Oncology Research, Vol. 25, pp. 617–627
Printed in the USA.

0965-0407/17 $90.00 + .00
DOI: https://doi.org/10.3727/096504016X14768374457986
E-ISSN 1555-3906
www.cognizantcommunication.com

MicroRNA-219-5p Represses the Proliferation, Migration, and Invasion of Gastric Cancer Cells by Targeting the LRH-1/Wnt/β-Catenin Signaling Pathway

Chunsheng Li,* Jingrong Dong,† Zhenqi Han,† and Kai Zhang‡

*Department of Gastrointestinal Colorectal and Anal Surgery, China–Japan Union Hospital of Jilin University, Changchun, Jilin, P.R. China
†Endoscopic Center, The Affiliated Hospital of Changchun University of Chinese Medicine, Changchun, Jilin, P.R. China
‡Department of Colorectal and Anal Surgery, The Second Hospital of Jilin University, Changchun, Jilin, P.R. China

MicroRNAs (miRNAs) are reportedly involved in gastric cancer development and progression. In particular, miR-219-5p has been reported to be a tumor-associated miRNA in human cancer. However, the role of miR-219-5p in gastric cancer remains unclear. In this study, we investigated for the first time the potential role and underlying mechanism of miR-219-5p in the proliferation, migration, and invasion of human gastric cancer cells. miR-219-5p was found to be markedly decreased in gastric cancer tissues and cell lines compared with adjacent tissues and normal gastric epithelial cells. miR-219-5p mimics or anti-miR-219-5p was transfected into gastric cancer cell lines to overexpress or suppress miR-219-5p expression, respectively. Results showed that miR-219-5p overexpression significantly decreased the proliferation, migration, and invasion of gastric cancer cells. Conversely, miR-219-5p suppression demonstrated a completely opposite effect. Bioinformatics and luciferase reporter assays indicated that miR-219-5p targeted the 3′-untranslated region of the liver receptor homolog-1 (LRH-1), a well-characterized oncogene. Furthermore, miR-219-5p inhibited the mRNA and protein levels of LRH-1. LRH-1 mRNA expression was inversely correlated with miR-219-5p expression in gastric cancer tissues. miR-219-5p overexpression significantly decreased the Wnt/β-catenin signaling pathway in gastric cancer cells. Additionally, LRH-1 restoration can markedly reverse miR-219-5p-mediated tumor suppressive effects. Our study suggests that miR-219-5p regulated the proliferation, migration, and invasion of human gastric cancer cells by suppressing LRH-1. miR-219-5p may be a potential target for gastric cancer therapy.

Key words: Gastric cancer; Liver receptor homolog-1 (LRH-1); miR-219-5p; Wnt/β-catenin signaling

INTRODUCTION

Gastric cancer is one of the most prevalent digestive malignancies, representing the second leading cause of cancer-related mortality worldwide[1,2]. Gastric cancer is difficult to diagnose at an early stage. As such, most of the patients are already at an advanced stage when they are diagnosed[3]. Despite the progress that has been made in cancer treatment, the prognosis of advanced gastric cancer is extremely poor, and the 5-year survival rate is less than 30%[4]. The main reason for this is the lack of biomarkers for early diagnosis and an insufficient understanding of the molecular pathogenesis of gastric cancer. Therefore, further understanding of gastric cancer can help improve the diagnosis, prognosis, and treatment of this malignancy.

MicroRNAs (miRNAs) are a class of conserved, noncoding RNA molecules consisting of ~22 nucleotides in length; miRNAs play an important role in regulating gene expression in a posttranscriptional manner[5,6]. Generally, miRNAs can directly target the 3′-untranslated region (3′-UTR) of the target mRNA to induce translation inhibition[5,6]. Cumulative evidence has indicated that miRNAs are critical regulators for cancer development and progression[7–9]. Various miRNAs are deregulated in cancer and can function as oncogenes or tumor suppressors, which are involved in regulating cancer cell proliferation, migration, and invasion[7–9]. Recent studies have proposed that numerous miRNAs participate in the progression of gastric cancer[10–12]. Targeting miRNAs may provide a novel strategy for gastric cancer treatment. However, the exact role of miRNAs in gastric cancer remains poorly understood.

Liver receptor homolog-1 (LRH-1), also named nuclear receptor subfamily 5 group A member 2 (NR5A2), is a nuclear receptor involved in a variety of biological

Address correspondence to Kai Zhang, Department of Colorectal and Anal Surgery, The Second Hospital of Jilin University, No. 218 Ziqiang Street, Changchun, Jilin 130041, P.R. China. Tel: +86-0431-88796555; E-mail: zhang_kaikz@163.com

processes including cell development, metabolism, and steroidogenesis[13]. Although the normal physiological functions of LRH-1 are critical for adult homeostasis, the deregulation of LRH-1 is implicated in cancer development and progression[14]. LRH-1 is involved in regulating breast cancer[15], colon cancer[16], gastric cancer[17], and others. LRH-1 regulates cell proliferation, apoptosis, migration, and invasion, as well as the whole cell cycle by modulating the Wnt/β-catenin and p53 signaling pathways[18–21]. Thus, LRH-1 has been considered to be a potential molecular target for cancer therapy[14].

Recent studies have reported that miR-219-5p is a tumor suppressor in several cancer types[22,23]. However, few studies have examined miR-873 in gastric cancer development. In the current study, we examined the expression of miR-219-5p in gastric cancer tissues and cell lines and found that miR-219-5p was frequently downregulated. Functional experiments revealed that miR-219-5p inhibited gastric cancer cell proliferation, invasion, and migration. Interestingly, LRH-1 was identified as a functional target of miR-219-5p. Moreover, miR-219-5p regulated the mRNA and protein expression of LRH-1 in gastric cancer cells, and an inverse correlation between miR-219-5p and LRH-1 expression was found in gastric cancer tissues. Furthermore, LRH-1 regulated the Wnt/β-catenin signaling pathway. Overall, our study suggests that miR-219-5p is a gastric cancer suppressor that regulates LRH-1 and its downstream signaling pathway.

MATERIALS AND METHODS

Tissue Specimens

Gastric cancer tissues and adjacent matched normal mucosal tissues were collected from 15 gastric cancer patients undergoing gastric cancer surgery at the Department of Gastrointestinal Colorectal and Anal Surgery, China–Japan Union Hospital of Jilin University (Changchun, P.R. China). Tumor resection was performed prior to any therapy. Surgically resected samples were immediately snap frozen in liquid nitrogen and stored at −80°C until use. Written informed consent was obtained from each patient prior to participation in this study. This study was approved by the Institutional Human Experiment and Ethics Committee of China–Japan Union Hospital of Jilin University. All experiments were performed in accordance with the Declaration of Helsinki.

Cell Lines

Human gastric cancer cell lines (MGC-803, BGC-823, MKN-45, and SGC-7901), normal human gastric epithelial GES-1 cells, and 293T cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, P.R. China). The cells were routinely cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum and 1% streptomycin/penicillin mix (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

To detect the mRNA expression, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (BioTeke, Beijing, P.R. China) in accordance with the manufacturer's instructions. To detect the miRNA expression, total RNA was isolated with RNeasy Mini Kit and then reverse transcribed into cDNA using the miScript Reverse Transcription Kit (QIAGEN, Dusseldorf, Germany) in accordance with the manufacturer's instruction. RT-qPCR assays were performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI7500 sequence detector (Applied Biosystems). The fold change of gene expression was determined via the $2^{-\Delta\Delta}$Ct method using GAPDH (mRNA) or U6 (miRNA) as the endogenous control. The primers used for the examination are listed as follows: LRH-1, 5′-GCACGGACTTACACCTATTGTG-3′ (forward) and 5′-TGTCAATTTGGCAGTTCTGG-3′ (reverse); cyclin D1, 5′-AACTACCTGGACCGCTTCCT-3′ (forward) and 5′-CCACTTGAGCTTGTTCACCA-3′ (reverse); cyclin E1, 5′-GCTGGGCAAATAGAGAGGAA-3′ (forward) and 5′-CTGGTGCAACTTTGGAGGAT-3′ (reverse); GAPDH, 5′-GAAATCCCATCACCATCTTCCAGG-3′ (forward) and 5′-GAGCCCCAGCCTTCTCCATG-3′ (reverse); miR-219-5p, 5′-ACACTCCAGCTGGGTGATTGTCCAAAC GCAAT-3′ (forward) and 5′-CTCAACTGGTGTCGTG GA-3′ (reverse); and U6, 5′-GCTTCGGCAGCACATA TACTAAAAT-3′ (forward) and 5′-CGCTTCACGAATT TGCGTGTCAT-3′ (reverse).

