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Decreased Expression of miR-138-5p by lncRNA H19 in Cervical Cancer Promotes Tumor Proliferation

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MicroRNAs (miRNAs) play important roles in the carcinogenesis of cervical cancer. However, the expression and underlying mechanisms of miRNA in cervical cancer progression remain unclear. In the present study, our data showed that the expression of miR-138-5p was significantly downregulated in cervical cancer tissues, and decreased expression of miR-138-5p was correlated with advanced FIGO stage, poor differentiation, lymph node metastasis, and poor overall survival of cervical cancer patients. Function assays showed that overexpression of miR-138-5p reduced cervical cancer cell proliferation, arrested cells in the G_0/G_1 phase, and induced cell apoptosis in vitro. Remarkably, SIRT1 was confirmed as a direct target of miR-138-5p in cervical cancer, and miR-138-5p exerted the reduced tumor functions by suppressing SIRT1 expression. Moreover, we further identified that lncRNA H19 could act as a molecular sponge of miR-138-5p in cervical cancer progression. Taken together, these results suggested that miR-138-5p could suppress cervical cancer cell progression by targeting SIRT1.

Key words: miR-138-5p; Long noncoding RNA (lncRNA) H19; SIRT1; Cervical cancer

INTRODUCTION

Cervical cancer is one of the most common gynecological cancers worldwide. According to statistics, there are over 300,000 deaths annually, and more than 85% of these deaths occur in developing countries[1,2]. Despite great progress in cervical cancer treatments, including surgical, chemotherapy, and radiotherapy in recent years, the clinical outcomes of cervical cancer remain poor[3]. Therefore, it is critical to identify new biomarkers and therapeutic targets to improve cervical cancer diagnosis and treatment.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs (ncRNAs) of 20–22 nucleotides that play important regulatory roles in biological processes through targeting mRNAs for cleavage and translational repression[4]. Through negatively mediating their target genes, miRNAs are involved in a variety of biological processes, such as cell proliferation, apoptosis, and differentiation[5]. Recently, miRNAs represent a promising new family of targets in the current era of molecular therapies in oncology[6]. For example, Song et al. showed that miR-630 controls cell growth and metastasis in lung cancer by targeting LIM domain only 3 (LMO3)[7]. Yang et al. showed that miR-506 inhibited cell growth and metastasis via flotilin 1 (FLOT1), but its expression is reduced in clear cell renal cell carcinoma[8]. Wang et al. showed that miR-98 targets collagen triple helix repeat containing 1 to reduce cell proliferation, migration, and invasion in hepatocellular carcinoma[9]. However, the expression and functions of miR-138-5p in cervical cancer are still unclear.

In the present study, our data showed that miR-138-5p was decreased in cervical cancer tissues. This decreased expression of miR-138-5p correlated with advanced International Federation of Gynecology and Obstetrics (FIGO) stage, poor differentiation, lymph nodes metastasis, and poor overall survival of cervical cancer patients. In function experiments, we showed that miR-138-5p reduced cervical cancer cell proliferation, arrested cells in the G_0/G_1 phase, and suppressed silent mating type information regulation 2 homolog 1 (SIRT1) expression to induce cell apoptosis in vitro. In addition, our data

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indicated that long noncoding RNA (lncRNA) H19 could act as a molecular sponge of miR-138-5p in cervical cancer progression.

MATERIALS AND METHODS

Clinical Specimens

A total of 56 pairs of cervical cancer and adjacent normal tissues were collected from cervical cancer patients who received treatment in Zhengzhou People's Hospital (Zhengzhou, P.R. China) from January 2010 to December March 2011. None of the patients received any chemotherapy or radiation before surgery. Histopathological diagnoses were based on the World Health Organization (WHO) classification, and clinical stages were in accordance with the FIGO criteria. Written informed consent was obtained from all participants, and the study was approved by the Board and Ethics Committee of Zhengzhou People's Hospital. The tissues were immediately frozen in liquid nitrogen following surgery and stored at −80°C until use.

Cell Culture and Transfection

Human cervical cancer cell lines (HeLa and SiHa) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin in a cell culture humidified incubator at 37°C with 5% CO_2. miR-138-5p mimics and miR-negative control (NC) were purchased from GeneCopoeia Co. Ltd. (Rockville, MD, USA). pcDNA-SIRT1 and H19 small interfering RNA (siRNA) were purchased from Applied Biosystems (Foster City, CA, USA). Transfection was performed in a six-well plate when cell confluence attained 70%–90% according to the Lipofectamine 2000 (Invitrogen) transfection manual.

