MiR-21 regulates biological behavior through the PTEN/PI-3 K/Akt signaling pathway in human colorectal cancer cells

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Abstract. The aim of this study was to determine a role of microRNA-21 (miR-21) in colorectal cancer (CRC) and to elucidate the regulation of phosphatase and tensin homologue (PTEN) gene by miR-21. MiR-21 expression was investigated in 30 CRC samples and five CRC cell lines. In this study, we show that the expression of miR-21 was overexpressed in CRC compared with adenomas and normal tissues. Patients with poor differentiation, lymph node metastasis and advanced TNM stage showed significantly high expression of miR-21. Inhibition of miR-21 in the HCT116 cell line reduced cellular proliferation, migration and invasion, induced apoptosis and inhibited cell cycle progression. The PTEN protein levels in CRC tissues and cells had an inverse correlation with miR-21 expression. Anti-miR-21-transfected cells increased PTEN protein expression without changing the PTEN mRNA level and increased a luciferase-reporter activity. MiR-21 targets PTEN at the post-transcriptional level and regulates cell proliferation and invasion in CRC. It may serve as a novel therapeutic target in CRC.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred in 2008 (1). In China, CRC remains the fifth most common cancer type and the fourth most common cause of cancer-related death (2). Moreover, the incidence of CRC is increasing rapidly in recent years in China (3). The development of CRC from normal epithelial cells to malignant carcinomas involves a multi-step process with accumulation of both genetic and epigenetic changes, leading to a temporal activation of oncogenes and inactivation of tumor suppressor genes (4). Despite research and resources dedicated to elucidating the molecular mechanisms of CRC, the molecular pathogenesis of CRC is complicated and remains unclear.

MiRNAs are a class of small non-coding 18- to 25-nucleotide-long RNAs that negatively regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of target messenger (m) RNAs; this causes translational repression or degradation (5). Up to October 2011, more than 1,400 human microRNAs have been reported (6,7). Studies have demonstrated that microRNAs play a crucial role in almost all cellular biological processes including development, metabolism, survival, differentiation, proliferation, apoptosis and the immune response (8-12). It may function as both oncomiR (oncogene-like miRNA) and tumor suppressors (anti-oncomiR) depending on the miRNAs and the tumor type (13-15).

MiRNAs have been demonstrated to play an important role in the multistep processes of carcinogenesis. Specifically, miR-21 is consistently overexpressed in diverse types of malignancy, including breast cancer (16-18), pancreatic cancer (16,19,20), cholangiocarcinoma (21), hepatocellular carcinoma (22), gastric cancer (16), esophagus cancer (23), colorectal cancer (16,24), brain tumor (25), cervical cancer (26), ovarian cancer (27), prostate cancer (16), lung cancer (16), leukemia (28,29) and osteosarcoma (30). The human miR-21 gene is located on chromosome 17q23-1 overlapping with the TMEM49 gene, a human homologue of rat Vacuole Membrane Protein 1 (VMP-1), and it has been shown to be implicated in multiple malignancy-related processes including cell proliferation, apoptosis, invasion and metastasis (16-44). According to recent reports, several significant miR-21 targets associated with malignancy have been described: phosphatase and tensin homologue (PTEN) (21,22,31,32), programmed cell death 4 (PDCD4) (23,33,34), reversion-inducing cysteine-rich protein (RECK) (35,36), maspin (37), tropomyosin 1 (TPM1) (38), heterogeneous nuclear ribonucleoprotein K (HNRPK) (39), and TAp63 (39), Sprouty (SPRY1 and 2) (40,41), ras homolog gene family member B (RhoB) (42), tissue inhibitor of metalloproteinase 3 (TIMP3) (34,35,43,44).

However, the expression of miR-21 and its target gene PTEN, as well as their relationship has not been established in CRC. The role and relevant pathway of miR-21 in carcinogenesis and development of CRC and whether its antagonism could be used as a potential treatment for CRC remains illusive. In this study,

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we analyzed the expression levels of miR-21 in samples of CRC tissues and correlated them with clinicopathological features of CRC. Subsequently, we used anti-miR-21 inhibitor (IN) antisense oligonucleotide (ASO) to transiently knockdown miR-21 in HCT116 colon cancer cells in order to evaluate the role of this miRNA in cell proliferation, apoptosis, cell cycle, invasion and migration, and investigated the expression of PTEN, its target gene, as well as its regulatory mechanism.

Materials and methods

Patients and tissue samples. CRC tissues were obtained from the 30 consecutive patients who underwent primary surgical resection of CRC between September 2008 and March 2009 at the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). No local or systemic treatment was conducted in these patients before the operation. A total of 10 adenomas as benign lesions with moderate dysplasia were resected by endoscopic mucosal resection (EMR). Then 10 specimens of normal colorectal mucosal tissues were evaluated as normal controls. Tissue samples were immediately frozen in liquid nitrogen after resection and stored at -80˚C until use. One section of each sample was stained with hematoxylin and eosin (H&E) and was used for histopathological evaluation. The study was approved by the Research Ethics Committee of Chongqing Medical University, China. Informed consent was obtained from all patients.

Cell culture. Human colorectal cancer cell lines Colo320, Lovo, HCT116, HT29 and SW480 cell lines were purchased from Shanghai Institute for Biochemistry, Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in humidified 5% CO_2 at 37˚C. Trypsin (0.25%) solution was used to detach the cells from the culture flask.

Oligonucleotide transfection. Anti-miR-21 inhibitor (5'-UCAA CAUCAGUCUGA UAA GCUA-3') and mismatched sequence negative control oligonucleotide (5'-CAGUACUUUUGUGUA GUACAA-3') were synthesized by GenePharma, Shanghai, transfected into HCT116 cells (200 nmol/well) using Oligofectamine reagent (Invitrogen, Carlsbad, CA, USA). RNA oligonucleotides were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfection efficiency was evaluated by GFP expression in control vector or real-time PCR. Medium was replaced 8 h later and cells were collected for the next experiments 48 h post-transfection. The transfection was performed in triplicates.

