miR-375 inhibits the invasion and metastasis of colorectal cancer via targeting SP1 and regulating EMT-associated genes

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Abstract. Accumulating evidence has shown that aberrantly expressed microRNAs (miRNAs) are associated with tumor development and progression. Our previous study found that microRNA-375 (miR-375) was downregulated in colorectal cancer (CRC), but little is known concerning the role of miR-375 and the related mechanism in CRC development. The proliferation, invasion and migration effects were investigated by Cell Counting Kit-8 (CCK-8), colony formation and Transwell assays with or without Matrigel. In addition, candidate target genes were screened and validated by luciferase reporter and western blot assays. In addition, western blot analysis was performed to explore the molecular mechanisms associated with epithelial-mesenchymal transition (EMT). It was found that miR-375 inhibited proliferation, invasion and migration in DLD1 and HCT8 cells. In addition, miR-375 negatively regulated Sp1 transcription factor (SP1) protein by directly binding to the 3'-untranslated region (3'-UTR). Furthermore, it was found that miR-375 regulated matrix metalloproteinase 2 (MMP2) and EMT-associated genes, E-cadherin, vimentin, snail, N-cadherin and β-catenin. In conclusion, miR-375 inhibited the proliferation, invasion and migration by directly targeting SP1 and regulating MMP2 and EMT-associated genes.

Introduction

Colorectal cancer (CRC) is the third most common malignant cancer diagnosed worldwide (1), and ranks as the second leading cause of cancer-related deaths in developed countries (2). Approximately 90% of CRC-related mortalities are a result of metastases (3). Metastasis is a complex, multi-step process whereby tumor cells first invade the surrounding tissues and intravasate into the vasculature, then translocate through the systemic circulation, and thus extravasate into the parenchyma of distant tissues, e.g. liver and lungs, eventually, establishing micrometastases and forming macroscopic secondary tumors (4).

Although much progress has been made in the identification and characterization of the genetic and epigenetic changes involved in CRC metastasis (5,6), the underlying mechanisms remain largely unclear. The search for predictive markers for CRC metastasis remains a priority, as it is the major cause of the high mortality rate. Recently, microRNAs (miRNAs) have become a hot spot due to their significant role in the regulation of gene expression. miRNAs are ~22-nucleotide conserved endogenous non-coding single-stranded RNA molecules (7), which bind to the 3'-untranslated region (3'-UTR) of target mRNAs and regulate the stability and translation of mRNAs, resulting in either inhibition of translation or degradation of target mRNAs (8,9). They are involved in a wide spectrum of biological processes, such as proliferation, metabolism, cellular differentiation and apoptosis (10,11). To date, a number of miRNAs have been found to be associated with cancer development and progression, including CRC. Among them, miR-375 is proven to be involved in early invasive CRC through downregulation of its expression (12-14); however, the functional role of miR-375 in CRC needs further investigation (15). In the present study, we attempted to reveal the mechanisms of miR-375 underlying the biological behavior of CRC. We investigated the biological functions of proliferation and invasion/migration in CRC cells and identified the target gene in order to explore the possible molecular mechanism involving CRC development, in hope of identifying a new predictor for prognosis or new target for diagnosis and therapy.

Materials and methods

Cell lines and transfection. DLD1 and HCT8 cells stored in our laboratory were maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Grand Island, NY, USA) at 37˚C under 5%

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CO_2. We had entrusted Beijing Microread Genetics Co., Ltd., Beijing, China for the identification of the 2 cell lines. The authentication reports (data not shown) showed no cross-contamination of other human cell lines, and 100% matched cell lines are found in ATCC banks with the DLD1 and HCT8 cell lines. Precursor miRNA (pre-miR-375) and precursor negative control (pre-neg) (Applied Biosystems, Foster City, CA, USA) were transfected into the two cell lines using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Additionally, following this method, the interfering RNA of sp1 messenger RNA (siSP1) and the negative control (si-neg) were transfected into the HCT8 cells.

