Upregulation of miR-598 promotes cell proliferation and cell cycle progression in human colorectal carcinoma by suppressing INPP5E expression

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Abstract. Colorectal cancer (CRC) is one of the most common types of cancer worldwide. Recently, microRNAs (miRs) have been considered as novel therapeutic targets for the treatment of cancer. miR-598 is a poorly investigated miR. The underlying mechanism of miR-598 in CRC cells remains to be elucidated. In the present study, miR-598 was demonstrated to be significantly upregulated in CRC tissue by analyzing data from The Cancer Genome Atlas and the Gene Expression Omnibus. The results of a polymerase chain reaction demonstrated that miR-598 expression was significantly upregulated in CRC tissues and cells. Gain of function and loss of function assays demonstrated that miR-598 significantly promoted cell proliferation and cell cycle progression. miR-598 was demonstrated to modulate cell functions by regulating 72 kDa inositol polyphosphate-5-phosphatase (INPP5E). In addition, knockdown of INPP5E counteracted the growth arrest caused by an miR-598-inhibitor. In conclusion, the present study demonstrated that miR-598 contributed to cell proliferation and cell cycle progression in CRC by targeting INPP5E.

Introduction

Colorectal cancer (CRC), the most common gastrointestinal malignant tumor, is one of the most common malignant tumors and the third leading cause of cancer-associated mortality worldwide (1,2). Despite the advances in diagnostic methods and therapy, the prognosis of patients with CRC remains poor (3,4). Therefore, it is urgent to investigate novel diagnostic, prognostic and treatment strategies for CRC.

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MicroRNAs (miRs) are a family of short noncoding RNAs (18-25 nucleotides long) that regulate diverse biological processes, including cell proliferation, cell migration, metastasis, invasion and apoptosis (5-8). It has been previously reported that miRs can act as tumor oncogenes or suppressors during cancer development, dependent on the role of their target genes (9-11). miR-598 was reported to be a biomarker for bile duct cancer (12). Zhao *et al* (13) indicated that miR-598 acted as a prognostic biomarker in esophageal cancer. However, the function and molecular mechanisms of miR-598 in human CRC remain to be fully elucidated. In the present study, miR-598 expression in CRC was investigated through analyzing data from two public datasets: The Caner Genome Atlas project (TCGA) and Gene Expression Omnibus (GEO; accession number GSE30454). In addition, in the present study miR-598 expression was demonstrated to be increased in CRC tissues and cell lines, and miR-598 may contribute to cell proliferation and cell cycle progression by targeting inositol polyphosphate-5-phosphatase E (INPP5E). The results of the present study indicated that miR-598 was frequently upregulated in CRC, may act as a tumor promoter and has potential as a prognostic biomarker for patients with CRC.

Materials and methods

Clinical specimens. Human CRC tissues and the matched adjacent non-tumor tissues were obtained from eight patients, including four males and four females, between 38 and 76 years old, with CRC and histopathologically diagnosed at the Department of General Surgery, Huizhou First Hospital (Huizhou, China) between 1 June 2015 and 31 December 2015. The present study was approved by the Ethics Committee of the Department of General Surgery, Huizhou First Hospital. Written informed consent was obtained from all patients. Tissue samples were collected during surgery, immediately frozen in liquid nitrogen and stored until total RNA or proteins were extracted.

Cell culture. Human CRC cell lines SW620, COLO320DM, SW403, SW480, HT-29 and COLO205 and normal colonic cell line FHC were purchased from the National Rodent Laboratory Animal Resources (Shanghai, China). All CRC

cell lines were grown in Dulbecco's modified Eagle medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and normal colon FHC cells were grown in DMEM/F-12 medium with 10% FBS, 10 ng/ml cholera toxin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 5 μg/ml transferrin, 5 μg/ml insulin, 100 ng/ml hydrocortisone and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Cell lines were cultured in a humidified 37˚C incubator with 5% CO_2.

Plasmids, small interfering RNA (siRNA) and transfection. SW480 cells ($5x10^5$) were transfected with 2.5 μg each of scrambled miRs as negative controls (NCs), miR-598 mimic and miR-598-in (miR-598-inhibitor; GeneCopoeia, Inc., Rockville, MD, USA), Mutant miR-598 was constructed by GeneCopoeia, Inc. INPP5E siRNAs and NCs (stQ0004501-1; http://www.ribobio.com/sitecn/product_info.aspx?id=338920) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Transfection of plasmids and siRNAs was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from culture cells and patient samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, then cDNA was synthesized with using reverse transcription reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) at 37˚C for 15 min, followed at 85˚C for 5 sec, and then cooling to 4˚C. RT-qPCR was carried out using an ABI 7900HT Fast Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR-Green PCR kit (Takara Biotechnology Co., Ltd.). The primers selected by GeneCopoeia, Inc., were miR-598 (cat. no. HmiRQP0702), U6 (cat. no. HmiRQP9001), cyclin D1 (cat. no. HQP016204), cyclin-dependent kinase inhibitor 1B (p27; cat. no. MQP028863) and GAPDH (cat. no. HQP006940). Thermocycling conditions were 95˚C for 30 sec, followed by 40 cycles of amplification at 95˚C for 5 sec, 59˚C for 30 sec and then 72˚C for 30 sec.

