

Long non-coding RNA H19 regulates cell growth and metastasis via the miR-22-3p/Snail1 axis in gastric cancer

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Abstract. Gastric cancer (GC) is the fifth most prevalent type of malignancy and the third leading cause of cancer-related mortality worldwide, with the prognosis of patients with late-stage GC remaining at poor levels. Long non-coding RNA (lncRNA) H19 (H19) is involved in the growth and metastasis of tumors, and it is upregulated under hypoxic conditions and in certain types of cancer; however, the underlying mechanisms of action of this lncRNA as regards the initiation and development of GC remain unknown. Thus, in the present study, we aimed to determine the role of lncRNA H19 in GC and to elucidate the underlying mechanisms. H19 was found to be upregulated in GC tissues and cells compared with the para-cancerous tissues, and an elevated expression of H19 was associated with lymph node metastasis and TNM stage. Furthermore, the downregulation of H19 suppressed the proliferation, invasion, migration and epithelial-mesenchymal transition of GC cells *in vitro* and suppressed tumor growth *in vivo*. H19 was also found to be able to bind with miR-22-3p, and H19-induced cell growth and metastasis were shown to be reversed by the upregulation of miR-22-3p; the miR-22-3p level was found to inversely correlate with H19 expression in GC tissues. Furthermore, the overexpression of miR-22-3p notably suppressed the proliferation, migration and invasion of GC cells, and these effects were enhanced by the downregulation of Snail1. In addition, cell growth and metastasis induced by miR-22-3p downregulation were partially reversed by the knockdown of Snail1. Furthermore, a negative correlation was observed between the expression levels of miR-22-3p and Snail1 in GC tissues. On the whole, the findings of the present study revealed that H19 was upregulated in GC tissues, which promoted tumor growth and metastasis via the miR-22-3p/Snail1 signaling pathway.

In summary, these findings provide novel insight into the potential regulatory roles of H19 in GC, and suggest that the H19/miR-22-3p/Snail1 axis may prove to be a promising therapeutic target for the treatment of patients with GC.

Introduction

Gastric cancer (GC) is the fifth most prevalent and aggressive type of cancer and the third leading cause of cancer-related mortality worldwide (1). The incidence and mortality rates associated with GC are increasing in East Asia and Eastern Europe. In China, the most recent statistical analysis has revealed that GC is the second most common type of cancer with a 5-year survival rate of ~25-30% (2). The development of endoscopy and surgical techniques has reduced the 5-year mortality rate associated with GC; however, for patients with late-stage GC, the 5-year mortality rate remains ~30-50% (3). Although the effectiveness of treatment strategies, such as surgery combined with radiotherapy and chemotherapy has somewhat improved, the prognosis of patients with advanced GC remains poor due to peritoneal dissemination, hematogenous spread and lymph node metastasis (4). Thus, it is crucial to explore the underlying molecular mechanisms responsible for the progression of GC.

Recent studies have indicated that non-coding RNAs (ncRNAs) are novel regulatory molecules that play essential roles in tumorigenesis (5,6). ncRNAs, including long non-coding RNAs (lncRNAs) and microRNAs (miRNAs or miRs) are not capable of coding proteins (7). miRNAs interact with associated proteins to form the active RNA-induced silencing complex, consequently suppressing the translation or inducing the degradation of target mRNAs (8). Aberrantly expressed miRNAs are associated with the proliferation, apoptosis, growth, migration and invasion of cancer cells (9). In recent years, the effects of lncRNAs on tumor development have been investigated. Previous studies have suggested that lncRNAs are involved in the regulation of various cellular functions and disease pathogenesis, such as cancer metastasis at the transcriptional and post-transcriptional level (10,11). The potential function of lncRNAs is to regulate the expression levels of mRNAs and miRNAs by altering chromatin modification and transcription (12). Furthermore, lncRNAs are capable of inhibiting the expression of miRNAs via direct

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binding, consequently producing competing endogenous RNAs (ceRNAs) that can induce protein destabilization at the post-transcriptional level (13). Accumulating evidence has suggested that the impaired expression of lncRNA H19 (hereon referred to as H19) plays an essential role in carcinogenesis. For example, H19 has been shown to promote the progression of GC (14), breast cancer (15), lung cancer (16) and colorectal cancer (17). In addition, a previous study revealed that the upregulated expression of H19 can affect the proliferation of GC cells and is associated with the initiation and development of GC (18). miR-22-3p, as a tumor suppressor miRNA, has been shown to be significantly downregulated in GC (19), colorectal cancer (20), hepatocellular carcinoma (21), cervical cancer (22), prostate cancer and lung cancer (23). The downregulation of miR-22-3p can promote tumor growth and metastasis. A recent study suggested that H19 functions as a ceRNA for miR-22-3p by regulating its downstream Wnt/ β -catenin signaling in nucleus pulposus cells (NPCs) (24). Although H19 functions as a molecular sponge of miR-22-3p in GC, the underlying mechanisms of this function remain unknown. The association between H19 and GC requires further investigation. Thus, the aim of the present study was to identify the functions of H19 in GC and to investigate whether miR-22-3p is the target of H19. The results of the present study reveal the regulatory mechanisms of action of H19 in GC, which may provide novel insight into potential therapeutic targets for the treatment of GC.

Materials and methods

Patient samples. A total of 40 pairs of primary tumor and para-cancerous tissues were obtained from patients with GC (28 males and 12 females; average age, 58.93 ± 9.17 years) undergoing surgical resection at the Second Affiliated Hospital of Chongqing Medical University (Chongqing, China). The study was approved by the Ethics Committee of Chongqing Medical University. Informed consent was obtained from each patient prior to surgery. The clinicopathological characteristics of the patient samples were confirmed by two pathologists according to the diagnostic criteria of World Health Organization (2010-2012). Metastasis was found in 28 cases, and 25 patients were diagnosed with stage I or II gastric cancer. All samples were immediately stored in liquid nitrogen until further use.

Cells, cell culture and transfection. AGS (cat. no. CRL-1739) and normal gastric mucous epithelium cells (GES-1; cat. no. 28200) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). BGC-823 (cat. no. TCHu 11), MKN-45 (cat. no. TCHu130) and SGC-7901 (cat. no. TCHu 46) were purchased from Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (HyClone; GE Healthcare Life Science, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Science) and incubated at 37°C in a humidified atmosphere of 5% CO₂. Short hairpin RNA (shRNA) sequences targeting H19 (sh-H19) and Snail1 (sh-Snail1), as well as the negative control (sh-NC) were obtained from Genepharma Co. Ltd. (Shanghai, China). Following annealing, shRNAs were integrated into the lentiviral pU6-Luc-Puro vector (Genepharma Co. Ltd.). To

generate the H19 overexpression model, the H19 fragment was amplified using PCR and then subcloned into the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). pcDNA3.1 vectors containing wild-type (WT) H19 or Snail1, mutant (MUT) H19 or Snail1 were obtained from RioBio (Guangzhou, China). miR-22-3p mimics, inhibitors and negative control (miR-NC) were synthesized by Genepharma Co. Ltd. (Shanghai, China). All transfections were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cells were harvested at 48 h post-transfection for use in further experiments.

Luciferase reporter assay. Targetscan (www.targetscan.org) and LncBase Predicted v.2 (<http://www.microna.gr/LncBase>) were employed to predict the potential targets of miR-22-3p or H19. Wild-type segments of the 3'UTR of H19/Snail1 containing potential binding sites of miR-22-3p were cloned into the pMIR-REPORT firefly luciferase vector (Applied Biosystems, Foster City, CA, USA). The mutant fragment of H19/Snail1 was used as a control. 293 cells (ATCC) were co-transfected with the luciferase reporter vector and miR-22-3p mimics or miRNA negative control (miR-NC) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h post-transfection, the luciferase activity was evaluated using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocols. The activity was normalized to *Renilla* luciferase.

