MicroRNA-582 promotes tumorigenesis by targeting phosphatase and tensin homologue in colorectal cancer

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Abstract. A number of studies have implicated that a class of non-coding RNAs named microRNAs (miRNAs or miRs) is associated with tumorigenesis and have identified miRNAs as promising targets for pharmaceutical intervention. Recently, the deregulated expression of miR-582 in tumor cells has been reported. However, the exact function of miR-582 in colorectal cancer (CRC) remains largely unknown. In this study, we demonstrate that miR-582 is extensively upregulated in CRC tissues and cell lines. The overexpression of miR-582 significantly enhanced the proliferation and migration ability of the CRC cells. However, the use of a specific miR-582 inhibitor counteracted these effects. miR-582 may also play an oncogenic role by promoting tumor growth in vivo. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) was identified as a putative target of miR-582; transfection of the cells with a lentivirus with miR-582 mimics substantially decreased both the mRNA and protein levels of PTEN. The restoration of PTEN expression in the CRC cells reversed the adverse effects of miR-582. Our findings therefore indicate that miR-582 promotes CRC progression by decreasing PTEN expression. These findings may also imply that miR-582 may be a target for therapeutic intervention in patients with CRC.

Introduction

Colorectal cancer (CRC) is considered one of the most malignant tumors affecting humans (1). The occurrence of CRC poses a serious threat to life and thousands of individuals succumb to the disease (2). Owing to substantial deficiencies in healthcare and physiological conditions, only a small fraction of patients are eligible for treatment. The onset of CRC has continually increased over the past decades; therefore, more and more attention has been paid to determining effective methods for the earlier diagnosis of CRC (3). However, the overall survival rate for patients with CRC remains low, largely due to the diagnosis being made at a relatively late stage. Therefore, an in-depth understanding of the underlying molecular mechanisms responsible for the disease is of utmost importance for the early diagnosis and potential therapeutic intervention for advanced CRC.

MicroRNAs (miRNAs or miRs) are well-characterized short-length non-coding RNAs capable of suppressing gene expression though base-pairing to the 3'-untranslated regions (3'-UTR) of their targets (4,5). Increasing evidence has suggested that miRNAs play important roles in the occurrence and development of CRC in recent years (6,7). Recently, miR-146 has been shown to direct the symmetric division of spheroid-derived CRC stem cells via feedback loops in Wnt signaling pathways (8). miR-130b is usually downregulated in tumor tissues and inhibits CRC progression by targeting integrin β1 (9). Zhang et al recently identified miR-520g as a potential miRNA contributing to multi-drug resistance (10). Further investigation demonstrated that the effect of miR-520g was largely mediated via the regulation of p21 expression (10). Kurihara et al recently provided direct evidence that the expression of a polycomb group protein named enhancer of zeste homolog 2 (EZH2) is highly associated with the expression of miR-31, a well-known oncogenic factor (11).

miR-582 has also been implicated in the progression of specific cancers. For example, the decreased expression of miR-582 has been implicated in the development of bladder cancer and the ectopic expression of miR-582-5p can inhibit the migration and invasion of bladder cancer cell lines (12). Another study also demonstrated that miR-582-5p was significantly upregulated in patients with tuberculosis and functioned as an anti-apoptotic factor (13). However, little information is available on the exact role of miR-582 in CRC.

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a tumor-suppressor gene located on human chromosome 10q23.3, which is both a lipid phosphatase and a protein phosphatase (14). The inactivation of PTEN results in the constitutive activation of the PI3K/AKT pathway with increased proliferation and survival (15). Of note, as previously demonstrated, the loss of PTEN may promote the progression
of CRC and PTEN can be used as a predictor of the outcome following treatment with bevacizumab (16). Therefore, PTEN may be critically involved in the pathogenesis of CRC.

In the present study, we found that the expression of miR-582 was frequently upregulated in CRC cell lines, as well as human specimens. In addition, the overexpression of miR-582 significantly promoted the colony-forming and the migration ability of HCT-116 cells. The adverse effects of the overexpression of miR-582 were partially reversed by synthetic miR-582 inhibitor. The systematic identification of miR-582 targets suggested that PTEN may be a direct target. The expression of PTEN transcript and protein was substantially inhibited by the overexpression of miR-582. miR-582 also promoted tumor xenograft growth and downregulated PTEN expression. Therefore, our results suggest that miR-582 may serve as a putative target for cancer intervention by dynamically regulating PTEN.

