miRNA-338-3p suppresses cell growth of human colorectal carcinoma by targeting smoothened

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Abstract

AIM: To investigate the regulative effect of miRNA-338-3p (miR-338-3p) on cell growth in colorectal carcinoma (CRC).

METHODS: The lentiviral vector pLV-THM-miR-338-3p and pLV-THM-miR-338-3p-inhibitor were constructed. The recombinant viral vector encoding the pre-miR-338-3p or miR-338-3p-inhibitor and the two packaging plasmids psPAX2 and pMD2.G were cotransfected into human embryonic kidney 293T cells to package lentivirus. The supernatant containing the lentivirus particles was harvested to determine the viral titer, and this supernatant was then used to transduce CRC-derived cell line, SW-620. Flow cytometry was utilized for sorting the green fluorescent protein (GFP) + cells to establish the SW-620 cell line stably expressing pre-miR-338-3p or miR-338-3p-inhibitor. Moreover, the expression of miR-338-3p was determined by real-time reverse transcriptase polymerase chain reaction, and Western blotting was used to detect the expression of the smoothened (SMO, the possible target of miR-338-3p) protein in SW-620 cells. Furthermore, the status of CRC cell proliferation and apoptosis were detected by 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide assay and flow cytometry, respectively.

RESULTS: Restriction enzyme digestion and DNA sequencing demonstrated that the lentiviral vector pLV-THM-miR-338-3p and pLV-THM-miR-338-3p-inhibitor were constructed successfully. GFP was expressed after the SW-620 cells were transduced by the lentivirus. Expression of miR-338-3p in SW-620 cells transduced with the lentivirus pLV-THM-miR-338-3p was significantly increased (relative expression 3.91 ± 0.51 vs 2.36 ± 0.44, P < 0.01). Furthermore, overexpression of miR-338-3p inhibited the expression of SMO protein in SW-620 cells, which showed obviously suppressed proliferation ability (cellular proliferation inhibition rate (CPIR) 61.9% ± 5.2% vs 41.6% ± 4.8%, P < 0.01). Expression of miR-338-3p in SW-620 cells transduced with the lentivirus pLV-THM-miR-338-3p-inhibitor was significantly decreased (relative expression 0.92 ± 0.29 vs 2.36 ± 0.44, P < 0.01). Moreover, the downregulated expression of miR-338-3p caused upregulated expression of the SMO protein in SW-620 cells, which showed significantly enhanced proliferation ability (CPIR 19.2% ± 3.8% vs 41.6% ± 4.8%, P < 0.01). However, anti-SMO-siRNA largely, but not completely, reversed the effects induced by blockage of miR-338-3p, suggesting that the regulative effect of miR-338-3p on CRC cell growth was indeed mediated by SMO.

CONCLUSION: miR-338-3p could suppress CRC growth by inhibiting SMO protein expression.

Key words: Colorectal carcinoma; Hsa-miRNA-338-3p; Smoothened; Lentivirus
miR-338-3p has recently been discovered and is involved in cell growth. Although miR-338-3p is known to be specifically expressed in neuronal tissue, little is known about its abundance and function during carcinogenesis. We have found that miR-338-3p might act as tumor suppressor in CRC, however, the targets that it regulates in CRC have not been established. Smoothened (SMO) protein is related to G-protein-coupled receptors, and is the key activator of the Hedgehog (Hh) signaling pathway. Upregulation of SMO in CRC is correlated with higher biological aggressiveness, advanced stage, poor differentiation, larger tumor size, and high proliferative activity. Furthermore, it is also well-known that SMO regulation, both in physiological and pathological conditions, is mostly at a post-transcriptional level. Moreover, with the application of bioinformatics predictions, we have found that miR-338-3p and SMO mRNA 3' untranslated region (UTR) have complementary binding sites. Thus, we inferred that the noncoding RNA, miR-338-3p, acts as a local regulator of SMO by binding to the 3'-UTR of its mRNA, thereby modulating CRC development. In order to verify this hypothesis, we investigated the regulatory effect of miR-338-3p on cell proliferation and apoptosis in CRC. We aimed to reveal a new regulatory mechanism of miR-338-3p in the development of CRC, and provide a new miRNA and target gene for clinical application.