Cell proliferation assay. Transfected AGS and SGC-7901 cells were seeded at a density of 1×10^4 /well in 96-well plates. Proliferation rates were determined by Cell Counting kit-8 assay (CCK-8 Assay; Beyotime, Shanghai, China) at days 1, 2, 3 and 4 post-transfection. The absorbance at 450 nm was measured using a microplate reader (9200, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

Transwell assay. The migration and invasion of the cells were evaluated by a Transwell assay. The AGS and SGC-7901 cells were harvested at 48 h post-transfection. For the migration assay, 5×10^4 cells in serum-free medium were inoculated onto the upper chamber (BD Biosciences, Franklin Lakes, NJ, USA) with an 8- μ m membrane. For the invasion assay, 2×10^5 cells in serum-free medium were inoculated onto the upper chamber pre-coated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA). Culture medium containing 10% FBS was added to the lower chamber. Non-migratory cells were removed using a cotton swab following overnight incubation at 37°C, while the migrated or invaded cells in the lower chamber were fixed in 4% paraformaldehyde for 10 min at room temperature, and stained using methanol and 0.1% crystal violet. Finally, the number of migratory or invasive cells were observed and counted using an inverted light microscope (magnification, $\times 100$; Olympus Corp., Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was used to determine the expression levels of H19, miR-22-3p, Snail1, E-cadherin (E-Cad), α -smooth muscle actin (α -SMA), vimentin (VI) and

fibronectin (FN). Total RNA was extracted from the tissues or cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. miRNA was extracted using the miRNeasy Mini kit (Qiagen, Shenzhen, China) according to the manufacturer's instructions. RNA was reverse transcribed using a PrimeScript RT kit (Takara, Dalian, China) according to the manufacturer's instructions. For the assessment of miR-22-3p, a TaqMan MicroRNA Assay kit (Applied Biosystems) was used and qPCR was performed using the Applied Biosystem 7500 Real-Time PCR System; U6 was used for normalization. For H19, Snail1, E-Cad, α -SMA, VI and FN, qPCR was performed using SYBR-Green PCR Master Mix (Takara). Relative expression was calculated and normalized to endogenous GAPDH. The forward and reverse primer sequences were as follows: H19 forward, 5'-ATCGGTGC CTCAGCGTTCCG-3' and reverse, 5'-CTGTCCTCG CCGTC ACACCG-3'; Snail1 forward, 5'-GAAGATGCACATCCGA AGC-3' and reverse, 5'-AGTGGGAGCGGAGAAAGG-3'; E-Cad forward, 5'-CAATGGTGTCCATGTGAACA-3' and reverse, 5'-CCTCCTACCCTCTGTTCG-3'; α -SMA forward, 5'-TCCCTTGAGAAGAGTTACGAGTTG-3' and reverse, 5'-ATGATGCTGTTGTAGGTGGTTTC-3'; VI forward, 5'-CGCTTCGCCAACTACAT-3' and reverse, 5'-AGGGCA TCCACTTCACAG-3'; FN forward, 5'-CCAAACCTCAAGC TCCCGTCA-3' and reverse, 5'-GAGATTCTGCACATCAC GGTC-3'; and GAPDH forward, 5'-GCAAGAGCACAAG AGGAAGA-3' and reverse, 5'-ACTGTGAGGAGGGGAG ATTC-3'. The PCR program used for the thermocycler was as follows: 95°C for 5 min, followed by 45 cycles of 95°C for 15 sec, 60°C for 20 sec and 72°C for 10 sec. Relative expression levels were evaluated via the $2^{-\Delta\Delta C_q}$ method (25). The results of significant difference tests between the relative and raw numerical value are the same.

Western blot analysis. Total protein was isolated from the tissues or cells using radioimmunoprecipitation assay buffer (Beyotime). Protein concentrations were evaluated by a BCA Protein Assay kit (Beyotime). Equal amounts (40 μ g) of extracted protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked with 5% (w/v) non-fat milk in TBST buffer (Beyotime) for 1 h at 37°C. The membranes were then incubated with anti-E-Cad (1:5,000; cat. no. ab40772), anti- α -SMA (1:2,000; cat. no. ab124964), anti-VI (1:1,000; cat. no. ab20346), anti-FN (1:1,000; cat. no. ab18265), anti-Snail1 (1:500; cat. no. ab82846) and anti-GAPDH (1:1000; cat. no. ab8245) (all from Abcam, Cambridge, MA, USA) at 4°C overnight. Following 3 washes in TBST for 10 min, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:1,000; cat. no. 7076) or goat anti-rabbit IgG (1:1,000; cat. no. 7074) (both from Cell Signaling Technology, Danvers, MA, USA) for 1 h at 37°C. The protein bands were visualized by an enhanced chemiluminescence kit and quantified by densitometric analysis using ImageJ software (NIH, Bethesda, MD, USA).

In vivo nude mouse xenograft and lung metastasis assays. All animal experiments were ethically approved by

the Research Ethics Committee of Chongqing Medical University (Chongqing, China). A total of 10 female BALB/C nude mice (5-6 weeks old) with a weight of 17-22 g were obtained from the Experimental Animal Centre of the Third Military Medical University (Chongqing, China). The mice were routinely housed in a humidity- (80%); and temperature-controlled (22±2°C) environment for at least 3 days prior to the experiments. A total of 2×10⁶ SGC-7901 cells transfected with sh-NC or sh-H19 were suspended in 200 μ l phosphate-buffered saline and injected subcutaneously into right side of the armpit regions of mice (5 mice/group). At 6 weeks post-injection, the mice were sacrificed, and the tumors were isolated and measured. Tumor volume was calculated using the following formula: $V \text{ (mm}^3\text{)} = 0.5 \times \text{length} \times \text{width}^2$. The tumor tissues were snap-frozen in liquid nitrogen until further use.

Statistical analysis. Data are presented as the means ± standard deviation unless otherwise indicated and were analyzed using Graphpad Prism v7.0 software (Graphpad Software Inc., La Jolla, CA, USA). A t-test (two-sided) and one-way analysis of variance (ANOVA) were used for statistical analysis. A student-Newman-Keuls test was performed as a post hoc test following ANOVA. The correlation between the expression levels of Snail1 and miR-22-3p was assessed using Spearman's correlation analysis, as appropriate. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

H19 is upregulated and miR-22-3p is downregulated in GC tissues and cells. The expression levels of H19 and miR-22-3p in 40 paired GC and para-cancerous samples were determined by RT-qPCR. The results revealed that H19 expression was significantly upregulated, whereas the expression level of miR-22-3p was decreased in the GC tissues compared with the para-cancerous tissues (Fig. 1A and D). Furthermore, the effects of H19 and miR-22-3p expression on metastasis and the prognosis of patients with GC were investigated. The results indicated that H19 expression was significantly elevated and that miR-22-3p expression was decreased in patients with GC with lymph node metastasis compared with those without metastasis (Fig. 1B and E). In addition, the expression level of H19 was significantly increased and that of miR-22-3p was decreased in aggressive GC tumors (TNM stage III/IV), compared with stage I/II tumors, suggesting that the upregulation of H19 and the downregulation of miR-22-3p are associated with the development of GC (Fig. 1C and F). Furthermore, the correlation between H19 and miR-22-3p expression was analyzed, and the results indicated that the expression levels of H19 and miR-22-3p inversely correlated in GC tissues (Fig. 1G). Additionally, H19 was significantly upregulated and miR-22-3p was downregulated in GC cells lines compared with GES-1 cells (Fig. 1H and I). These results suggested that the expression of H19 and miR-22-3p is upregulated and downregulated in GC respectively, which is also associated with metastasis in patients with GC. Furthermore, the miR-22-3p level is downregulated by H19 in GC cells, suggesting that H19 may exert its regulatory function in GC via miR-22-3p.

