

The oncogenic role of microRNA-500a in colorectal cancer

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Abstract. Colorectal cancer (CRC) is a common and lethal disease, and microRNAs (miRNAs/miRs) serve an important role in the pathogenesis of CRC. miR-500a is a novel miRNA, and although its function has been studied in hepatocellular carcinoma, the function of miR-500a in CRC remains unknown. In the present study, the function of miR-500a in CRC was investigated. The expression levels of miR-500a in cells and tissues were investigated using reverse transcription-quantitative PCR. Cell proliferation was tested using MTT assay and migration was assessed using Transwell systems. The results revealed that there were higher levels of miR-500a in tumor tissue compared with in normal tissue. Inhibition of miR-500a suppressed cell growth and migration, whereas overexpression of miR-500a promoted cell growth and migration. Additionally, it was revealed that miR-500a may target the 3'-untranslated region of the phosphatase and tensin homolog gene. In conclusion, the present study demonstrated that miR-500a may serve an oncogenic role in CRC.

Introduction

Colorectal cancer (CRC) is a common and lethal disease, and CRC incidence and mortality rates vary markedly around the world. CRC is the third most commonly diagnosed type of cancer in men and the second most commonly diagnosed type of cancer in women, with global statistics identifying 1.65 million new cases and ~835,000 cases of CRC-associated mortality in 2015 (1). In China, CRC was the fifth most common cancer in men and fourth in women, with 245,000 new cases and 139,000 cases of CRC-associated mortality in 2012 (2). Additionally, the incidence rate of CRC greatly increases with age, particularly from 40-45 years onwards, in rural and urban areas in China. To reduce the morbidity and

mortality associated with this disease, targeted prevention and treatment are recommended (3).

MicroRNAs (miRNAs/miRs) are a class of non-coding small RNAs, ~22 nucleotides in length. miRNAs function in RNA silencing and post-transcriptional regulation of gene expression via base pairing with complementary sequences within mRNA molecules (4). Previous studies have demonstrated that miRNAs serve multiple roles in the pathogenesis of various types of cancer (5-9). Numerous miRNAs have been identified to be associated with the pathogenesis of CRC (10-14).

miR-500a is a novel miRNA. The function of miR-500a has been studied in hepatocellular carcinoma (HCC) (15), and it has been determined that miR-500a promotes the progression of HCC by post-transcriptionally targeting the BH3 interacting domain death agonist gene. In addition, miR-500a expression is upregulated in HCC tissues, and high miR-500a expression is significantly correlated with poor prognosis of patients with HCC (15). However, the function of miR-500a in CRC remains unknown. In the present study, the function of miR-500a in CRC was investigated.

Materials and methods

Tissue samples. For the present study, 14 CRC tissue samples and matched adjacent normal tissues (age range, 45-78; sex male:female, 8:6) were acquired from the Department of Gastrointestinal Surgery, West China Hospital, Sichuan University (Chengdu, China) (between July 2012 and May 2013). The pathological diagnosis of all patients with CRC was confirmed by senior pathologists at the West China Hospital, Sichuan University. Tissues were immediately frozen at -80°C. Written informed consent was obtained from all patients, and the present study was approved by the Ethics Committee of the West China Hospital, Sichuan University.

Cell culture. CRC cell lines (SW620 and SW1417) and a normal human colorectal cell line (FHC; cat. no. CRL-1831), were acquired from the Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China). SW620, SW1417 and FHC cells were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium/Ham's F-12 (Sigma-Aldrich; Merck KGaA), supplemented with 10% fetal bovine serum (FBS; cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc.), antibiotic-antimycotic (1:100, cat. no. 15240096; Thermo Fisher Scientific, Inc.)

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Detection of miR-500a in CRC tissue samples and cell lines.

The expression levels of miR-500a in the 14 CRC tissue samples and FHC, SW620 and W1417 cells were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In detail, total RNA was extracted from the 14 specimens and three cell lines using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The expression levels of miR-500a were then detected by TaqMan miRNA RT-Real Time PCR, as previously described according to the manufacturer's protocol (16). Single-stranded cDNA was synthesized using the TaqMan miRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), and then amplified using TaqMan Universal PCR Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.), with miRNA-specific TaqMan Minor Groove Binder probes (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol (17). The following primers were used: PCR miR-500a forward, 5'-ACACTCCAGCTGGGTAATCCTTGCTACCTGG-3' and reverse, 5'-TGGTGTCTGGAGTCG-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGCAT-3', and small nuclear RNA was used for normalization (18). The conditions were as follows: 40 cycles of three-step PCR (95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec) following an initial denaturation at 95°C for 10 min.