Materials and methods

Human samples and cell lines. The CRC cell lines used in the present study (HCT-116, SW-480, SW-620, SW-948, DLD-1 and HT-29) were all commercially obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The control epithelial colon cell line, FHC, was also purchased from ATCC. These cells were cultured in RPMI-1640 medium (Gibson, Gaithersburg, MD, USA). The medium was supplemented with 3% fetal bovine serum (FBS) and penicillin (200 U/ml) (both from Gibson) in a humidified atmosphere 5% CO2 at 37°C. The CRC specimens and adjacent normal tissues (n=83) were all surgical archives from patients at the First Affiliated Hospital, Xi’an Jiaotong University (Shaanxi, China) obtained between October 2013 and June 2015. All specimens were kept in liquid nitrogen at -80°C following resection prior to use in the experiments. All patients provided formal and signed consent forms. The protocols of the experimental procedures for the use of human samples were formally approved by the Research Ethics Committee of the First Affiliated Hospital, Xi’an Jiaotong University (no. 2013H015).

Target prediction. We used algorithms for target gene prediction TargetScan (http://genes.mit.edu/targetscan), and miRDB (www.mirdb.org), as previously described (17,18). Briefly, putative targets were ranked by Z scores. The top ranked overlapping targets were selected for experimental verification.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). The gene expression of miR-582 and PTEN in the CRC cells and tissues was measured by RT-qPCR. In brief, RNA was extracted from both the CRC cells and CRC human specimens using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcribed into cDNA using the miScript II RT kit (Qiagen GmbH, Hilden, Germany) according to the instructions provided by the manufacturer. The corresponding cDNA was then used as a template for qPCR performed using the SYBR Green PCR Master Mix kit (Applied Biosystems). All kinetic reactions for RT-qPCR were carried out using the ABI Prism™ 7000 Sequence Detection System (Applied Biosystems). Relative expression was determined. The primer sequences were as follows: PTEN forward, 5'-AGCAATGTTAAGCCCGAG-3' and reverse, 5'-TACGCCCGACTATTTAGTAGCA-3'; miR-582 forward, 5'-ACACTCGAGCTGGGCTTTGTCGACTCAT-3' and reverse, 5'-TGTAGCTTCTTAGTCG-3'; and GAPDH forward, 5'-CTCGATCTCATACGGCGTGC-3' and reverse, 5'-ATGTCGTTCTCA GCGTTGAC-3'.

Cell line transfection. The lentiviral system to ectopically express miR-582 in the HCT-116 cell line was used in the present study. The lentiviruses with miR-582 mimics (Lenti-miR-582) and negative controls (Lenti-NC) were synthesized and purchased both from Sigma-Aldrich (Shanghai, China). The miR-582 precursor, miR-582 inhibitor and the scramble control were all obtained from Sigma-Aldrich. The pcDNA3.1-PTEN plasmids were used for transfection. A scramble pcDNA3.1 plasmid was used as a control. The Lipofectamine™ 2000 system (Invitrogen, Shanghai, China) was used for viral transfection. At 24 h post-transfection, the culture medium was replaced with fresh medium. The transfection efficiency of all plasmids was experimentally verified by RT-qPCR.

Dual luciferase reporter assay. The recombinant psi-CHECK-remiR-582-WT and psiCHECK-PTEN-3'-UTR-WT vectors were constructed by linking the seed sequences with the 3'-UTR of PTEN or reverse complement of miR-582 (rcmiR-582). The combined sequences were inserted into the psi-CHECK vector. Mutant constructs were similarly obtained. The mutation spots were indicated. The constructs were verified by DNA sequencing. 293T cells (Institute of Biochemistry and Cell Biology, Shanghai, China) were loaded into a 96-well plate 24 h prior to transfection and then co-transfected with 1 μg recombinant vectors alone or vectors with additional 20 nM precursors or inhibitors. Transfection was carried out using the Lipofectamine™ 2000 system (Invitrogen). The luciferase activities were measured using the Dual-Luciferase reporter system (Promega, Madison, WI, USA) as relative luciferase units following the manufacturer’s instructions.

Wound healing and migration assay. The wound healing assay was used to quantify the migration ability of the cancer cells. The experiments were performed as previously described (19). Briefly for wound healing assay, the HCT-116 cells were seeded on 25-mm dishes and cultured in RPMI-1640 medium (Gibson). A scratch with a fixed width was formed in the cell monolayer using a 200 μl pipette tip. Subsequently, all cells were washed twice with fresh phosphate-buffered saline (PBS) and the suspended ones were then removed. Wound healing was monitored and images were acquired at 0 and 48 h after the scratch was made using a bright-field microscope (Olympus, Tokyo, Japan).

