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miR-339-3p inhibits proliferation and metastasis of colorectal cancer

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Abstract. MicroRNAs (miRNAs) serve important roles in regulating cancer cell proliferation and metastasis. The same hairpin RNA structure may produce mature products from each strand, termed miR-5p and miR-3p, which can bind different mRNAs. Previously, the present authors reported that miR-339-5p could inhibit cell proliferation and migration by targeting the 3'-untranslated region (3'-UTR) of PRL-1 mRNA. The present study analyzed the expression, function and preliminary regulatory mechanism of miR-339-3p in colorectal cancer (CRC). The results of reverse transcription-quantitative polymerase chain reaction analysis demonstrated that miR-339-3p is downregulated in CRC specimens and highly invasive cell lines. Furthermore, the low-level expression of miR-339-3p was significantly associated with lymph node metastasis in patients with CRC; however, reduced miR-339-3p expression was not associated with age, gender or the differentiation status of the tumor. Overexpression of miR-339-3p was sufficient to suppress tumor growth and metastasis *in vitro*. In addition, the present study demonstrated that unlike miR-339-5p, PRL-1 expression was not regulated by miR-339-3p. The findings of the present study indicate that miR-339-5p and miR-339-3p may target different mRNA. The target gene of miR-339-3p requires future identification.

Introduction

Colorectal cancer (CRC) is one of most common malignant cancers and is associated with a poor prognosis and a high mortality rate (1). Previous studies have indicated that oncogene and tumor suppressor genes are involved in CRC initiation and progression (2,3). However, the molecular biological mechanism of CRC is complicated and is not yet fully understood. A recently discovered class of non-protein-coding small RNA, microRNA (miRNA), has extended the understanding of the process of oncogenesis, which offers a novel perspective to solve this problem. Over 1,000 miRNAs have been identified in human cells and their diverse functions in normal cell homeostasis and numerous different diseases have been thoroughly investigated during the past decade (4-6). To date, abnormal expression of a number of miRNAs, including miR-21 (7), miR-31 (8), and miR-137 (9), have been identified in CRC and may contribute to the development and progression of CRC.

miRNA are a class of small molecule RNA, with a size of ~19-22 nucleotides (10). The miRNA maturation process is divided into 2 steps: i) The pre-miRNA is cleaved by the RNase III enzyme Dicer-1 producing a short doublestranded miRNA duplex, which is ii) unwound by a helicase, creating a mature miRNA (11). In humans, the 2 mature miRNA sequences, miR-339-5p and miR-339-3p, are excised from opposite arms of the stem-loop pre-miR-339. The present authors previous study indicated that miR-339-5p inhibited tumorigenesis and metastasis of colorectal cancer through the regulation of PRL-1 expression and activation of the ERK1/2 signalling pathway (12). However, to the best of our knowledge, there have been no previous reports investigating the role of miR-339-3p in CRC. Although 2 mature miRNAs can be excised from the same stem-loop pre-miRNA, the resulting miRNAs may have different effects. The miRNA miR-34c-3p exhibits tumor-suppressing effects in SiHa cells through reducing the expression of Notch pathway members, however miR-34c-5p overexpression does not result in tumor suppressive effects in this cell line (13). miR-28 suppresses proliferation but activates metastasis, since the 5p and 3p forms of miR-28 target different mRNAs. miR-28-5p altered expression of CCND1 and HOXB3, whereas miR-28-3p bound NM23-H1 (14). The present study investigated the role of miR-339-3p in CRC: miR-339-3p expression was detected in CRC cells and tissue samples. Functional assays were used to analyze the effect of miR-339-3p on cell behaviors. In addition, the preliminary molecular mechanisms underlying the function of miR-339-3p were investigated.

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Materials and methods

Clinical specimens. A total of 30 fresh-frozen CRC tissue and matched normal colorectal tissue samples were collected from Nanfang Hospital of Southern Medical University (Guangzhou, China). Tumor and non-tumor samples were confirmed by pathological examinations. Pathological diagnoses were performed based on the system of the International Union Against Cancer (15). The study was approved by the Ethics Committee of Southern Medical University, and written informed consent was obtained from all patients for the use of their tissues. The pathological stage, grade and nodal status were evaluated by an experienced pathologist. Clinicopathological characteristics, including gender, age, lymph node metastasis and differentiation were also recorded.