Plasmid construct. The 3'-UTR of the human PTEN gene was PCR amplified from human genomic DNA (5'-GCTCTAGATC CCGCCCAAGCATGAAC-3' and 5'-GCTCTAGACCATTTT ATAAATGTCATCATC-3'), and cloned into the Xba1-site of pGL3-control vector (Promega, Madison, WI, USA), which is designated pGL3-PTEN-wt after sequencing. Site-directed mutagenesis of the miR-21 target-site in the PTEN 3'-UTR was carried out using site-directed mutagenesis kit (Takara, Dalian, China), with pGL3-PTEN-wt as a template, and named pGL3-PTEN-mut (FW, 5'-TTCTCGCGATGATGTATACAGT TTTTTATG-3', RV, 5'-CTTTTATGTAAACATCATAAG CTCA-3').

Cell proliferation assay. Cells were seeded in 96-well plate at 4,000 cells per well the day before transfection. The HCT116 cells were transfected with miR-21 negative control (NC) and anti-miR-21 inhibitor (IN). Cell Counting Kit 8 (CCK8, Dojindo, Tokyo, Japan) assay was used to measure the viable, proliferating cells at 24, 48, 72 and 96 h after transfection. The absorbance at 450 nm was measured using a thermo spectrophotometer. Each cell group was measured in triplicate.

Luciferase assay. Cells were seeded in 24-well plates 24 h before transfection. HCT-116 cells were transiently transfected with wild-type (pGL3-PTEN-wt) or mutant (pGL3-PTEN-mut) reporter plasmid containing miR-21 potential binding sites in the presence or absence of miR-21 using Lipofectamine 2000. Luciferase assays were performed 36 h post-transfection using the Dual-luciferase assay system (Promega, Madison, WI, USA) and they were normalized for transfection efficiency with cotransfected Renilla luciferase. All experiments were performed in triplicate.

Matrigel invasion assay. Twenty-four hours after transfection, $2x10^4$ HCT116 cells were suspended in 0.25 ml of culture medium with 1% FBS and plated in the top chamber with matrigel-coated membrane (Becton Dickinson, USA). The cells were incubated for 36 h, after which the cells that did not invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were stained with H&E for visualization, and counted under a light microscope in 5 random fields with magnification, x400.

Scratch wound-healing motility assay. The scratch wound assay was performed as previously described (36). When HCT116 cells were seeded and grown to confluence, a scratch was set with a pipette tip running though the dish and cultured under standard conditions for 24 h. Plates were washed twice with fresh medium to remove non-adherent cells and then photographed. The cell migration was evaluated by counting cells that migrated from the wound edge. Each cell group was measured in triplicate.

Colony formation assay. HCT116 cells were transfected with negative control (50 nM) and anti-miR-21 inhibitor (50 nM) in 24-well plates at a density of $5x10^5$ cells/well. Cells were subsequently trypsinized at 24 h and seeded in 6-well culture plates (500 cell for each well) to form colonies. After 7 days, colonies were fixed in 4% paraformaldehyde and stained in a 0.1% crystal violet solution. Each cell group was measured in triplicate.

Cell cycle assay. For cell cycle analysis, parental and transfected cells in the log phase of growth were stained with propidium iodide (PI; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and examined with a fluorescence-activated cell sorting (FACS) flow cytometer (FCM) and DNA histograms were analyzed with modified software. Each test was repeated in triplicate.

Apoptosis assay. For apoptosis assays, floating and adherent cells were harvested 24 or 48 h after transfection, and then combined and washed with PBS. Annexin V in combination with PI (Santa

Cruz Biotechnology) was added to the cells and samples were analyzed within 30 min after staining. Quantification of fluorescence was done by flow cytometry as described above. Each test was repeated in triplicate.

RNA isolation and qRT-PCR. Total-RNA was extracted using TRIzol reagent (Invitrogen) for both miR-21 and PTEN-mRNA analyses according to the manufacturer's instructions.

For detection of miR-21 expression, stem-loop RT-PCR was performed as previously described (45). qPCR was carried out using SYBR-Premix Ex Taq™ (Takara) according to the manufacturer's protocol. For detection of PTEN-mRNA expression, qPCR was performed using Quantitect SYBR-Green PCR kit (Takara). β-actin was used to normalize PTEN-mRNA expression levels.

Western blot analysis. Western blot analysis was performed according to standard procedures. Total protein was isolated from HCT116 cells. Protein concentrations were determined by BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). The membrane was first incubated with antibody against PTEN, phosphorylated AKT (p-AKT), PI3K p85α (Tyr458), p-mTOR and MMP-9 (Santa Cruz Biotechnology), then with anti-β-actin antibody (Sigma, St. Louis, MO, USA) as a loading control. Signals were detected by secondary antibodies labeled with HRP and signal intensity was determined by Quantity One software.

Immunohistochemistry. For immunohistochemistry, a mouse monoclonal antibody (Zhongshan Technology, Beijing, China) were used as primary antibodies for overnight incubation at 4˚C. The sections were subsequently treated with goat anti-mouse secondary antibody, followed by further incubation with streptavidin-horseradish peroxidase complex (Zhongshan Technology). Diaminobenzidine (Zhongshan Technology) was used as a chromogen and sections were lightly counterstained with hematoxylin. The proportion of PTEN immunostaining tumor cells varied from 0 to 100%, and a four-grade scoring system was used to evaluate the degree of immunostaining: score 0, <5%; score 1, 5-25%; score 2, 25-50%; score 3, >50% of tumor cells with positive immunostaining.

Statistical analysis. All values in the present study were reported as mean ± standard deviation (SD) from three independent experiments. Statistics was determined using ANOVA, χ^2 test or Student's t-test using SPSS17.0 (Windows). The inverse correlation of PTEN protein and miR-21 expression levels was examined by Spearman correlation analysis. P-values <0.05 were considered statistically significant.