Quantitative Real-Time Reverse Transcriptase PCR (qRT-PCR)

Total RNA was extracted from tumor samples and cell lines using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA (500 ng) was reverse transcribed to complementary DNA using PrimeScript™ RT Master Mix (Takara, Dalian, P.R. China). The qRT-PCR was performed in a total reaction volume of 20 ml using SYBR Green qPCR Master Mix (Takara) in the ABI PRISM 7900HT Sequence Detection System. The U6 short nuclear RNA (snRNA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The relative fold expressions were calculated with the $2^{-\Delta\Delta}$CT method. All the qRT-PCRs were run in triplicate.

Western Blotting

Cells were collected and lysed using radioimmunoprecipitation assay (RIPA) protein extraction reagent (Beyotime, Shanghai, P.R. China) supplemented with a protease inhibitor cocktail (Roche, Carlsbad, CA, USA). Then protein concentrations were determined by a Bio-Rad assay kit (Carlsbad, CA, USA). Next, the protein was boiled after adding equal amounts of loading buffer. Protein samples were loaded onto the sodium dodecyl sulfate (SDS)-polyacrylamide gel, electrophoresed, and then transferred onto 0.22-μm nitrocellulose membrane before blocking and probing with primary antibodies at 4°C for 12 h. Then the membranes were subsequently incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The signals from Western blotting were quantified by densitometry, and the statistical analyses were conducted from three independent experiments.

Cell Proliferation Assay

The proliferation of cervical cancer cells was determined using the Cell Counting Kit-8 (CCK-8; Dojindo, Osaka, Japan) according to the manufacturer's instructions. Cells were seeded at a density of 3×10^3 cells per well in 96-well plates. CCK-8 (10 μl) was then added to each well at 24, 48, 72, and 96 h and incubated for 2 h at 37°C. The absorbance was measured at a wavelength of 450 nm using a Model 680 microplate reader (Bio-Rad).

Flow Cytometry Analysis

For cell apoptosis analysis, cells were harvested and resuspended in staining buffer at a concentration of 1×10^6 cells/ml. The cells were stained with annexin V and propidium iodide (PI) staining using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Apoptotic cells were measured using a BD FACSCanto™ Flow Cytometer (BD Biosciences).

For cell cycle analysis, cells were harvested by trypsin in the logarithmic growth phase and fixed in 75% ethanol at 4°C overnight. Then cells were resuspended in phosphate-buffered saline (PBS) and incubated with BD PharMingen™ PI/RNase staining buffer (BD Biosciences) at room temperature for 30 min in the dark. Finally, cell cycle distribution and DNA content were analyzed using a BD FACSCanto™ Flow Cytometer (BD Biosciences).

Target Gene Identification

The bioinformatics online software program TargetScan (http://www.targetscan.org) was utilized to determine likely target genes for miR-138-5p. One likely target was SIRT1. Starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php) was used to identify complementary lncRNA binding partners for miR-138-5p.

Luciferase Reporter Assay

The fragment from H19 containing the predicted miR-138-5p binding site was amplified by PCR and then cloned into a pmirGlO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to form the reporter vector H19-wild type (H19-Wt). To mutate the putative binding site of miR-138-5p in the H19, the sequence of the putative binding site was replaced and named as H19-mutated-type (H19-Mut). Similarly, the fragment from SIRT1 3′-UTR (untranslated region) sequences were amplified by PCR and cloned into pmiR-RB-Report™ vector (RiboBio, Guangzhou, P.R. China) to form the reporter vector SIRT1-Wt. To mutate the putative binding site of miR-138-5p in the 3′-UTR-containing vector, the sequence of putative binding site was replaced and named as SIRT1-Mut. Then the vectors and miR-138-5p mimics were cotransfected into HeLa cells, and