Cell Transfection

miR-219-5p mimics, inhibitors (anti-miR-219-5p), and corresponding negative controls (miR-NC and anti-miR-NC) were synthesized by GenePharma (Shanghai, P.R. China). The LRH-1 cDNA without the 3′-UTR region was cloned into pcDNA3.0 vector (Invitrogen). Cell transfections were performed using Lipofectamine 2000 (Invitrogen) in accordance with the recommended protocols by the manufacturer. After 48 h of transfection, cells were harvested for RT-qPCR or Western blot analysis.

MTT Assays

Cells were plated into 96-well plates (5×10^3 cells/well) and cultured for 24 h. After indicated transfection for 48 h,

20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Sigma-Aldrich) was added to each well. After incubation for 4 h, the medium was discarded, and dimethyl sulfoxide (200 µl/well) was added to dissolve the formazan product. After agitation for 15 min, the absorbance at a wavelength of 490 nm was determined by an ELISA reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assays

Cells were transfected with miR-219-5p mimics, anti-miR-219-5p, or NCs for 48 h. Afterward, the transfected cells were resuspended and seeded into six-well plates at a density of 1,000 cells/well in growth medium containing 0.3% noble agar for 14 days. Subsequently, the cells were stained with 0.1% crystal violet (Sigma-Aldrich). The number of colonies was counted under a microscope (Olympus, Tokyo, Japan).

Cell Cycle Assays

For cell cycle analysis, the transfected cells were harvested and fixed with 70% ethanol overnight at 4°C. The cells were then treated with propidium iodide (PI; 100 µg/ml; Sigma-Aldrich) and RNase A (10 µg/ml) for 30 min in the dark. Cell cycle distribution was analyzed by flow cytometry with BD FACSCalibur (BD Biosciences, San Jose, CA, USA). Data were analyzed using CellQuest software (BD Biosciences).

Cell Migration and Invasion Assays

For cell migration assay, the transfected cells (1×10^5 cells) were resuspended in 500 µl of serum-free medium and placed in the upper chamber. Meanwhile, 500 µl of growth medium containing 10% FBS was placed in the lower chamber. For cell invasion assay, the upper chamber of the Transwell inserts was precoated with Matrigel (BD Biosciences). After culturing for 24 h, the cells that neither migrated nor invaded were gently erased by a cotton swab. The cells at the bottom of the membrane were fixed with 95% ethanol and stained with 0.1% crystal violet. The cells were then counted under a microscope (Olympus).

Western Blot Analysis

Cells were harvested and lysed in a cell lysis buffer (Beyotime, Haimen, P.R. China). Protein concentration was quantified using a BCA kit (Beyotime). Up to 50 µg of proteins was separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked by 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST buffer) for 1 h at 37°C, followed by incubation with primary antibodies (anti-LRH-1 and anti-GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight at appropriate dilutions in accordance with the recommended instructions. After washing with TBST three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000; Santa Cruz Biotechnology) for 1 h at 37°C. The protein bands were visualized by an ECL Western blotting kit (Millipore, Boston, MA, USA). The intensities of the bands were analyzed by the Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Luciferase Reporter Assays

To detect the prediction between miR-219-5p and LRH-1-3′-UTR, a cDNA fragment of the LRH-1-3′-UTR mRNA containing the seed sequence of the mature miR-219-5p binding site or a mutated binding site of the 3′-UTR sequence was cloned into the pmirGLO dual-luciferase vector (Promega, Madison, WI, USA). The constructed pmirGLO dual-luciferase vector was cotransfected with miR-219-5p mimics into 293T cells using Lipofectamine 2000 (Invitrogen). After incubation for 48 h, cells were harvested, and the relative luciferase activity was measured using the dual-luciferase assay kit (Promega). To detect the Wnt signaling activity, cells were cotransfected with phRL-TK *Renilla* luciferase vectors (Promega), TOPFlash firefly luciferase reporter vector (Addgene, Cambridge, MA, USA), and miR-219-5p mimics or anti-miR-219-5p. After 48 h, the relative luciferase activity was determined using the dual-luciferase assay kit (Promega).

Data Analysis

All results were expressed as means ± standard deviation, and statistical analysis was performed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Differences were analyzed by Student's *t*-test and one-way analysis of variance followed by Bonferroni post hoc test on the basis of the data characteristics. The relationship between miR-219-5p and LRH-1 in gastric cancer tissues was examined by Pearson correlation analysis. Differences were considered statistically significant at $p<0.05$.

RESULTS

Downregulated Expression of miR-219-5p in Gastric Cancer

To assess the relevance of miR-219-5p in gastric cancer, we examined the expression of miR-219-5p in gastric cancer and normal adjacent tissues using RT-qPCR. The results showed that the miR-219-5p expression in malignant tumor tissues was significantly lower than that in normal adjacent tissues (Fig. 1A). To further confirm this result, we detected the expression level of miR-219-5p in normal human gastric epithelial GES-1 cells and a panel of gastric cancer cell lines, including MGC-803, BGC-823, MKN-45, and SGC-7901, using RT-qPCR. We observed

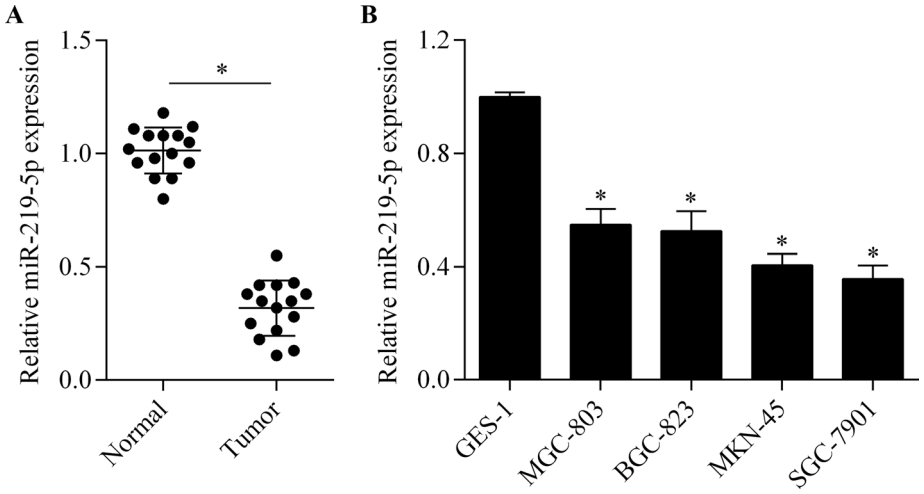

Figure 1. Analysis of miR-219-5p in gastric cancer tissues and cell lines. (A) Relative expression levels of miR-219-5p in 15 pairs of tumor tissues and adjacent normal counterparts were detected by RT-qPCR. *$p<0.05$. (B) Relative expression levels of miR-219-5p were examined in normal human gastric epithelial GES-1 cells and four gastric cancer cell lines (MGC-803, BGC-823, MKN-45, and SGC-7901) using RT-qPCR. *$p<0.05$ versus GES-1.

that normal gastric epithelial GES-1 cells expressed significantly more miR-219-5p than gastric cancer cell lines (Fig. 1B). These results indicate that miR-219-5p is frequently downregulated in gastric cancer and may act as a tumor suppressor.