Downregulation and overexpression of miR-500a in SW620 and SW1417 cells. miR-500a expression was upregulated by miR-500a mimic and downregulated by miR-500a antisense oligonucleotides (ASO). miR-500a mimics, miR-500a ASO, and control miRNA were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The sequence of miR-500a mimic, miR-500a ASO were listed as follows: miR-500a mimic, 5'-UAAUCCUUGCUACCUGGGUGAGA-3'; miR-500a ASO, 5'-AUUAGGAACGAUGGACCCACUCUAAAA-3. miRNAs (50 ng) were transfected into SW620 and SW1417 cells (5×10^5) using Lipofectamine[®] 2000 transfection reagent (Thermo Fisher Scientific, Inc.). Subsequent experiments were performed 24 h post-transfection.

Cell proliferation assay. Cell growth was analyzed by MTT assay as described previously (19-22). Briefly, SW620 and SW1417 cells were cultured in flat 96-well plates overnight, at a density of 5×10^5 cells/well. MTT reagent (0.1 mg/ml) was added to the medium for 5 min at room temperature, followed by the addition of 100 μ l dimethyl sulfoxide at room temperature. The optical density value was measured on a microplate reader at a wavelength of 570 nm.

Cell migration assay. Transwell systems were used to assess cell migration. In detail, the Transwell chambers (8.0 μ m pore size; Sigma-Aldrich, Merck KGaA) were placed in 24-well plates. The miR-500a mimic-transfected or ASO-transfected SW620 or SW1417 cells (1×10^6 cells/ml) were FBS-deprived for 12 h, and subsequently added to the upper chamber. Medium containing 10% FBS was placed in the lower chamber at 37°C. After 6 h, migratory cells (SW620 or SW1417) were counted using an inverted Leica DM IL microscope (magnification, x200; Leica Microsystems GmbH).

Prediction of the putative targets of miR-500a. The putative targets of miR-500a were predicted by the online software TargetScan (http://www.targetscan.org/vert_71/). TargetScan predicts biological targets of miRNAs by searching for the presence of 8-mer, 7-mer, and 6-mer sites that match the seed region of each miRNA (23-25).

Dual luciferase reporter assays. SW620 cells were seeded in a 24-well plate at 1×10^5 cells/well and were serum-starved for 6 h prior to transfection. Mutants of the 3'-untranslated region (3'-UTR) of phosphatase and tensin homolog (PTEN) were generated using the Site-Directed Mutagenesis kit (cat. No. F701; Thermo Fisher Scientific, Ltd.). The 3'-UTR of PTEN and mutated controls were cloned and inserted into the reporter plasmid (500 ng; Promega Corporation). miR-500a mimics (500 ng) were then transfected into the plasmids (mutant group), separately, using Lipofectamine[®] 2,000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). miR-NC (500 ng) was also transfected into the SW620 cells, containing either the wild-type (WT group) or mutant 3'-UTR plasmids (Mutant group) as a control. Cells were harvested 24 h later, and the luciferase activity was measured using the Dual-Luciferase[®] Reporter Assay system (cat. no. 16186, Thermo Fisher Scientific, Inc.). Firefly luciferase were normalized to *Renilla* luciferase activity.

Cell apoptosis analysis. Cells (5×10^5 cells/ml) were suspended in Annexin V-fluorescein isothiocyanate (FITC; Abcam, Cambridge, UK) binding buffer. Subsequently, Annexin V-FITC was added, and the suspension was incubated for 15 min at room temperature. Subsequently, propidium iodide (PI; Abcam) was added to each sample for 5 min prior to FACS analysis, at room temperature. Next, the samples were analyzed using a fluorescence-activated cell sorting instrument at 488 nm excitation (using an argon-ion laser or solid-state laser), and emission was detected at 530 nm (green; FITC) and 575-610 nm (orange; PI) using a FACSverse scanner (BD Biosciences). The FACS data was analyzed using FACSuite Version 1.0.0.1477 (BD Biosciences).