RNA extraction and quantitative RT-PCR (qRT-PCR). Total RNA of the cells was extracted using mirVana isolation kit according to the manufacturer's instructions (Applied Biosystems). For validation of miRNA and target gene mRNA expression, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) using TaqMan miRNA assays and quantitative RT-PCR using SYBR-Green PCR Master Mix kit (Applied Biosystems) were performed according to the manufacturer's instructions. The expression level of U47 small nuclear RNA was used for miRNA, and GAPDH was used for mRNA as the endogenous controls. All assays were carried out in triplicate.

Cell colony formation assay. For the colony formation assay, $1x10^3$ cells were plated in 6-well plates for each well in medium with 20% FBS for 72 h, and then fixed and stained with crystal violet. Then, the colonies were photographed and counted under a microscope. All experiments were performed in 3 replicates and were repeated 3 times, independently.

Cell Counting Kit-8 (CCK-8) assay. DLD1 ($2x10^3$) and HCT8 cells ($3x10^3$) were suspended in RPMI-1640 medium (100 μl) containing 10% FBS and cultured into 96-well plates overnight, and then transfected with pre-miR-375 and pre-neg. Cell proliferation was determined using CCK-8 assay at 0, 1, 2 and 3 days after transfection, respectively, and the absorbance of the samples was measured with a spectrophotometer reader at 450 nm.

Cell migration and invasion assays. DLD1 ($2x10^5$) and HCT8 cells ($2x10^5$) were cultured into 6-well plates, at 24 h after transfection of pre-miR-375 and pre-neg, as previously described. Migration and invasion assays were performed both using 24-well Transwell migration chambers with 8-μl pore size (Corning Costar, Inc., Corning, NY, USA). DLD1 ($1x10^5$) and HCT8 cells ($5x10^4$) suspended in 100 μl corresponding culture medium without FBS were loaded into the top chamber, and the bottom chamber contained 600 μl medium with 20% FBS. For the migration assay, the cells were allowed to migrate for 12 h. In addition, for the invasion assay, Transwell wells were pre-coated with Matrigel, and the cells were allowed to invade for 48 h. When both assays were stopped, the cells that migrated or invaded into the bottom chamber were fixed with methanol for 5 min, and then stained with crystal violet (0.05%) for 4 min. The cells that migrated or invaded were photographed and counted under a microscope. In addition, the migration and invasion rates were assessed by the formula: (motile cells transfected with pre-miR-375/motile cells transfected with pre-neg). All experiments were independently repeated in triplicate.

Luciferase reporter assay. To elucidate the molecular mechanisms involved in the effects of miR-375 on CRC cells, putative miR-375 target genes were predicted through the gateway miRecords (http://mirecords.biolead.org/). To improve the accuracy of the prediction, the genes that were predicted by at least 4 of 11 databases (DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid and TargetScan) were selected as candidate targets. Wild and mutant putative targets of the Sp1 transcription factor (SP1) 3'-UTR (Fig. 3A) were cloned into pmiReport vector (Ambion, Carlsbad, CA, USA). 293T cells ($2x10^4$) were co-transfected with 500 ng of wild or mutant constructs of the SP1 3'-UTR with pre-miR-375 or pre-neg. Each sample was cotransfected with 50 ng of pRL-TK plasmid expressing *Renilla* luciferase to monitor the transfection efficiency. A luciferase activity assay was performed 48 h after transfection with the dual-luciferase reporter assay system. The relative luciferase activity was normalized to *Renilla* luciferase activity.