Overexpression of miR-219-5p Inhibits Gastric Cancer Cell Proliferation

To explore the potential biological role of miR-219-5p in gastric cancer cells, two gastric cancer cell lines, MKN-45 and SGC-7901, were transfected with miR-219-5p mimics or anti-miR-219-5p. The levels of miR-219-5p were significantly increased in MKN-45 and SGC-7901 cells after transfection with miR-219 mimics (Fig. 2A). By contrast, miR-219-5p expression was markedly suppressed after transfection with anti-miR-219 (Fig. 2B). We then analyzed the gastric cancer cell proliferation by performing colony formation assays. The results demonstrated that miR-219-5p overexpression can markedly reduce the colony-forming capacity of gastric cancer cells (Fig. 3A), whereas miR-219-5p suppression increased the colony-forming capacity (Fig. 3B).

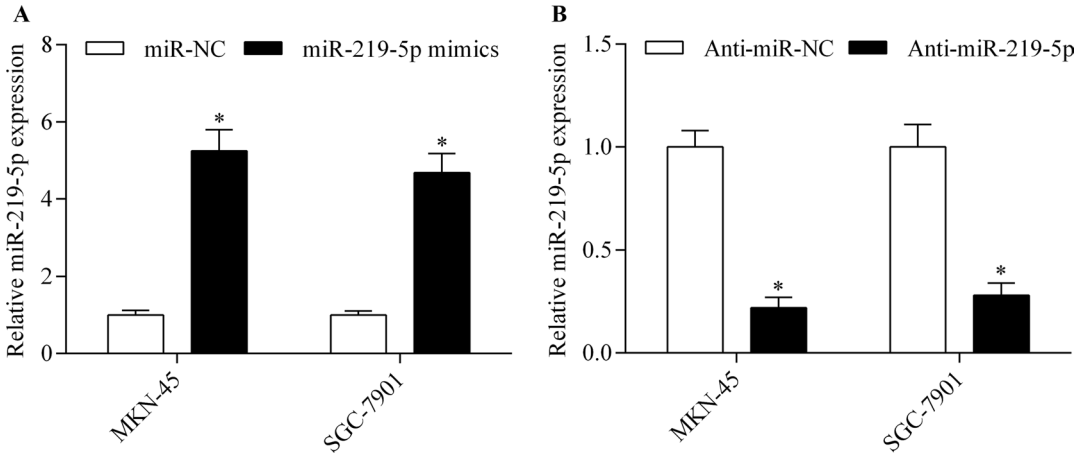

Figure 2. Transfection of miR-219-5p mimics or anti-miR-219-5p in gastric cancer cells. The relative expression levels of miR-219-5p in MKN-45 and SGC-7901 cells were examined after the cells were transfected with miR-219-5p mimics (A) or anti-miR-219-5p (B) for 48 h using RT-qPCR. *$p<0.05$.

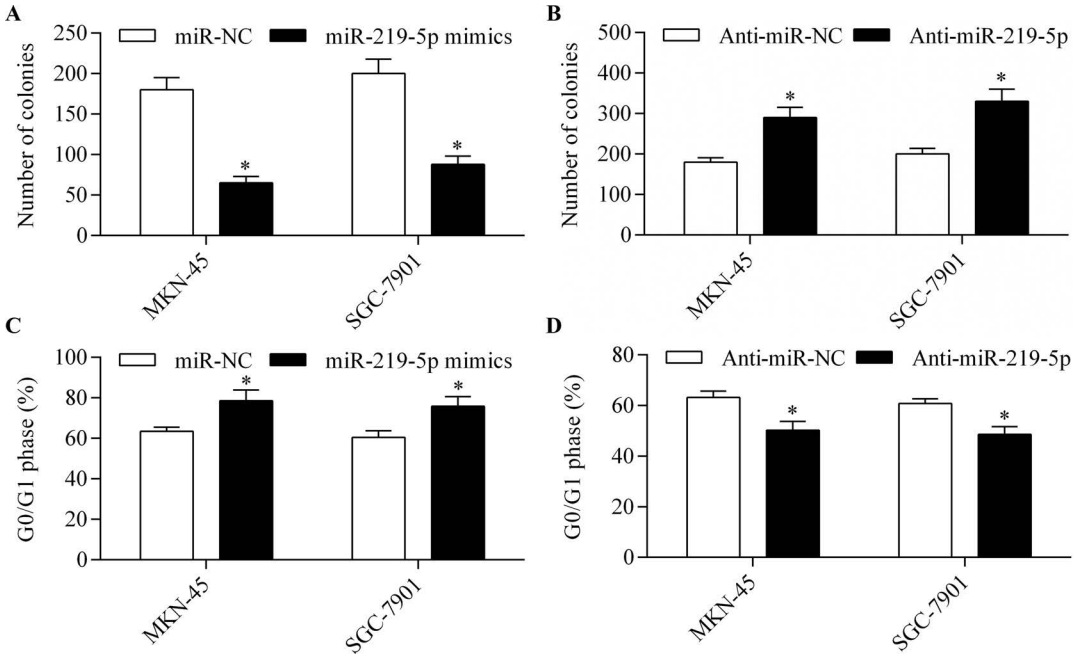

Figure 3. miR-219-5p inhibits colony formation and induces G_0/G_1 arrest. The colony formation of MKN-45 and SGC-7901 cells was quantified by colony formation assays after transfection with miR-219-5p mimics (A) or anti-miR-219-5p (B). Cells were cultured for 14 days and then harvested for analysis. The cell cycle distribution of MKN-45 and SGC-7901 cells was detected by cytometry assay after transfection with miR-219-5p mimics (C) or anti-miR-219-5p (D) for 48 h. *$p < 0.05$.

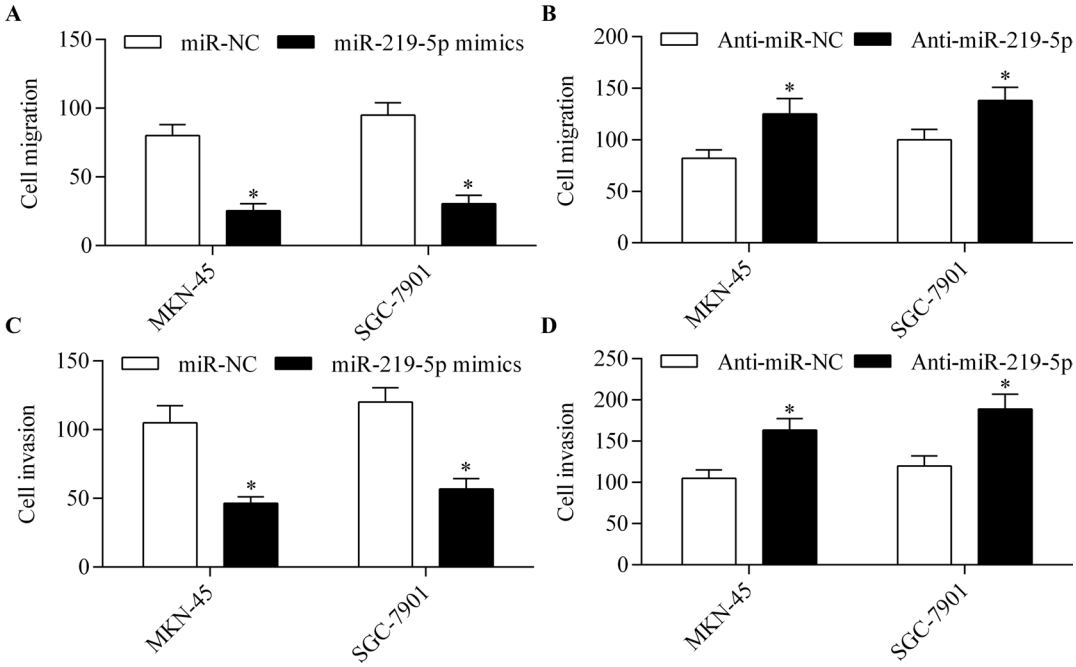

Figure 4. miR-219-5p inhibits gastric cancer cell migration and invasion. The effect of miR-219-5p overexpression (A) or anti-miR-219-5p (B) on gastric cancer cell migration of MKN-45 and SGC-7901 cells was detected by Transwell migration assay. The effect of miR-219-5p overexpression (C) or anti-miR-219-5p (D) on gastric cancer cell invasion of MKN-45 and SGC-7901 cells was detected by Transwell invasion assay. *$p < 0.05$.