Proliferation assay. The HCT-116 cells were seeded in 96-well plates (10^3 cells/well) for 5 days. The Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was used. At an interval of 24 h, the 3-(4,5-dimethylthi-
azol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to the culture medium at a final concentration of 5 mg/ml. After 6 h, the medium was removed and crystalline formazan was dissolved in 100 µl SDS (10%; Gibson) solution for 1 day. The optical density (OD, 490 nm) was evaluated using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Colony formation assay. The cells were trypsinized to obtain a single cell suspension. Subsequently, 400 HCT-116 cells were seeded in a 12-well plate and the medium was refreshed with fresh medium every 2 days. After 6 days, the medium was discarded and the cells were stained with crystal violet (Sigma-Aldrich, Shanghai, China) (0.2% in 20% methanol). Images were acquired using a digital camera.

Xenograft implantation. In total, 10^7 HCT-116 cells transfected with the empty vector control, lentiviral miR-582 or Lenti-NC were injected subcutaneously into BALB/c nude mice into the right flank. In total, 27 mice (aged 4‑5 weeks; average weight, 19.6 g; males, 0; females, 27) were used. The mice were obtained from the Model Animal Research Center (Nanjing, China). The mice were housed in an environment with a temperature of 20˚C, 55‑60% humidity and a light‑dark cycle of 12 h. The access to food and water was provided ad libitum. The protocols for the current study (no. 15-006) were approved by the Animal Research Committee of the First Affiliated Hospital of Xi’an Jiaotong University (Xi’an, China). The mice were divided into 3 groups (n=8 per group) according to the transfected cells injected. The tumor volume was measured at an interval of 3 days for a total of 30 days. By the end of the implantation, all mice were sacrificed.

Immunochemistry. All specimens were fixed with 15% formalin and loaded in paraffin. The sections (2-µm-thick) were then examined. Deparaffinized sections were then subjected to antigen retrieval by autoclaving in 20 mmol/l citrate buffer (pH 6.0) at 120°C for 15 min. The sections were immunostained using the Histofine stain kit (Nichirei, Tokyo, Japan). The antibody against PTEN (Cat. no. P3487) used in this study was diluted at a ratio of 1:100 (Sigma-Aldrich). The stained sections were then visualized with diaminobenzidine and counterstained with hematoxylin.

Western blot analysis. The HCT-116 cells were harvested with lysis buffer (10% glycerol and 3% NP-40) obtained from Sigma-Aldrich. The protein extracts (equally 100 μg for each) were dissolved in 10% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were then coated with monoclonal anti-PTEN (Cat. no. P3487) and anti-GAPDH (Cat. no. G8795) antibodies (both from Sigma-Aldrich) over night. The HRP-conjugated secondary antibodies (1:1,000) were then added followed by incubation at 20°C for 1.5 h. The blots were then visualized using the chemiluminescence film system (Amersham Pharmacia Biotech, Shanghai, China).

Statistical analysis. The results were all analyzed using SPSS version 15.0 software (SPSS, Inc., Chicago, IL, USA). All experiments were carried out at least 3 times. P-values <0.05 were considered to indicate statistically significant differences. A paired U test was used for pair-wise comparisons.

Results

miR-582 is frequently upregulated in human CRC specimens and cell lines. We aimed to determine whether miR-582 plays a role in CRC. We compared the expression of miR-582 in 83 cases of CRC tissues and adjacent normal tissues using RT-qPCR. The results revealed that the miR-582 level was substantially increased in the cancer tissues compared with the adjacent normal tissues (Fig. 1A). We also quantified the expression of miR-582 in 6 well-characterized CRC cell lines, as well as in a normal epithelial colon cell line. The results revealed that miR-582 was also upregulated in the CRC cell lines (Fig. 1B). These results suggested that miR-582 was ubiquitously elevated in CRC in comparison with the normal controls. The upregulation of miR-582 may also suggest that miR-582 contributes to the progression of CRC. Since the HCT-116 cells displayed the relatively highest expression of miR-582, we selected the HCT-116 cell line for further in vitro analysis.
miR-582 enhances the malignant potential of CRC cells. To verify whether miR-582 has a direct affect on CRC cell proliferation, we transfected the HCT-116 cells with miR-582 precursor or inhibitor. The efficiency of transfection was verified (Fig. 2A). The results of MTT assay revealed that the overexpression of miR-582 markedly increased cell proliferation (Fig. 2B). However, using miR-582 specific inhibitors, we found that the adverse effects of miR-582 transfection were effectively reversed (Fig. 2B). Subsequently, colony formation assays were performed. The result revealed that transfection with miR-582 precursor significantly increased the number of colonies (Fig. 3C and D). However, miR-582 inhibitor markedly decreased colony formation compared with the controls (Fig. 3C and D). Finally, wound healing assay was used to evaluate the migration ability of the CRC cells. The results revealed that the migratory capacity of the tumor cells was substantially enhanced following transfection with miR-582 precursor, as evidenced by the decreased wound width (Fig. 2E and F). We also confirmed the efficacy of miR-582 inhibitor transfection owing to the significant reduction in migration (Fig. 2E and F). These results collectively suggest that miR-582 enhances the proliferative and migration ability of the CRC cells.