Western blot analysis. DLD1 and/or HCT8 cells ($1x10^5$) were incubated in 6-well plates for 72 h. Then, cell proteins were harvested and homogenized with lysis buffer (Tiangen, Shanghai, China). Proteins from the cells were resolved by 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). The membrane was incubated with primary antibody at 4˚C overnight, then the secondary antibody for 1 h at 37˚C and finally visualized by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The antibodies used were: SP1 (1:2,000; Abcam); matrix metalloproteinase 2 (MMP2) (1:1,000), E-cadherin (1:2,000), vimentin (1:500), snail (1:1,000), β-catenin (1:1,000) [all from Cell Signaling Technology (CST), Beverly, MA, USA]; β-actin (1:1,000) and GAPDH (1:2,000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Immunofluorescence analysis. HCT8 cells ($5x10^4$) were seeded into 6-well plates containing 13-mm collagen (Sigma, St. Louis, MO, USA)-coated coverslips. After 12 h, the cells were transfected with pre-miR-375 and pre-neg. Then, for another 48 h, the cells on the coverslips were washed using cooled PBS on ice twice with PBS for 5 min. Primary antibodies were diluted in 1% (w/v) BSA/PBS and applied to the coverslips and incubated on ice for 1 h. After 3 washes with PBS, coverslips were incubated with cy3-conjugated secondary antibody diluted in 1% (w/v) BSA/PBS on ice for 30 min. The cells were washed twice with PBS before coverslips were mounted onto glass slides using Gelvatol mounting medium. Slides were viewed using an Olympus IX71 microscope fitted with a U-RFL-T fluorescent lamp, and images were captured and analyzed using DP Controller software (Olympus Corporation, Tokyo, Japan).

Expression of miR-375 in metastatic CRC tissues. Fifteen snap-frozen tumor tissues from 5 patients who were surgi-

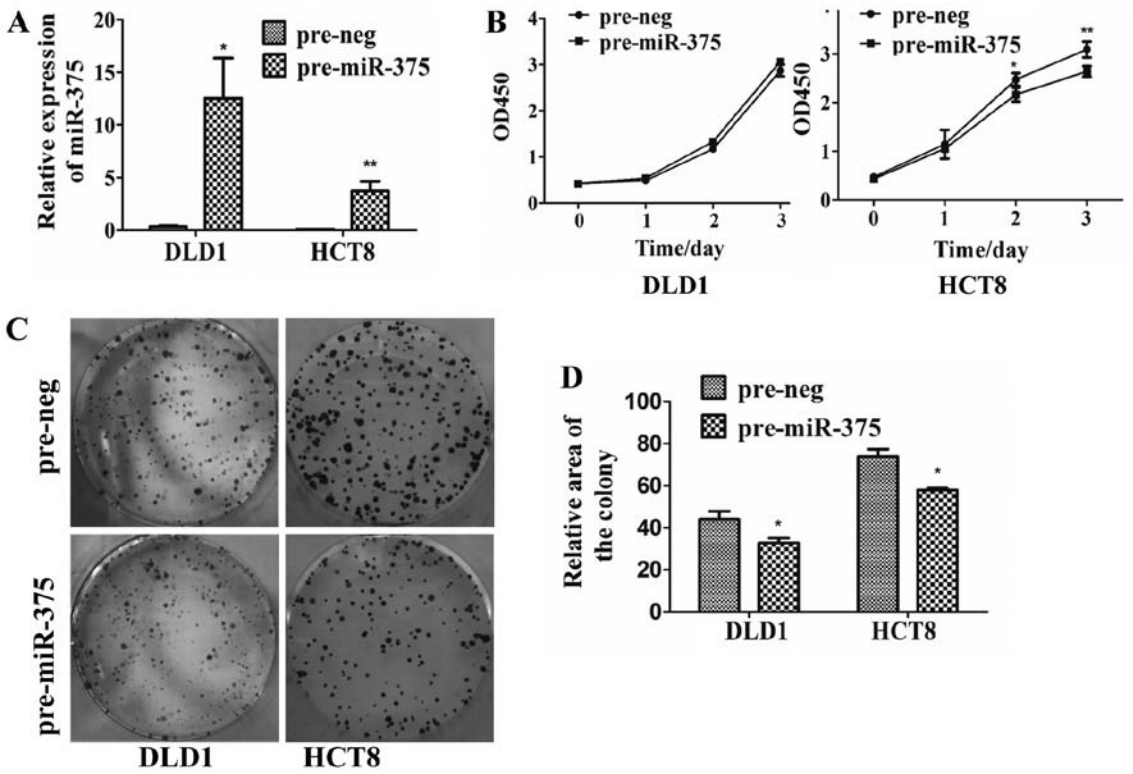

Figure 1. miR-375 inhibits CRC cell proliferation. (A) miR-375 was overexpressed in DLD1 and HCT8 cells transfected with pre-miR-375 compared with its expression in cells transferred with pre-neg. (B) By CCK-8 analysis, overexpression of miR-375 reduced the growth of HCT8 cells, but not obviously in the DLD1 cells. (C and D) Colony formation assay showed that overexpression of miR-375 decreased the number and mean area of the colonies in the DLD1 and HCT8 cells.

cally resected at Shanghai Huashan Hospital in 2008 were obtained. Each group included primary cancer (T), lymphatic metastasis (N) and liver metastasis tissues (M) from the same patient. The present study was approved by the Institutional Review Board of Shanghai Medical College, Fudan University and informed consent was obtained from all patients. The RNA extraction and qRT-PCR protocols are referred to as above.