Western blot analysis. The transfected SW620 cells were thawed and lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100 and 0.1% SDS) with Protease Inhibitor Cocktail (Sigma-Aldrich; Merck KGaA) and Phosphatase Inhibitor Cocktail (Sigma-Aldrich; Merck KGaA). The total protein was quantified using a bicinchoninic acid protein kit (cat. no. ab102536, Abcam). Total protein (30 μ g per lane) was separated by SDS-PAGE on a 10% gel and subsequently transferred onto a polyvinylidene difluoride membrane. Subsequently, the membrane was blocked using 5% bovine serum albumin buffer (1.0 g BSA in 20 ml 1x TBST; cat. no. A1933; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. For PTEN analysis, an anti-PTEN antibody (cat. no. ab32199; 1:500 dilution; Abcam) was prepared in 5% BSA. The membrane was incubated overnight with anti-PTEN antibody at 4°C. The membranes were washed using TBST for three times, prior to incubation with a peroxidase-linked anti-rabbit secondary antibody (cat. no. ab7090; 1:2,000 dilution; Abcam) at room temperature for 2 h. Proteins were detected with Enhanced

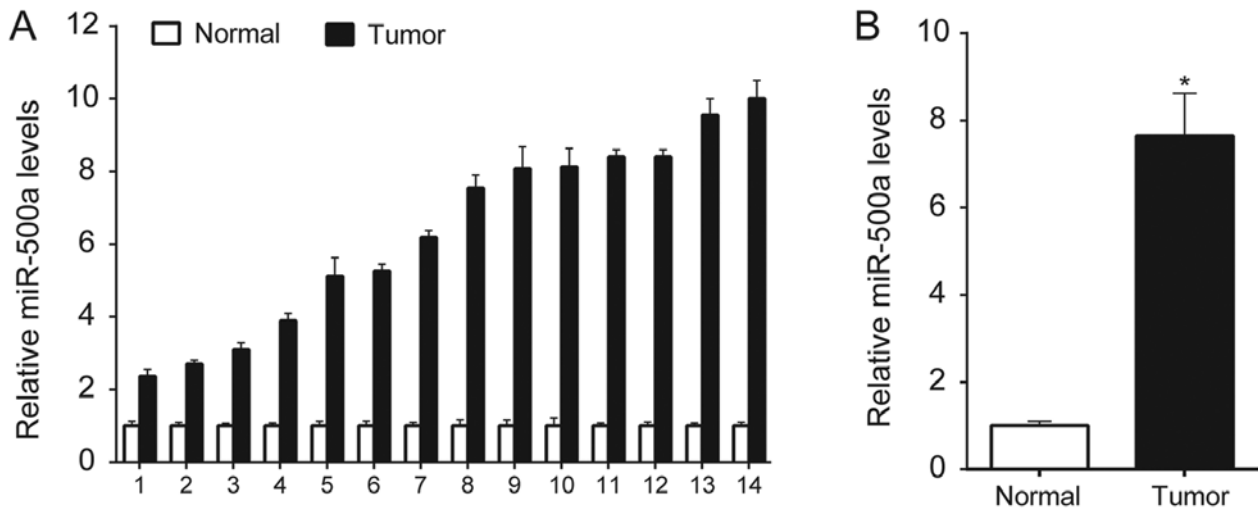


Figure 1. Overexpression of miR-500a in CRC tissue samples. (A) miR-500a expression levels in 14 CRC tissues and matched adjacent normal tissues were assessed by reverse transcription-quantitative polymerase chain reaction. (B) Mean values of miR-500a expression levels in the CRC tissues and their matched adjacent normal tissues were calculated. These experiments were performed in triplicate. * $P < 0.05$. CRC, colorectal cancer; miR-500a, microRNA-500a.

Chemiluminescence Western Blotting Detection reagents (GE Healthcare, Chicago, IL, USA) and images were analyzed using ImageJ software (Windows v. 1.8.0_122; National Institutes of Health). β -actin was used as an internal control. For β -actin detection, an anti- β -actin antibody (cat. no. ab1801; 1:2,000 dilution; Abcam) was prepared in 5% BSA buffer and TBST. The remaining steps were identical to the aforementioned PTEN detection steps.

Statistical analysis. All experiments were repeated three times. The data are presented as the means \pm standard deviation. A two-tailed Student's t-test was used to analyze the differences between two groups. One-way analysis of variance was used to analyze the differences among three or more groups, with a Student-Newman-Keuls post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. All calculations were performed using SPSS v16.0 software (SPSS).