Identification of the potential target of miR-582. miRNAs can post-transcriptionally regulate gene expression and exert their biological effects (20). In this study, to identify the molecular mechanisms of action of miR-582 in CRC, we used multiple algorithms to predict the target of miR-582 (microRNA.org, http://www.microrna.org/microrna/home.do and MIRDB, www.mirdb.org). It was suggested that PTEN may be a direct target. To confirm the targeting effect of miR-582, luciferase reporters were used. Vectors containing the seed sequences were used (3'-UTR) (Fig. 3A). The psiCHECK
Figure 3. Identification of the target of miR-582. (A) Alignment of miR-582 with rcmiR-582 (rcmiR-582-WT), PTEN (PTEN-3'-UTR WT), mutant rcmiR-582 (rcmiR-582-MUT) and mutant PTEN (PTEN-3'-UTR MUT). (B) Dual luciferase activity using empty plasmids alone or in combination with miR-582 precursor and miR-582 inhibitor. The rcmiR-582-WT and rcmiR-582-MUT were used as controls. (C) Dual luciferase activity using empty plasmids alone or in combination with miR-582 precursor and miR-582 inhibitor. The PTEN-3'-UTR WT or PTEN-3'-UTR MUT were used separately. (D) The mRNA level of PTEN was determined by RT-qPCR. (E) The protein expression of PTEN was evaluated by western blot analysis. GAPDH was used as a control. Error bars denote the means ± SD. **P<0.01 vs. control.

Figure 4. miR-582 exerts its effects by targeting PTEN. (A) MTT assay was performed to evaluate the proliferation of transfected HCT-116 cells as indicated. (B) Wound healing assays for HCT-116 migration. Wound healing was determined at 48 h after the scratch was made. The width of the gap was determined to evaluate cell migration. (C) The results in (B) were quantified. Statistical significance is shown, "**"P<0.01 vs. control.
SONG et al: miR-582 IN COLORECTAL CANCER

empty vector (negative control) and reverse complementary miR-582 (rmiR-582, positive control) were utilized (Fig. 3A). The efficiencies for positive and negative controls were verified (Fig. 3B). The results revealed that miR-582 precursor significantly inhibit the luciferase activities of the constructs containing PTEN 3'-UTR WT (Fig. 3C). However, the effect of miR-582 was severely reduced when the 3'-UTR of PTEN was mutated (Fig. 3C). Moreover, miR-582 can directly target PTEN in CRC cells, as miR-582 was found to decrease the transcripts of PTEN (Fig. 3D). The protein level of PTEN was also downregulated following transfection with miR-582 precursor (Fig. 3E). Transfection with miR-582 inhibitor reversed the effects of miR-582 on PTEN expression at both the mRNA and protein level (Fig. 3D and E). These results thus suggest that miR-582 directly targets PTEN for tumorigenesis.

miR-582 targets PTEN to promote malignant phenotypes in CRC. To further evaluate the effects of miR-582 in CRC, we transfected the HCT-116 cells with pcDNA3.1-PTEN alone or together with miR-582 precursor. The results revealed that transfection with pcDNA3.1-PTEN substantially decreased the proliferation of the HCT-116 cells (Fig. 4A). However, the effect of pcDNA3.1-PTEN transfection was counteracted by transfection with miR-582 precursor (Fig. 4A). Wound healing assays also demonstrated that the migratory ability of the HCT-116 cells was decreased by transfection with pcDNA3.1-PTEN (Fig. 4B and C). Consistently, transfection with miR-582 precursor promoted cell migration (Fig. 4B and C). However, the adverse effects of transfection with miR-582 precursor were somehow neutralized by co-transfection with pcDNA3.1-PTEN, as co-transfection with miR-582 precursor and pcDNA3.1-PTEN increased cell migration compared to that observed with transfection with pcDNA3.1-PTEN alone, but not to the extent observed with miR-582 precursor transfection (Fig. 4B and C). These data suggest that miR-582 promotes the progression of CRC by directly targeting PTEN.