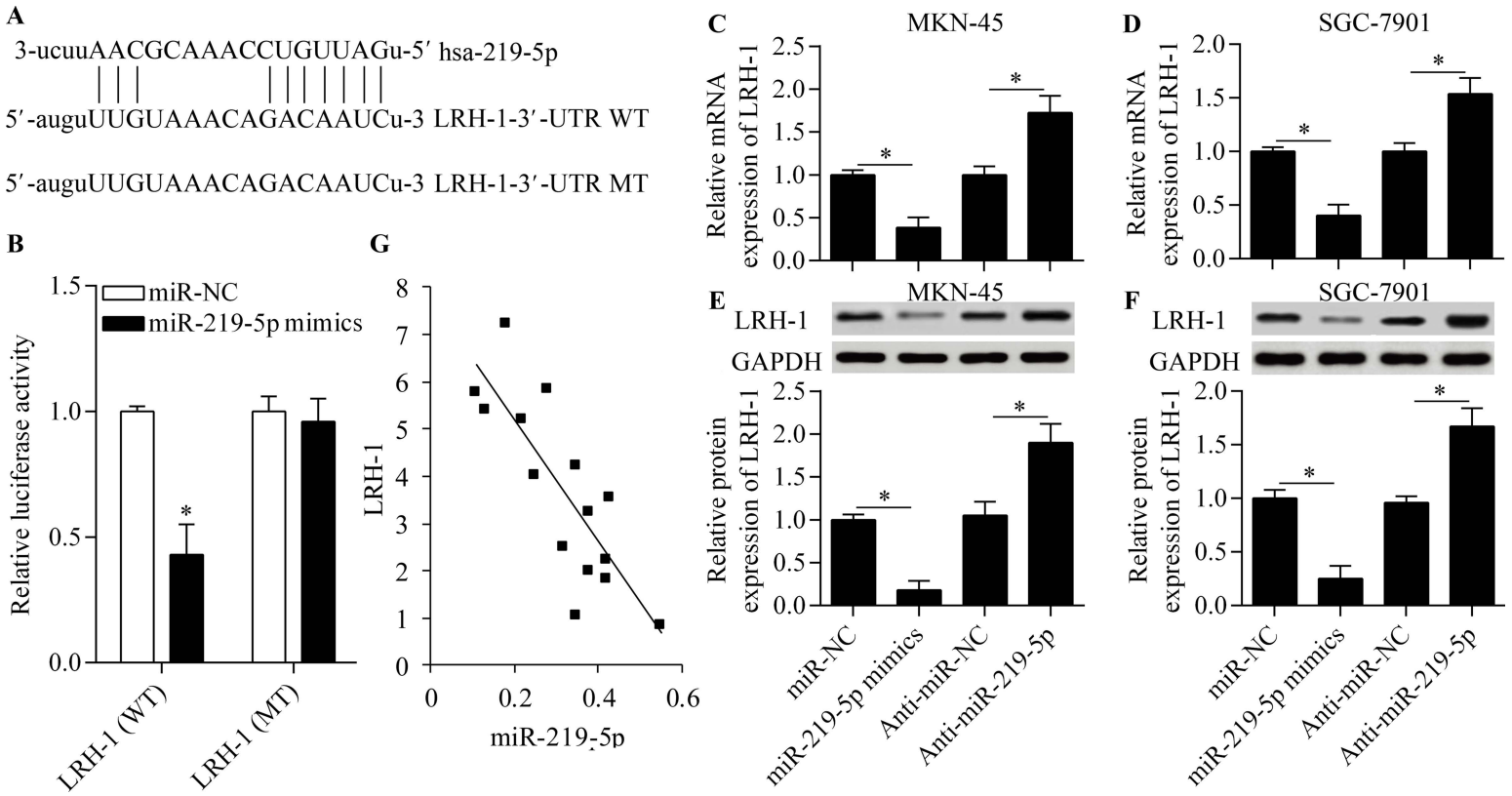

Figure 5. miR-219-5p directly targets the 3′-UTR of LRH-1. (A) Schematic representation of the seed sequences between miR-219-5p and LRH-1-3′-UTR. (B) The relative luciferase activity generated by the luciferase reporter plasmids containing LRH-1-3′-UTR WT or LRH-1-3′-UTR MT in 293T cells with miR-219-5p mimics was determined using dual-luciferase assays. After incubation for 48 h, the luciferase activity was analyzed using a dual-luciferase assay kit. *$p<0.05$ versus miR-NC. The mRNA expression of LRH-1 in MKN-45 (C) and SGC-7901 (D) cells was detected by RT-qPCR, and the protein expression of LRH-1 in MKN-45 (E) and SGC-7901 (F) cells was detected by Western blot analysis after transfection with miR-219-5p mimics or anti-miR-219-5p for 48 h. *$p<0.05$. (G) The correlation of LRH-1 mRNA and miR-219-5p expression in gastric cancer tissues was examined by Pearson correlation analysis. $r=-0.8186$, $p<0.0001$.

Cell cycle distribution was analyzed by flow cytometry, which demonstrated that miR-219-5p overexpression significantly induced cell cycle arrest in the G_0/G_1 phase (Fig. 3C). However, miR-219-5p suppression showed an opposite effect on cell cycle arrest (Fig. 3D). Therefore, these data suggest that miR-219-5p inhibited the proliferation of gastric cancer cells.

Overexpression of miR-219-5p Inhibits Gastric Cancer Cell Migration and Invasion

To confirm whether miR-219-5p plays a tumor suppressor role in gastric cancer, we performed Transwell assays to examine the effect of miR-219-5p on cell migration and invasion. The migration levels of the MKN-45 and SGC-7901 cells transfected with miR-219-5p mimics significantly decreased (Fig. 4A). Similarly, the invasive potential of MKN-45 and SGC-7901 cells transfected with miR-219-5p mimics was also markedly decreased (Fig. 4C). Conversely, suppression of miR-219-5p significantly promoted cell migration (Fig. 4B) and invasion (Fig. 4D) of MKN-45 and SGC-7901 cells. These data indicated that miR-219-5 can suppress migration and invasion of gastric cancer cells.

LRH-1 Is Characterized as a Target of miR-219-5p

To identify the functional target gene of miR-219-5p, we performed bioinformatics analysis to predict the potential targets of miR-219-5p. Among these genes, LRH-1, a well-known oncogene in a large number of malignancies, has elicited our interest. The putative binding sites of miR-219-5p in the 3′-UTR of LRH-1 (LRH-1-3′-UTR WT) are shown in Figure 5A. The complementary seed sequences were mutated to generate the 3′-UTR mutant (LRH-1-3′-UTR MT), which should not bind miR-219-5p. To confirm this prediction, we conducted luciferase reporter assays. The luciferase activity generated by the reporter vector with LRH-1-3′-UTR WT significantly decreased after cotransfection with miR219-5p mimics compared with the control group (Fig. 5B). However, the activity generated by the reporter vector with LRH-1-3′-UTR MT was not significantly affected by miR-219-5p overexpression (Fig. 5B). These results suggest that miR-219-5p inhibits the expression of LRH-1 by directly binding to the 3′-UTR of LRH-1. The following RT-qPCR and Western blot assays showed that the relative mRNA and protein levels of LRH-1 were significantly decreased by miR-219-5p overexpression (Fig. 5C–F). However, suppression of miR-219-5p markedly increased the LRH-1 expression level (Fig. 5C–F). In addition, LRH-1 mRNA expression was inversely correlated with miR-219-5p expression in gastric cancer tissues (Fig. 5G). Therefore, these results suggest that miR-219-5p inhibits LRH-1 expression by directly targeting its 3′-UTR in gastric cancer.