Cell culture and miRNA transfection. The 293FT human embryonic kidney cell line and 6 human colorectal cancer cell lines (HCT116, HT29, LS174T, SW480, SW620 and LOVO) with different metastatic abilities were purchased from The American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured and stored according to the ATCC's instructions. 293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Gaithersburg, MD, USA). All CRC cells were maintained in RPMI 1640 (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% FBS at a humidity of 5% CO_2 at 37˚C. The miR-339-3p mimic, a non-specific miR mimics control (miR mimics control), miR-339-3p inhibitor (As-miR-339-3p), and a nonspecific inhibitor-negative control (As-miR-control) were all purchased from GenePharma (Shanghai, China). miRNAs were transfected at a working concentration of 100 nmol/l using Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. After 48 h, cells were harvested for analysis of protein and RNA.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays. For detection of miR-339-3p expression, stem-loop RT-qPCR was performed using an All-in-OneTM miRNA RT-qPCR Detection Kit (GeneCopoeia, Rockville, MD, USA), as previously described. Briefly, total RNA was extracted from cells using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. RT reactions were performed using 1 mg of total RNA extracted from clinical specimens and also from cell lines. RT-qPCR reactions were performed using the Hairpin-it TM miRNAs qPCR kit (GeneCopoeia, Inc., Rockville, MD, USA), and U6 for normalization to endogenous control. The thermal profile for amplification of miR-339-3p was conducted at 95˚C for 10 min, followed by 40 cycles of 95˚C for 20 sec, 60˚C for 20 sec and 72˚C 10 sec. For PRL-1 mRNA detection, reverse transcription was performed using the Reverse Transcriptase System (Takara Biotechnology Co., Ltd.). PRL-1 expression was measured by SYBR green qPCR assay (Takara Biotechnology Co., Ltd.). The primer sequences were as follows: PLR-1, F 5'-GACCTGGATGGGGTAAACCT-3' and R 5'-TGTGACTTCCACAGGAGCTG-3'; GAPDH, F 5'ACCCACTCCTCCACCTTTG-3' and R 5'-CACCACCCTGTTGCTGTAG-3'. The data were collected with ABI PRISM 7500 Fast Real-time PCR system (Applied Biosystems, Foster, CA, USA). The comparative threshold cycle method was used to calculate the amplification factor, where the threshold cycle (Ct) was defined as the cycle number at which the fluorescence passes the fixed threshold intensity level. The n-fold change in miR-339-3p and PRL-1 expression was obtained using $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay. Cells were transfected with 100 nM miR-339-3p inhibitor or mimics. 24 h later, cells were seeded in 96-well plates ($2x10^3$ cells/well). The viability of cells was examined by CCK8 (3-2,5-diphenyl tetrazolium bromide) assay (Dojindo Laboratories, Kumamoto, Japan) daily for 4 days. Each well was measured at 450 nm with a GloMaxTM96 Microplate Luminometer (Promega Corporation, Madison, WI, USA). All experiments were repeated 3 times.

Cell cycle analysis. A total of $1x10^6$ cells were harvested, washed twice with cold PBS and fixed in 70% cold ethanol. They were then processed with a Cell Cycle Detection Kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. The samples were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Cell migration and invasion assay. The migration and invasion capabilities of the cells were evaluated using Chemicon QCM 24-Well Collagen-Based Cell Invasion assay (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. For the invasion assay, $2x10^5$ cells in serum-free medium were added into the upper compartment of the Transwell chambers and 600 μl RPMI 1640 medium containing 10% FBS was added into the lower compartment. The cells were incubated for 48 h at 37˚C. All non-migratory cells were then removed from the interior of the insert and the migratory cells were stained with Wright-Giemsa (Nanjing KeyGen Biotech Co., Ltd.) and images were captured under the microscope (Olympus DP26; Olympus Corporation, Tokyo, Japan) in 5 random visual fields (magnification, x200). For the invasion assay, the procedures were similar, except that the cells were seeded into the top chamber, which was pre-coated with 20% matrigel matrix (BD Biosciences, San Jose, CA, USA).