Results

Expression levels of miR-500a are higher in CRC tissues compared with in normal tissues. Initially, 14 CRC tissues and corresponding adjacent normal tissues were collected, and the expression levels of miR-500a were detected by RT-qPCR. miR-500a was overexpressed in tumor tissues compared with in normal tissues (Fig. 1A). The average expression levels of miR-500a in tumor and normal tissues were calculated, and CRC tumor tissues exhibited higher expression levels than normal tissues (Fig. 1B).

Inhibition of miR-500a suppresses cell proliferation and migration, and increases apoptosis rates. To investigate the role of miR-500a in CRC, miR-500a expression in two CRC cell lines (SW620 and SW1417) was assessed. The normal human colorectal cell line FHC was used as a control. The present study revealed that higher miR-500a expression levels were observed in SW620 and SW1417 cells compared with in FHC cells (Fig. 2A). Additionally, miR-500a expression was downregulated in SW620 and SW1417 cells by miR-500a

ASO. After 24 h, the miR-500a levels were tested by RT-qPCR. The data revealed that miR-500a ASO decreased miR-500a expression levels (Fig. 2B).

Subsequently, cellular proliferation following miR-500a ASO transfection was assessed. The present study demonstrated that transfection with miR-500a ASO inhibited proliferation of SW620 and SW1417 cells (Fig. 2C). Testing of the migratory ability of CRC cells revealed that miR-500a ASO transfection decreased the number of migratory cells (Fig. 2D). An assay to determine the apoptosis rate of miR-500a ASO-transfected CRC cells revealed that miR-500a ASO increased the apoptosis rate of SW620 and SW1417 cells (Fig. 2E and F).

Overexpression of miR-500a promotes cell proliferation and migration, and decreases cell apoptosis. miR-500a expression was upregulated in SW620 and SW1417 cells by miR-500a mimic transfection. The expression levels of miR-500a in transfected SW620 and SW1417 cells were analyzed by RT-qPCR. miR-500a mimic transfection upregulated the miR-500a expression levels in the two cell lines (Fig. 3A). Next, proliferation of SW620 and SW1417 cells was analyzed by MTT assay, and it was demonstrated that overexpression of miR-500a promoted cell proliferation (Fig. 3B). Testing the migratory ability of CRC cells revealed that miR-500a mimic transfection increased the number of migratory cells (Fig. 3C). Additionally, the apoptosis rate of miR-500a mimic-transfected CRC cells was assessed. miR-500a mimic transfection decreased the apoptosis rate of SW620 and SW1417 cells (Fig. 3D and E).

miR-500a targets PTEN. The present study attempted to determine whether PTEN, a classical tumor suppressor gene, is a target gene of miR-500a (26). A previous study demonstrated that upregulated miR-500a enhances HCC metastasis by repressing PTEN expression (27). The potential binding sites of the 3'-UTR of PTEN were identified using bioinformatics methods, and the mutated version of the 3'-UTR of PTEN is shown in Fig. 4A. The mutated sites were cloned into a luciferase reporter plasmid. miR-500a mimics and the reporter