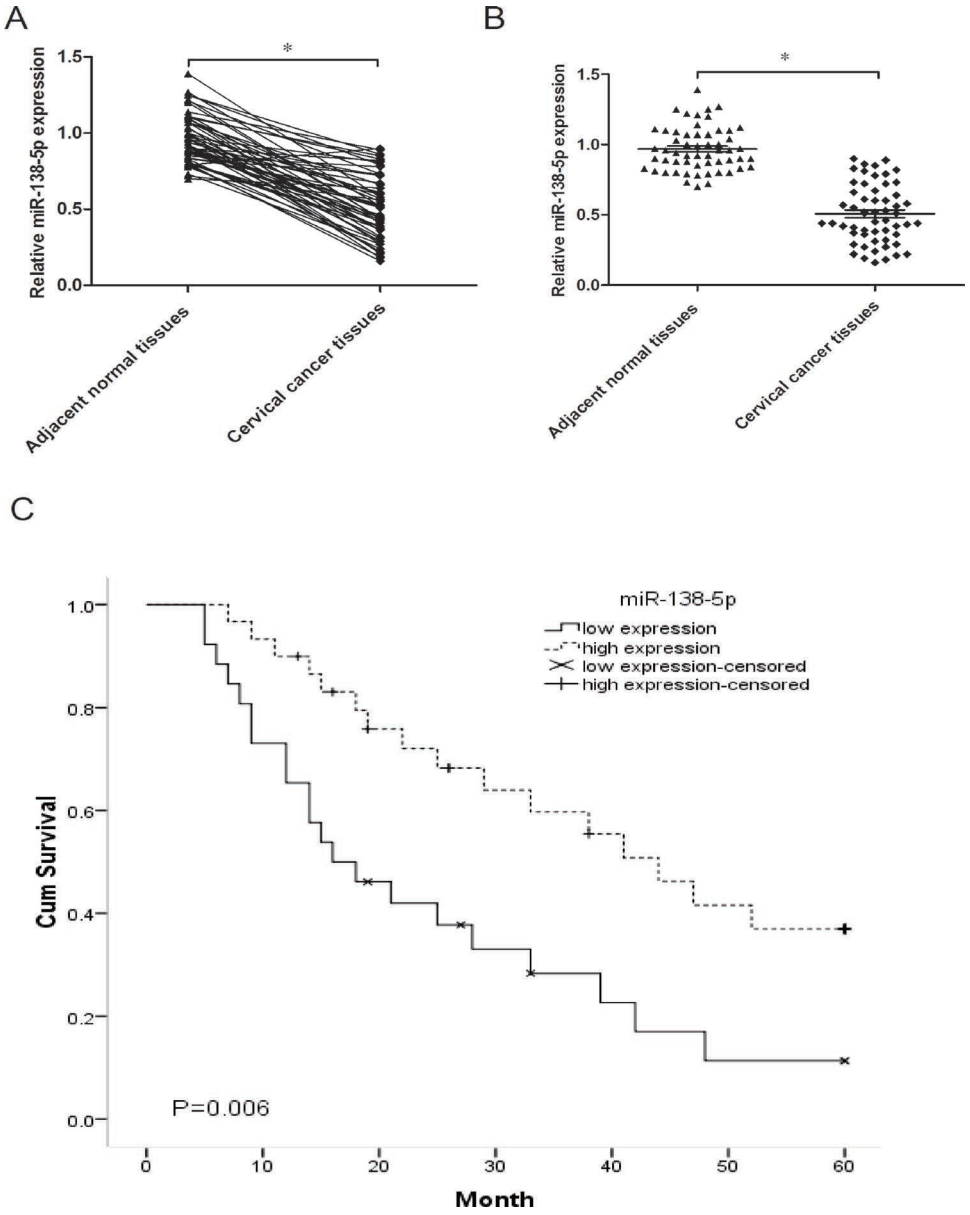

Figure 1. MicroRNA-138-5p (miR-138-5p) is downregulated in cervical cancer tissues and predicts poor overall survival in cervical cancer patients. (A, B) Expression of miR-138-5p was decreased in cervical cancer tissues compared to adjacent normal tissues as determined by quantitative real-time reverse transcriptase PCR (qRT-PCR). (C) Kaplan–Meier analysis showed that cervical cancer patients with low miR-138-5p expression (<median) have shorter overall survival compared to patients with high miR-138-5p expression (>median). *$p<0.05$.

the dual-luciferase reporter assay system (Promega) was used for testing the luciferase activity.

Statistical Analysis

The data were expressed as mean ± standard deviation (SD) and analyzed by SPSS17.0. The difference between two groups was evaluated using two-tailed Student's *t*-test or one-way ANOVA. Overall survival was defined by Kaplan–Meier and analyzed by log-rank test. Person correlation analysis was used to evaluate the relationship between miR-138-5p and H19. A value of $p < 0.05$ was considered statistically significant.