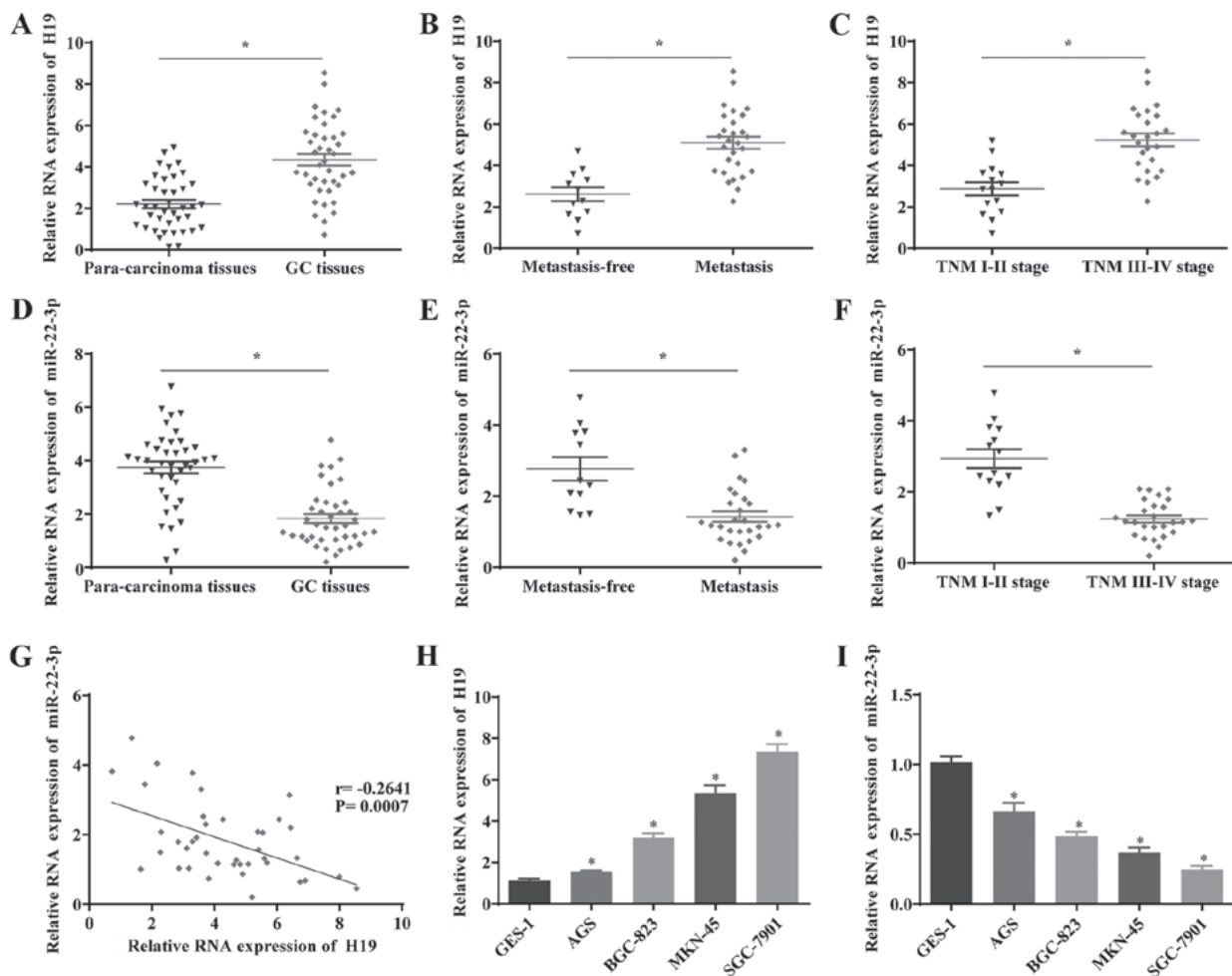


Figure 1. H19 is upregulated and miR-22-3p is downregulated in GC tissues and cells. (A and D) The expression levels of H19 and miR-22-3p were examined in forty GC tumor samples and paired para-carcinoma controls using RT-qPCR. (B and E) H19 and miR-22-3p expression levels were determined in 28 patients with GC with lymph node metastasis compared with 12 samples without metastasis. (C and F) The levels of H19 and miR-22-3p were evaluated in GC tissues with various TNM stages by RT-qPCR. (G) The RNA levels of H19 and miR-22-3p inversely correlated in GC tissues ($r = -0.2641$; $P = 0.0007$). (H and I) The expression levels of H19 and miR-22-3p were determined in normal gastric mucous epithelium cells (GES-1) and GC cells (AGS, BGC-823, MKN-45 and SGC-7901). * $P < 0.05$ vs. GES-1. Each experiment was repeated 3 times. GC, gastric cancer; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Downregulation of H19 suppresses cell proliferation, invasion, migration and epithelial-mesenchymal transition (EMT). To examine the effects of H19 on the proliferation, invasion and migration of GC cells, the expression of H19 was silenced by sh-H19 in AGS (the lowest expression of H19) and SGC-7901 (the highest expression of H19) cells. The knockdown efficiency of H19 was determined by RT-qPCR (Fig. 2A). The results of CCK-8 assay revealed that the proliferative ability of the AGS and SGC-7901 cells transfected with sh-H19 was decreased compared with the controls (Fig. 2B and C). In addition, Transwell assay indicated that the invasion and migration of the AGS and SGC-7901 cells transfected with sh-H19 were significantly suppressed (Fig. 2D-G). Furthermore, the results of RT-qPCR and western blot analysis revealed that the expression level of E-Cad was notably increased, whereas the expression levels of α -SMA, VI and FN were decreased in the sh-H19-transfected cells compared with the controls (Fig. 2H-J). On the whole, these results suggested that the downregulation of H19 suppressed the growth, metastasis and EMT of GC cells.

miR-22-3p is the potential target of H19 in GC cells. To determine whether H19 functions by suppressing its target miRNA in GC, the potential binding sites of miR-22-3p in H19 transcripts were predicted using LncBase Predicted v.2 (Fig. 3A). Luciferase reporter plasmids containing the wild-type H19 (H19-WT) and mutant H19 (H19-MUT) of predicted miR-22-3p binding sites were constructed. The results revealed that transfection with miR-22-3p mimics significantly reduced the luciferase activity in H19-WT, while the luciferase activity in 293 cells transfected with H19-MUT was not affected by miR-22-3p mimics (Fig. 3B). In order to further examine the effects of H19 on the expression level of miR-22-3p, the AGS and SGC-7901 cells were transfected with sh-H19. The cells transfected with sh-H19 exhibited a marked increase in miR-22-3p levels (Fig. 3C). Furthermore, H19 knockdown resulted in a prominent decrease in Snai1 protein expression in the AGS and SGC-7901 cells (Fig. 3D). Additionally, a miR-22-3p overexpression model was established by transfecting the AGS and SGC-7901 cells with miR-22-3p mimics, and the transfection efficiency was confirmed by RT-qPCR (Fig. 3E).