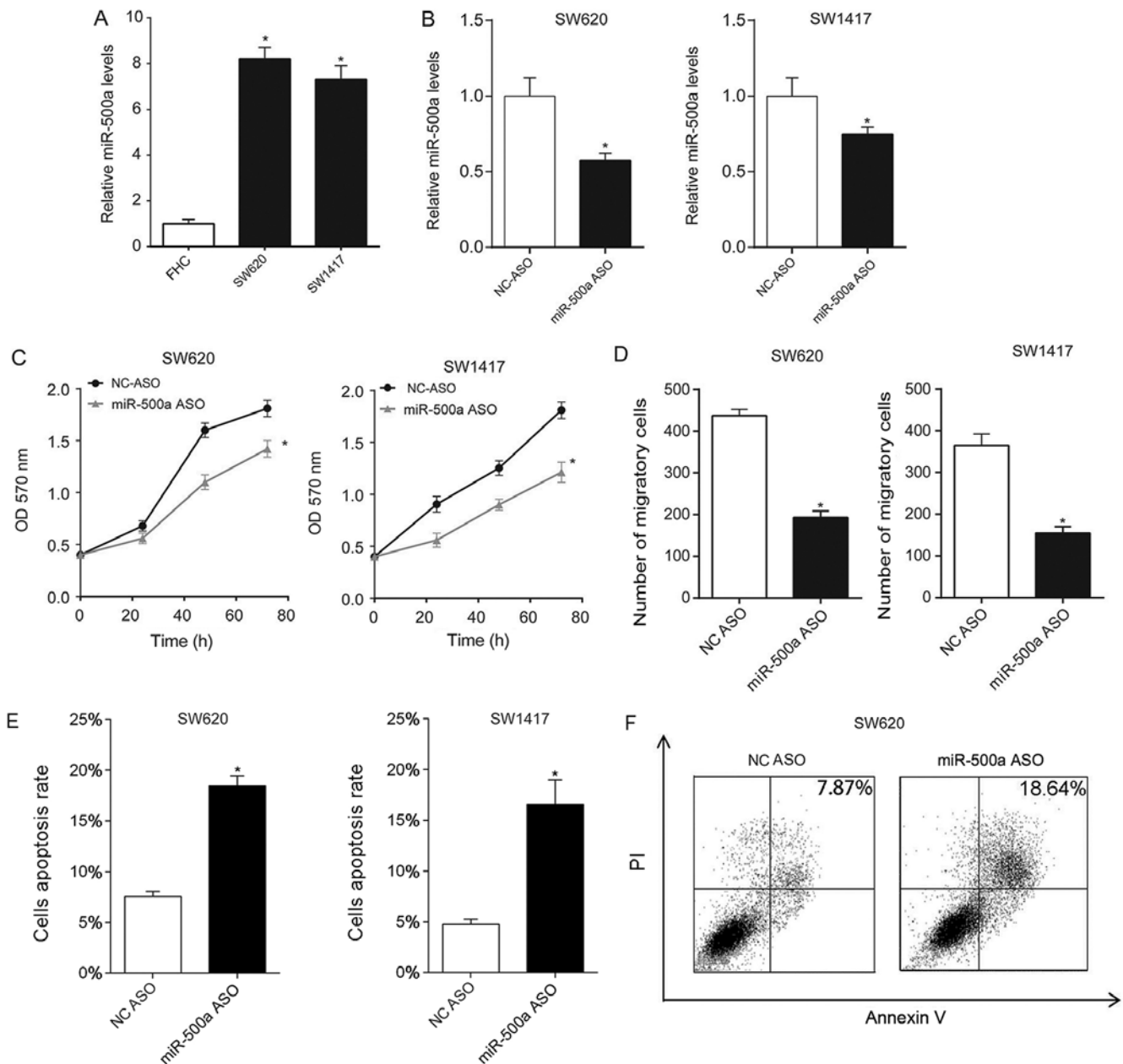


Figure 2. Downregulation of miR-500a inhibits SW620 and SW1417 cell proliferation and migration. (A) miR-500a levels in FHC, SW620 and SW1417 cells were analyzed using RT-qPCR. The expression levels of miR-500a in FHC were arbitrarily defined as 1. (B) In SW620 and SW1417 cells, miR-500a was downregulated by miR-500a ASO transfection. After 24 h, the miR-500a expression levels were assessed by RT-qPCR. (C) Proliferation of SW620 and SW1417 cells, following transfection, was assessed by MTT analysis. (D) To assess cellular migration, SW620 or SW1417 cells from each group were added to the upper uncoated chamber of a Transwell assay system. After 24 h, the cells in the lower chamber were counted. (E and F) Transfected cells were stained with Annexin V-FITC and PI, and were processed by fluorescence-activated cell sorting using 488 nm excitation. Annexin V-FITC-positive and PI-negative cells were defined as apoptotic cells. These experiments were performed in triplicate. * $P < 0.05$ vs. miR-500a ASO. ASO, antisense oligonucleotide; FITC, fluorescein isothiocyanate; miR-500a, microRNA-500a; NC, negative control; OD, optical density; PI, propidium iodide; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

plasmid were co-transfected into SW620 cells. The luciferase activity was assessed 24 h after transfection (Fig. 4B). The upregulation of miR-500a inhibited luciferase activity in wild-type 3'-UTR-transfected cells, whereas miR-500a had no effect on luciferase activity in cells transfected with the mutated 3'-UTR, indicating that miR-500a targets PTEN in SW620 cells. PTEN protein expression levels were measured following miR-500a mimic transfection. The present study revealed that miR-500a mimic transfection inhibited PTEN protein expression in SW620 cells (Fig. 4C).