Luciferase activity. 293FT and HCT116 cells were seeded in triplicate in 24-well plates ($1x10^5$/well), respectively, and cultured for 24 h. PRL-1 3'-UTR and mutant PRL-1 3'-UTR plasmid were constructed in previous experiment (12). Subsequently, co-transfections of PRL-1 3'-UTR or mutant PRL-1 3'-UTR plasmid with miR-339-3p mimics into 293FT cells were accomplished using Lipofectamine 2000. The same procedure were proceeded in HCT116 cells. Firefly and renilla luciferase activities were measured using the Dual-LuciferaseH Reporter Assay System (Promega Corporation) 48 h after transfection as previously described (12). Renilla luciferase activity was used as a transfection normalization control.

Western blot analysis. Protein expression was assessed by immunoblot analysis of cell lysates (20-60 μg) in RIPA buffer with 1% PMSF (Nanjing KeyGen Biotech Co., Ltd.).

Table I. miR-339-3p expression levels and clinicopathological characteristics in colorectal cancer patients.

Characteristics	n	$2^{-\Delta\Delta Ct}$ (mean)	P-value
Differentiation			0.204
Good	3	0.3287±0.0750	
Moderate	24	0.6861±0.1999	
Poor	3	0.2547±0.3250	
Lymph node status			0.032
Negative	18	0.6926±0.9219	
Positive	12	0.3591±0.4807	
Gender			0.138
Male	18	0.4733±0.3651	
Female	12	0.7901±1.2156	
Age, years			0.312
<50	7	0.8229±1.2191	
≥50	23	0.5350±0.6731	

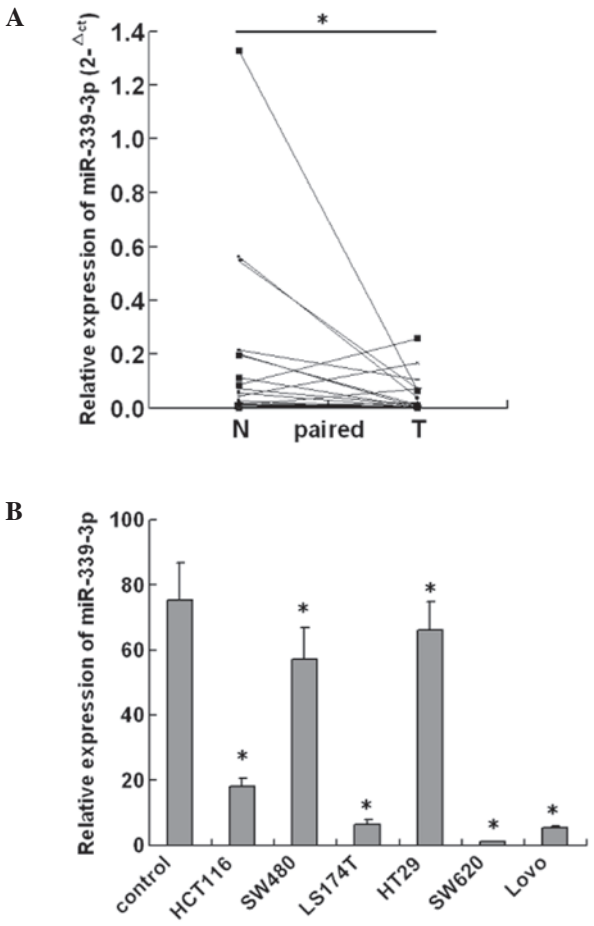

Figure 1. The expression levels of miR-339-3p in CRC tissues and cell lines. (A) Expression levels of miR-339-3p were examined by RT-qPCR in in 30 CRC tissues and their pair-matched adjacent normal colonic tissues. Each sample was analyzed in triplicate and normalized to U6 (*P<0.05). (B) Expression levels of mature miR-339-3p were detected by RT-qPCR in 6 human CRC cell lines. The relative expression of miR-339-3p was normalized to the endogenous control U6. Each sample was analyzed in triplicate. The relative miR-339-3p expression in CRC cell lines was reduced compared with 3 non-cancerous colonic tissues (N1, N2 and N3) (*P<0.05). CRC, colorectal cancer; T, tumor tissues; N, adjacent normal tissues; RT-qPCR, reverse transcription-quantative polymerase chain reaction.