Results

MiR-21 is overexpressed in CRC tissues and clinicopathological characteristics. The results indicate that, among the 30 CRC samples analyzed, the relative expression of miR-21 ($2^{-\Delta\Delta Ct}$=4.956±1.892) was significantly upregulated compared with the adenomas (1.662±0.496) and normal tissues (1.024±0.043) (p<0.01, respectively). MiR-21 expression was significantly higher in adenomas than it in the normal tissues determined by post-hoc analyses (p<0.05). Moreover, the high miR-21 group was significantly associated with invasion depth, lymph node metastasis (present), poor differentiation and advanced TNM stage (III, IV) (p<0.05, respectively). On the other hand, no significant differences were observed regarding gender, histological type, tumor size (p>0.05, respectively), implying miR-21 might be involved in the development and metastasis of cancer, and has a prognostic implication for CRC. This result is consistent with the previous findings (46-53).

Table I. Correlation between miR-21 overexpression and PTEN in CRC.

	PTEN	
MiR-21 (n=30)	Negative (n=16)	Positive (n=14)
3.32±0.29 (n=13)	4 (30.77)	9 (69.23)
6.12±1.86 (n=17)	12 (70.59)	5 (29.41)

Spearman's rank correlation coefficient test was used for the data. The statistically significant negative correlation between miR-21 and PTEN was r=-0.396, p<0.05.

Low PTEN expression correlates with clinicopathological variables. As shown in Table I, 14 of 30 (46.67%) CRC tissues were positive for PTEN, mainly weakly positive and expressed in the cytoplasm (Fig. 1), which was distinctly lower than that in adenomas and normal controls (p<0.01). Moreover, the low PTEN was significantly associated with invasion depth, lymph node metastasis (present), poor differentiation and advanced TNM stage (III, IV) (p<0.05, p<0.05, p<0.05, p<0.01 and p<0.05, respectively). On the other hand, no significant differences were observed regarding gender, histological type (p>0.05, respectively).

PTEN protein correlates inversely with miR-21 in CRC cell lines and tissues. We determined expression levels of miR-21 and PTEN (protein and mRNA) in 5 different colorectal cancer cell lines (Fig. 2). In cell lines with high endogenous miR-21 (for example, HCT116, Colo320, Fig. 2B) as measured by miRNA qRT-PCR, a low amount of PTEN protein was observed (Fig. 2A and C), whereas cell lines with low miR-21 (for example, SW480, HT29, Fig. 3B) showed high amounts of PTEN protein (Fig. 2A and C). Across all 5 cell lines tested, we found a significant inverse correlation between miR-21 and PTEN protein levels (r=-0.972, p<0.01). However, the differences in PTEN protein are higher than for PTEN-mRNA among the cells (Fig. 2C and D) (p=0.000 for PTEN protein, p=0.010 for PTEN mRNA). These data suggest that miR-21 expression is inversely correlated with PTEN expression in human colorectal cancer cells, and it is possible that miR-21 negatively regulates PTEN post-transcriptionally.

We then surveyed correlation between miR-21 expression and PTEN expression in CRC tissues, we found 9 cases were positive for PTEN among the 13 cases of CRC tissues with relatively low miR-21 expression (3.32±0.29), but only 5 cases were positive for PTEN among the 17 cases of CRC tissues with relatively high miR-21 expression (6.12±1.86). Spearman's rank

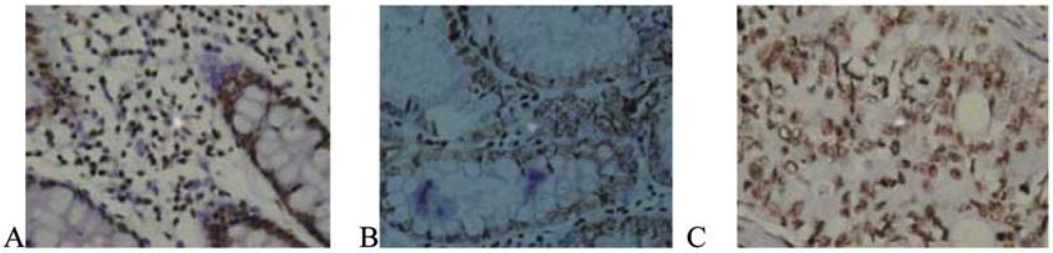

Figure 1. Representative immunohistochemistry of PTEN expression in CRC. The expression of PTEN was generally localized in the cytoplasm of the tissue specimens; (A) positive staining in adjacent normal tissue and (B) mutinous adenoma (+++, x200); (C) negative expression in CRC (x200).