miR-582 promotes tumor growth in vivo. To further verify the effects of miR-582 in vivo, HCT-116 cells were first transfected with empty control, Lenti-NC or Lenti-miR-582. Subsequently, the HCT-116 cells were subcutaneously injected into nude mice into the right flank. The xenograft model comprised of 3 groups each containing 9 mice. The tumor volume was monitored every 3 days. After 30 days, all mice were sacrificed. We found that the injection of cells transfected with Lenti-miR-582 led to a significantly increased tumor weight (P<0.01; Fig. 5A). Moreover, the growth rate of the Lenti-miR-582-transfected xenografts was also substan-
PTEN is decreased in patients with cancer and its deficiency miRNAs can target PTEN to regulate tumorigenesis (28,36-38). Inhibiting tumorigenesis in the progression of CRC, the systematic delivery of miR-582 has been proven to be effective in inhibiting lung cancer (30). The administration of formulated miRNAs has also been investigated in osteosarcoma (31). A number of studies have suggested that even the same miRNA can play different roles under specific manner. For example, Jiang et al. found that miR-492-5p inhibited bladder cancer progression (12). A more recent study suggested that miR-582 targets Rab27a and promotes tumor cell progression, although their findings were not conclusive (26). In glioblastoma stem cells, miR-582-5p was shown to inhibit apoptosis by targeting caspase-9, caspase-3 and possibly Bim (27). Therefore, the exact function of miR-582, particularly in CRC remains largely elusive. In this study, we report that miR-582 targets PTEN and promote tumorigenesis in CRC. Both in vitro and in vivo experiments demonstrated that miR-582 increased the oncogetic potential of CRC cells. The inhibition of miR-582 expression by transfection with miR-582 inhibitor counteracted the adverse effects of CRC cells, suggesting that targeting miR-582 may provide a potentially effective strategy with which to annihilate malignant tumors, particularly CRC. Notably, miRNAs can both function as oncogenic factors or tumor suppressors in a cell type-specific manner. For example, Jiang et al. found that miR-492-5p promoted tumorigenesis in hepatocarcinoma by targeting PTEN (28). However, the decreased expression of miR-492 was also shown to contribute to oxaliplatin resistance by increasing CD147 expression in colon cancer cell lines, implying that miR-492 may serve as a tumor suppressor (29). These findings suggest that even the same miRNA can play different roles under different genetic backgrounds.

miRNA-based therapeutics have been extensively reported in recent years. For example, the systematic delivery of miR-34a has been proven to be effective in inhibiting lung cancer (30). The administration of formulated miRNAs has also been proven not to significantly affect blood chemistry, guaranteeing the safety of miRNA delivery (30). In addition, recent study demonstrated that the delivery of synthetic miR-143 inhibited the migration of osteosarcoma (31). A number of studies have suggested that the delivery of miRNAs alters the tumorigenic potential of various tumors (32-35). Given the role of miR-582 in the progression of CRC, the systematic delivery of miR-582 inhibitor may provide an alternative method with which to inhibit tumorigenesis.

PTEN is an important tumor suppressor gene and various miRNAs can target PTEN to regulate tumorigenesis (28,36-38). PTEN is decreased in patients with cancer and its deficiency is usually associated with tumorigenesis (39). Accumulating evidence has suggested that PTEN inhibits tumor progression by abolishing the PI3K/AKT pathway through the dephosphorylation of PIP3 (40). Moreover, PTEN has also been implicated in a positive feedback loop mediated through the tumor suppressor p53 (41). In this study, we confirmed that PTEN may be a direct target of miR-582 via multiple strategies. Therefore, miR-582 may also participate in the regulation of other tumor suppressor pathways via intrinsic feedback loops. These implications may provide the basis for designing effective miR-582 inhibitors for the targeting of miR-582 to promote tumor suppression.

In conclusion, our study demonstrates that miR-582 promotes CRC progression and serves as an oncogenic factor. PTEN was predicted to be the direct target of miR-582. By regulating PTEN expression, miR-582 substantially increases the proliferation and migration of CRC cells. Our findings suggest that miR-582 may serve as a potential target for CRC intervention in the future.