Protein lysates were extracted and separated in SDS-PAGE gels (Nanjing KeyGen Biotech Co., Ltd.), transferred onto 0.22 μm polyvinylidene difluoride membranes (EMD Millipore, Boston, MA, USA), and the membranes were blocked for 1 h in Tris-buffered saline with Tween (TBST) containing 5% milk powder (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). The membranes were then incubated overnight at 4˚C with rabbit anti-PRL-1 monoclonal antibody (dilution, 1:800; Proteintech Group, Inc., Chicago, IL, USA) and rabbit anti-β-tubulin antibody (dilution, 1:2,000; Epitomics, Burlingame, CA, USA). The membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (dilution, 1:2,000) Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 1 h at room temperature, prior to visualization by enhanced chemiluminescence (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's instructions. The protein expression was normalized to β-tubulin.

Statistical analysis. The results of all the experiments are presented as the mean ± standard deviation (SD) of ≥3 independent experiments. All statistical analyses were performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA) software packages. The Shapiro-Wilk test was used to verify the clinical samples' distribution. Differences were analyzed using the Mann-Whitney-Wilcoxon non-parametric test. For *in vitro* studies, the Student's *t*-test or analysis of variance were used to compare values of test and control samples. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-339-3p is downregulated in human colon cancer. To study the expression pattern of miR-339-3p in CRC, RT-qPCR was used to assess miR-339-3p expression in 30 CRC samples and the pair-matched adjacent normal colonic tissue, as well as in 6 CRC-derived cell lines. As presented in Fig. 1A, the level of miR-339-3p expression is modestly reduced in CRC tissues (86.7%) compared with that observed in the adjacent normal colon mucosa tissues (P<0.05). The association between miR-339-3p expression and clinicopathological factors was examined in tumor tissues. The results demonstrated that reduced levels of miR-339-3p expression was more strongly associated with CRC samples with lymph node metastasis compared with CRC without lymph node metastasis (Table I, P<0.05). However, miR-339-3p expression was not associated with the other clinicopatholical factors assessed, including differentiation status, gender or age. In addition, the expression of miR-339-3p was also assessed in 6 human CRC cell lines. All 6 CRC cell lines demonstrated a statistically significant reduction in miR-339-3p expression, whereas the control normal colonic mucosa pooled from 3 healthy individuals (N1, N2 and N3) expressed high levels of miR-339-3p. Notably, miR-339-3p expression was expressed at the lowest levels in cancer cell lines SW620 and LOVO, which possess the highest metastatic abilities compared with HCT116, HT29, LS174T, SW480, which possess lower metastatic abilities (Fig. 1B). These results indicated that miR-339-3p may serve a role in CRC metastasis and invasion.