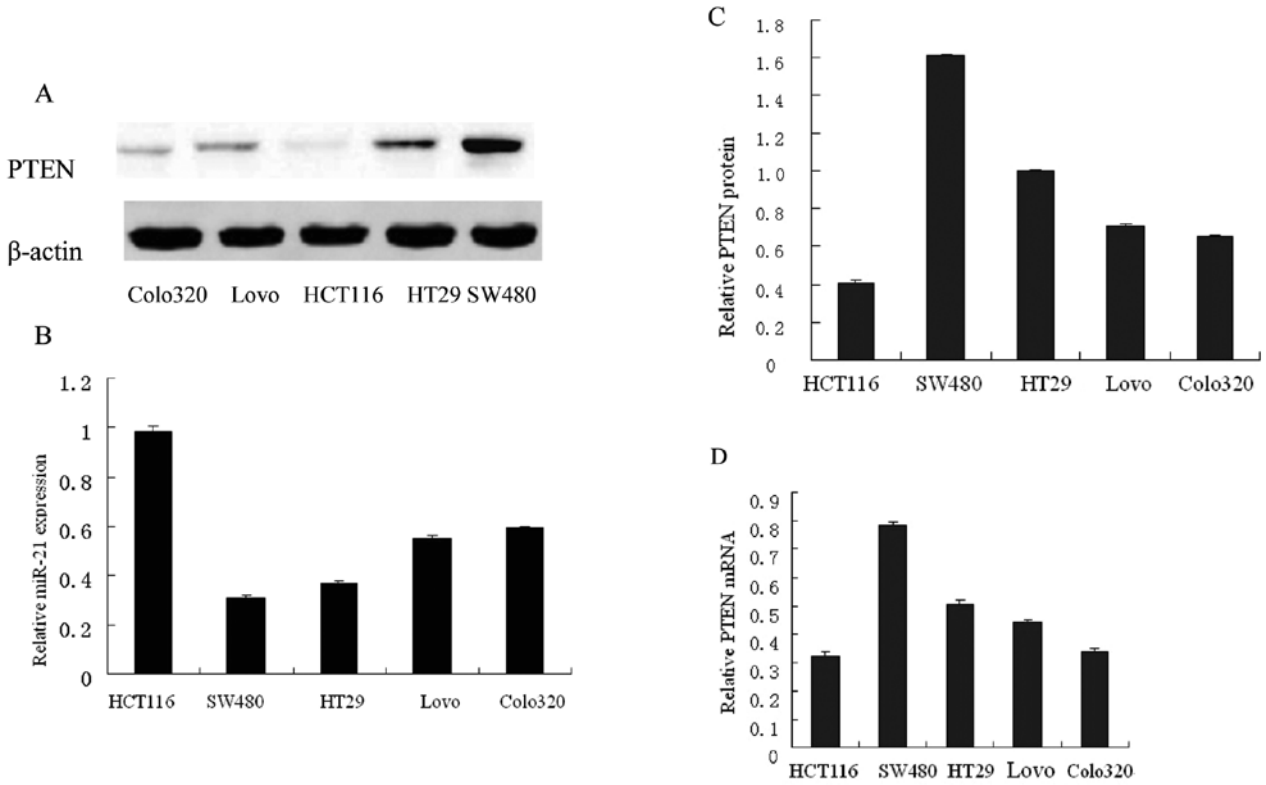

Figure 2. MiR-21 expression is inversely correlated with PTEN expression in CRC cell lines. (A) Western blot analysis for PTEN protein in 5 colorectal cancer cell lines. (B) qRT-PCR analysis for miR-21 expression in 5 colorectal cancer cell lines. The miR-21 expression was normalized to U6 expression using the 2-DDCT method. (C) The intensities of blots for PTEN protein in (A) were quantified by Quantity one soft. PTEN protein expressions are inversely correlated with miR-21 expressions ($p<0.01$). (D) qRT-PCR analysis for PTEN mRNA expression in 5 colorectal cancer cell lines. The differences in PTEN protein are higher than for PTEN-mRNA, and PTEN protein and mRNA levels inversely correlate with miR-21. Data are representative of three independent experiments performed in triplicate.

correlation analysis showed that PTEN expression was negatively correlated with miR-21 expression ($r=-0.396$, $p<0.05$) (Table I).

Anti-miR-21 inhibits CRC cell growth, proliferation and migration, and induces apoptosis. HCT116 cell line was selected to investigate miR-21 functions and targets by using sequence-specific functional inhibition of miR-21, because the cell line expressed higher levels of miR-21. Knockdown of miR-21 with ASO reduced significantly miR-21 levels in HCT116 cells (Fig. 3B). Anti-miR-21 inhibitor led to a significant decrease in HCT116 cell growth and proliferation ($p<0.01$, Fig. 3A). As shown in Fig. 3B, the number of colonies from HCT116 cells transfected with anti-miR-21 inhibitor was significantly lower than that of negative control and MOCK group ($p=0.05$). Furthermore, the size of the colonies from the cells transfected with anti-miR-21 inhibitor was much smaller than those of the control group (Fig. 3C). *In vitro* matrigel invasion assay showed that anti-miR-21 inhibitor significantly suppressed HCT116 cell invasion ($p<0.05$), compared with anti-miR-21NC and MOCK group (Fig. 4A and B). Wound healing assay showed that wound repair was delayed in anti-miR-21 inhibitor compared with anti-miR-21NC and MOCK (Fig. 5A). Anti-miR-21 inhibitor significantly suppressed HCT116 cell migration ($p<0.05$), compared with anti-miR-21NC at 24 h after wound scratch (Fig. 5B). These data demonstrate the tumorigenic properties of miR-21 in regulating cell growth, proliferation, invasion and migration.

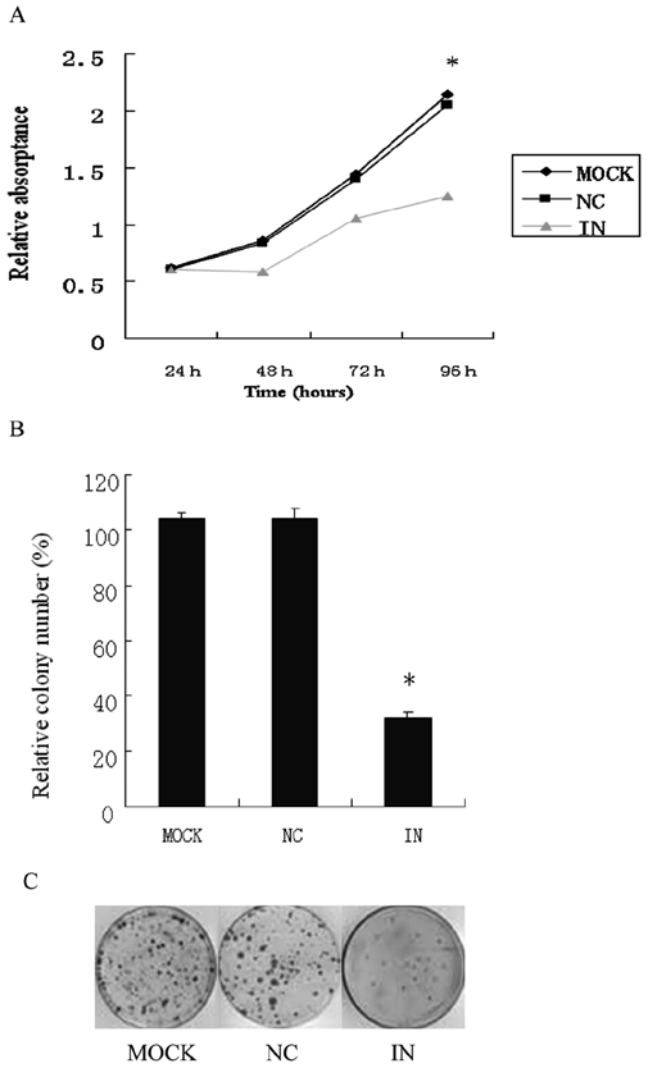

Figure 3. Inhibition of miR-21 suppresses HCT116 cell proliferation and colony formation. (A) Cell growth curve. HCT116 cells were transfected with either the negative control (NC) or anti-miR-21 inhibitor (IN). Relative cell growth was determined at indicated times by the cell proliferation assay. Values are the average ± SD of three replicates. *p<0.05. (B) The bar graph summarizes the number of colonies counted (mean ± SD, n=3, *p<0.05.) (C) Representative pictures of colonies at low magnification.