MATERIALS AND METHODS

Construction of transfer vector pLV-THM-miR-338-3p and pLV-THM-miR-338-3p-inhibitor

The lentiviral vectors used in this study were pLV-THM, psPAX2, and pMD2.G, which were a transfer vector, packaging plasmid, and envelope plasmid, respectively. The sequences of interest were inserted into the transfer vector between the CMV-driven enhanced green fluorescence protein (GFP) reporter and an H1 promoter upstream of the restriction sites (MluI and ClaI). The pre-miR-338-3p and miR-338-3p-inhibitor oligonucleotides were chemically synthesized by Sangon Biotech Co. Ltd. (Shanghai, China) and were inserted between the MluI and ClaI sites of the pLV-THM plasmid. The pre-miR-338-3p and miR-338-3p-inhibitor lentiviral-based vector were transformed into competent Escherichia coli DH5α cells.

Core tip: The previous study has shown that loss of miR-338-3p expression is associated with clinical aggressiveness of colorectal carcinoma (CRC). In this study, the authors demonstrated that forced expression of pre-miR-338-3p in CRC cells suppressed cell proliferation and induced apoptosis, whereas inhibition of miR-338-3p in CRC cells promoted growth. We described miR-338-3p as a direct regulator of smoothened (SMO) expression in CRC, showing a new mechanism responsible for SMO upregulation in CRC. This study provides evidence for antiangiogenic activity of miR-338-3p in the development of CRC and it may develop as a useful biomarker or therapeutic target in CRC.

INTRODUCTION

Colorectal carcinoma (CRC) is one of the leading causes of cancer-related death worldwide with an estimated one million new cases and 500,000 deaths annually. The CRC incidence and mortality in China have increased rapidly in the past few decades. Screening for CRC allows early-stage diagnosis of the malignancy and potentially reduces mortality. New targeted therapies directed against molecules involved in the pathogenesis of CRC have recently been reported to be safe and effective. With the advent of new chemotherapeutic agents, such as angiogenesis inhibitor and transforming growth factor-α inhibitors, there is growing interest to identify new prognostic biomarkers and therapeutic targets for this disease.

miRNAs are a new class of small noncoding RNAs that regulate the expression of target genes through translational repression or mRNA cleavage/decay. Genome-wide studies have demonstrated that miRNA genes are frequently located at cancer-associated genomic regions or in fragile sites, and in minimal regions of loss of heterozygosity or of amplification, or in common breakpoint regions, indicating the potential roles of miRNAs in tumorigenesis. miRNAs have been demonstrated to play an important role in the multistep processes of carcinogenesis, either by oncogenic or tumor suppressor function. Studies of miRNAs have been extended to many types of tumors, including CRC. These studies have revealed that miRNAs may be potential diagnostic or prognostic tools for cancer, and the identification of target miRNAs is a key step for assessing the role of aberrantly expressed miRNAs in human cancer.
using the calcium chloride method, antibiotic-resistant colonies were selected on LB-ampicillin agar plates. After colony selection and further propagation, the plasmid was extracted using the alkaline lysis method. The plasmid DNA was then analyzed by restriction enzyme digestion and sequence analysis. The plasmid containing the target gene was digested with the restriction enzymes and amplified by polymerase chain reaction (PCR). The clones with positive PCR results were subjected to DNA sequencing.