Statistical analysis. The data are expressed as mean ± SD. Student's t-test was used to compare test samples with the controls. Two-way analysis of variance was used to compare differences among three or more experimental groups. Statistical analyses were performed using SPSS 11.0 software. $P<0.05$ was considered to indicate a statistically significant result.

Results

miR-375 inhibits the proliferation of CRC cells. To further determine the role of miR-375 in CRC cells, expression of miR-375 was upregulated in DLD1 cells by 40.9-fold (P=0.0040) and in HCT8 cells by 89.2-fold (P=0.0052) (Fig. 1A) compared with the negative control. Overexpression of miR-375 repressed the proliferation of CRC cells as detected by CCK-8 and colony formation assays. CRC HCT8 cells transfected with pre-miR-375 were observed to grow more slowly by 18.8% (P=0.0096) on day 3 (Fig. 1B). The cell colony average area in the DLD1 cells transfected with pre-miR-375 was reduced by 25% (P=0.0201), and by 21.5% in the HCT8 cells (P=0.0136) (Fig. 1C and D).

miR-375 inhibits the migration and invasion of CRC cells. To validate the involvement of miR-375 in metastasis, migration and invasion assays were performed in the DLD1 and HCT8 cells. In the migration assay, the numbers of migrating cells transfected with pre-miR-375 were significantly reduced, and the migration rate decreased by 33% in the DLD1 cells (P=0.0138), and 36% in the HCT8 cells (P=0.0140) compared with the pre-neg groups (Fig. 2A). In the invasion assay, the numbers of invading cells transfected with pre-miR-375 were significantly reduced, and the invasion rate decreased by 46% in the DLD1 cells (P=0.0479), and 52.3% in the HCT8 cells (P=0.0422) compared with the pre-neg groups (Fig. 2B).

SP1 is identified as a target of miR-375. SP1 was found to be one of the candidate target genes, which bears a miR-375 binding sites in its 3'-UTR. To determine whether miR-375 can regulate the expression of SP1, luciferase reporter assay was performed with a vector containing the wild-type (WT) or mutant (MUT) (Fig. 3A) putative SP1 3'-UTR target site downstream of the luciferase reporter pMIR-REPORT vector. When pre-miR-375 was co-transfected, the relative luciferase activity of the reporter containing WT-3'-UTR was significantly suppressed by 28.5% (P=0.0203) compared with that of the reporter containing NC-3' UTR. In contrast, the luciferase activity of the reporter containing MUT-3'-UTR was unaffected by simultaneous transfection with NC-3'-UTR (Fig. 3B). Then, western blotting and qRT-PCR results showed that overexpression of miR-375 decreased the SP1 expression at the protein (Fig. 3D) but not the messenger RNA level (Fig. 3C).