RESULTS

miR-138-5p Was Downregulated in Cervical Cancer

To determine whether miR-138-5p was dysregulated in cervical cancer, we explored miR-138-5p expression in cervical cancer tissues and pair-matched adjacent normal tissues. Using qRT-PCR, we showed that miR-138-5p was significantly decreased in cervical cancer tissues compared with the adjacent normal tissues ($p < 0.05$) (Fig. 1A and B). The aberrant expression level of miR-138-5p in the cancer tissues indicated that miR-138-5p might play an important role in the development and progression of cervical cancer.

Correlation Between miR-138-5p Expression and Clinical Characteristics and Prognosis

To assess whether miR-138-5p expression was correlated with clinical pathological features and prognosis of cervical cancer, the 56 cervical cancer patients were classified into two groups according to the median of miR-138-5p in tumor tissues: the high miR-138-5p group ($n = 28$, >median value) and the low miR-138-5p group ($n = 28$, ≤median value). Correlation analysis showed that low miR-138-5p expression was significantly associated with advanced FIGO stage, poor differentiation, and lymph nodes metastasis (Table 1). However, the expression of miR-138-5p was not related to other clinical parameters, such as age, tumor size, histology, and vascular invasion. Furthermore, Kaplan–Meier analysis showed that patients with low miR-138-5p expression had poor overall survival from cervical cancer compared to patients with high miR-138-5p expression ($p < 0.05$) (Fig. 1C).

miR-138-5p Suppressed Cervical Cancer Cell Proliferation by Affecting Cell Apoptosis and Cell Cycle

To explore the cellular functions of miR-138-5p in cervical cancer, miR-138-5p mimics were transfected into HeLa and SiHa cells, and the overexpression efficiency was determined by qRT-PCR ($p < 0.05$) (Fig. 2A). To explore whether miR-138-5p affects the growth of

Table 1. Association of MicroRNA-138-5p (miR-138-5p) Expression With Clinicopathological Features in Cervical Cancer

		miR-138-5p Expression		
Parameters	Total	High	Low	*p* Value
Age (years)				0.420
<50	25	14	11	
≥50	31	14	17	
Tumor size (cm)				0.415
<4	23	10	13	
≥4	33	18	15	
Histology				0.397
Squamous	37	17	20	
Adenocarcinoma	19	11	8	
FIGO stage				0.016
Ib–IIa	29	19	10	
IIb–IIIa	27	9	18	
Differentiation				0.026
Well + moderate	36	22	14	
Poor	20	6	14	
Lymph nodes metastasis				0.002
No	35	23	12	
Yes	21	5	16	
Vascular invasion				0.131
No	41	23	18	
Yes	15	5	10	

FIGO, International Federation of Gynecology and Obstetrics.