**Cell lines and culture**

Human embryonic kidney 293T (HEK-293T) cells (Invitrogen, Carlsbad, CA, United States) and the human CRC-derived cell line SW-620 (Shanghai Institutes for Biological Science, CAS, China) were cultured in Dulbecco's Modified Eagle's Medium high glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, United States) at 37°C in a humidified incubator with 5% CO₂. The medium was changed every 3 d, and the cells were trypsinized with trypsin/ethylene diamine tetraacetic acid when 80%-90% confluence was reached. Cells at passages 4-8 were used for the experiments.

**Lentiviral packaging and virus collection**

Twenty-four hours prior to transfection, the HEK-293T cells in logarithmic growth phase were transduced, and the cell density was adjusted to 1.0 × 10⁶ cells/mL with complete culture medium. The cells were reseeded into 15-cm cell culture dishes and cultured for 24 h prior to transfection. The cells were 90%-95% confluent on the day of transfection. The recombinant viral vector encoding the miR-338-3p or miR-338-3p-inhibitor and the two packaging plasmids pSPAX2 and pMD2.G were extracted with a plasmid extraction kit (Invitrogen) and cotransfected into HEK-293T cells according to the manufacturer's instructions. After 8 h transfection, the cell culture medium was replaced with fresh complete medium. After 24 h transfection, the expression of GFP was determined. After 48 h transfection, the culture medium was collected and centrifuged at 4000 × g at 4°C for 10 min to remove any cellular debris. The supernatant was filtered through a 0.45-μm filter into a Plus-20 cytometer. The lentivirus without the transgene was used as the negative control for the experiments.

**Detection of miR-338-3p expression by real-time reverse transcriptase RT-PCR**

Total RNA from SW-620 cells was prepared using the TRIzol reagent (Invitrogen) after viral transduction. The precipitate was dissolved in diethylpyrocarbonate-treated water, and a nucleic acid protein analyzer (Beckman Coulter, Fullerton, CA, United States) was used to determine the RNA concentration. The purity and integrity of the RNA were identified as follows: the A₂₆₀/A₂₈₀ was ≥ 1.8, and the band ratio of 28 S RNA to 18 S RNA was ≥ 1.5 in formaldehyde denaturing gel electrophoresis. Accurate quantitation of the mature miR-338-3p was obtained using the TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, United States). The reverse transcription reaction was performed using 10 ng total RNA and the looped primers. Real-time PCR was performed using the standard TaqMan MicroRNA Assays protocol on the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States). The PCR reaction (20 μL) included 1.33 μL reverse transcription product, 1 × TaqMan Universal PCR Master Mix, No AmpErase UNG, 0.2 μmol/L TaqMan probe, 1.5 μmol/L forward primer, and 0.7 μmol/L reverse primer. The reactions were incubated in a 96-well plate at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The miR-338-3p expression level was measured using the Ct (threshold cycle) method. Ct is the fractional cycle number at which the fluorescence of each sample passes the fixed threshold. The ΔΔCT method for relative quantitation of gene expression was used to determine the miR-338-3p expression levels. The ΔCT was calculated by subtracting the Ct of U6 from the Ct of the miR-338-3p. The ΔΔCT was calculated by subtracting the ΔCT of the reference sample from the ΔCT of each sample. The fold change was calculated using the equation 2^{-ΔΔCT}. The TaqMan MicroRNA Assays for U6 RNA was used to normalize the relative abundance of miR-338-3p.

**miRNA target prediction**

The analysis of miR-338-3p-predicted targets was performed using the algorithms TargetScan (http://targetsan.org/), PicTar (http://pictar.mdc-berlin.de/) and MiRanda (http://www.microrna.org/microrna/home.do).