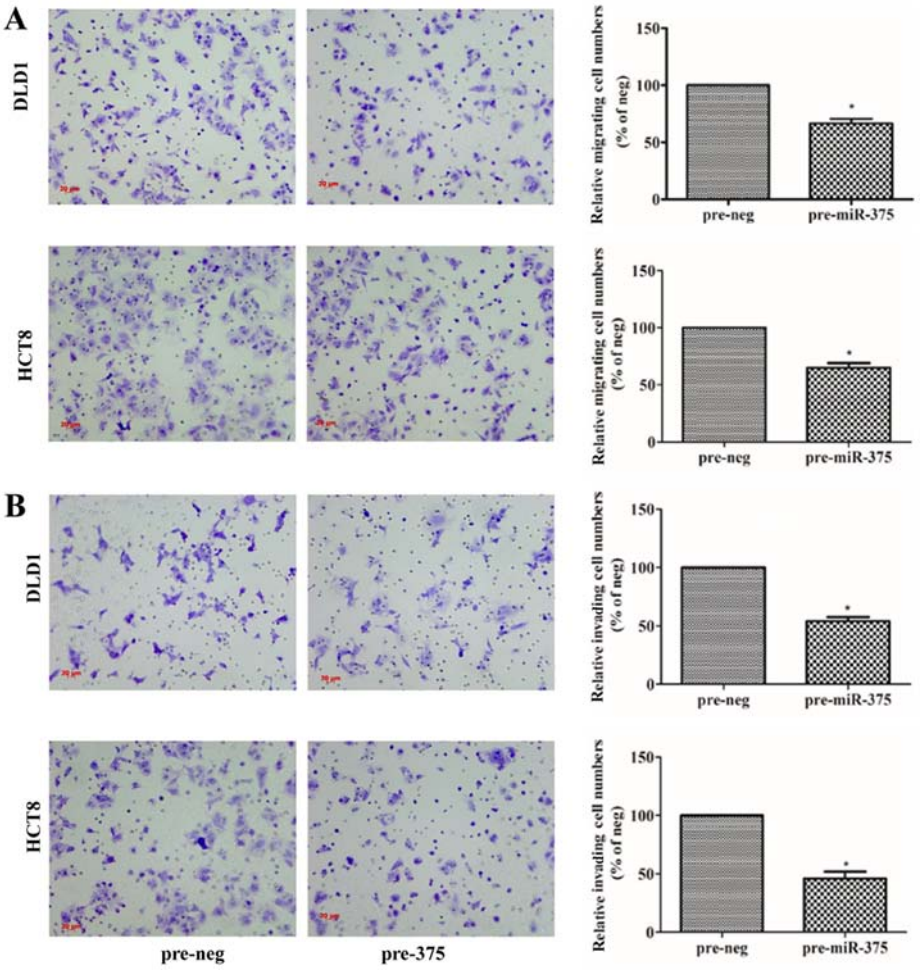

Figure 2. Overexpression of miR-375 inhibits CRC cell invasion and migration. (A) The migration of the DLD1 and HCT8 cells transfected with pre-miR-375 was reduced compared with that noted in the cells transfected with pre-neg. (B) Invasion of the DLD1 and HCT8 cells transfected with pre-miR-375 was reduced compared with that noted in the cells transfected with pre-neg. The motile cells were stained with Gentian violet, and photographed at magnification (x200). The migration or invasion rates were calculated as: Motile cells transfected with pre-miR-375/motile cells transfected with pre-neg.