Relative miR-598 or cyclin D1 and p27 mRNA expression were normalized to U6 or GAPDH, respectively. Relative quantification was calculated as $2^{-\Delta\Delta Cq}$ (14) and was used to calculate fold-changes.

Western blotting. Protein lysates were lysed with radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology, Haimen, China), equal quantities (50 μg) of protein were separated on a 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were incubated with primary antibodies as follows: Rabbit anti-INPP5E monoclonal antibody (ab191520; 1:1,000); rabbit anti-cyclin D1 monoclonal antibody (ab134175; 1:1,000); rabbit anti-p27 monoclonal antibody (ab32034; 1:1,000) overnight at 4˚C, and anti-α-tubulin antibody (ab7291; 1:5,000; all from Abcam, Cambridge, MA, USA) was used as a reference protein. The next day, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (ab205718; 1:10,000; Abcam) for 2 h at room temperature and bound antibodies were visualized using an enhanced chemiluminesence kit (Beyotime Institute of Biotechnology) with the SuperSignal West PICO chemiluminescent detection system (Pierce; Thermo Fisher Scientific, Inc.).

MTT and colony formation assays. Cell growth was measured by MTT assay (Sigma-Aldrich; Merck KGaA) according to the manufacturers' protocol. Transfected SW480 cells were seeded in 96-well plates at $5x10^3$ cells/well. Cells were allowed to grow for 0-5 days. A total of 20 μl, 5 mg/ml MTT solution was then added to each well and incubated for 4 h, and then 150 μl DMSO (Sigma-Aldrich; Merck KGaA) was added. Absorbance was measured at 490 nm using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Anchorage-independent growth assay. The soft agar colony-forming assay was performed using transfected SW480 (2,000 cells/well), which were seeded into each well of a 6-well plate with 0.5% agar (Sigma-Aldrich; Merck KGaA). After 14 days, three fields were randomly selected and cell colonies >0.1 mm^2 were counted with an inverted microscope at a magnification of x200.

Cell cycle analysis. Following 48 h of incubation post-transfection, SW480 cells were removed from culture plates by trypsinization and were washed with PBS. Cells were fixed with 75% ice-cold ethanol at 4˚C. Following overnight incubation at 4˚C and washing with PBS, the cells were treated with RNase A (300 μg/ml) at 37˚C for 30 min and stained with propidium iodide (PI) at 4˚C for 30 min in the dark, and the cell cycle distribution was analyzed using a flow cytometer (BD FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA). The percentages of cells in G_0/G_1, S and G_2/M phases were counted by BD CellQuest™ Pro Software version 3.3 and compared. Experiments were repeated three times.

Luciferase assays. The INPP5E open reading frame with the 3'-untranslated region (UTR) was cloned into pGL3 vectors were purchased from GeneCopoeia, Inc. (Guangzhou, China). SW480 cells ($5x10^4$/well) were cultured in 24-well plates. Transient co-transfection with either the INPP5E 3'-UTR wild type and miR-598 or miR-598- in or control mimics were performed using Lipofectamine 2000 reagent. A total of 48 h following transfection, firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) according to manufacturer's protocol.

Bromodeoxyuridine (BrdU) labeling and immunofluorescence. Following transfection cells were grown on cover slips (Thermo Fisher Scientific, Inc.) and were incubated with BrdU for 1 h and then stained with an anti-BrdU antibody (20-BS17; 1:500; Upstate Biotechnology, Inc., Lake Placid, NY, USA) according to the manufacturer's protocol. Gray level images were acquired using a laser-scanning microscope (Axioskop 2 plus; Carl Zeiss AG, Oberkochen, Germany).