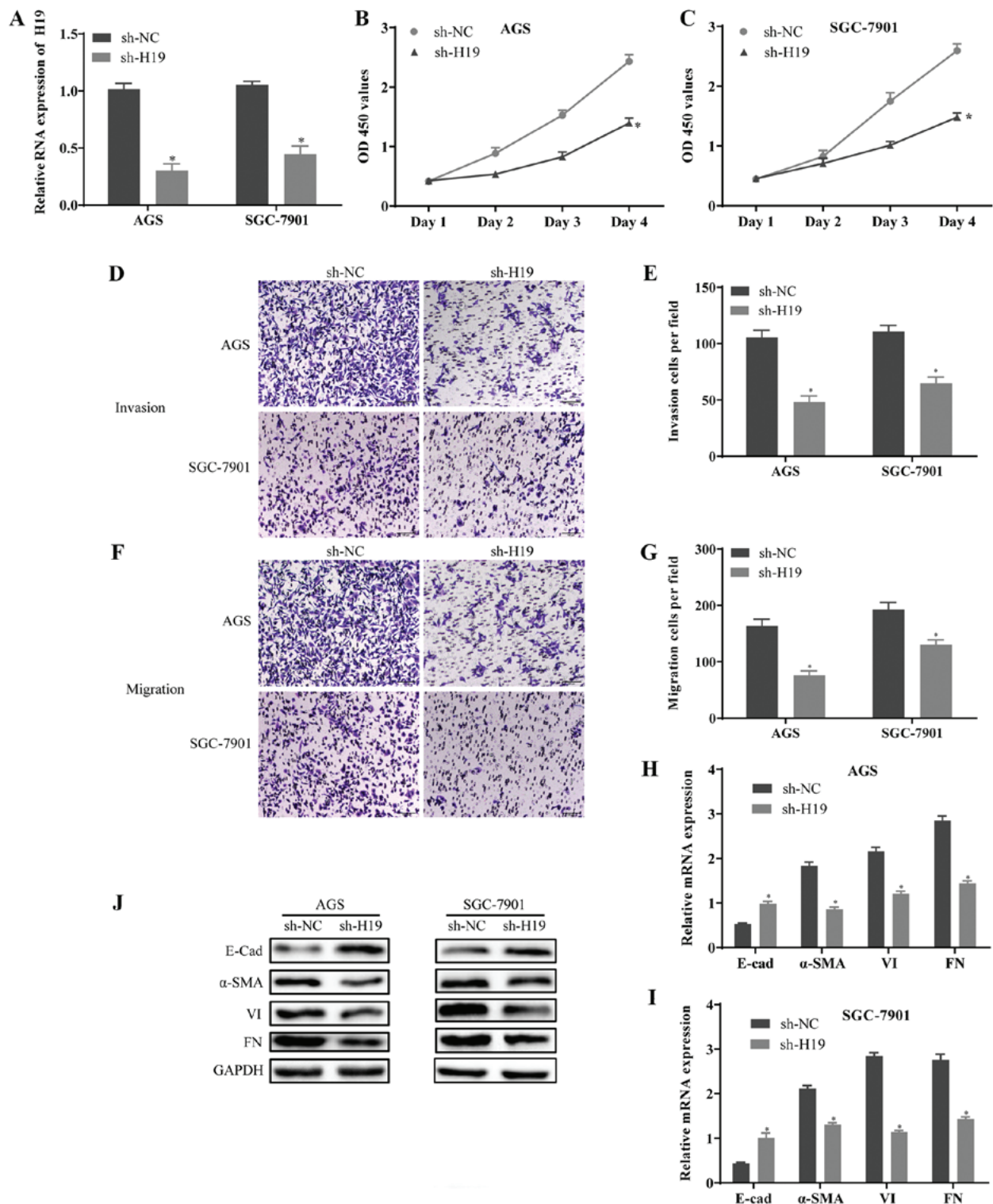


Figure 2. Downregulation of H19 suppresses the proliferation, invasion, migration and EMT of GC cells. (A) Transfection efficiency of sh-H19 was determined by RT-qPCR. (B and C) The proliferation of AGS and SGC-7901 cells transfected with sh-NC or sh-H19 was evaluated using Cell Counting kit-8 assay. (D and E) The invasive abilities of transfected AGS and SGC-7901 cells were determined by a Transwell assay (magnification, x100). (F and G) The migration of AGS and SGC-7901 cells transfected with sh-NC or sh-H19 were evaluated using a Transwell assay (magnification, x100). (H and I) The expression levels of EMT-associated molecules in transfected AGS and SGC-7901 cells were determined using RT-qPCR. (J) The levels of EMT-associated proteins in AGS and SGC-7901 cells transfected with sh-NC or sh-H19 were evaluated by western blot analysis. *P<0.05 vs. sh-NC. Each experiment was repeated 3 times. GC, gastric cancer; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Furthermore, the overexpression of miR-22-3p inhibited the expression level of Snail1 in the AGS and SGC-7901 cells (Fig. 3F), suggesting that the miR-22-3p/Snail1 axis may be a potential target of H19 in GC cells.

Overexpression of H19 promotes cell proliferation, migration, invasion and EMT by regulating miR-22-3p. To further investigate the association between H19 and miR-22-3p, the AGS and SGC-7901 cells were transfected with pc-NC,

