Southern Medical University, 6600, Nan Feng Road, Shanghai 201499, P. R. China. Tel: +86-21-57420702; E-mail: lejing1996@aliyun.com (XLZ); navylhj@aliyun.com (HJL)

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