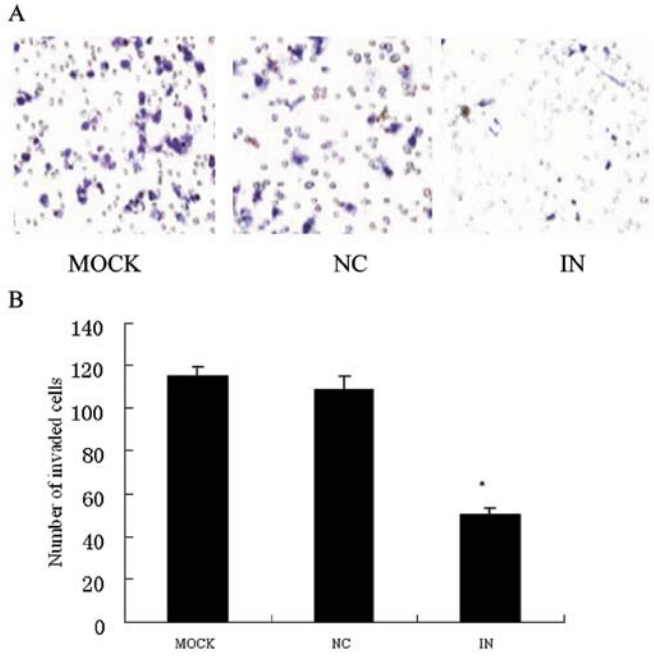

Figure 4. MiR-21 regulates cell invasion ability in HCT116 cells. (A) HCT116 cells that invaded through Matrigel-coated membrane after transfection with anti-miR-21 negative control (NC) and anti-miR-21 inhibitor (IN). (B) Quantification of HCT116 cells that invaded through Matrigel-coated membrane after transfection with anti-miR-21 inhibitor or negative control (mean ± SD, n=3, *p<0.05).

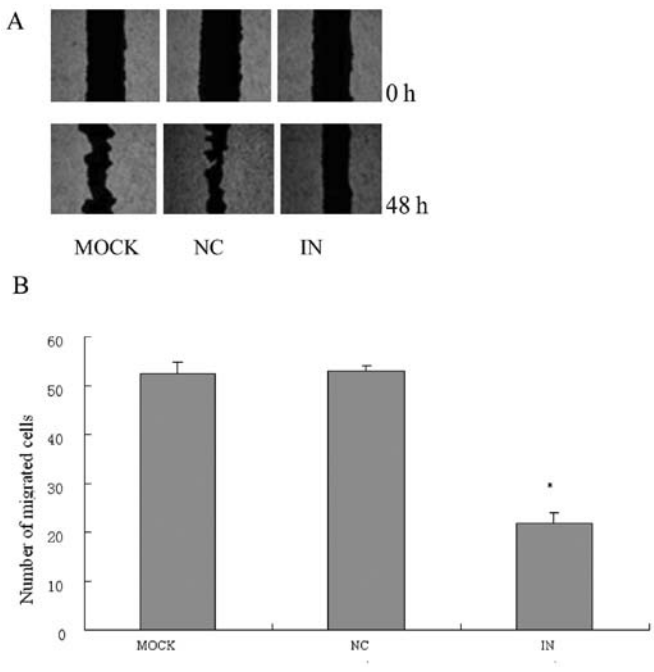

Figure 5. MiR-21 influences HCT116 cell migration ability. (A) Migration of HCT116 cells transfected with anti-miR-21 negative control (NC) and anti-miR-21 inhibitor (IN). (B) Quantification of HCT116 cells that migrated from the edge of scratch transfected with anti-miR-21 inhibitor and negative control (mean ± SD, n=3, *p<0.05).

Induced apoptosis in vitro. To further analyze whether decreased viability was a result of cell cycle arrest, the cell cycle distribution was analyzed using FCM. At 48 h after transfection, FCM analysis showed a 25% increase in G_0/G_1 phase cells and a 20% decrease in S-phase cells and a 20% decrease in G_2/M phase cells in HCT116 cells transfected with a miR-21 inhibitor ($p<0.05$, Fig. 6A and B). We also analyzed the effect of miR-21 inhibitor on apoptosis by conducting Annexin V and PI double staining. The rate of apoptosis significantly increased in cells transfected with anti-miR-21 inhibitor (48 h after transfection) compared with blank and NC control ($p<0.05$, Fig. 6C and D). It suggested that miR-21 may function as a strong antiapoptotic factor in human colorectal cancer cells.

PTEN is a direct target of miR-21 in CRC. To verify that PTEN may be a tumor-suppressor gene in CRC and miR-21 may directly target PTEN, we constructed luciferase-reporter plasmids that contain the wt or mutant 3'-UTR segments of PTEN (Fig. 7A). The wt or mutant reporter plasmid was cotransfected into HCT116 cells along with miR-21 inhibitor or negative