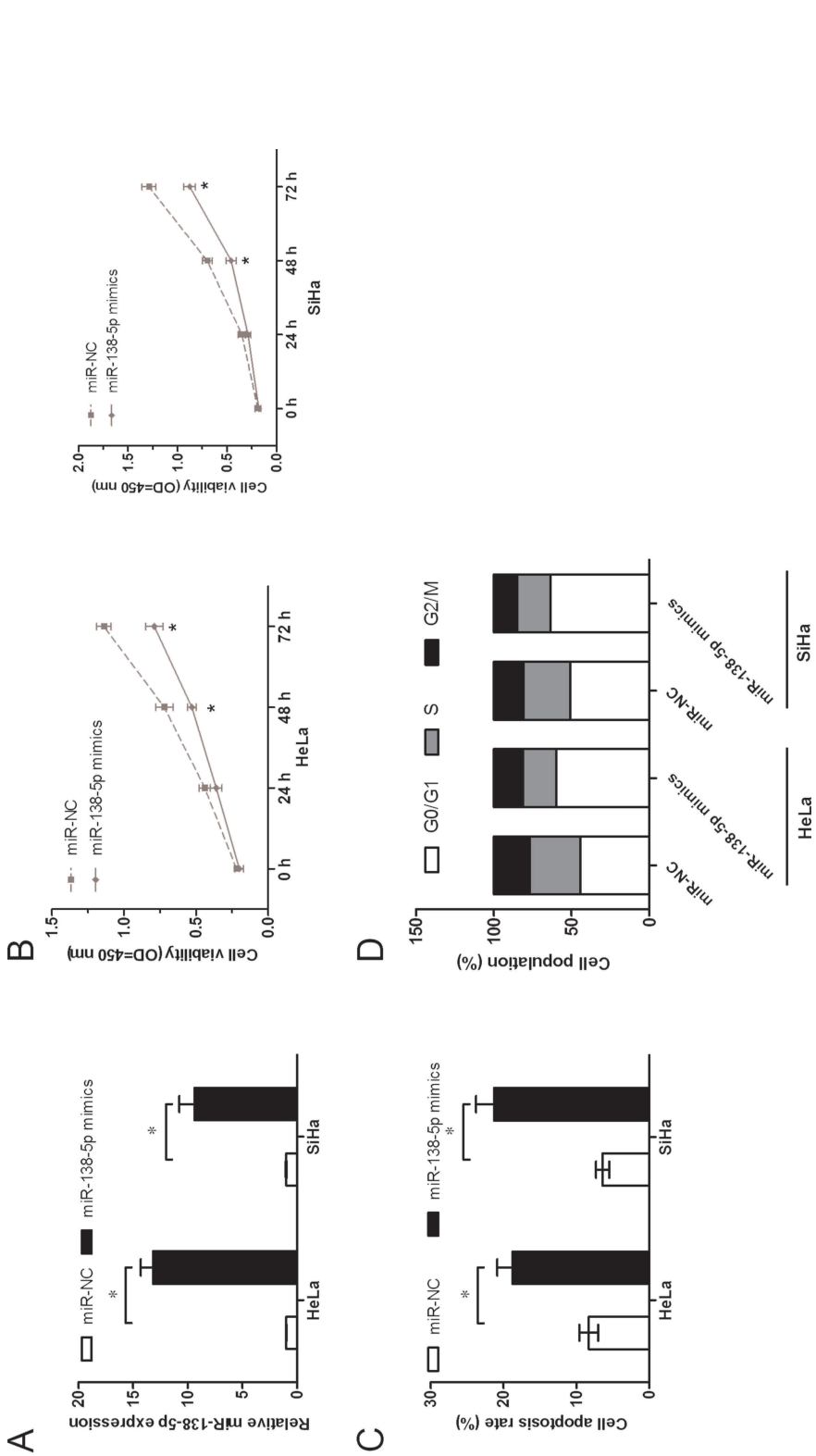

Figure 2. The role of miR-138-5p in regulating the proliferative abilities of cervical cancer cells in vitro. (A) qRT-PCR was used to determine the expression of miR-138-5p in HeLa and SiHa cells transfected with miR-138-5p mimics and miR-negative control (NC). (B) Cell proliferation was measured by cell counting kit-8 (CCK-8) assay in HeLa and SiHa cells transfected with miR-138-5p mimics and miR-NC at indicated time. (C) Flow cytometry showed that the miR-138-5p mimics induced cell apoptosis using fluorescein isothiocyanate (FITC)-annexin V and propidium iodide (PI) staining. (D) Flow cytometry showed that miR-138-5p mimics arrested cervical cancer cell in the G_0/G_1 phase. *$p<0.01$.

cervical cancer cells, cell proliferation was detected using CCK-8 assays. We found that overexpression of miR-138-5p significantly suppressed the proliferation of cervical cancer cells compared with the miR-NC group ($p<0.05$) (Fig. 2B). To investigate whether cell apoptosis and cell cycle contributed to the growth of cervical cancer cells in vitro, we analyzed the effects of miR-138-5p on cell apoptosis and cell cycle of HeLa and SiHa cells. Flow cytometry assay revealed that the apoptosis of HeLa and SiHa cells was greatly enhanced after overexpression of miR-138-5p ($p<0.05$) (Fig. 2C). Furthermore, the percentage of cells in the G_0/G_1 phase significantly increased with miR-138-5p overexpression in HeLa and SiHa cells ($p<0.05$) (Fig. 2D). These results indicated that miR-138-5p suppressed cell proliferation by regulating both cell apoptosis and the cell cycle.

SIRT1 Was a Direct Target of miR-138-5p in Cervical Cancer

Using the bioinformatics software TargetScan for target gene prediction, SIRT1 was identified as one of the potential targets of miR-138-5p (Fig. 3A). The target sequences of SIRT1 3′-UTR Wt or the mutant sequences (Mut) were cloned into the luciferase reporter. After cotransfection of the reporters with miR-138-5p mimics or miR-NC into HeLa cells, the luciferase activity was recorded. Our data showed that miR-138-5p induced a remarkable decrease in the firefly luciferase activity when compared with the miR-NC group. However, the same effect could not be observed in the Mut group ($p<0.05$) (Fig. 3B). Additionally, we investigated the regulatory effect of miR-138-5p on SIRT1 by qRT-PCR and Western blotting in HeLa and SiHa cells. We found that the ectopic expression of miR-138-5p suppressed both SIRT1 mRNA and protein levels in HeLa and SiHa cells using qRT-PCR and Western blot ($p<0.05$) (Fig. 3C and D). Taken together, it was suggested that miR-138-5p directly targeted SIRT1 in cervical cancer cells.