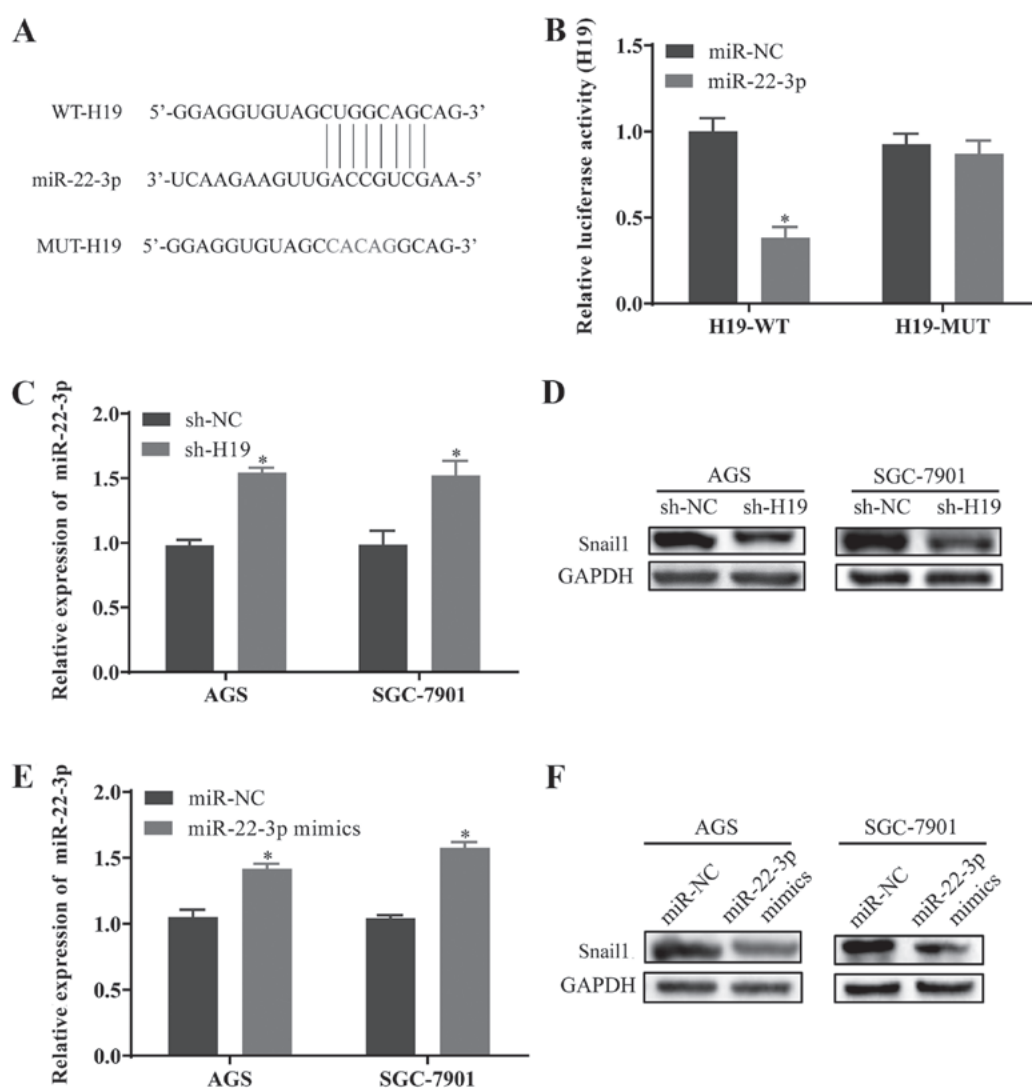


Figure 3. miR-22-3p is the potential target gene of H19 in GC cells. (A) The putative binding sites of miR-22-3p on H19 transcript. (B) Overexpression of miR-22-3p led to a notably reduced luciferase activity of H19-WT, while no change was observed in H19-MUT. (C) The expression level of miR-22-3p in AGS and SGC-7901 cells transfected with sh-NC or sh-H19 was evaluated by RT-qPCR. (D) The protein level of Snail1 in transfected AGS and SGC-7901 cells was determined by western blot analysis. (E) The expression of miR-22-3p in AGS and SGC-7901 cells transfected with miR-NC or miR-22-3p mimics was assessed by RT-qPCR. (F) The expression levels of Snail1 in AGS and SGC-7901 cells transfected with miR-NC or miR-22-3p mimics was determined by western blot analysis. * $P < 0.05$ vs. sh-NC. Each experiment was repeated 3 times. GC, gastric cancer; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control; MUT, mutant; WT, wild-type.

pc-H19 or co-transfected with pc-H19 and miR-22-3p mimics. The expression of H19 was significantly increased (Fig. 4A) and the level of miR-22-3p decreased (Fig. 4B) in the AGS and SGC-7901 cells transfected with pc-H19. Furthermore, the overexpression of H19 promoted the proliferation (Fig. 4C and D), invasion (Fig. 4E), migration (Fig. 4F) and EMT (Fig. 4G-I) of the AGS and SGC-7901 cells, whereas these effects were substantially abrogated by the upregulation of miR-22-3p. In addition, the overexpression of H19 increased the level of Snail1, which was inhibited by the upregulation of miR-22-3p (Fig. 4J). These results revealed that H19 may promote the proliferation, invasion, migration and EMT of GC cells by downregulating miR-22-3p and upregulating Snail1.

Snail1 is the target gene of miR-22-3p in GC cells. Using the TargetScan database, the binding sites of Snail1 on miR-22-3p was predicted (Fig. 5A). To investigate whether

Snail1 is the potential target of miR-22-3p, the WT and MUT fragments of Snail1 were cloned into the downstream of the Firefly luciferase coding domain in pGL-3 vector, and the results revealed that the co-transfection of miR-22-3p and Snail1-WT reduced the relative luciferase activity, whereas the activity remained unaltered in the cells co-transfected with miR-22-3p and Snail1-MUT (Fig. 5B). To further determine whether miR-22-3p can regulate the expression level of Snail1, the AGS and SGC-7901 cells were transfected with miR-22-3p inhibitors. The results confirmed that miR-22-3p was significantly downregulated in the cells transfected with miR-22-3p inhibitors (Fig. 5C), whereas the protein level of Snail1 was elevated (Fig. 5D). Furthermore, Snail1 expression was upregulated in the GC tissues compared with the paired para-cancerous tissues (Fig. 5E), and Snail1 expression inversely correlated with miR-22-3p expression in GC tissues (Fig. 5F), suggesting that Snail1 may be a potential target of

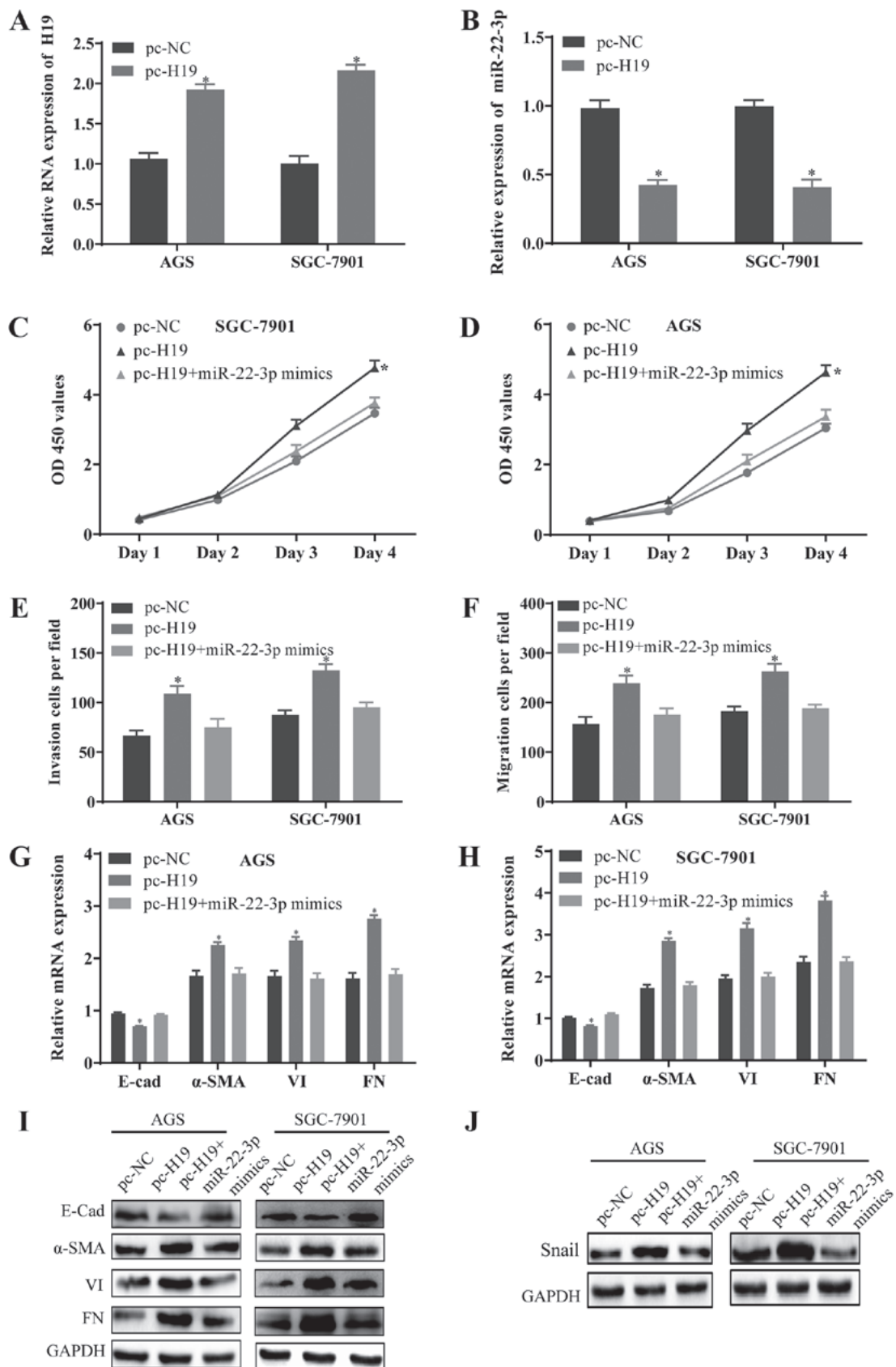


Figure 4. Overexpression of H19 promotes the proliferation, migration, invasion and EMT of GC cells via miR-22-3p. (A and B) The expression levels of H19 and miR-22-3p in AGS and SGC-7901 cells transfected with pc-NC or pc-H19 were evaluated by RT-qPCR. (C and D) The proliferation of AGS and SGC-7901 cells transfected with pc-NC, pc-H19 or co-transfected with pc-H19 and miR-22-3p mimics was evaluated using Cell Counting kit-8 assay. (E and F) The invasion and migration of transfected AGS and SGC-7901 cells were evaluated by a Transwell assay. (G and H) The levels of EMT-associated molecules in AGS and SGC-7901 cells transfected with pc-NC, pc-H19 or co-transfected with pc-H19 and miR-22-3p mimics were determined using RT-qPCR. (I) The expression levels of EMT-associated proteins in transfected AGS and SGC-7901 cells were evaluated by western blot analysis. (J) The protein level of Snail in AGS and SGC-7901 cells transfected with pc-NC, pc-H19 or co-transfected with pc-H19 and miR-22-3p mimics was determined by western blot analysis. * $P < 0.05$ vs. pc-NC. Each experiment was repeated 3 times. GC, gastric cancer; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control.