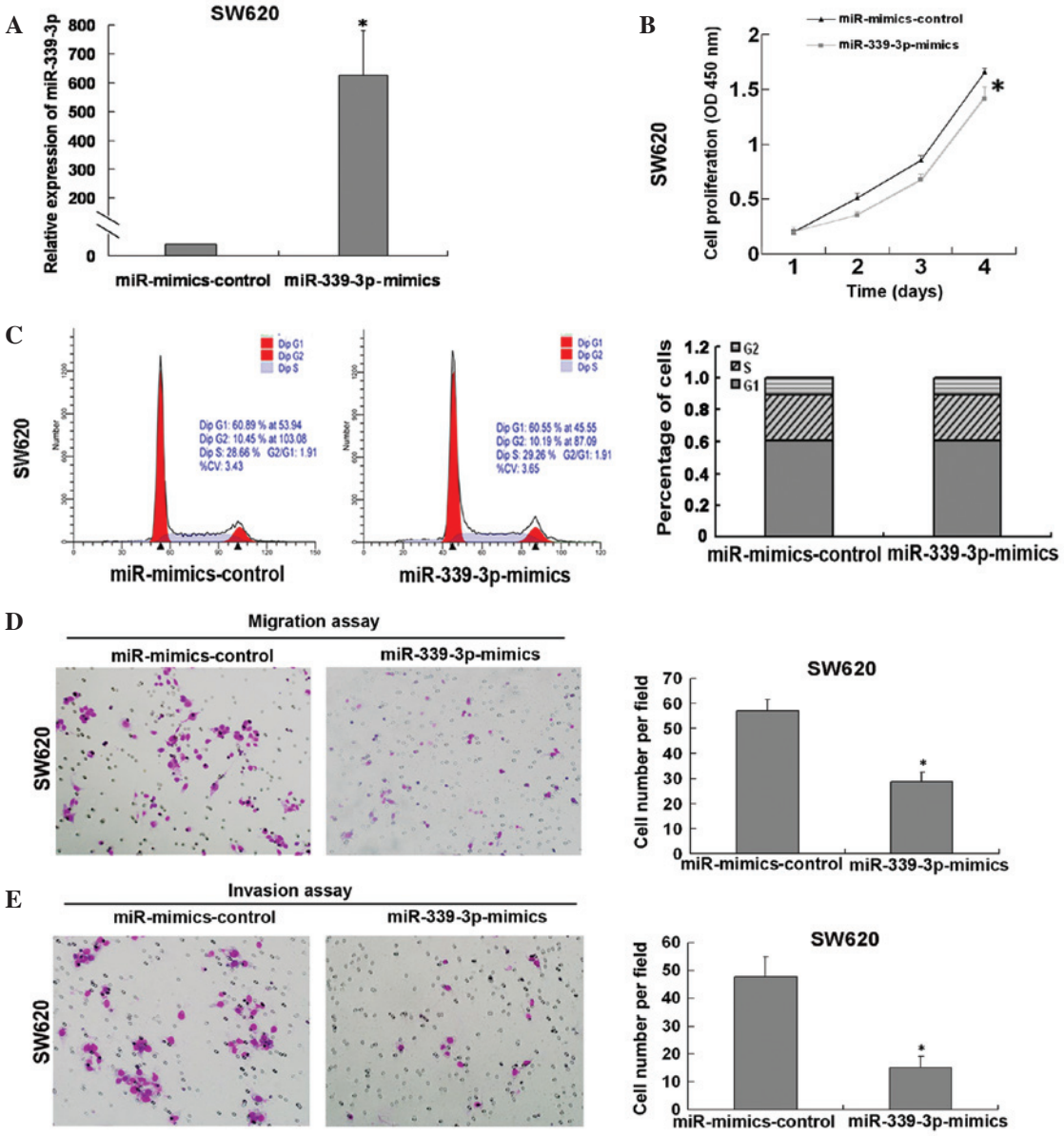

Figure 2. Overexpression of miR-339-3p inhibited cell proliferation, migration and invasion of SW620 cells. (A) The expression of miR-339-3p was analysis in SW620 cells transfected with miR-mimics control or miR-339-3p-mimics by reverse transcription-quantitative polymerase chain reaction. (B) The vitality of cells infected with miR-mimics control or miR-339-3p-mimics was detected using the CCK-8 assay. (C) Cell-cycle distribution of CRC cells infected with miR-339-3p-mimics was detected by flow cytometry analysis. Transwell assay was employed to evaluate (D) migration and (E) invasion of SW620 cells infected with miR-mimics-control or miR-339-3p mimics. Data are presented as mean ± SD. The results were reproducible in 3 independent experiments $^{*}P<0.05$.