SIRT1 Overexpression Attenuated the Suppressive Effect of miR-138-5p

We further investigated whether overexpression of SIRT1 could reverse the suppressive effect of miR-138-5p. CCK-8 assay ($p<0.05$) (Fig. 4A), cell apoptosis assay ($p<0.05$) (Fig. 4B), and cell cycle assay ($p<0.05$) (Fig. 4C) showed that overexpression of SIRT1 significantly reversed the suppressive effect of miR-138-5p on HeLa and SiHa cells. The effect of pcDNA-SIRT1 was confirmed by qRT-PCR ($p<0.05$) (Fig. 4D).

lncRNA H19 Serves as an Endogenous Sponge of miR-138-5p in Cervical Cancer

Recent studies have shown that lncRNAs acted as an endogenous sponge of miRNAs. We used Starbase v.2.0 to predict that lncRNA H19 had a complementary sequence for miR-138-5p (Fig. 5A). Dual-luciferase reporter assay was performed to explore whether H19 was a functional target of miR-138-5p. Our data showed that the luciferase activity was significantly decreased by the cotransfection of miR-138-5p mimics and H19-Wt rather than the cotransfection of miR-NC and H19-Wt, whereas cotransfection of miR-138-5p mimics and H19-Mut did not change the luciferase activity (Fig. 5B). Furthermore, we found that the expression of H19 was upregulated in cervical cancer tissues compared with adjacent normal tissues ($p<0.05$) (Fig. 5C and D). Moreover, a negative relationship was identified between miR-138-5p and H19 expression ($r^2=0.2369$) (Fig. 5E). In addition, we silenced H19 expression by siRNA in HeLa and SiHa cells. qRT-PCR results showed that H19 knockdown increased miR-138-5p expression in cervical cancer cells ($p<0.05$) (Fig. 5F). These findings might explain why miR-138-5p expression was lower in cervical cancer.

DISCUSSION

Cervical cancer is one of the most common gynecological cancers worldwide. Although widespread implementation of screening programs in recent years has decreased the incidence and mortality of this cancer, it continues to be a major public health problem, specifically in advanced cases[10]. Thus, understanding the molecular mechanism of cervical cancer is urgently needed for the development of effective therapeutic strategies.

miRNAs are regulated in several diseases including cancers, where they have been characterized as oncogenes, tumor suppressors, or components of regulatory pathways that are critical for tumorigenesis[6]. miR-138-5p is commonly dysregulated in diverse cancers and is involved in various biological processes, including proliferation, migration, invasion, angiogenesis, and metabolism by targeting multiple mRNAs. For example, Yang et al. showed that miR-138-5p suppressed cell proliferation and invasion by targeting survivin in bladder cancer cells[11]. Zhao et al. found that miR-138-5p acted as a tumor suppressor in colorectal cancer by targeting programmed death ligand 1 (PD-L1)[12]. Yu et al. revealed that miR-138-5p reduced pancreatic cancer cell growth in a forkhead box protein C1 (FOXC1)-dependent fashion[13].

In this study, for the first time, we determined that the expression of miR-138-5p was downregulated in cervical cancer tissues. Decreased expression of miR-138-5p was associated with advanced clinical features and poor overall survival in cervical cancer patients. The data suggested that the decreased expression of miR-138-5p was an unfavorable factor and might lead to the disorder of cell growth in cervical cancer. To confirm this hypothesis, in vitro function assays were designed to explore the biological roles of miR-138-5p in cervical cancer.