Statistical analysis. All statistical analyses were performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL,

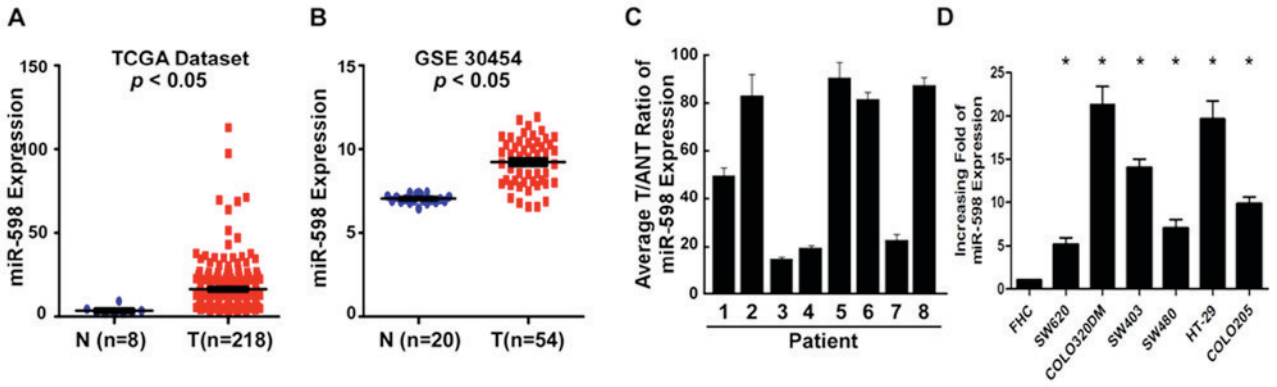

Figure 1. Expression of miR-598 in human CRC tissues and cell lines. (A) The expression levels of miR-598 in CRC tissues from the TCGA dataset. (B) Analysis of miR-598 data from the Gene Expression Omnibus database. (C) Relative miR-598 mRNA expression levels in eight paired primary CRC tissues and the matched ANT from the same patients were analyzed by PCR. (D) Reverse transcription-quantitative PCR analysis of miR-598 expression in FHC cells and CRC cell lines, including SW620, COLO320DM, SW403, SW480, HT-29 and COLO205. *P<0.05 vs. FHC. CRC, colorectal cancer; PCR, polymerase chain reaction; miR, microRNA; TCGA, The Cancer Genome Atlas; ANT, adjacent non-tumor tissues; GSE30454; Gene Expression Omnibus dataset; T, primary CRC tissues.

USA). All data are expressed as the mean ± standard deviation. Student's t-test was used to evaluate the significance of the differences between two groups of data and one-way analysis of variance followed by Tukey's post hoc test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-598 expression was elevated in CRC tissues and cell lines. To investigate the potential roles of miR-598 in CRC, the expression data downloaded from TCGA and GEO (accession number GSE30454) was analyzed and indicated that miR-598 expression was notably upregulated in CRC tissues (n=218) compared with normal colorectal tissue (n=8; Fig. 1A). The results of GEO analysis demonstrated that compared with normal colorectal tissue (n=20), the expression of miR-598 was significantly upregulated in CRC samples (n=54; P<0.05; Fig. 1B). In addition, RT-qPCR was performed to analyze the expression of miR-598 in CRC and demonstrated that compared with the matched tumor adjacent tissues, miR-598 expression was upregulated in the CRC tissues (Fig. 1C) and in all six tested CRC cell lines (SW620, COLO320DM, SW403, SW480, HT-29 and COLO205) had significantly upregulated expression of miR-598 compared with the normal colonic cell line FHC (P<0.05; Fig. 1D). These results indicated that miR-598 was upregulated in CRC cell lines and tissues. miR-598 expression in SW480 cells was decreased compared with COLO320DM, HT-29, SW403 and COLO205, but it was increased compared with SW620. SW480, therefore appeared to be a suitable cellular model for processing up and down-regulation of miR-598.

miR-598 promoted CRC cell proliferation whereas miR-598-in inhibited proliferation. In order to investigate the role of miR-598 in CRC, an miR-598 mimic, miR-598-in or the relative controls were transiently transfected into SW480 cells. Relative miR-598 expression in SW480 cells was confirmed using RT-qPCR (Fig. 2A and B). Using MTT and anchorage-independent growth assays, it was observed that miR-598 promoted cell proliferation (Fig. 3A and C), while the growth rate of SW480 cells following transfection with miR-598-in was decreased (Fig. 3B and D), compared with respective NC-transfected cells. The results of the BrdU analysis indicated that miR-598 overexpression significantly increased the percentage of BrdU positive cells (P<0.05; Fig. 4A), while BrdU positive cells were significantly reduced in SW480 cells with miR-598-in (P<0.05; Fig. 4B). Additionally, flow cytometry indicated an increase in the percentage of cells in the S phase and a decrease in the percentage of cells in G_1/G_0 phase in miR-598 transfected SW480 cells (Fig. 4C). In SW480 cells transfected with miR-598-in, the number of cells in the S phase of the cell cycle decreased and the number in G_1/G_0 phase increased (Fig. 4D). These results suggested that miR-598 serves an important role in cell proliferation and cell cycle progression in CRC cells.