Overexpression of miR-219-5p Inhibits the Wnt/β-Catenin Signaling Pathway in Gastric Cancer Cells

To further elucidate the molecular mechanism of miR-219-5p-mediated tumor suppression, we analyzed the influence of miR-219-5p on the Wnt/β-catenin signaling pathway. Luciferase reporter assay results showed that miR-219-5p overexpression markedly repressed Wnt signaling, whereas miR-219-5p suppression promoted Wnt signaling in gastric cancer cells (Fig. 6A). Furthermore, the transcription levels of cyclin D1 (Fig. 6B) and cyclin

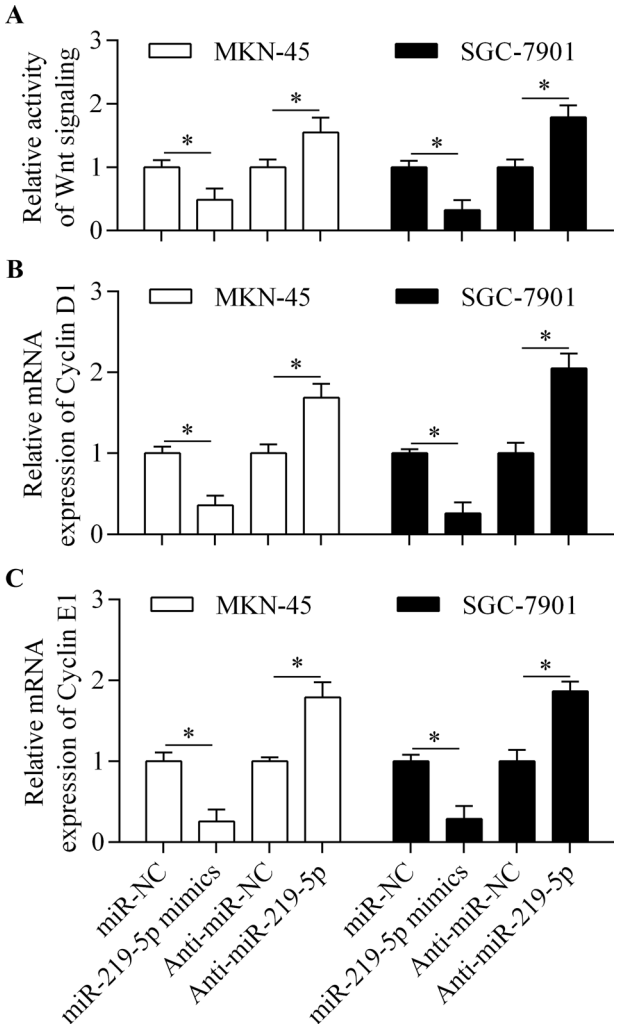

Figure 6. miR-219-5p suppresses the Wnt/β-catenin signaling pathway. (A) The effect of miR-219-5p overexpression or suppression on Wnt signaling in gastric cancer cells was examined by TCF-dependent TOPFlash reporter activity assay. MKN-45 and SGC-7901 cells were transfected with phRL-TK *Renilla* luciferase vectors and TOPFlash firefly luciferase reporter vectors in the presence of miR-219-5p mimics or anti-219-5p for 48 h. The expression of cyclin D1 (B) and cyclin E1 (C) was detected by RT-qPCR in MKN-45 and SGC-7901 cells transfected with miR-219-5p mimics or anti-miR-219-5p for 48 h. $^*p<0.05$.

E1 (Fig. 6C), which are the target genes of Wnt signaling, were significantly decreased by miR-219-5p overexpression or increased by miR-219-5p suppression. These data indicate that miR-219-5p inhibited the Wnt/β-catenin signaling pathway.

Overexpression of LRH-1 Rescues miR-219-5p-Mediated Tumor Suppression

To confirm whether LRH-1 is involved in miR-219-5p-mediated tumor suppression, we performed a rescue experiment by overexpressing LRH-1. To overexpress LRH-1 in gastric cancer cells, we transfected the cells with pcDNA3/LRH-1 vectors. The protein level of LRH-1 significantly increased in gastric cancer cells cotransfected with mir-219-5p mimics and pcDNA3/LRH-1 vectors (Fig. 7A). These data indicated that overexpression of LRH-1 using the pcDNA3/LRH-1 vector can partially rescue the downregulated expression of LRH-1 induced by miR-219-5p overexpression. Accordingly, LRH-1 overexpression significantly restored the inhibitory effect of miR-219-5p on cell proliferation (Fig. 7B and C), as detected by MTT and cell cycle assays. Moreover, the miR-219-5p-mediated inhibitory effect on Wnt signaling was significantly reversed by LRH-1 overexpression (Fig. 7D). Transwell assays also showed that the inhibitory effect of miR-219-5p overexpression on migration (Fig. 8A) and invasion (Fig. 8B) of gastric cancer cells was significantly abrogated by LRH-1 overexpression. Overall, these results indicated that LRH-1 directly contributed to miR-219-5p-mediated tumor suppression.