**Detection of SMO protein expression by Western blotting**

SW-620 cells were rinsed twice with cold PBS and were then lysed in ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.6), 0.1% SDS, 1% Nonidet P-40, and protease inhibitor cocktail (Boehringer Mannheim, Lewes, United Kingdom). The samples were cleared by centrifugation at 13 000 × g for 10 min. The cellular protein (50 μg) was subjected to SDS-PAGE and electrophoresed to polyvinylidene fluoride membranes (Immobilon, Bedford, MA, United States). After blocking in 20 mmol/L Tris-HCl, (pH 7.6) containing 150 μg/mL, the membranes were incubated with the primary antibody for SMO (1:2000) and horseradish peroxidase-conjugated secondary antibody (1:30000). The bands were visualized using enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Piscataway, NJ, United States).
mmol/L NaCl, 0.1% Tween-20, and 5% nonfat dry milk, the membranes were incubated with primary antibodies against SMO or β-actin, which was used as a sample loading control, overnight at 4 °C. The membranes were then incubated with horseradish-peroxidase-conjugated secondary antibody. The blot was developed using the ECL detection kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, United States) according to the manufacturer’s instructions.

**Cell proliferation assay**

The status of cell proliferation was determined by 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Amresco, Solon, OH, United States) assay. Exponentially growing SW-620 cells were adjusted to 2.5 × 10^5 cells/mL with DMEM, plated in 96-well plates (Corning, Corning, NY, United States) at 200 μL/well and then incubated for 12 h according to routine procedure. After being transduced with each lentivirus stock and incubated for 48 h (5 duplicate wells for each sample), 20 μL/well MTT (5 g/L) was added to each well. The medium was then removed after 4 h incubation and 100 μL/well dimethyl sulfoxide was added to dissolve the reduced formazan product. Finally, the plate was read in an enzyme-linked immunosorbent microplate reader (Bio-Rad 2550) at 490 nm. The cellular proliferation inhibition rate (CPIR) was calculated using the following formula: CPIR = (1 - average A value of experimental group/average A value of control group) × 100%.

**Apoptosis assay**

The effects of miR-338-3p on CRC cell cycle and apoptosis were examined by flow cytometry. Pretreated SW-620 cells were harvested and washed twice with PBS, fixed with 70% ethanol at −20 °C for 30 min, and stored at 4 °C overnight, then washed with PBS against SMO or β-actin, which was used as a sample loading control, overnight at 4 °C. The membranes were then incubated with horseradish-peroxidase-conjugated secondary antibody. The blot was developed using the ECL detection kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, United States) according to the manufacturer’s instructions.

**RESULTS**

**Lentivirus package and transduction**

HEK-293T cells were cotransfected with the transfer plasmid, pLV-THM-transgene, the packaging plasmid, psPAX2, and the envelope plasmid, pMD2.G. The higher-titer lentivirus was harvested as the stock virus solution. GFP was expressed 48 h after the SW-620 cells were transduced by the lentivirus, and the cells were observed under a fluorescence microscope (Figure 1A, B). This suggests that the miR-338-3p or miR-338-3p-inhibitor vector was successfully transduced into the SW-620 cells, which provides the basis for further studies regarding the molecular function of miR-338-3p in CRC cells. The GFP fluorescence cells were then identified and harvested using flow cytometry for the next experiment (Figure 1C-E).

**Real-time reverse transcriptase-PCR detecting miR-338-3p expression in CRC cells after lentivirus transduction**

To study the expression pattern of miR-338-3p in SW-620 cells after lentivirus transduction, we performed real-time reverse transcriptase (RT)-PCR to detect miR-338-3p expression in the SW-620 cells. Real-time RTPCR indicated that the miR-338-3p EDNA increased exponentially and then reached a plateau. The miR-338-3p amplification curve was a typical reverse S pattern (Figure 2A) and showed higher amplification efficiency. The miR-338-3p PCR product was 72 bp long, the corresponding Tm was 84.09 ± 0.15 °C, the melting temperature was even, and the shape of the peak was sharp (Figure 2B). As shown in Figure 2C, the expression level of miR-338-3p in the pLV-THM-miR-338-3p group was more than one-third of the expression in the control cells that were transduced with the blank pLV-THM vector, whereas the expression level of miR-338-3p in the pLV-THM-miR-338-3p-inhibitor group decreased significantly compared with the control group (P < 0.01).