miR-598 directly targets INPP5E by binding to its 3'-UTR and alters levels of proteins associated with cell proliferation and cell cycle in CRC cells. Using the miR target prediction website (www.targetscan.org), INPP5E was demonstrated to act as the potential target of miR-598. The wild type INPP5E 3-UTR mRNA contained a length of conserved sequence with miR-598 and a mutant miR-598 was constructed (Fig. 5A). Western blot analysis of the INPP5E expression levels demonstrated that INPP5E levels in the miR-598 mimic transfection group were significantly lower than those in the NC transfection group, whereas cells in the miR-598-in group demonstrated significantly increased INPP5E levels compared with the NC (P<0.05; Fig. 5B).

To confirm that INPP5E is directly targeted by miR-598, the present study investigated whether miR-598 recognizes the 3'UTR of INPP5E mRNA using a dual-luciferase reporter assay. The relative luciferase activity of the reporter containing the wild-type INPP5E 3'-UTR was significantly decreased following co-transfection with miR-598; however, this was notably increased following co-transfection with miR-598-in. The luciferase activity was not significantly affected by co-transfection with the miR-598-mut (P<0.05; Fig. 5C).

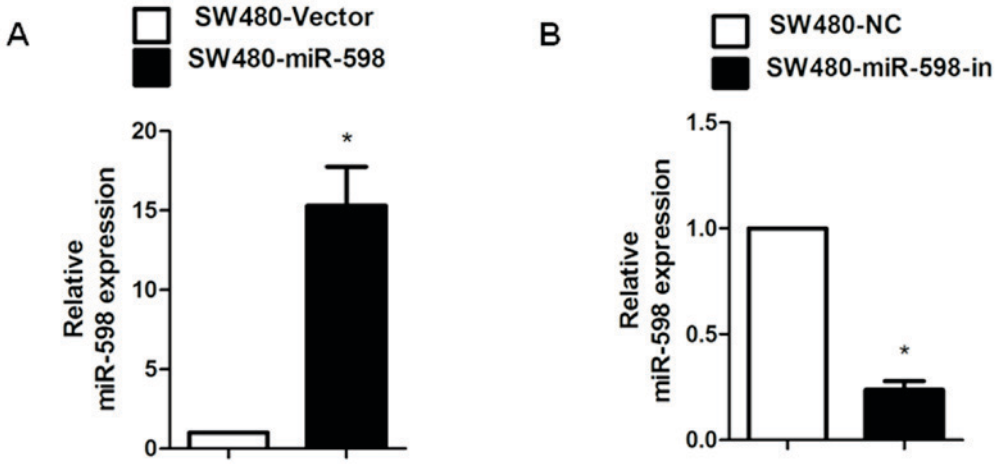

Figure 2. Investigation of miR-598 expression levels in SW480 following transfection with (A) miR-598 or (B) miR-598-in, using reverse transcription-quantitative polymerase chain reaction. *P<0.05.