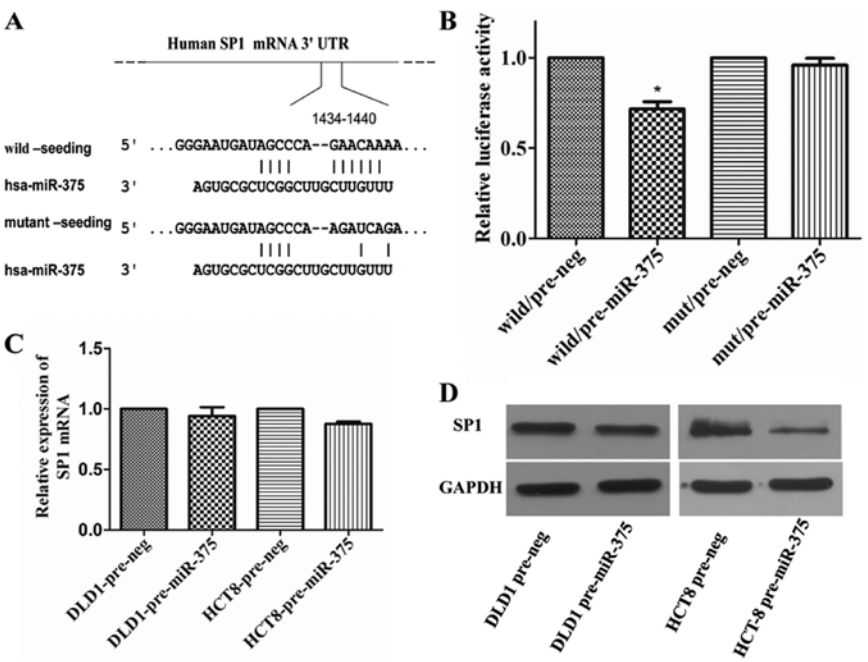

Figure 3. Prediction and verification of the target gene of miR-375. (A) The seeding sequence of the miR-375 target on the 3'-UTR of SP1. (B) Luciferase reporter assay analysis. Overexpression of miR-375 reduced the luciferase activity of wild-type 3'-UTR-SP1, but not the mutant 3'-UTR-SP1. (C and D) Upregulation of miR-375 decreased the SP1 protein level but not the SP1 mRNA in the CRC cells.

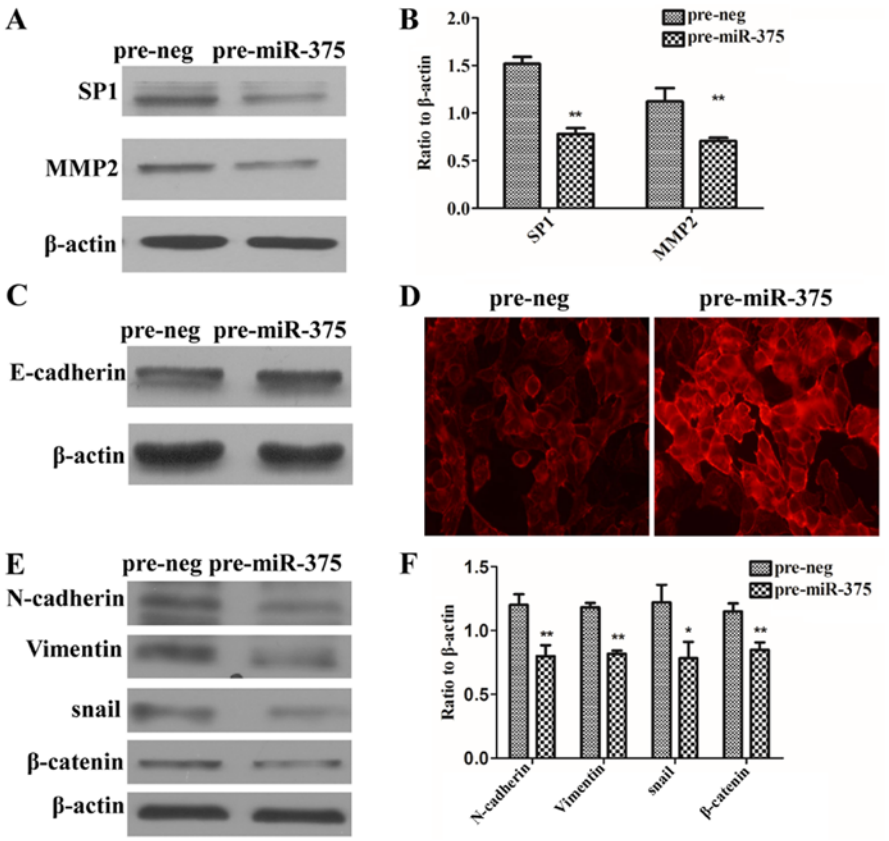

Figure 4. miR-375 regulates MMP2 and EMT-associated genes. (A and B) Western blot analysis showed that overexpression of miR-375 reduced SP1 and MMP2 protein but (C) did not increase the whole cell E-cadherin protein obviously. (D) Immunofluorescence analysis showed that upregulation of miR-375 increased E-cadherin on the HCT8 cell membrane, and (E and F) inhibited expression of EMT-associated proteins: N-cadherin, vimentin, snail and β-catenin. *P<0.05, **P<0.01.