Thus, we established the SW-620-miR-338-3p and SW-620-miR-338-3p-inhibitor cell lines successfully to observe the corresponding biological effect.

**SMO is a target of miR-338-3p in CRC**

Most miRNAs are thought to control gene expression by base-pairing with the miR-recognition elements found in their messenger target. We then used all three currently available major prediction programs, including TargetScan, Miranda and PicTar, to analyze the potential interaction between miR-338-3p and SMO. SMO mRNA was predicted by all of the algorithms and revealed potential miR-338-3p target sites in its 3′-UTR (Figure 3A).

To check if miR-338-3p actually affected SMO expression in CRC cells, we analyzed the consequence of the ectopic expression of miR-338-3p. We transfected the pre-miR-338-3p and miR-338-3p-inhibitor into SW-620 cells by lentivirus transduction as described above, and we searched for changes in SMO protein expression.
levels by Western blotting analysis. Introduction of pre-miR-338-3p caused a significant increase of miR-338-3p value and decreased SMO protein levels in SW-620 cells. Conversely, miR-338-3p-inhibitor caused a significant decrease of miR-338-3p value and increased SMO protein level (Figure 3B). This result strongly validates a post-transcriptional regulation of SMO protein by miR-338-3p.

**miR-338-3p suppresses proliferation and induces apoptosis in CRC cells**

SMO has a key role in the cell cycle, particularly in the growth arrest at the G1/S transition, therefore, we further tested if the cell growth potential of stably transduced CRC cells expressing miR-338-3p or miR-338-3p-inhibitor was modified as a consequence of the demonstrated SMO alteration. First, to evaluate the effect of miR-338-3p on CRC cell proliferation, growing SW-620 cells were transduced with lentivirus pLV-THM-miR-338-3p or pLV-THM-miR-338-3p-inhibitor for 48 h and the cell proliferation was determined by MTT assay. We observed a significant increase in proliferation after transduction of pLV-THM-miR-338-3p-inhibitor (Figure 4A, \( P < 0.01 \)). In contrast, pre-miR-338-3p significantly inhibited cell proliferation (Figure 4A, \( P < 0.01 \)). These data indicate that cell proliferation can
be significantly suppressed by increased miR-338-3p expression. Second, we performed flow cytometry analysis after exposure to miR-338-3p or miR-338-3p-inhibitor to investigate CRC cell-cycle phase distribution. SW-620 cells overexpressing miR-338-3p had a significant decrease in the S-phase population and an increase in the G0/G1 population compared with cells transduced with negative control lentivirus (Figure 4B, \( P < 0.01 \)). On the contrary, miR-338-3p-inhibitor significantly increased the S-phase and decreased the G0/G1 population (Figure 4B, \( P < 0.01 \)). Third, we investigated the effect of miR-338-3p on apoptosis by flow cytometry and found that apoptosis increased dramatically in SW-620 cells after transduction with lentivirus pLV-THM-miR-338-3p, suggesting that miR-338-3p may function as a strong apoptotic inducer in human CRC cells (Figure 4C-F). These
results confirm the potential tumor-suppressor activity of miR-338-3p in CRC.