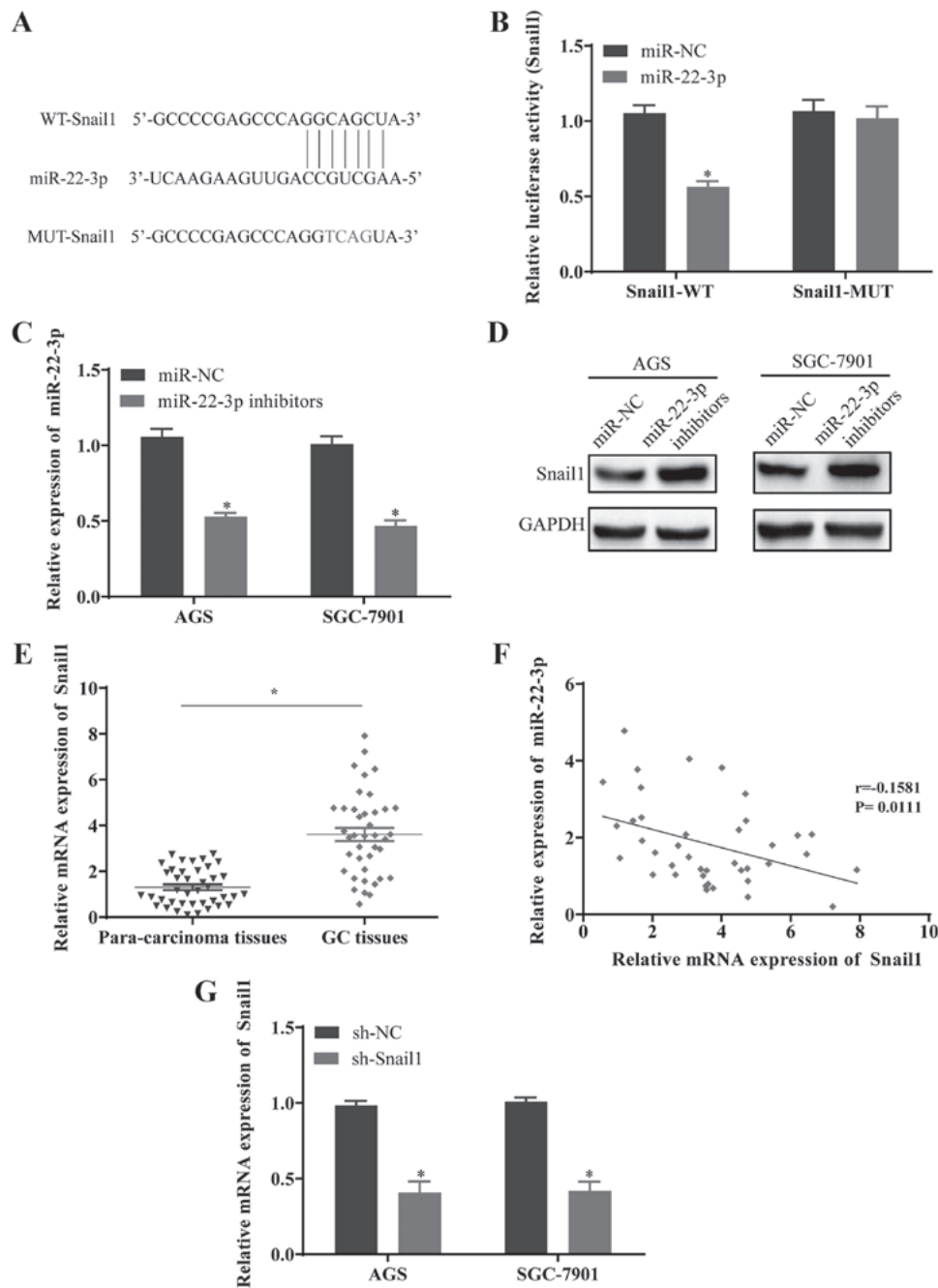


Figure 5. Snail1 is the potential target gene of miR-22-3p in GC cells. (A) The putative binding sites of Snail1 on miR-22-3p transcript. (B) Overexpression of miR-22-3p resulted in a markedly decreased luciferase activity of Snail1-WT, whereas no change was observed in Snail1-MUT. (C) The expression levels of miR-22-3p in AGS and SGC-7901 cells transfected with miR-NC or miR-22-3p inhibitors were evaluated by RT-qPCR. (D) The protein level of Snail1 in transfected AGS and SGC-7901 cells was determined by western blot analysis. (E) Snail1 expression was assessed in 40 GC tumor samples and paired para-carcinoma controls. (F) The mRNA expression levels of miR-22-3p and Snail1 inversely correlated in GC tumor tissues ($r = -0.1581$; $P = 0.0111$). (G) The expression levels of Snail1 in AGS and SGC-7901 cells transfected with sh-NC or sh-Snail1 were evaluated by RT-qPCR. * $P < 0.05$ vs. miR-NC. Each experiment was repeated 3 times. GC, gastric cancer; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control.

miR-22-3p in GC. To further verify the role of Snail1, the AGS and SGC-7901 cells were transfected with sh-Snail1 and Snail1 was significantly downregulated in the cells transfected with sh-Snail1 (Fig. 5G).

Downregulation of Snail1 enhances the effects exerted by the overexpression of miR-22-3p and reverses the effects exerted by the silencing of miR-22-3p in GC cells. To investigate whether the functions of Snail1 as regards the growth, metastasis and EMT of GC cells are regulated by miR-22-3p,

the AGS and SGC-7901 cells were transfected with miR-NC, miR-22-3p mimics or co-transfected with miR-22-3p mimics and sh-Snail1, miR-22-3p inhibitors and sh-Snail1, respectively. The results revealed that the overexpression of miR-22-3p suppressed the proliferation (Fig. 6A and B), invasion (Fig. 6E) and migration (Fig. 6F) of the AGS and SGC-7901 cells, while these effects were enhanced by the silencing of Snail1 expression. Conversely, the downregulation of miR-22-3p promoted the proliferation (Fig. 6C and D), invasion (Fig. 6G) and migration (Fig. 6H) of GC cells, whereas these effects