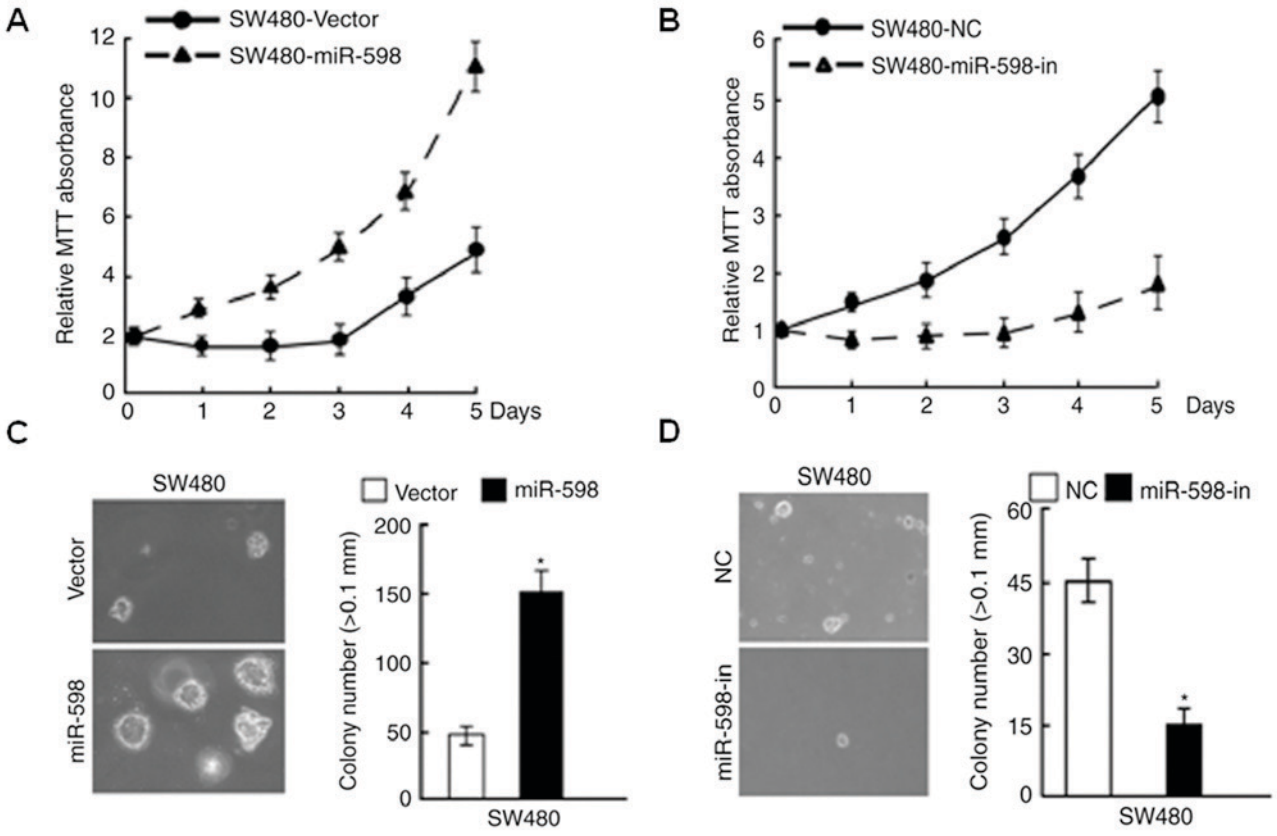

Figure 3. miR-598 upregulation promoted, while miR-598-in inhibited, CRC cell proliferation. (A) MTT assays revealed that upregulation of miR-598 promoted growth of SW480 cells. (B) MTT assay revealed that inhibition of miR-598 suppressed growth of SW480 cells. (C) Upregulation of miR-598 promoted the anchorage-independent growth of SW480 cells. Representative micrographs and quantification of colonies that were >0.1 mm. (D) Inhibition of miR-598 suppressed the anchorage-independent growth of SW480 cells. Representative micrographs and quantification of colonies that were >0.1 mm. Each bar represents the mean of three independent experiments. *P<0.05 vs. vector.

To analyze the effect of miR-598 on the expression levels of mRNA and proteins associated with cell proliferation and cell cycle progression in CRC cells, miR-598 or miR-598-in were transfected into SW480 cells and RT-qPCR and western blot analysis were performed. The results indicated that mRNA and protein expression of cyclin D1 expression was significantly upregulated and p27 was downregulated by miR-598 (P<0.05; Fig. 5D). In contrast, the expression level of cyclin D1 was downregulated and p27 was upregulated in SW480 cells transfected with the miR-598-in (Fig. 5E). These results confirmed that INPP5E is a target of miR-598.

Silencing of INPP5E counteracted the cell growth arrest caused by an miR-598-in. To further investigate the role of INPP5E in CRC, specific siRNAs against INPP5E were designed and synthesized. Western blot analysis indicated