**CRC cell proliferation suppression by miR-338-3p is mediated by SMO**

If miR-338-3p suppression of CRC cell proliferation was indeed mediated by SMO, we would expect that the SMO-specific and irreversible antagonist anti-SMO-siRNA would abolish this effect. To test this hypothesis, we measured the changes in proliferation induced by pre-miR-338-3p or miR-338-3p-inhibitor in CRC cells previously transfected with anti-SMO-siRNA. The aim was to study if and how the SMO-depleted cellular environment responded to pre-miR-338-3p or miR-338-3p-inhibitor. SW-620 cells were pretreated with or without anti-SMO-siRNA (50 nmol/L) for 24 h prior to transduction with lentivirus pLV-THM-miR-338-3p or pLV-THM-miR-338-3p-inhibitor, and cell proliferation was determined by MTT assay. A reduction in SMO protein levels, by means different from miR-338-3p overexpression, led to analogous outcomes. When we transfected SW-620 cells with anti-SMO-siRNA, SMO protein was reduced by about 80% (Figure 5A), and we observed the SMO-specific and irreversible antagonist anti-SMO-siRNA and pre-miR-338-3p-inhibitor, into SW-620 cells. A reduction in SMO protein levels was determined by MTT assay. A reduction in SMO protein levels, by means different from miR-338-3p overexpression, led to analogous outcomes. When we transfected SW-620 cells with anti-SMO-siRNA, SMO protein was reduced by about 80% (Figure 5A), and we observed the SMO-specific and irreversible antagonist anti-SMO-siRNA and pre-miR-338-3p-inhibitor, into SW-620 cells. A reduction in SMO protein levels was determined by MTT assay. A reduction in SMO protein levels, by means different from miR-338-3p overexpression, led to analogous outcomes. When we transfected SW-620 cells with anti-SMO-siRNA, SMO protein was reduced by about 80% (Figure 5A), and we observed the SMO-specific and irreversible antagonist anti-SMO-siRNA and pre-miR-338-3p-inhibitor, into SW-620 cells. A reduction in SMO protein levels was determined by MTT assay. A reduction in SMO protein levels, by means different from miR-338-3p overexpression, led to analogous outcomes. When we transfected SW-620 cells with anti-SMO-siRNA, SMO protein was reduced by about 80% (Figure 5A), and we observed the SMO-specific and irreversible antagonist anti-SMO-siRNA and pre-miR-338-3p-inhibitor, into SW-620 cells. A reduction in SMO protein levels was determined by MTT assay. A reduction in SMO protein levels, by means different from miR-338-3p overexpression, led to analogous outcomes. When we transfected SW-620 cells with anti-SMO-siRNA, SMO protein was reduced by about 80% (Figure 5A), and we observed the SMO-specific and irreversible antagonist anti-SMO-siRNA and pre-miR-338-3p-inhibitor, into SW-620 cells. A reduction in SMO protein levels was determined by MTT assay. A reduction in SMO protein levels, by means different from miR-338-3p overexpression, led to analogous outcomes. When we transfected SW-620 cells with anti-SMO-siRNA, SMO protein was reduced by about 80% (Figure 5A), and we observed the SMO-specific and irreversible antagonist anti-SMO-siRNA and pre-miR-338-3p-inhibitor, into SW-620 cells. A reduction in SMO protein levels was determined by MTT assay. A reduction in SMO protein levels, by means different from miR-338-3p overexpression, led to analogous outcomes. When we transfected SW-620 cells with anti-SMO-siRNA, SMO protein was reduced by about 80% (Figure 5A), and we observed the SMO-specific and irreversible antagonist anti-SMO-siRNA and pre-miR-338-3p-inhibitor, into SW-620 cells. A reduction in SMO protein levels was determined by MTT assay. A reduction in SMO protein levels, by means different from miR-338-3p overexpression, led to analogous outcomes. When we transfected SW-620 cells with anti-SMO-siRNA, SMO protein was reduced by about 80% (Figure 5A), and we observed the SMO-specific and irreversible antagonist anti-SMO-siRNA and pre-miR-338-3p-inhibitor, into SW-620 cells.

When lentivirus pLV-THM-miR-338-3p was transduced into SW-620 cells previously treated with anti-SMO-siRNA, we observed that the enhancement of cell proliferation by miR-338-3p-inhibitor was largely abrogated by anti-SMO-siRNA (Figure 5B, P < 0.01). These results indicated that the promotive effect of miR-338-3p-inhibitor on CRC cell growth was largely, but not completely, mediated by SMO, suggesting that miR-338-3p-inhibitor could also activate some SMO-independent signaling pathway to promote CRC cell growth in addition to upregulation of SMO.