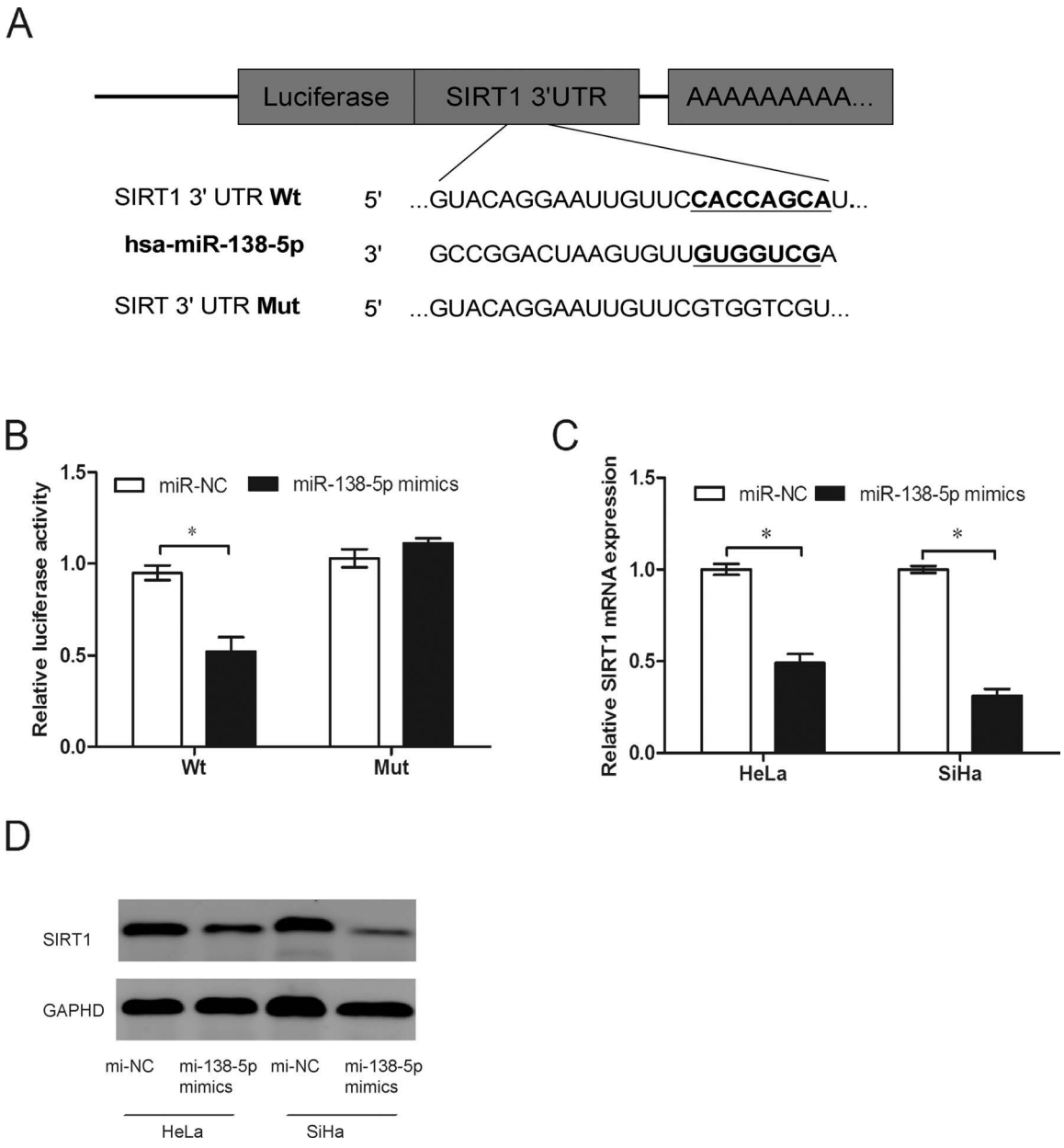

Figure 3. Suppressed silent mating type information regulation 2 homolog 1 (SIRT1) is the direct target of miR-138-5p in cervical cancer. (A) The potential miR-138-5p binding sites of the SIRT1 3′-untranslated region (UTR) and the mutated sequences. (B) Luciferase activity assay showed that miR-138-5p suppressed wild-type (Wt) SIRT1 3′-UTR luciferase activity, while it had no effect on mutated (Mut) SIRT1 3′-UTR luciferase activity compared to negative control in HeLa cells. (C) qRT-PCR analysis showed that the relative mRNA levels of SIRT1 were decreased after miR-138-5p mimic transfection in HeLa and SiHa cells. (D) Western blot analysis showed that SIRT1 protein levels were significantly reduced after transfection with miR-138-5p mimics. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. *$p<0.05$.

Our data showed that miR-138-5p mimics significantly reduced cervical cancer cell proliferation, induced cell apoptosis, and arrested cell in the G_0/G_1 phase in vitro. Our results were consistent with the previous reports in other cancers[14].