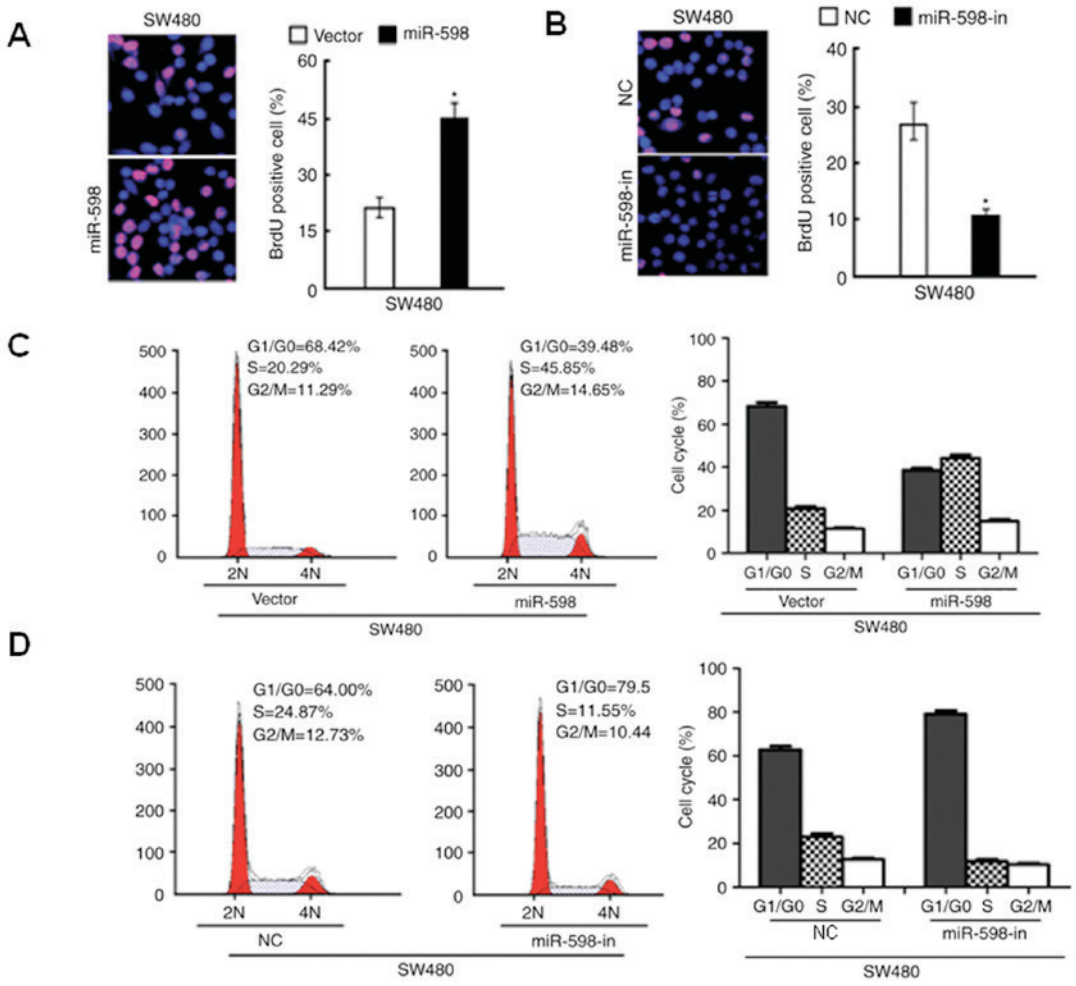

Figure 4. miR-598 upregulation promoted, while miR-598-in inhibited CRC cell proliferation and cell cycle. Representative micrographs and quantification of the BrdU incorporation assay in SW480 cells following (A) transfection with miR-598. (B) Representative micrographs and quantification of the BrdU incorporation assay in SW480 cells after transecton with miR-598-in. (C) Flow cytometric analysis of the indicated CRC cell line SW480 cells transfected with vector or miR-598. (D) Flow cytometric analysis of the indicated CRC cell line SW480 cells transfected with NC or miR-598-in. Each bar represents the mean of three independent experiments. *P<0.05 vs. vector. CRC, colorectal cancer; NC, negative control; miR, microRNA; BrdU, bromodeoxyuridine.

that INPP5E protein level was decreased following treatment with siRNAs in SW480 cells transfected with miR-598-in (Fig. 6A). The results of anchorage-independent growth assays demonstrated that knockdown of INPP5E counteracted the growth arrest by miR-598-in (Fig. 6B). In addition, silencing of INPP5E also increased the percentage of BrdU positive cells in SW480 cells transfected with miR-598-in compared with the NC (Fig. 6C).

Discussion

In the present study, miR-598 was demonstrated to be upregulated in CRC tissues in comparison with matched non-tumor tissues. Additional experiments using RT-qPCR indicated that the expression of miR-598 was upregulated in CRC tissues and cells compared with the matched tumor adjacent tissues and the normal colonic cell line FHC. Biological functions of miR-598 in CRC were investigated using gain or loss of function studies. miR-598 was determined to act as a tumor promoter by targeting INPP5E.