**DISCUSSION**

With the advent of new chemotherapeutic agents, clarification of the molecular pathogenesis of CRC is crucial for developing effective therapeutic strategies to improve patient outcome[21,22]. The miR-338 gene is located on chromosome 17 and produces two mature forms (miR-338-3p and miR-338-5p). Tsuchiya et al[19] have reported that miR-338-3p contributes to the formation of epithelial basolateral polarity by facilitating the translocalization of β1-integrin to the basolateral membrane, which highlights a potentially important role for miR-338-3p in the process of epithelial cell differentiation. Huang et al[20] have demonstrated that a decrease in miR-338-3p expression in hepatocellular carcinoma, which is another type of epithelial-cell-derived cancer, was significantly associated with TNM stage, vascular invasion, intrahepatic metastasis, tumor size, and tumor grade. Our previous study has also shown that loss of miR-338-3p expression is associated with clinical aggressiveness of CRC. Moreover, the miR-338-3p expression was not only related to TNM stage but also to tumor invasion and migration. The level of miR-338-3p expression at TNM stages III and IV was lower than that at stages I and II, and the tumors which invaded adjacent tissues or organs had less miR-338-3p expression than those limited to the wall of the colon and rectum (data not shown). Thus, miR-338-3p may be an important tumor suppressor, which can cleave or inhibit the targeted mRNAs of tumor promoters, and play a role in the progression of CRC. However, lack of knowledge about the targets for miR-338-3p hampers a full understanding of the biological functions deregulated by miR-338-3p aberrant expression. To confirm the molecular mechanism of miR-338-3p in CRC, it is necessary to observe the biological effects of the up- and down-regulation of miR-338-3p. Thus, we constructed a CRC-derived cell line in which miR-338-3p was stably over- or under-expressed by transducing the lentivirus vector, pLV-THM-miR-338-3p or pLV-THM-miR-338-3p-inhibitor, into SW-620 cells[19]. Notably, we showed that our combined lentivirus specifically enhanced or inhibited endogenous miR-338-3p to induce the corresponding biological effect. The successful construction of the lentiviral vector provides the basis for further studies regarding the molecular function of miR-338-3p in CRC[17,18].

To extend our previous observation, we focused on the role of miR-338-3p in regulation of proliferation by miR-338-3p-inhibitor was largely abrogated by anti-SMO-siRNA (Figure 5B, P < 0.01). These results indicated that the promotive effect of miR-338-3p-inhibitor on CRC cell growth was largely, but not completely, mediated by SMO, suggesting that miR-338-3p-inhibitor could also activate some SMO-independent signaling pathway to promote CRC cell growth in addition to upregulation of SMO.
and apoptosis in CRC. We found that the proliferative potential was suppressed after restoration of miR-338-3p expression in CRC cells transduced by lentivirus vector, pLV-THM-miR-338-3p. However, the downregulation of miR-338-3p, due to transducing by lentivirus vector pLV-THM-miR-338-3p-inhibitor into SW-620 cells, induced CRC cell proliferation. Cell cycle status and apoptosis are usually closely associated. Cells failing to progress to mitosis are destined for apoptosis. Besides cell-cycle arrest, the inhibition of cell growth observed in CRC cells with pre-miR-338-3p may also be a result of increased apoptosis. In this study, treatment of lentivirus pLV-THM-miR-338-3p caused G0/G1 phase arrest and blocked cells from entering S phase. Interestingly, as seen in other tumor cells, we clearly demonstrated that pre-miR-338-3p induced significant apoptosis in CRC cells, as demonstrated by flow cytometry. These data demonstrate that miR-338-3p is a potential tumor suppressor for CRC. However, the exact mechanisms of miR-338-3p remain unknown.