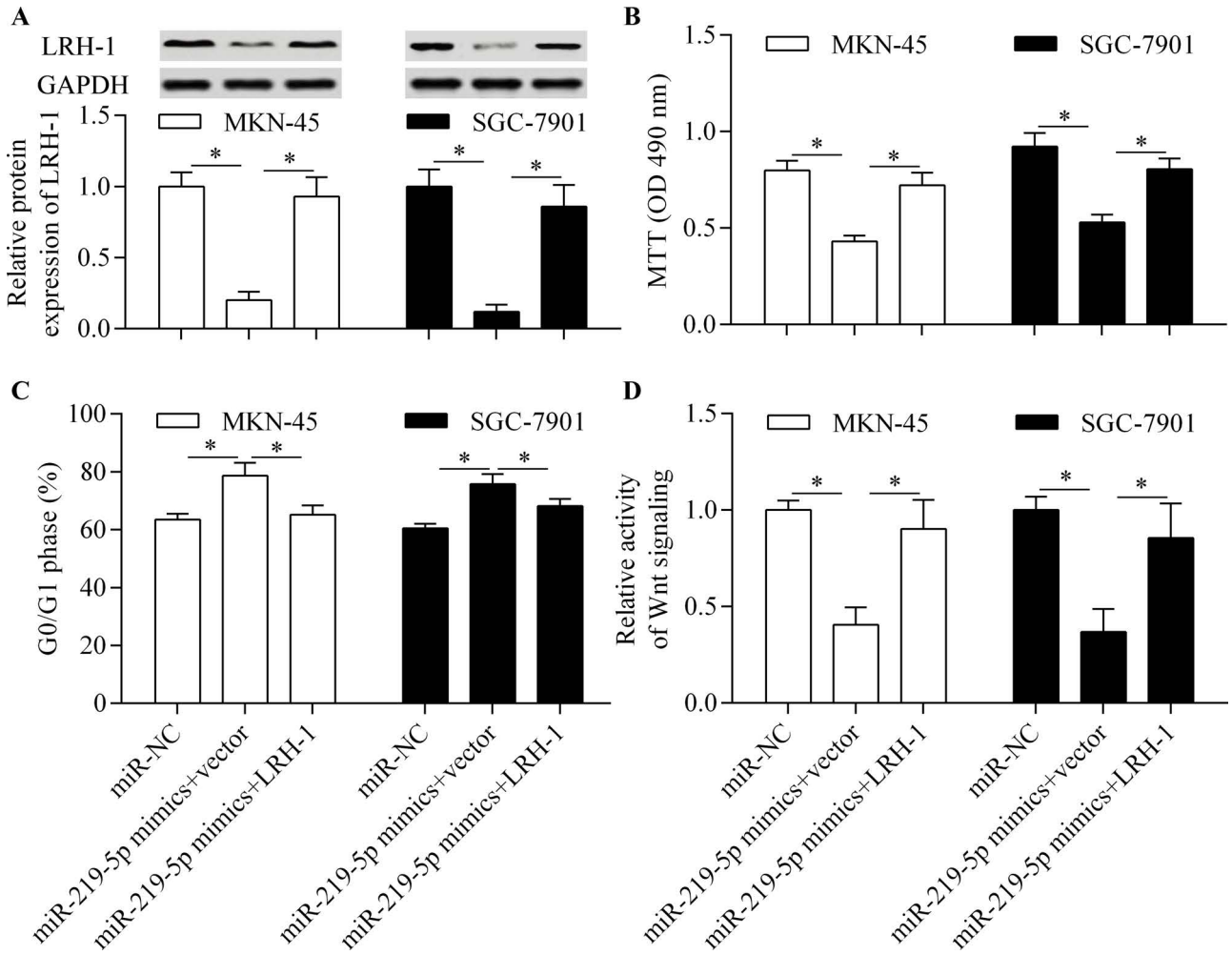

Figure 7. Restoration of LRH-1 reverses miR-219-5p-mediated inhibitory effect on gastric cancer cell proliferation. MKN-45 and SGC-7901 cells were cotransfected with miR-219-5p mimics and pcDNA3/LRH-1 vectors (without 3′-UTR) and incubated for 48 h. miR-219-5p mimics + vector, cells were transfected with miR-219-5p mimics and pDNA3.0 empty vector; miR-219-5p mimics + LRH-1, cells were transfected with miR-219-5p mimics and pcDNA3/LRH-1 vectors. (A) The protein expression of LRH-1 was detected by Western blot. Cell proliferation was detected by MTT (B) and cell cycle (C) assays. (D) Wnt signaling was detected by TCF-dependent TOPFlash reporter activity assay. *$p < 0.05$.

DISCUSSION

Increasing evidence has indicated that the dysregulation of miRNAs is involved in the development of gastric cancers[11,24,25]. Identification of miRNAs related to gastric cancer may help improve the diagnosis, prognosis, and treatment of this malignancy. In this study, we found that miR-219-5p was a novel miRNA that regulated gastric cancer development. We found that miR-219-5p expression was significantly reduced in gastric cancer tissues and cell lines. The overexpression of miR-219-5p inhibited gastric cancer cell proliferation, migration, and invasion by targeting LRH-1, a well-known oncogene[14]. Our data revealed a novel miRNA-based molecular mechanism for gastric cancer pathogenesis.

Moreover, we found that miR-219-5p expression was decreased in gastric cancer. This finding indicates a tumor suppressor role for miR-219-5p. As expected, miR-219-5p overexpression suppressed gastric cancer cell proliferation, migration, and invasion. Similar findings have been obtained in several other types of human cancers. Huang et al. reported that miR-219-5p suppressed hepatocellular carcinoma cell proliferation by targeting glypican-3[26]. In glioblastoma, miR-219-5p overexpression inhibited cell growth and migration by targeting epidermal growth factor receptor[27] or Roundabout 1[23]. Forced expression of miR-219-5p inhibited papillary thyroid carcinoma cell growth by targeting estrogen receptor α[28]. miR-219-5p has also been reported to be a tumor suppressor in colon cancer by targeting platelet-derived growth factor receptor α[29] and Sall4[22]. These reports together with our findings support the tumor suppressor role of miR-219-5p.

miRNAs posttranscriptionally regulate gene expression by targeting the 3′-UTR of target mRNAs[5,6]. To investigate the mechanism responsible for miR-219-5p-mediated tumor suppression, we performed bioinformatics analysis and identified LRH-1 as a functional target of miR-219-5p in gastric cancer cells. LRH-1 is an oncogene in various cancers[14]. LRH-1 is abnormally expressed in 45% of all breast cancers and promotes local estrogen biosynthesis[30]. LRH-1 also promotes proliferation, motility, and invasion of breast cancer cells by upregulating estrogen-responsive genes and matrix metalloproteinase-9, remodeling actin, and inactivating E-cadherin[18,19,31]. The overexpression of LRH-1 promotes wound healing, migration, and invasion, as well as sphere formation of pancreatic cancer cells[32–34]. Deficiency of LRH-1 impedes intestinal tumorigenesis by regulating inflammation, as shown by a mouse model in vivo[16]. Silencing of LRH-1 inhibits colon cancer cell proliferation by inhibiting oncogenic genes and signaling pathways[21,35]. LRH-1 also plays an important role in gastric cancer. Wang et al. have reported that LRH-1 is highly expressed in gastric cancer tissues, and LRH-1 overexpression promotes gastric cancer cell proliferation[17]. In the present study, we found that miR-219-5p can target and inhibit LRH-1. This finding implied a novel endogenic inhibitor for LRH-1.

Considering the important role of LRH-1 in tumorigenesis, current efforts have been made to develop the inhibitors for LRH-1. Two compounds, Cpd3 and Cpd3d2, have been reported to block the transcriptional activity of LRH-1, leading to an inhibitory effect on cell growth and proliferation of cancer cells[36]. Corzo et

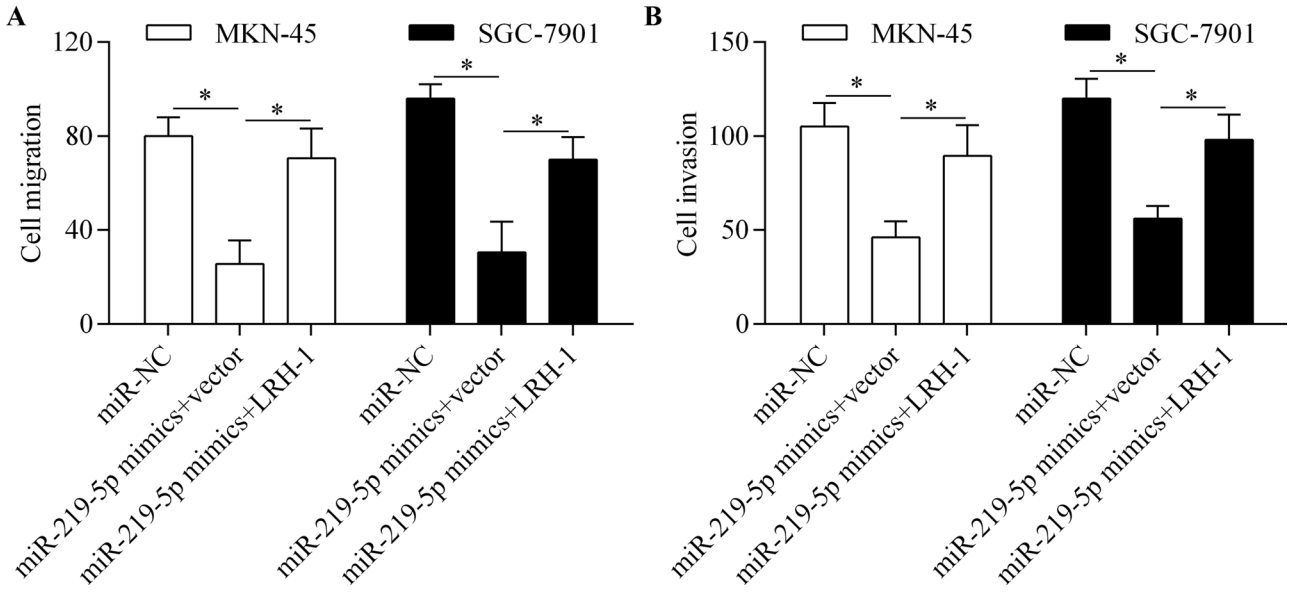

Figure 8. Restoration of LRH-1 reverses miR-219-5p-mediated inhibitory effect on gastric cancer cell migration and invasion. The effect of LRH-1 overexpression on cell migration (A) and invasion (B) of MKN-45 and SGC-7901 cells cotransfected with miR-219-5p mimics and pcDNA3/LRH-1 vectors was detected by Transwell assays. *$p < 0.05$.