Effect of miR-339-3p on colon cancer cells growth, migration and invasion. To further confirm whether miR-339-3p is involved in regulating CRC cell growth, a proliferation assay was performed by transfecting miR-339-3p mimics or mimics control into SW620 cells, which were observed to express low endogenous levels of miR-339-3p (Fig. 1B). The results of the CCK8 assay demonstrated that miR-339-3p was successfully overexpressed in SW260 cells (Fig. 2A) and that this resulted in inhibition of cell growth (Fig. 2B). The analysis of cell cycle distribution demonstrated that there was no significant difference in the percentage of cells in S and G1 phase distribution between the miR mimics control group and miR-339-3p mimics group (Fig. 2C). To investigate the role of miR-339-3p in the migration and invasion of tumor cells, a Transwell assay was performed with the SW620 cells transfected with the miR-339 mimics and control. A reduction in the migratory and invasive activity of SW620 cells overexpressing miR-339-3p was observed (reduced by 50.5% and 31.8%, respectively; Fig. 2D and E; both $P<0.05$). Inversely, miR-339-3p was downregulated in HCT116 cells using a miR-339-3p inhibitor (Fig. 3A), and this resulted in increased levels of cell growth compared with the inhibitor control (Fig. 3B). Flow cytometry and cell cycle analysis revealed no significant changes in the percentage of HCT116 cells treated with miR-339-3p in the G1 or S phase (Fig. 3C). In addition the number of migratory and invasive cells transfected with the miR-339-3p inhibitor was assessed by Transwell assay and demonstrated that treatment with miR-339-3p inhibitor promoted migration and invasion of the HCT116 cells by 3.23 fold and 2.15 fold, respectively (Fig. 3D