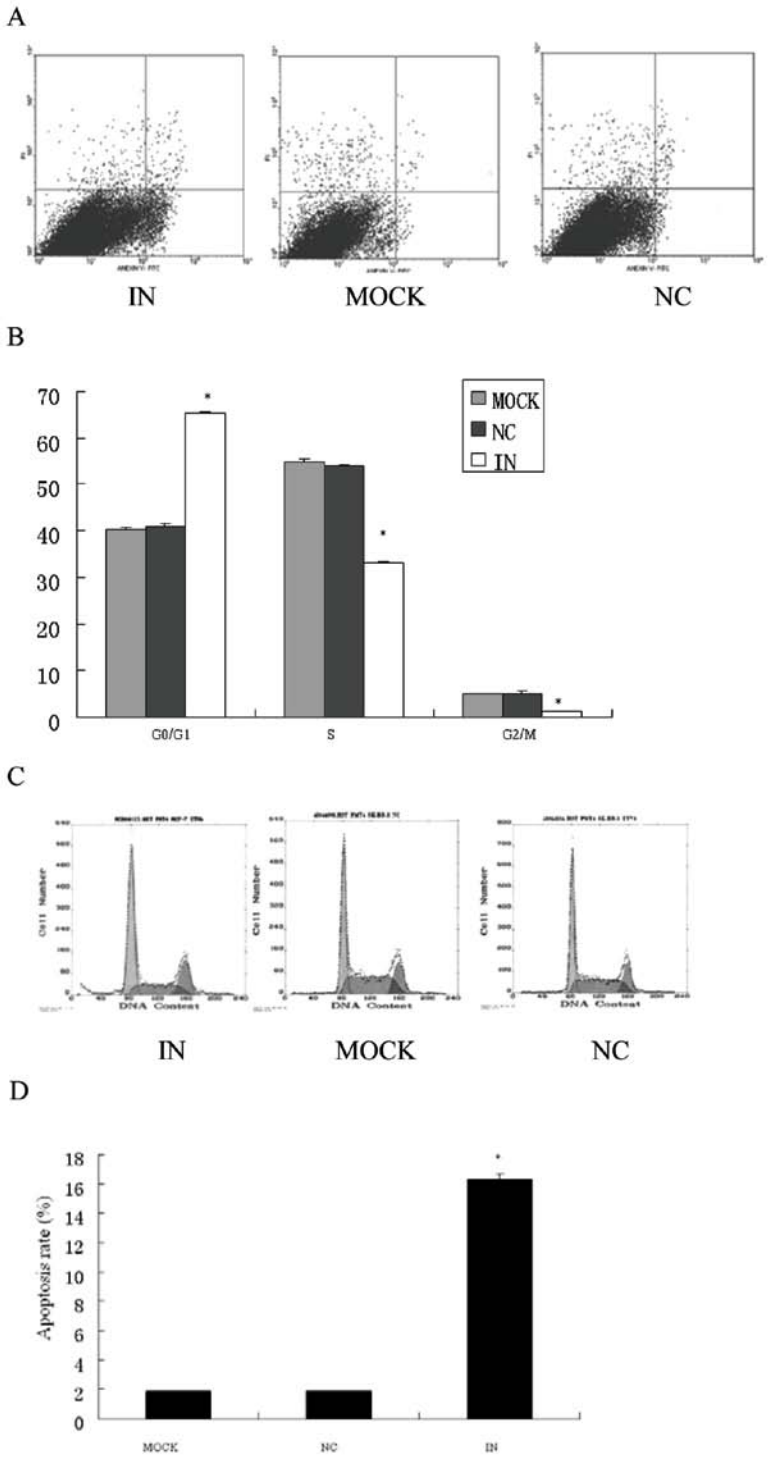

Figure 6. The effect of miR-21 on cell cycle distribution and apoptosis of HCT116 cells. (A) Proportion of cells in various phases of the cell cycle. (B) Representative histograms depicting cell cycle profiles of HCT116 cells transiently transfected with anti-miR-21 negative control (NC) and anti-miR-21 inhibitor (IN). The results are the mean of three independent experiments ± SD ($^*p<0.05$). (C) Cells staining positive for Annexin V-TITC and negative for PI at 48 h post-transfection were considered to have undergone apoptosis. (D) Representative histograms depicting apoptosis of HCT116 cells transiently transfected with anti-miR-21 negative control (NC) and anti-miR-21 inhibitor (IN). The results are the mean of three independent experiments ± SD ($^*p<0.05$).

control (NC). Compared with NC, miR-21 inhibitor significantly increased the relative luciferase activity when cotransfected with the wt reporter plasmid. However, the mutant reporter plasmid abolished miR-21 inhibitor-mediated increase in luciferase activity (Fig. 7B). These findings suggest that miR-21 suppresses PTEN by direct binding to the 3'-UTR of PTEN.

MiR-21 regulates expression of PTEN and its downstream targets. We assessed whether the downregulation of miR-21 affected PTEN protein expression in HCT116 cell lines. HCT116 cells were transiently transfected with miR-21 inhibitor, inhibitor negative control or blank control culture medium. The decrease in endogenous miR-21 levels with miR-21 inhibitor

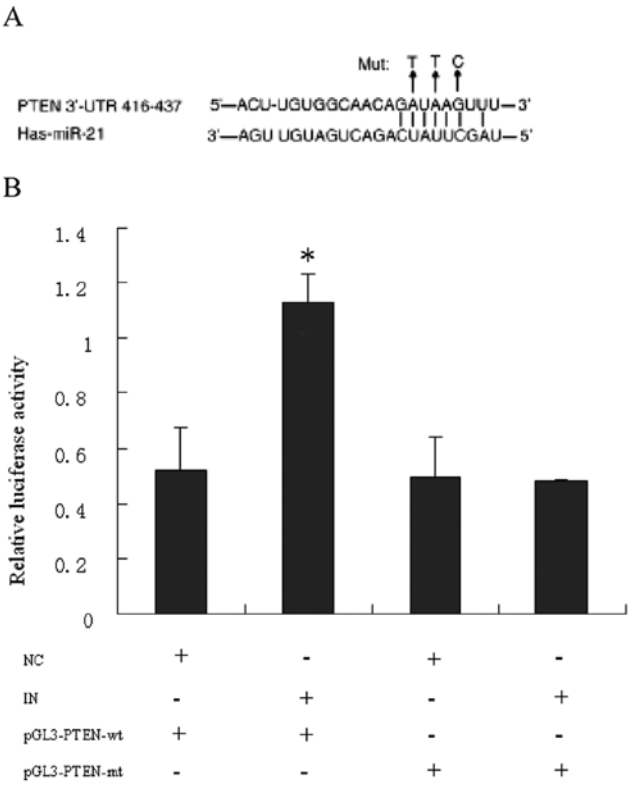

Figure 7. The 3'-UTR of PTEN mRNA is a target for miR-21. (A) Predicted miR-21 binding sites within the 3'-UTR of PTEN mRNA. The arrows display the mutational nucleotides. (B) The wt or mutant reporter plasmid was cotransfected into HCT116 cells with miR-21 inhibitor (IN) or negative control (NC). The normalized luciferase activity in the control group was set as relative luciferase activity. Luciferase activity of pGL3-PTEN-wt was significantly increased by miR-21 inhibitor (*p<0.05). However, luciferase activity of pGL3-PTEN-mut was not affected by miR-21 inhibitor (p>0.05). The data are representative of three independent experiments.