al. synthesized a new nonphospholipid small-molecule repressor of LRH-1, SR1848, which can impede the nuclear translocation of LRH-1 and inhibit the expression of oncogenes involved in promoting cancer cell proliferation[37]. In addition to chemical inhibitors, miRNAs are emerging as novel inhibitors for gene expression. Various miRNAs have been found to target and inhibit LRH-1. In particular, miR-451 can inhibit osteosarcoma cell proliferation by targeting LRH-1[38]. Suppression of LRH-1 by miR-381 inhibited cell proliferation and the invasion of colon[39] and hepatocellular carcinoma cells[40]. Jiang et al. recently reported that miR-376c targets and inhibits LRH-1 in lung cancer cells, leading to reduced cell proliferation and invasion[41]. Other miRNAs, such as miR-134[42], miR-139-5p[43], miR-27[44], miR-186, and miR-326[45], are reported to posttranscriptionally regulate the LRH-1 expression. In the current study, we demonstrated that miR-219-5p was a novel miRNA for inhibiting LRH-1. We found that miR-219-5p inhibited gastric cancer cell proliferation, migration, and invasion by targeting and inhibiting LRH-1. Restoration of the LRH-1 expression can significantly reverse the miR-219-5p-mediated tumor suppressive effects. Our findings indicate that miR-219-5p acted as an endogenous inhibitor for LRH-1. Therefore, miR-219-5p might be used as a potential anticancer drug target for LRH-1-related cancers, at least in gastric cancer.

LRH-1 has been suggested as a coactivator of the Wnt/β-catenin signaling pathway[14,46]. LRH-1 can interact with transcription factor 4 and β-catenin to promote the expression of c-Myc and cyclin D1/E1[46]. LRH-1 regulates the proliferation of pancreatic and colon cancer cells via the Wnt/β-catenin signaling pathway[16,32]. The Wnt/β-catenin signaling pathway plays an important role in gastric carcinogenesis by regulating cell proliferation, migration, invasion, and epithelial–mesenchymal transition[47–49]. In this study, we demonstrated that LRH-1 suppression by miR-219-5p inhibited the Wnt/β-catenin signaling pathway in gastric cancer cells. These findings suggest that targeting LRH-1 by miR-219-5p is a novel strategy for inhibiting the Wnt/β-catenin signaling pathway in gastric cancer.

In conclusion, our study is the first to report that miR-219-5p was downregulated in gastric cancer. In particular, miR-219-5p functions as a gastric cancer suppressor by inhibiting cell proliferation, migration, and invasion by targeting the LRH-1 and Wnt/β-catenin signaling pathways. These results indicated that miR-542-3p may represent a novel molecular therapeutic target for gastric cancer.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

REFERENCES

1. Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: Defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol. 2006;24:2137–50.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016;66:7–30.
3. Orditura M, Galizia G, Sforza V, Gambardella V, Fabozzi A, Laterza MM, Andreozzi F, Ventriglia J, Savastano B, Mabilia A, Lieto E, Ciardiello F, De Vita F. Treatment of gastric cancer. World J Gastroenterol. 2014;20:1635–49.
4. Terry MB, Gaudet MM, Gammon MD. The epidemiology of gastric cancer. Semin Radiat Oncol. 2002;12:111–27.
5. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
6. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: MicroRNA biogenesis pathways and their regulation. Nat Cell Biol. 2009;11:228–34.
7. Manikandan J, Aarthi JJ, Kumar SD, Pushparaj PN. Oncomirs: The potential role of non-coding microRNAs in understanding cancer. Bioinformation 2008;2:330–4.
8. Pang JC, Kwok WK, Chen Z, Ng HK. Oncogenic role of microRNAs in brain tumors. Acta Neuropathol. 2009; 117:599–611.
9. Silber J, James CD, Hodgson JG. microRNAs in gliomas: Small regulators of a big problem. Neuromolecular Med. 2009;11:208–22.
10. Jiang C, Chen X, Alattar M, Wei J, Liu H. MicroRNAs in tumorigenesis, metastasis, diagnosis and prognosis of gastric cancer. Cancer Gene Ther. 2015;22:291–301.
11. Wang G, Fu Y, Liu G, Ye Y, Zhang X. miR-218 inhibits proliferation, migration, and EMT of gastric cancer cells by targeting WASF3. Oncol Res. 2017;25:355–64.
12. Lin F, Li Y, Yan S, Liu S, Qian W, Shen D, Lin Q, Mao W. MicroRNA-181a inhibits tumor proliferation, invasiveness, and metastasis and is downregulated in gastric cancer. Oncol Res. 2015;22:75–84.
13. Fayard E, Auwerx J, Schoonjans K. LRH-1: An orphan nuclear receptor involved in development, metabolism and steroidogenesis. Trends Cell Biol. 2004;14:250–60.
14. Nadolny C, Dong X. Liver receptor homolog-1 (LRH-1): A potential therapeutic target for cancer. Cancer Biol Ther. 2015;16:997–1004.
15. Chand AL, Wijayakumara DD, Knower KC, Herridge KA, Howard TL, Lazarus KA, Clyne CD. The orphan nuclear receptor LRH-1 and ERalpha activate GREB1 expression to induce breast cancer cell proliferation. PLoS One 2012;7:e31593.
16. Schoonjans K, Dubuquoy L, Mebis J, Fayard E, Wendling O, Haby C, Geboes K, Auwerx J. Liver receptor homolog 1 contributes to intestinal tumor formation through effects on cell cycle and inflammation. Proc Natl Acad Sci USA 2005;102:2058–62.
17. Wang SL, Zheng DZ, Lan FH, Deng XJ, Zeng J, Li CJ, Wang R, Zhu ZY. Increased expression of hLRH-1 in human gastric cancer and its implication in tumorigenesis. Mol Cell Biochem. 2008;308:93–100.
18. Annicotte JS, Chavey C, Servant N, Teyssier J, Bardin A, Licznar A, Badia E, Pujol P, Vignon F, Maudelonde T, Lazennec G, Cavailles V, Fajas L. The nuclear receptor liver receptor homolog-1 is an estrogen receptor target gene. Oncogene 2005;24:8167–75.