With the application of bioinformatics prediction programs, such as TargetScan, PicTar and MiRanda, we found that miR-338-3p and the 3’-UTR of SMO mRNA had complementary binding sites. From this, we hypothesized that SMO may be a new target of miR-338-3p in CRC; however, this finding has not yet been reported. SMO, a seven-membrane-spanning receptor is a fundamental component of the Hh signaling pathway and an important anticancer drug target[25-27]. Once activated, SMO triggers a series of intracellular events with resultant activation of the zinc finger transcription effectors including Gli, which in turn regulates cell proliferation, differentiation, apoptosis and invasion[28-30]. It has been reported that 3-Keto-N-(aminoethyl-aminocaproyl-dihydrocinnamoyl) cyclopamine (KAAD-cyclopamine), a synthetic specific antagonist of SMO, markedly inhibits hepatocellular carcinoma cell growth and motility by binding to SMO[31]. Indeed, in our study, downregulation of SMO occurred in response to lentivirus vector pLV-THM-miR-338-3p transduction into CRC cells, and significant upregulation of SMO occurred in response to lentivirus vector pLV-THM-miR-338-3p-inhibitor transduction. Consistent with Huang et al.[32], our results suggest that SMO is a direct target of miR-338-3p in CRC cells.

We deduced that miR-338-3p inhibited CRC cell proliferation, likely through downregulating SMO. To confirm this, we performed RNA interference to knock down SMO in CRC cells before transduction with miR-338-3p-inhibitor. We showed that anti-SMO-siRNA could significantly, but not completely, inhibit miR-338-3p-inhibitor-induced proliferation of CRC cells.
In summary, we have described miR-338-3p as a direct regulator of SMO expression in CRC, showing a new mechanism responsible for SMO upregulation in CRC. These findings further outline the importance of miR-338-3p in CRC carcinogenesis. However, it should be emphasized that our results were generated from cultured CRC cells and that they might not necessarily and comprehensively reflect the situation in vivo[37]. Further experiments, beyond the scope of this study, are required to elucidate the antitumor mechanisms of miR-338-3p in athymic mice.

**COMMENTS**

**Background**
miRNAs regulate gene expression by mainly binding to the 3’-untranslated region (UTR) of the target mRNAs, leading to mRNA degradation or translation inhibition. miRNAs are aberrantly expressed in various cancers, suggesting that they play a vital role as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate.

**Research frontiers**
Colorectal carcinoma (CRC) is one of the most serious malignancies in China. Our previous study has shown that loss of miRNA-338-3p (miR-338-3p) expression is associated with clinical aggressiveness of CRC. In this study, the authors report the regulatory effect of miR-338-3p on proliferation and apoptosis of CRC cells.

**Innovations and breakthroughs**
Some human miRNAs are consistently deregulated in human cancer, suggesting a role for these genes in tumorigenesis. Authors previous study has also shown that loss of miR-338-3p expression is associated with clinical aggressiveness of CRC. The authors demonstrated that forced expression of miR-338-3p in CRC cells suppressed cell growth, whereas inhibition of miR-338-3p promoted cell growth. Furthermore, silenced (SMO) was identified as a direct target of miR-338-3p. The antiangiogenic role of miR-338-3p was determined as tumor suppressor.

**Applications**
This study indicates that miR-338-3p suppresses cell growth by targeting the SMO gene in CRC in vitro and miR-338-3p might be a novel potential strategy for CRC treatment.

**Terminology**
Most miRNAs are thought to control gene expression by base-pairing with the miR-recognizing elements, 3’-UTR, found in their messenger target. Not surprisingly, with the application of bioinformatics predictions, we find that miR-338-3p and SMO mRNA 3’-UTR has complementary binding sites.

**Peer review**
miR-338-3p could suppress CRC growth ability by inhibiting SMO protein expression. This study provides evidence for antiangiogenic activity of miR-338-3p in the development of CRC, and may be developed as a useful biomarker or therapeutic target in CRC.

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