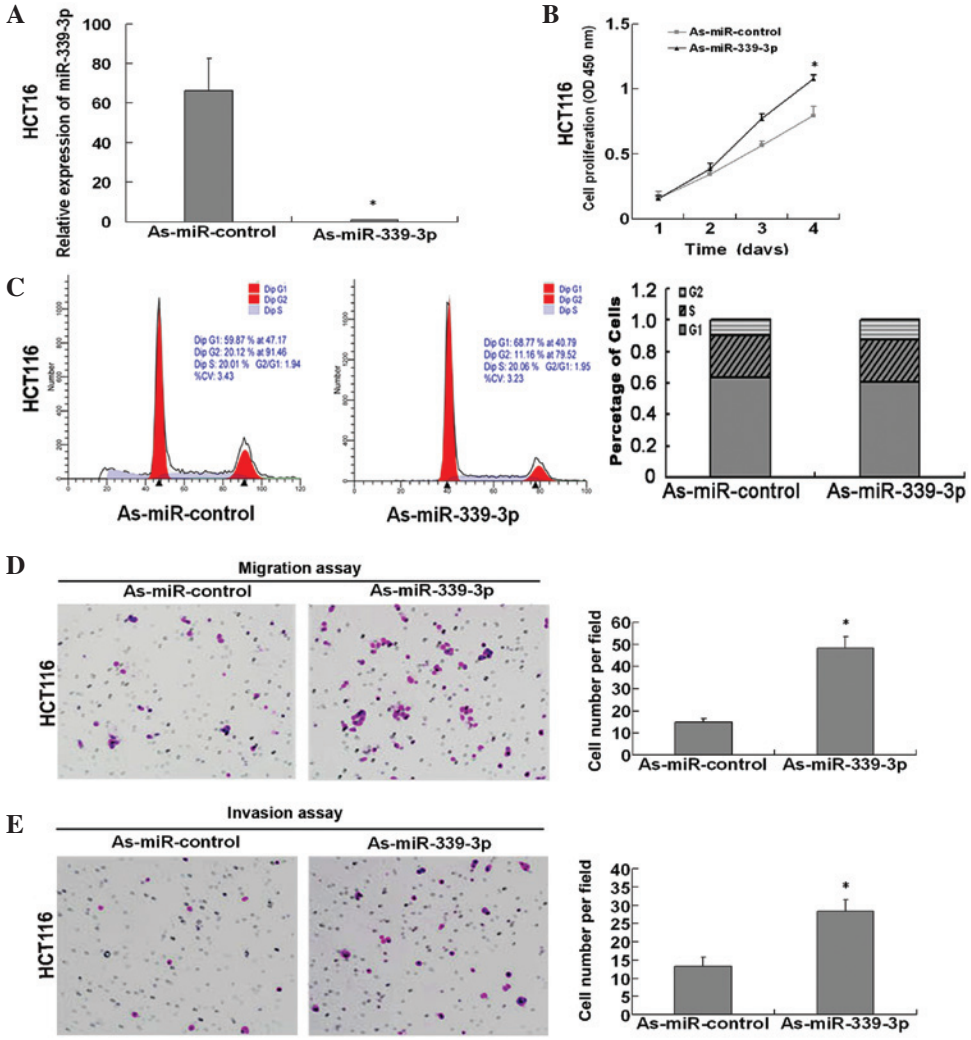

Figure 3. Inhibition of miR-339-3p promoted the growth, migration and invasion of HCT116 cells. (A) The expression of miR-339-3p was analysis in HCT116 cells transfected with miR-339-3p inhibitor or negative control by reverse transcription-quantitative polymerase chain reaction. (B) The viability of cells transfected with the miR-339-3p inhibitor or negative control was detected using the CCK-8 assay. (C) Cell-cycle distribution of CRC cells infected with miR-339-3p inhibitor was detected by flow cytometry analysis. Transwell assay was employed to evaluate (D) migration and (E) invasion of HCT116 cells transfected with miR-339-3p inhibitor or negative control. Data are presented as mean ± SD. The results were reproducible in 3 independent experiments *P<0.05. CRC, colorectal cancer.

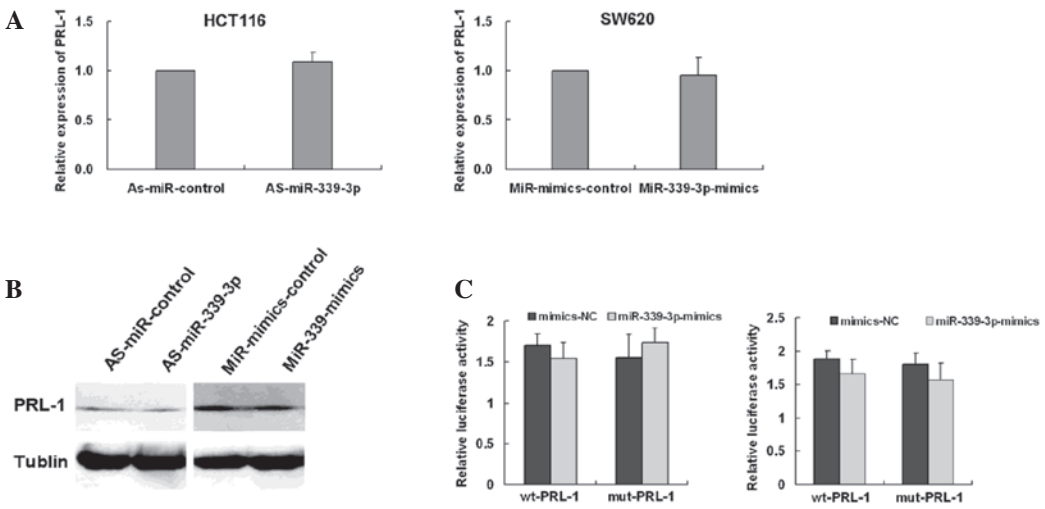

Figure 4. PRL-1 is not a direct target of miR-339-3p in CRC cells. (A) The expression of PRL-1 mRNAs were analysis by reverse transcription-quantitative polymerase chain reaction. (B) The protein expression levels of PRL-1 were detected using western blot analysis. (C) Analysis of luciferase activity. 293FT cells and HCT116 cells were co-transfected with psiCHECK™-2 luciferase reporter plasmid containing either wt or mut PRL-1 3'-UTR and either the miR-339-3p mimics or non-specific miR mimic control (NC). Luciferase activity was assayed 48 h after transfection. Renilla luciferase activity of each sample was normalized by Firefly luciferase activity. Data are presented as mean ± SD from 3 independent experiments. CRC, colorectal cancer; wt, wild-type; mut, mutant.

and E; both P<0.05). Taken together, these results indicated that miR-339-3p may function as a tumor suppressor by inhibiting CRC cells proliferation and CRC cells mobility.