19. Chand AL, Herridge KA, Thompson EW, Clyne CD. The orphan nuclear receptor LRH-1 promotes breast cancer motility and invasion. Endocr Relat Cancer 2010;17:965–75.
20. Kramer HB, Lai CF, Patel H, Periyasamy M, Lin ML, Feller SM, Fuller-Pace FV, Meek DW, Ali S, Buluwela L. LRH-1 drives colon cancer cell growth by repressing the expression of the CDKN1A gene in a p53-dependent manner. Nucleic Acids Res. 2016;44:582–94.
21. Bayrer JR, Mukkamala S, Sablin EP, Webb P, Fletterick RJ. Silencing LRH-1 in colon cancer cell lines impairs proliferation and alters gene expression programs. Proc Natl Acad Sci USA 2015;112:2467–72.
22. Cheng J, Deng R, Zhang P, Wu C, Wu K, Shi L, Liu X, Bai J, Deng M, Shuai X, Gao J, Wang G, Tao K. miR-219-5p plays a tumor suppressive role in colon cancer by targeting oncogene Sall4. Oncol Rep. 2015;34:1923–32.
23. Jiang Y, Yin L, Jing H, Zhang H. MicroRNA-219-5p exerts tumor suppressor function by targeting ROBO1 in glioblastoma. Tumour Biol. 2015;36:8943–51.
24. Yu B, Lv X, Su L, Li J, Yu Y, Gu Q, Yan M, Zhu Z, Liu B. MiR-148a functions as a tumor suppressor by targeting CCK-BR via inactivating STAT3 and Akt in human gastric cancer. PLoS One 2016;11:e0158961.
25. Wu G, Zheng K, Xia S, Wang Y, Meng X, Qin X, Cheng Y. MicroRNA-655-3p functions as a tumor suppressor by regulating ADAM10 and beta-catenin pathway in hepatocellular carcinoma. J Exp Clin Cancer Res. 2016;35:89.
26. Huang N, Lin J, Ruan J, Su N, Qing R, Liu F, He B, Lv C, Zheng D, Luo R. MiR-219-5p inhibits hepatocellular carcinoma cell proliferation by targeting glypican-3. FEBS Lett. 2012;586:884–91.
27. Rao SA, Arimappamagan A, Pandey P, Santosh V, Hegde AS, Chandramouli BA, Somasundaram K. miR-219-5p inhibits receptor tyrosine kinase pathway by targeting EGFR in glioblastoma. PLoS One 2013;8:e63164.
28. Huang C, Cai Z, Huang M, Mao C, Zhang Q, Lin Y, Zhang X, Tang B, Chen Y, Wang X, Qian Z, Ye L, Peng Y, Xu H. miR-219-5p modulates cell growth of papillary thyroid carcinoma by targeting estrogen receptor alpha. J Clin Endocrinol Metab. 2015;100:E204–13.
29. Xiong GB, Zhang GN, Xiao Y, Niu BZ, Qiu HZ, Wu B, Lin GL, You L, Shu H. MicroRNA-219-5p functions as a tumor suppressor partially by targeting platelet-derived growth factor receptor alpha in colorectal cancer. Neoplasma 2015;62:855–63.
30. Miki Y, Clyne CD, Suzuki T, Moriya T, Shibuya R, Nakamura Y, Ishida T, Yabuki N, Kitada K, Hayashi S, Sasano H. Immunolocalization of liver receptor homologue-1 (LRH-1) in human breast carcinoma: Possible regulator of insitu steroidogenesis. Cancer Lett. 2006;244:24–33.
31. Thiruchelvam PT, Lai CF, Hua H, Thomas RS, Hurtado A, Hudson W, Bayly AR, Kyle FJ, Periyasamy M, Photiou A, Spivey AC, Ortlund EA, Whitby RJ, Carroll JS, Coombes RC, Buluwela L, Ali S. The liver receptor homolog-1 regulates estrogen receptor expression in breast cancer cells. Breast Cancer Res Treat. 2011;127:385–96.
32. Benod C, Vinogradova MV, Jouravel N, Kim GE, Fletterick RJ, Sablin EP. Nuclear receptor liver receptor homologue 1 (LRH-1) regulates pancreatic cancer cell growth and proliferation. Proc Natl Acad Sci USA 2011;108:16927–31.
33. Lin Q, Aihara A, Chung W, Li Y, Chen X, Huang Z, Weng S, Carlson RI, Nadolny C, Wands JR, Dong X. LRH1 promotes pancreatic cancer metastasis. Cancer Lett. 2014;350:15–24.
34. Lin Q, Aihara A, Chung W, Li Y, Huang Z, Chen X, Weng S, Carlson RI, Wands JR, Dong X. LRH1 as a driving factor in pancreatic cancer growth. Cancer Lett. 2014;345:85–90.
35. Kramer HB, Lai CF, Patel H, Periyasamy M, Lin ML, Feller SM, Fuller-Pace FV, Meek DW, Ali S, Buluwela L. LRH-1 drives colon cancer cell growth by repressing the expression of the CDKN1A gene in a p53-dependent manner. Nucleic Acids Res. 2016;44:582–94.
36. Benod C, Carlsson J, Uthayaruban R, Hwang P, Irwin JJ, Doak AK, Shoichet BK, Sablin EP, Fletterick RJ. Structure-based discovery of antagonists of nuclear receptor LRH-1. J Biol Chem. 2013;288:19830–44.
37. Corzo CA, Mari Y, Chang MR, Khan T, Kuruvilla D, Nuhant P, Kumar N, West GM, Duckett DR, Roush WR, Griffin PR. Antiproliferation activity of a small molecule repressor of liver receptor homolog 1. Mol Pharmacol. 2015;87:296–304.
38. Li Z, Wu S, Lv S, Wang H, Wang Y, Guo Q. Suppression of liver receptor homolog-1 by microRNA-451 represses the proliferation of osteosarcoma cells. Biochem Biophys Res Commun. 2015;461:450–5.
39. Liang Y, Zhao Q, Fan L, Zhang Z, Tan B, Liu Y, Li Y. Down-regulation of MicroRNA-381 promotes cell proliferation and invasion in colon cancer through up-regulation of LRH-1. Biomed Pharmacother. 2015;75:137–41.
40. Zhang Q, Zhao S, Pang X, Chi B. MicroRNA-381 suppresses cell growth and invasion by targeting the liver receptor homolog-1 in hepatocellular carcinoma. Oncol Rep. 2016;35:1831–40.
41. Jiang W, Tian Y, Jiang S, Liu S, Zhao X, Tian D. MicroRNA-376c suppresses non-small-cell lung cancer cell growth and invasion by targeting LRH-1-mediated Wnt signaling pathway. Biochem Biophys Res Commun. 2016;473:980–6.
42. Tay YM, Tam WL, Ang YS, Gaughwin PM, Yang H, Wang W, Liu R, George J, Ng HH, Perera RJ, Lufkin T, Rigoutsos I, Thomson AM, Lim B. MicroRNA-134 modulates the differentiation of mouse embryonic stem cells, where it causes post-transcriptional attenuation of Nanog and LRH1. Stem Cells 2008;26:17–29.
43. Liu R, Yang M, Meng Y, Liao J, Sheng J, Pu Y, Yin L, Kim SJ. Tumor-suppressive function of miR-139-5p in esophageal squamous cell carcinoma. PLoS One 2013;8:e77068.
44. Fuchs H, Theuser M, Wruck W, Adjaye J. miR-27 negatively regulates pluripotency-associated genes in human embryonal carcinoma cells. PLoS One 2014;9:e111637.
45. Zhang ZL, Bai ZH, Wang XB, Bai L, Miao F, Pei HH. miR-186 and 326 predict the prognosis of pancreatic ductal adenocarcinoma and affect the proliferation and migration of cancer cells. PLoS One 2015;10:e0118814.
46. Botrugno OA, Fayard E, Annicotte JS, Haby C, Brennan T, Wendling O, Tanaka T, Kodama T, Thomas W, Auwerx J, Schoonjans K. Synergy between LRH-1 and beta-catenin induces G1 cyclin-mediated cell proliferation. Mol Cell 2004;15:499–509.
47. Song X, Xin N, Wang W, Zhao C. Wnt/beta-catenin, an oncogenic pathway targeted by H. pylori in gastric carcinogenesis. Oncotarget 2015;6:35579-88.
48. Taketo MM. Wnt signaling and gastrointestinal tumorigenesis in mouse models. Oncogene 2006;25:7522–30.
49. Kolligs FT, Bommer G, Goke B. Wnt/beta-catenin/tcf signaling: A critical pathway in gastrointestinal tumorigenesis. Digestion 2002;66:131–44.