(Fig. 8B) significantly increased PTEN protein expression (Fig. 8C and A), but it almost caused no alternations in PTEN mRNA (Fig. 8D). These findings showed that miR-21 inhibits PTEN protein production, rather than degrading its mRNA. It further suggests that, in CRC, miR-21 regulates PTEN at the level of protein translation. This finding strongly suggests that PTEN is regulated by miR-21 in CRC at post-transcription level.

As AKT was an important downstream target of PTEN and expression of PTEN resulted in the reduced levels of phosphorylated AKT (p-AKT), and PTEN suppressed the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 through FAK dephosphorylation (22,31), the levels of p-AKT, PI3K, p-mTOR and MMP-9 were also examined at the same time. As shown in Fig. 8C, the expression of p-AKT, PI3K, p-mTOR and MMP-9 was concomitantly decreased by miR-21 inhibitor. In addition, siRNA to PTEN abrogated the reduction in p-Akt, PI3K, p-mTOR and MMP-9 by anti-miR-21 (Fig. 8C). In combination, these studies define an important role for PTEN as a mediator of the biological effects of miR-21 on cell proliferation and invasion in human CRC.

Discussion

Although miRNA signatures for CRC cancer have been established, the molecular mechanism of the mir-21-mediated tumorigenesis, development and metastases of CRC needs to be elucidated. In the present study, we identified miR-21 overexpression in adenomas and CRC. *In vitro*, miR-21 blockade with ASO inhibits growth and induces apoptosis in CRC cell lines, which is mediated by retrieving PTEN expression. *In vivo*, injection of miR-21 ASO into CRC xenografts implanted subcutaneously in nude mice suppressed tumor growth.

MiR-21 is one of the most prominent miRNAs implicated in human cancer. It has been proposed that miR-21 acts as a potential oncogene. MiR-21 is upregulated in various cancer types and has been associated with poor survival and poor therapeutic outcome (46-48). Its expression is obviously upregulated in diverse types of malignancies, including CRC. We found the expression levels of miR-21 were significantly higher in tumor tissues than the levels in adenomas and normal tissues (p=0.000).

Moreover, overexpression of miR-21 was closely correlated with advanced TNM clinical stage (p<0.05) and poor differentiation (p<0.05), and lymph node metastases (p<0.05) which are the main prognostic factors for CRC, implying miR-21 might be involved in the development and metastasis of cancer, and has a prognostic implication for CRC. This result is consistent with the previous findings (46-53). We further demonstrated that knockdown of miR-21 with ASO could significantly decrease cell proliferation, invasion and migration abilities in HCT116 cells. Higher expression levels of miR-21 in adenomas and carcinomas relative to normal surrounding colonic tissue suggest that this represents an early cellular event in the progression to cancer. These findings suggest that the aberrant expression of miR-21 maybe a marker for early diagnosis of CRC, directly affects invasive and metastatic potential of tumor cells and is implicated in the progression of CRC.

However, it was contradicted by another study (54), which discovered that the level of miR-21 was underexpressed in a subset of Cdc25A-overexpressing colon cancers. The reason may lie in the amount and quality of tissue samples in the latter study or the specific cell line and culture condition in the former study. Folini *et al* (55) also argued that the oncogenic properties of miR-21 could be cell and tissue dependent and that the potential role of a given miRNA as a therapeutic target should be contextualized with respect to the disease. Cheng *et al* (56) reported that inhibition of miR-21 significantly promotes proliferation in HeLa cervical carcinoma cells, which may suggest its role as a tumor suppressor. It has been considered that miR-21 may have completely different effects, depending on the cellular environment or context (26,57). It suggested that miRNAs could participate in many signal pathways that have been proven to be important in tumorigenesis, inflammation and cell growth, and miRNAs seem to be involved in a complicated network of epigenetics of cancer.

Various studies have reported that miR-21 plays an important role not only in tumor growth but also in the invasion and metastasis by targeting multiple tumor suppressor genes including PTEN, PDCD4, BCL-2, TPM1, and RECK (32-38). PTEN (phosphatase and tensin homolog deleted on chromosome 10) (58), also called MMAC1 (59) or TEP1 (60), is a tumor suppressor gene. It is localized to chromosome 10q23.2 and is often lost in late-stage human cancers, especially that of the prostate, brain and endometrium. PTEN negatively regulates intracellular levels of PI-(3,4,5)P3, most