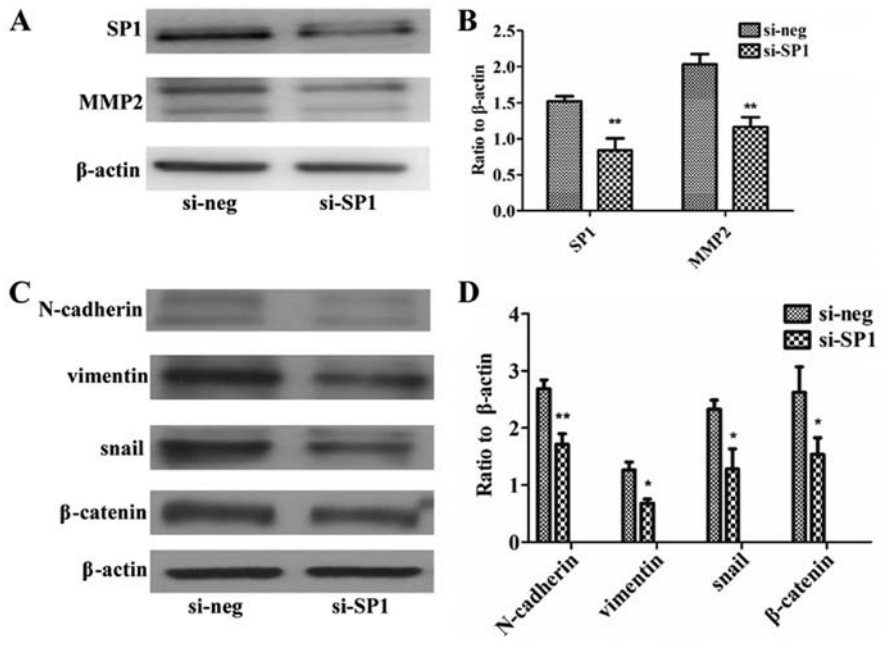

Figure 5. Downregulation of SP1 protein by siSP1 regulates MMP2 and EMT-associated genes. Transfection of siSP1 in HCT8 cells reduced SP1 protein successfully, and at the same time reduced MMP2 (A and B), and also decreased the expression of EMT-associated genes in the HCT8 cells (C and D). *P<0.05, **P<0.01.

miR-375 is involved in the epithelial-mesenchymal transition (EMT) of CRC cells via regulation of SP1. To further explore the molecular mechanisms of how miR-375 regulates migration and invasion development, we tested whether miR-375 regulates the expression of MMP-2 and EMT-related genes through SP1. The data showed that overexpression of miR-375 in the HCT8

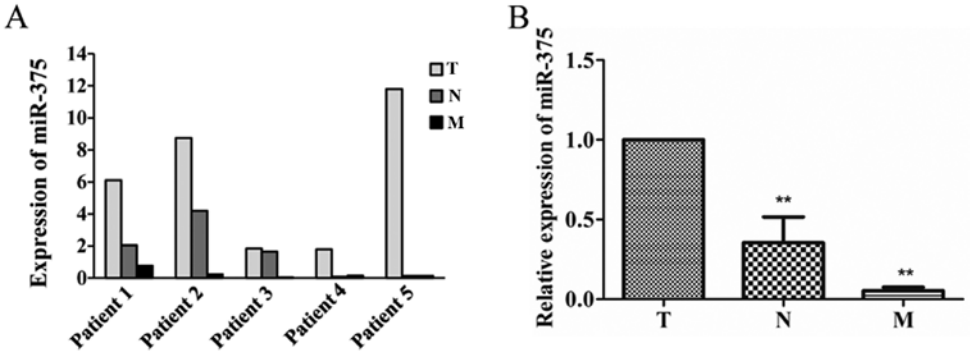

Figure 6. qRT-PCR analysis of miR-375 in metastastic CRC tissues. (A) Expression of miR-375 in 15 samples from 5 patients: T, primary CRC; N, lymphatic metastasis and M, liver metastasis samples. (B) miR-375 was significantly downregulated in N and M compared with T.

cells reduced SP1 and MMP2 protein (Fig. 4A and B). In addition, immunofluorescence assay showed that upregulation of miR-375 predominantly increased E-cadherin protein on the HCT8 cell membrane (Fig. 4D), even though the whole protein of E-cadherin did not increase obviously (Fig. 4C). Upregulation of miR-375 reduced N-cadherin, vimentin, snail and β-catenin proteins in the HCT8 cells ($P<0.05$, $P<0.01$) (Fig. 4E and F).