Discussion

In the present study, the function of miR-500a in CRC was investigated and it was revealed that miR-500a may be involved in the oncogenesis of CRC. Higher expression levels of miR-500a were observed in tumor tissues compared with in adjacent normal tissues. Inhibition of miR-500a suppressed cell growth and migration, whereas overexpression of miR-500a promoted cell growth and migration. Additionally, it was determined that miR-500a may target PTEN.

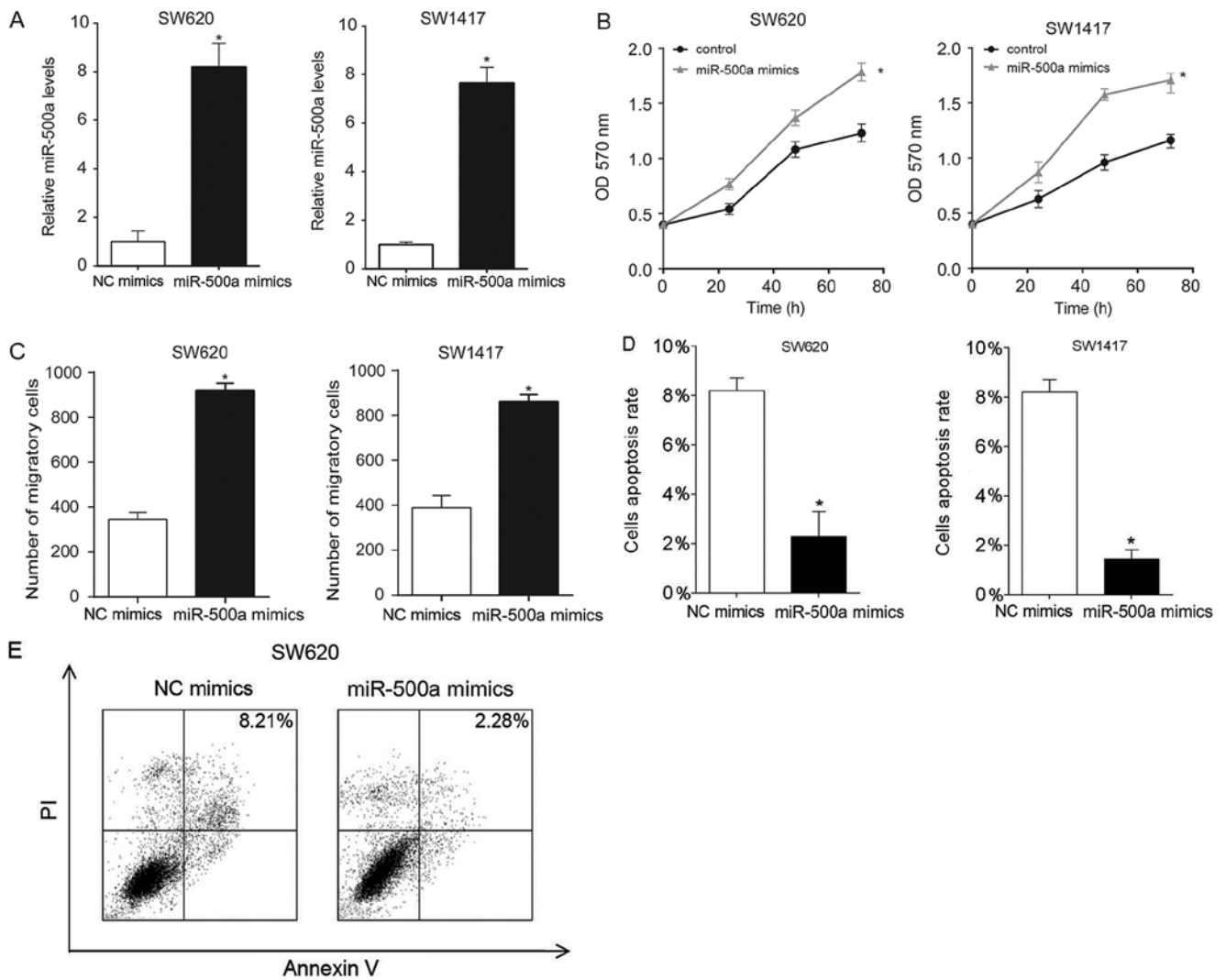


Figure 3. Overexpression of miR-500a promotes SW620 and SW1417 cell proliferation and migration. (A) In SW620 and SW1417 cells, miR-500a was overexpressed using miR-500a mimic transfection. After 24 h, the miR-500a levels were assessed by reverse transcription-quantitative polymerase chain reaction. (B) Proliferation of SW620 and SW1417 cells, following transfection, was assessed by MTT analysis. (C) To assess cellular migration, SW620 or SW1417 cells from each group were added to the upper uncoated chamber of a Transwell assay system. After 6 h, the cells in the lower chamber were counted. (D and E) Transfected cells were stained with Annexin V-FITC and PI, and were processed by fluorescence-activated cell sorting using 488 nm excitation. Annexin V-FITC-positive and PI-negative cells were defined as apoptotic cells. These experiments were performed in triplicate. * $P < 0.05$ vs. miR-500a mimics. FITC, fluorescein isothiocyanate; miR-500a, microRNA-500a; NC, negative control; OD, optical density; PI, propidium iodide; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

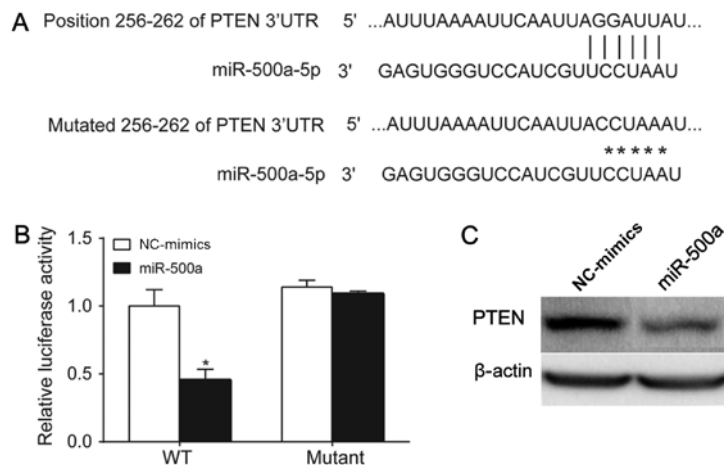


Figure 4. miR-500a targets PTEN. (A) Binding sites and location of the mutations (position 256-262) are listed. (B) miR-500a mimics and a plasmid containing either WT or mutated 3'-UTR sequence of PTEN were transfected into SW620 cells, and 24 h later, the luciferase activity was analyzed. (C) miR-500a mimics were transfected into SW620 cells, and the protein expression levels of PTEN were determined by western blotting. Each experiment was repeated at least three times. * $P < 0.05$ vs. miR-500a mimics, miR, microRNA; NC, negative control; PTEN, phosphatase and tensin homolog; UTR, untranslated region; WT, wild-type.