Recently, a number of studies have indicated dysfunction of miRs may lead to a variety of disorders, as a result of the impact on the target genes regulated by the miRs (15-17). It has been previously reported that a number of miRs serve essential roles in CRC aggression (18-20). The discovery of miRs provided novel insight into understanding the molecular mechanisms and treatment of cancer. MiR-219-5p was reported to serve a tumor suppressive role in colon cancer by targeting oncogene Sal-like protein 4 (21). miR-320b was demonstrated to suppress cell proliferation of human CRC by targeting c-Myc (22). Cai *et al* (23) indicated that miR-211 expression promoted CRC cell growth by targeting CHD5. Zhang *et al* (24) demonstrated that miR-638 suppresses cell proliferation, invasion and regulated cell cycle by targeting tetraspanin-1 in human colorectal carcinoma. Liang *et al* (25) demonstrated that miR-892a promoted cell proliferation in human CRC by regulating serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B α isoform expression. In the present study, the expression of miR-598 in CRC samples from TCGA and GEO was analyzed, which demonstrated that miR-598 expression was upregulated in CRC. In addition, significantly higher miR-598 expression was demonstrated in CRC tissues in comparison with matched non-tumor tissues. To investigate the association of the miR-598 upregulation and

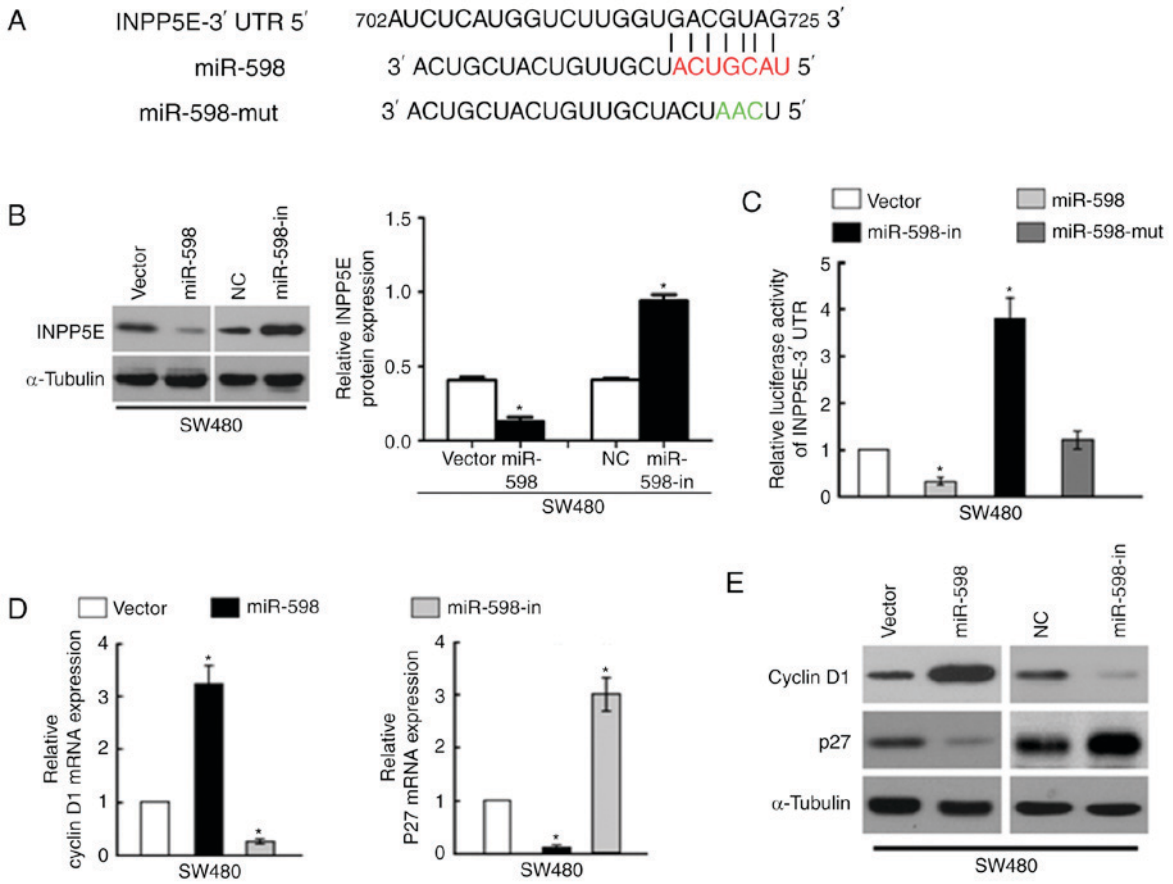

Figure 5. MiR-598 suppressed INPP5E expression by directly targeting the INPP5E 3'-UTR and altered levels of proteins associated with proliferation in SW480 cells. (A) Predicted miR-598 target sequence in the INPP5E-3'-UTR and positions of three mutated nucleotides (green) in the 3'-UTR of miR-598. (B) INPP5E protein expression in SW480 cells transfected with miR-598 or miR-598-in were detected by western blotting analysis. α-Tubulin served as the loading control. (C) Luciferase reporter assay of SW480 cells transfected with the pGL3-INPP5E-3'-UTR reporter and miR-598, miR-598-in or miR-598-mut. (D) Reverse transcription-quantitative polymerase chain reaction analysis of the expression of cyclin D1 and p27 in SW480 cells. (E) Western blotting analysis of protein expression of cyclin D1 and p27 in SW480 cells. α-Tubulin was used as the loading control. *P<0.05 vs. vector. UTR, untranslated region; mut, mutant; INPP5E, 72 kDa inositol polyphosphate-5-phosphatase; miR, microRNA; p27, cyclin-dependent kinase inhibitor 1B; in, inhibitor; NC, negative control.