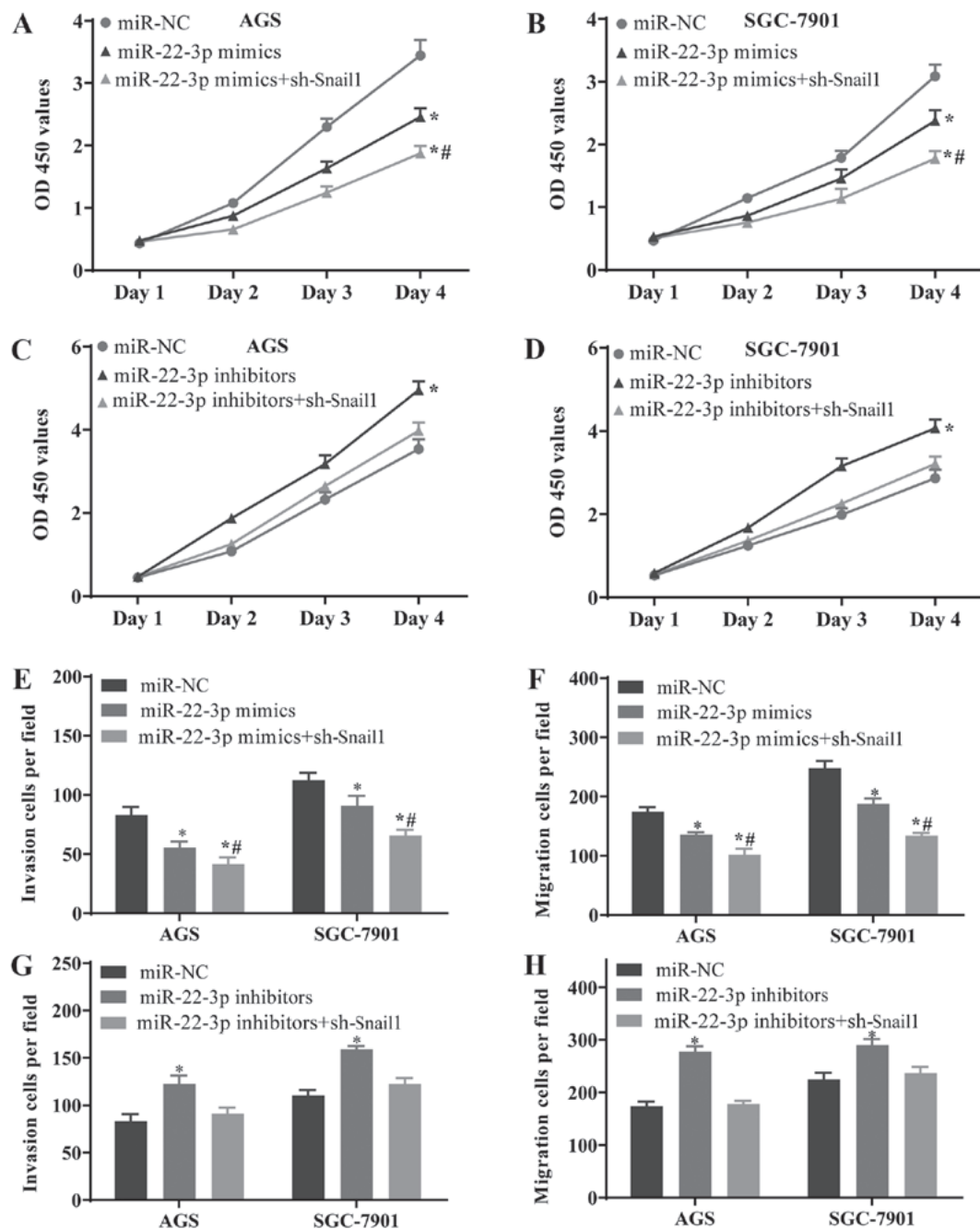


Figure 6. Downregulation of Snail1 enhances the effects exerted by the overexpression of miR-22-3p and reverses the effects exerted by the silencing of miR-22-3p in GC cells. (A and B) The proliferation of AGS and SGC-7901 cells transfected with miR-NC, miR-22-3p mimics or co-transfected with miR-22-3p mimics and sh-Snail1 was evaluated by Cell Counting kit-8 assay. (C and D) The proliferation of AGS and SGC-7901 cells transfected with miR-NC, miR-22-3p inhibitors or co-transfected with miR-22-3p inhibitors and sh-Snail1 was assessed. (E and F) The invasion and migration of AGS and SGC-7901 cells transfected with miR-NC, miR-22-3p mimics or co-transfected with miR-22-3p mimics and sh-Snail1 were determined using a Transwell assay. (G and H) The invasion and migration of AGS and SGC-7901 cells transfected with miR-NC, miR-22-3p inhibitors or co-transfected with miR-22-3p inhibitors and sh-Snail1 were evaluated. *P<0.05 vs. miR-NC. #P<0.05 vs. miR-22-3p mimics. Each experiment was repeated 3 times. GC, gastric cancer; miR, microRNA; NC, negative control.

were abrogated by the silencing of Snail1 expression. On the whole, the results of the present study suggest that miR-22-3p may inhibit the growth of GC by downregulating Snail1. In summary, H19 may regulate the proliferation, invasion, migration and EMT of GC cells via the miR-22-3p/Snail1 signaling pathway.

Downregulation of H19 suppresses the development of GC in vivo. To investigate whether sh-H19 suppresses the

growth and metastasis of gastric tumors, GC cells transfected with sh-NC or sh-H19 were subcutaneously injected into BALB/C nude mice. At 6 weeks post-injection, the mice were sacrificed and the isolated tumors were measured (Fig. 7A). The mean value of the tumor volume in the sh-H19 group was significantly reduced compared with that in the sh-NC group (Fig. 7B). In addition, the tumor weight in the sh-H19 group was notably decreased compared with that in the sh-NC group (Fig. 7C). Furthermore, the numbers of macroscopic nodules