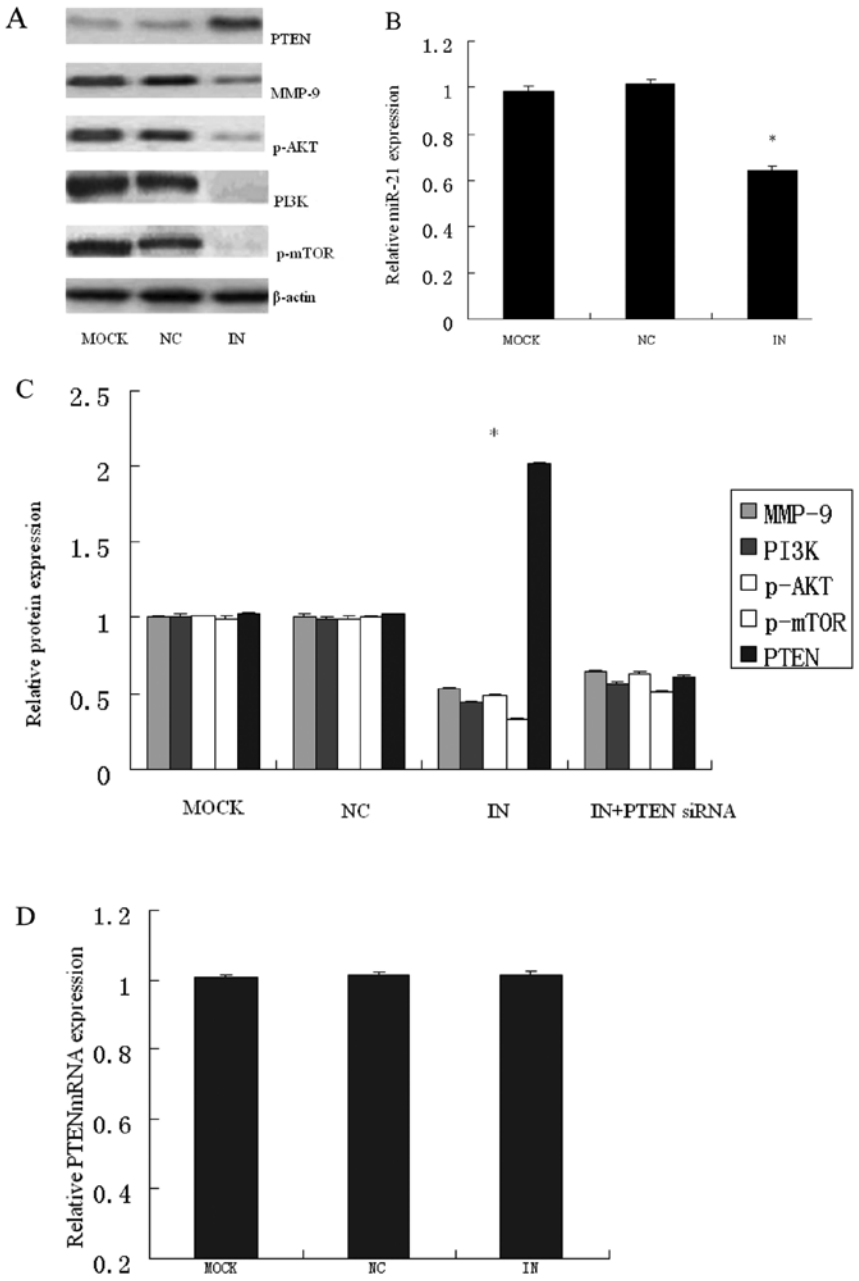

Figure 8. MiR-21 regulates PTEN expression at the post-transcriptional level and influences the phosphorylation of AKT. Cells were transfected with miR-21 inhibitor (IN), inhibitor-negative control (NC) or blank control culture medium (MOCK). Cell lysates were obtained after 48 h for analysis. (A) Representative immunoblots show that miR-21 inhibitor can increase PTEN protein expression and decrease the levels of phosphorylated AKT, MMP-9, PI3K and p-mTOR. (B) MiR-21 expression was detected by qRT-PCR. The results were normalized to U6 expression and expressed as fold change relative to the corresponding negative control. (C) The expression of PTEN, p-AKT, MMP-9, PI3K and p-mTOR protein was examined by western blot analysis. The results were normalized to β-actin protein expression and expressed as fold change relative to the corresponding negative control. In addition, siRNA to PTEN abrogated the reduction in p-Akt, PI3K, p-mTOR and MMP-9 by anti-miR-21; (D) PTEN-mRNA expression was detected by qRT-PCR. The results were normalized to β-actin mRNA expression and expressed as fold change relative to the corresponding negative control. All data are representative of three independent experiments. $^{*}p<0.05$.

likely via direct dephosphorylation, suggesting that it exerts its role as a tumor suppressor by negatively regulating the PI-3 K/PKB/Akt signaling pathway (61). This pathway is known to play a key role in numerous cellular functions including proliferation, adhesion, angiogenesis, migration, invasion, metabolism, survival and chemoresistance (62). Our study shows that 46.67% of CRC samples were positive for PTEN expression and 53.33% were negative that is consistent with previous studies (63,64).

In the present study, PTEN expression showed a negative correlation with the expression of miR-21 in CRC tissues and cell lines. It was shown that the ability of HCT116 cell proliferation and migration was inhibited significantly after transfection with miR-21 inhibitor. Furthermore, we demonstrated that inhibition of miR-21 can significantly increase PTEN expression in HCT116 cell lines, suggesting CRC might enhance malignant biological behavior by inactivating PTEN genetically and upregulating miR-21. As shown in Fig. 7, luciferase activity of

the wt, but not mutant, PTEN-3'-UTR reporter was significantly increased in HCT116 cells transfected with miR-21 inhibitor compared with negative control. Furthermore, miR-21 expression inversely correlated with PTEN protein levels, however, without obvious change in PTEN mRNA level and decreased phosphorylated AKT expression. Thus, it can be demonstrated that miR-21 negatively regulated the PTEN at the post-translational level, performing as oncogene in the CRC.

Asangani *et al* (33) reported that miR-21 may target PDCD4 directly in human CRC RKO cells and demonstrated that miR-21 induced invasion, intravasation and metastasis. Taken together, miR-21 may negatively regulate PDCD4 and PTEN, which in turn alter focal adhesion kinase phosphorylation and expression of several MMPs and thereby contribute to cancer cell migration and invasion.

Although PTEN as a target gene of miR-21 has been validated in HCC (22), breast cancer (65) and non-small cell lung cancer (31), and gastric cancer (32), Hatley *et al* (66) confirmed that PTEN was not regulated by miR-21 in non-small cell lung cancer and miR-21 high expression was not sufficient in the non-small cell lung cancer tumorigenesis model. These findings further demonstrate that miR-21, along with its known targets and a few associated genes, forms a complex regulatory network and mechanism that plays an important role in CRC formation and progression.

In conclusion, miR-21 is overexpressed in CRC, and aberrant expression of miR-21 can alter multiple biological processes of human CRC cells such as proliferation, apoptosis, migration, and invasion, probably through regulating PTEN and other critical target genes. Our data suggest that miR-21 can serve as a biomarker for CRC, and can possibly become a potential therapeutic target for CRC.

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