The role of miR-500a has been studied in various types of cancer, including HCC (27) and breast cancer (28). In breast cancer, miR-500a-5p regulates oxidative stress response genes and predicts cancer survival. In the present study, miR-500a promoted cell growth and migration and it was hypothesized that the expression of miR-500a in CRC tissues is negatively associated with the survival rates of patients with CRC.

Notably, a previous study demonstrated that the nuclear localization of PTEN is regulated by oxidative stress and mediates p53-dependent tumor suppression (29). It is possible that miR-500a regulates PTEN and oxidative stress response genes, and oxidative stress also regulates PTEN. Therefore, miR-500a may be associated with two pathways, which can be used to regulate PTEN.

PTEN is a well-known tumor suppressor gene. Notably, PTEN is frequently mutated or deleted in various human types of cancer (30-34). PTEN could function as a lipid phosphatase, thereby negatively regulating the phosphatidylinositol 3-kinase (PI3K)-protein kinase B signaling pathway. PTEN can also localize to the nucleus, where it binds and regulates the p53 protein level and transcription activity (35). Therefore, miR-500a may regulate PI3K and p53 function via PTEN, and this possibility will be investigated in future studies. Additionally, more CRC tissues will be collected for immunohistochemistry analysis of PTEN, and its mechanisms will be further investigated.

In conclusion, the present study demonstrated the possible oncogenic function of miR-500a in CRC. Therefore, miR-500a may represent a potential molecular target for the treatment of CRC and warrants further investigation.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YL collected patient data and performed cell experiment, PCR, western blotting and other molecular experiments. ZC contributed to study design and manuscript writing.

Ethics approval and consent to participate

Written informed consent was obtained from all patients, and the present study was approved by the Ethics Committee of the West China Hospital, Sichuan University.

Patient consent for publication

All patients have provided their consent for the use of their information and samples for scientific research and publication.

Competing interests

The authors declare that they have no competing interests.

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