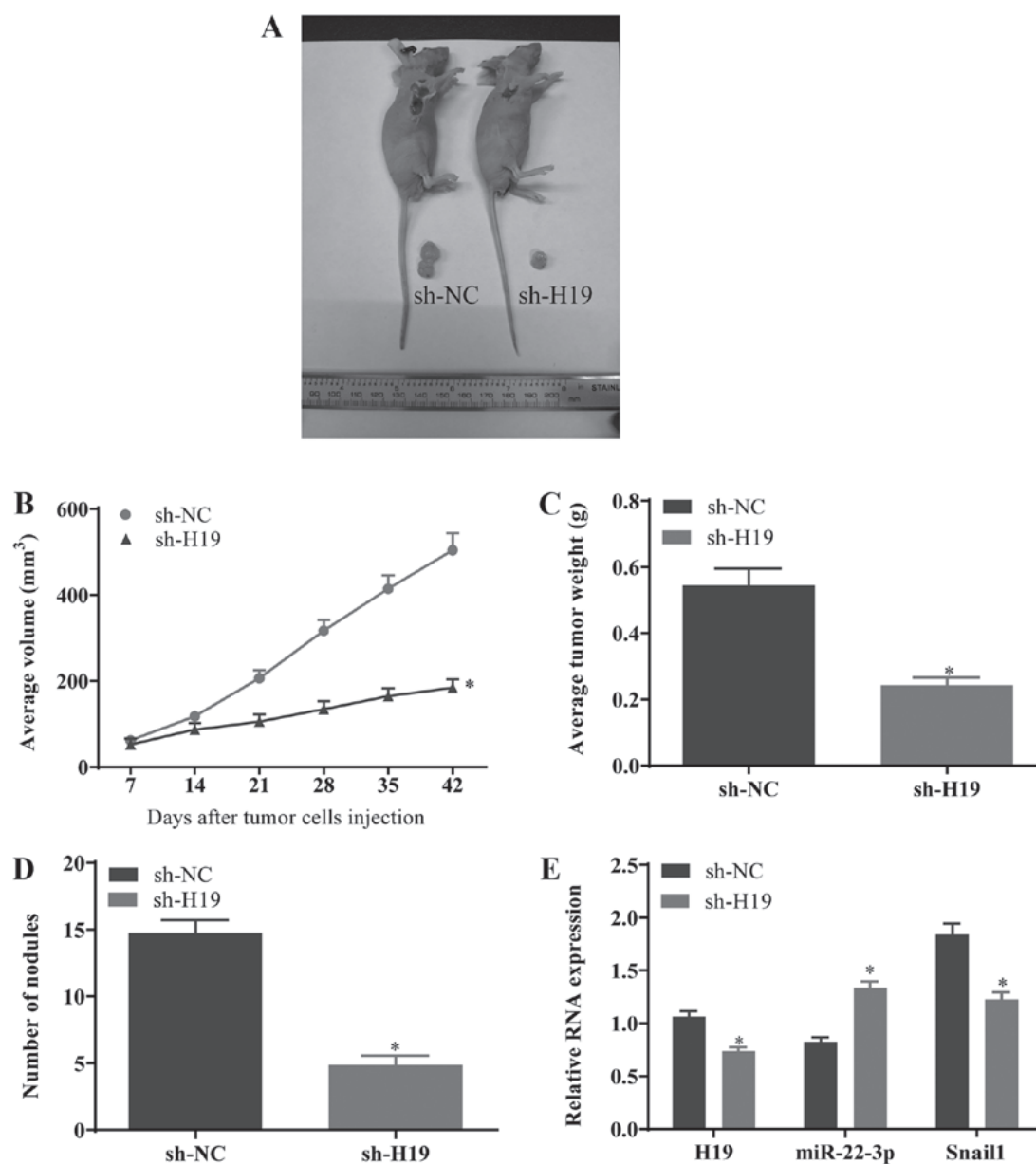


Figure 7. Downregulation of H19 suppresses the growth of GC *in vivo*. (A) Images of tumors in mice. (B) The mean tumor volumes were measured in tumors derived from cells transfected with sh-NC and cells transfected with sh-H19. (C) Orthotopic tumor weights at day 42 post-inoculation were calculated. (D) The numbers of nodules in nude mice at day 42 post-inoculation were calculated. (E) The expression levels of H19, miR-22-3p and Snail1 mRNA in xenograft tumors were determined by reverse transcription-quantitative polymerase chain reaction. * $P < 0.05$ vs. sh-NC. Five mice were used per each experimental group. GC, gastric cancer; miR, microRNA.

were significantly reduced in the sh-H19 group (Fig. 7D). Additionally, RT-qPCR revealed that the mRNA levels of H19 and Snail1 were downregulated in the sh-H19 group, whereas the expression level of miR-22-3p was elevated (Fig. 7E) in these tumors, suggesting that the downregulation of H19 may suppress the growth of gastric tumors via the miR-22-3p/Snail1 axis *in vivo*.

Discussion

lncRNAs are RNA transcripts >200 nt in length, with no capacity of coding proteins. Studies on lncRNAs have revealed their significance over the past decade, and there is adequate evidence to suggest that lncRNAs are important regulators of the proliferation and migration of cancer cells; aberrant

lncRNA expression plays important roles in the progression of numerous types of cancer; however, the regulatory functions and underlying mechanisms of various lncRNAs remain unclear (26,27). A previous study revealed that a high expression of lncRNAs may competitively bind to miRNAs, consequently inhibiting miRNA expression and promoting the progression of GC; for example, downregulated lncRNA-SNHG5 was shown to inhibit the proliferation and migration of GC cells via the miR-32/KLF4 axis (28). Additionally, lncRNA-BC032469 competitively binds with miR-1207-5p and upregulates human telomerase reverse transcriptase (hTERT), subsequently promoting the proliferation of GC cells (29). Furthermore, lncRNAs regulate their target genes by interacting with its mRNA directly. For example, Xu *et al* (30) reported that lncRNA-TINCR promoted the proliferation and apoptosis

of GC cells by binding to STAU1 protein and interfering with the stability of Krüppel-like factor 2 (KLF2) mRNA. Recent studies have also indicated that lncRNAs, such as H19 regulated the proliferation, migration and invasion of GC cells by binding to their target miRNAs (14,31).

Accumulating evidence indicates that H19 is overexpressed in a variety of tumors and is associated with the proliferation and metastasis of tumors by functioning as a ceRNA (32,33). H19 regulates the migration and invasion of breast cancer cells by targeting miR-675 (34). H19 also controls the expression level of FADD via miR-675, consequently promoting the proliferation and cell cycle progression and inhibiting the apoptosis of GC cells (31). In addition, previous studies have suggested that miR-22-3p is a potential tumor suppressor, which is downregulated in various human tumors, including GC (35,36). The aim of the present study was to further investigate the underlying mechanisms of action of H19 and miR-22-3p as regards the initiation and development of GC.

In the present study, H19 was significantly upregulated and miR-22-3p was downregulated in GC tissues, and the elevated expression of H19 and reduced level of miR-22-3p in GC were associated with a poor prognosis of patients with GC. Furthermore, the results of present study revealed negative correlations between the expression of H19, miR-22-3p and Snail1 in GC samples. Further experiments confirmed the association among the H19, miR-22-3p and Snail1. Wang *et al* (24) reported that H19 could regulate HO-induced deregulation in nucleus pulposus cell senescence, proliferation via miR-22-3p. Additionally, the present study indicated that miR-22-3p was the potential target gene of H19, and that the downregulation of H19 suppressed the proliferation, invasion, migration and EMT of GC cells. Furthermore, the overexpression of H19 promoted the proliferation, invasion, migration and EMT of GC cells, while these effects were substantially abrogated by the upregulation of miR-22-3p, suggesting that H19 may promote cell growth, metastasis and EMT in GC via miR-22-3p.

It has been well established that EMT plays critical roles in the development of tumor metastasis (37), and Snail is one of the major activators of EMT signaling (38). The upregulation of Snail may be a potential biomarker of EMT (39). The activation of the EMT program leads to the transformation of epithelial cells into interstitial cells (40). E-Cad, as a member of transmembrane glycoprotein family, is an EMT marker. Numerous EMT inducers, including α -SMA, VI and FN suppress the transcription of the E-Cad gene. The process of EMT is induced in tumor development with the reduced expression of E-Cad and increased levels of α -SMA, VI and FN (41). In the present study, the downregulation of H19 and the overexpression of miR-22-3p suppressed cell invasion and migration by inhibiting EMT, whereas the silencing of Snail1 enhanced these effects.

Furthermore, luciferase reporter assay revealed that Snail1 was the promising target gene of miR-22-3p, and the overexpression of miR-22-3p suppressed cell proliferation, invasion and migration by targeting Snail1. The downregulation of miR-22-3p induced the proliferation, invasion and migration of GC cells, while these effects were abolished by the silencing of Snail1 expression. In addition, Snail1 was significantly upregulated in GC tissues compared

with the paired para-cancerous tissues and its expression inversely correlated with that of miR-22-3p in GC tissues. Furthermore, Zuo *et al* (36) reported that miR-22-3p inhibited the growth, migration and invasion of GC cells via Snail1. In addition, the results of the present study revealed that the knockdown of H19 in a xenograft tumor model suppressed the growth of tumors *in vivo*, suggesting that H19 may upregulate Snail1 expression by inhibiting miR-22-3p and consequently promoting the development of GC.

In conclusion, the present study revealed that H19 is a potential oncogene that may increase Snail1 mRNA transcripts and promote the growth and metastasis of GC cells via miR-22-3p. Snail1 induced EMT by regulating the expression levels of α -SMA, VI, FN and E-Cad. Although there are some limitations to this study, in that the migratory and invasive activity of the cells could be further confirmed by examining cell proliferation- and invasion-related genes, these findings revealed the essential role of H19 and its functional mechanisms the proliferation, invasion, migration and EMT of GC cells. The findings of this study suggest that the H19/miR-22-3p/Snail1 axis may be a promising therapeutic target for the treatment of GC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL designed the present study. LG, SL and LL performed the experiments. LG analyzed the data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Chongqing Medical University. Informed consent was obtained from each patient prior to surgery. All animal experiments were ethically approved by the Research Ethics Committee of Chongqing Medical University (Chongqing, China).

Patient consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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