Downregulation of SP1 by siSP1 regulates MMP2 and EMT-associated genes. To further explore whether SP1 regulates MMP2 and EMT-associated genes, SP1 was interfered by siSP1 in the HCT8 cells. In addition, the results showed that interference with siSP1 reduced SP1 protein, and at the same time reduced expression of MMP2 (Fig. 5A and B) and EMT-associated genes (Fig. 5C and D) ($P<0.05$, $P<0.01$).

miR-375 is downregulated in metastatic CRC. Expression of miR-375 in primary cancer (T), lymphatic metastasis (N) and liver metastasis tissues (M) were examined. In addition, miR-375 was downregulated in all the 5 lymphatic metastasis and liver metastasis tissues compared with primary cancer tissues (Fig. 6A). In addition, miR-375 was significantly downregulated in lymphatic metastasis by 65% ($P=0.0163$) and liver metastasis tissues by 94.7% ($P=0.0000$) compared with primary cancer (Fig. 6B).

Discussion

MicroRNAs (miRNAs) are considered as potential specific biomarkers and play important roles in the diagnosis, progression and prognosis of many types of cancers (16-18). miR-375 was first identified as a pancreatic islet-specific miRNA regulating insulin secretion (19,20). Current studies show that miR-375 is decreased in liver, lung and gastric cancer, and could participate in the regulation of cancer development as a tumor-suppressor gene by targeting YAP1 and JAK2 (21,22), but its association with colorectal cancer (CRC) development was not carefully investigated. Our previous study showed that miR-375 was weakly expressed in CRC, and coupled with another two miRNAs could distinguish early invasive CRC from high-grade intraepithelial neoplasms (13). In the present study, the functions and underlying mechanism of miR-375 in the regulation of CRC development were explored. miR-375 was found to inhibit CRC cell growth to some extent, and particularly suppress CRC cell migration and invasion ability. One study found that miR-375 could inhibit CRC cell growth by targeting PIK3CA (15), but there were no data documented concerning whether miR-375 could regulate CRC metastasis. The present study firstly found that miR-375 expression was negatively associated with metastasis, and also could suppress CRC cell migration and invasion ability. The functional research found that miR-375 expression in the metastatic CRC samples was less than that in the primary ones.

The molecular mechanisms underlying the function was further explored. SP1 was predicted and validated as a direct target of miR-375. SP1 is a ubiquitous nuclear transcription factor that regulates gene expression via multiple mechanisms (23), for example, by regulating cell cycle (24,25), activating MMP2 (26) and promoting EMT process (27), to participate in the development of cell proliferation and invasion. In the present study, SP1 was verified as a direct target gene of miR-375. Thus, we predicted that miR-375 inhibited CRC development via targeting SP1.

In the present study, we found that miR-375 was significantly downregulated in CRC tissues from lymphoma and liver metastasis compared with the primary tumors. In addition, MMPs and the EMT process are important molecular events in cancer metastasis (28-31). In addition, we found that HCT8 cells had weak expression of miR-375, but higher invasion ability compared the DLD1, HCT116, HT29, LS174T cell lines stored in our laboratory, thus HCT8 was selected for further study of EMT (data not shown). EMT is a complicated process, with the most obvious feature: loss of E-cadherin and increased vimentin (32). In addition, genes such as snail, N-cadherin, β-catenin, are involved in the process (33-36). Further study was carried out to ascertain whether miR-375 could regulate MMP2 and EMT-associated genes. The results showed that overexpression of miR-375 inhibited the expression of SP1, MMP2, and also vimentin, snail, β-catenin, N-cadherin, but increased E-cadherin, particularly on the cell membrane. In addition, the similar results were found when SP1 mRNA was interfered in CRC cells. Thus, we recognized that miR-375 is a key factor to inhibit CRC migration and invasion, by targeting SP1 through downregulating MMP2 and inhibiting the EMT process.

In conclusion, miR-375 inhibited proliferation, invasion and migration in CRC via directly targeting SP1 through

EMT. In addition, miR-375 could be metastasis predictor and a treatment target for clinical application.

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