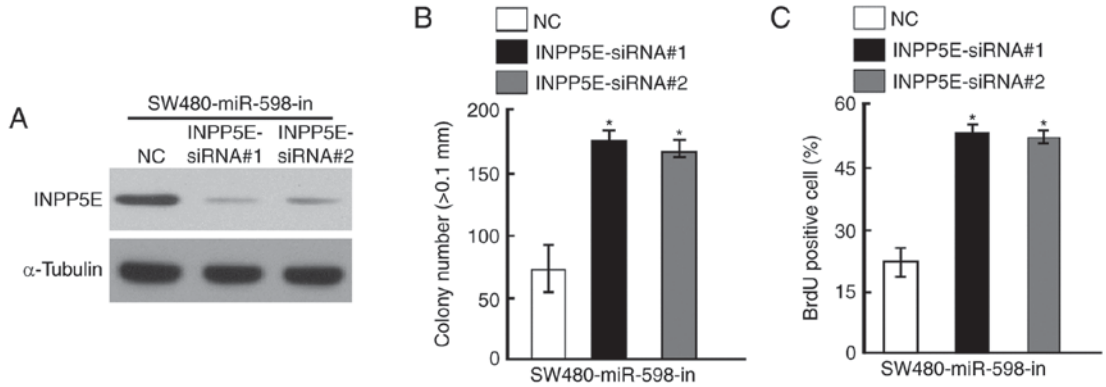

Figure 6. Silencing of INPP5E counteracted cell growth arrest caused by miR-598-in. (A) Western blot analysis verified that silencing INPP5E decreased the expression of INPP5E in miR-598-in-transfected SW480 cells. (B) miR-598-in-transfected SW480 cells following transfection with INPP5E-siRNAs promoted anchorage-independent growth. Quantification of colonies that were >0.1 mm in anchorage-independent growth assays. (C) Quantification of the BrdU incorporation assay. Each bar represents the mean of three independent experiments. *P<0.05 vs. NC. INPP5E, 72 kDa inositol polyphosphate-5-phosphatase; miR, microRNA; NC, negative control; in, inhibitor; si, small interfering.

cell proliferation in CRC, miR-598 gain or loss of-function studies were performed, the results indicated that overexpression of miR-598 promoted proliferation, whereas miR-598-in CRC inhibited cell proliferation.

To further investigate the mechanisms via which miR-598 to promoted cell proliferation and cell cycle progression in CRC, INPP5E was identified as a potential target gene of miR-598 using bioinformatics analysis. INPP5E is an inositol

polyphosphate 5-phosphatase that hydrolyzes the 5-phosphate of phosphatidylinositol 3,4,5 phosphatase 3 [PtdIns(3,4,5)P3] and PtdIns(4,5)P2 and serves an essential role in cancer, diabetes and inflammation (26-28). INPP5E was reported to act as an essential inhibitor of the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin C1 signaling axis and regulated the development of renal epithelial cells (29). In the present study, bioinformatics analysis predicted INPP5E as a target of miR-598. The bioinformatics prediction was verified and the association between INPP5E and miR-598 investigated using western blotting that demonstrated INPP5E protein expression was downregulated by miR-598. In addition, miR-598 was demonstrated to recognize the 3'-UTR of INPP5E, as investigated using a dual luciferase reporter gene assay. Further functional experiments indicated that silencing of INPP5E counteracted the arrest of cell growth caused by an miR-598-in in CRC. The results of the present study suggested that miR-598 promoted CRC cell proliferation by suppressing INPP5E.

In conclusion, the present study indicated that miR-598 was frequently upregulated in CRC. miR-598 promoted CRC cell proliferation and cell cycle progression by silencing INPP5E, providing novel insight into the pathogenesis of CRC.

Acknowledgements

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