PRL-1 is not a functional target for miR-339-3p in CRC cells. In the present authors previous study, PRL-1 was identified as a downstream target of miR-339-5p. Since miR-5p and miR-3p forms can target the same mRNAs (16,17), the present study investigated whether there was an association between miR-339-3p and PRL-1 expression. To examine this hypothesis, miR-339-3p mimics were transfected into SW620 cells and HCT116 cells were treated with an miR-339-3p inhibitor. The mRNA and protein expression levels were examined by RT-qPCR and western blot analysis. The results demonstrated that miR-339-3p had no effect on PRL-1 mRNA and protein expression in SW620 or HCT116 cells (Fig. 4A and B; both P>0.05). To determine whether miR-339-3p directly interacts with the 3'-UTR region of PRL-1 luciferase reporter assays were performed. miR-339-3p mimics and luciferase reporter constructs containing wild type or mutant PRL-1 3'-UTR were co-transfected into 293FT cells. The same procedure was used for HCT116 cells. Luciferase activity of the reporter containing PRL-1 3'-UTR in 293FT and HCT116 cells was not affected in the presence of miR-339-3p when compared with its negative miRNA control. In addition, miR-339-3p did not alter the activity of the mutant PRL-1 luciferase reporter in 293FT and HCT116 cells (Fig. 4C). These results indicated that miR-339-3p did not directly bind to the 3'-UTR of PRL-1 under these conditions.

Discussion

Expression patterns of miRNAs are systematically altered in a number of types of human cancer (18,19). Previous studies have demonstrated that a number of mature miRNA serve essential roles in cellular transformation and tumorigenesis, angiogenesis, invasion, and metastasis (20,21). Previous work on miR-339-5p reported that miR-339-5p may be abnormally downregulated in colon cancer. The present authors previous study indicated that miR-339-5p inhibits CRC cell proliferation and migration by regulation of PRL-1 expression and the ERK1/2 signaling pathway. Two mature miRNAs may be generated from the same stem-loop pre-miR-339 and generate 2 different miRNAs, miR-339-5p and miR-339-3p. Although miR-339-3p is generated from the same primary transcript as miR-339-5p, it has a different sequence and its expression, the modulation of its expression profiles and the exact functional mechanism of miR-339-3p is unknown in CRC cells.

In the present study, the potential role of miR-339-3p was investigated in CRC cel lines and patient samples. The results demonstrated that the downregulation of miR-339-3p was apparent in CRC tissue and cell lines, indicating a tumor suppressive role for miR-339-3p in CRC development. Furthermore, the potential association between the expression level of miR-339-3p and various clinicopathological characteristics of CRC patients were investigated: Low miR-339-3p expression levels were demonstrated to be significantly associated with cases with lymph node metastases; however, reduced miR-339-3p expression was not associated with age, gender or the differentiation status of the tumor. In addition, to the best of our knowledge, no evidence of the influence of miR-339-3p expression on the behavior of CRC cells has been documented. The gain- and loss-of-function assays in the present study demonstrated that miR-339-3p overexpression inhibited cell proliferation and migration *in vitro*. However, miR-339-3p overexpression did not affect the cell-cycle distribution of CRC cells. miR-339-3p expression may inhibit the proliferation of CRC cells through other ways, such as apoptosis, but this was not examined in the present study. The results indicated miR-339-3p serves a suppressive role in CRC progression.

miRNAs generally exert their biological function by suppressing their specific target genes. A single miRNA may regulate a number of different targets. The present authors previous study previous studies demonstrated that the suppressive effects of miR-339-5p on CRC cells may be partially due to its regulation of PRL-1 expression and activation of the ERK1/2 signaling pathway. Previous studies have revealed that both strands of miR-5p and miR-3p can target the same mRNAs (16,17). As the exact functional mechanism of miR-339-3p remains unclear, the present study also investigated whether PRL-1 is the target of miR-339-3p. miR-339-3p overexpression in SW620 cells and knockdown of miR-339-3p in HCT116 cells had no effect on PRL-1 levels respectively, at both the mRNA and protein levels. In addition, mRNA expression of miR-339-3p and PRL-1 were not demonstrated to be significant in CRC cells. It was also demonstrated that miR-339-3p did not bind directly to 3'-UTR of PRL-1. Therefore, these results indicate that miR-339-3p may function as a tumor suppressor by regulating a gene other than PRL-1 to inhibit the progression of CRC.

The results of the present study indicated that miR-339-3p may participate in the modulation of malignant biological behavior in CRC. However, PRL-1 is not regulated by miR-339-3p in the CRC pathological processes and future experiments should aim to identify the target gene of miR-339-3p in CRC.

Acknowledgements

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