SIRT1, the human homolog of Sir2, is a member of the sirtuins family[15]. SIRT1 is a nicotinamide adenine dinucleotide 1 (NAD1)-dependent class III histone deacetylase (HDAC), which plays an important role in the regulation of critical biological processes such as metabolism, oncogenesis, and cancer progression[16,17]. Increasing studies showed that SIRT1 contributed to cell growth, drug resistance, invasion, metastasis, and recurrence[18,19]. In the present study, bioinformatics software indicated that

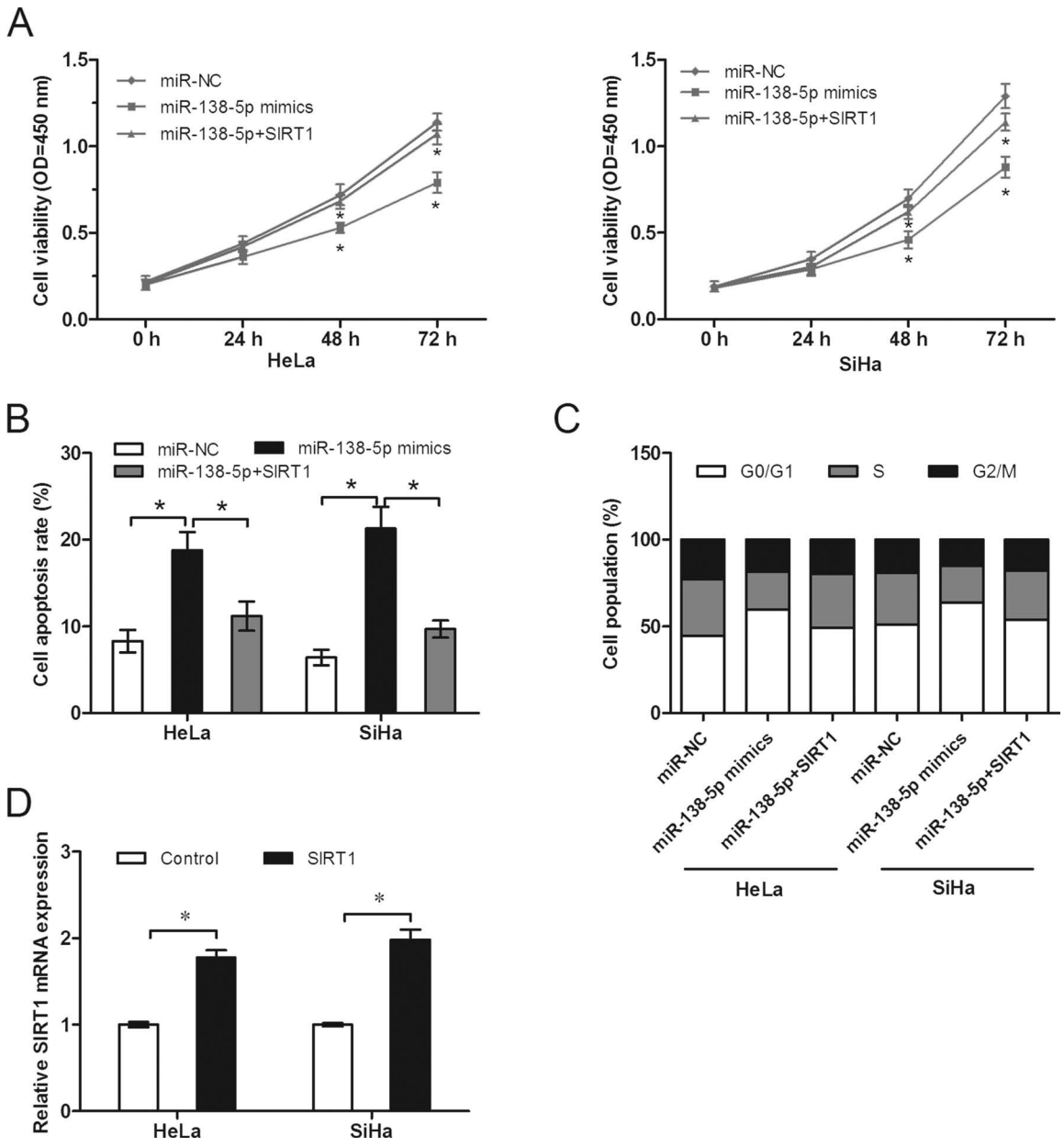

Figure 4. Overexpression of SIRT1 attenuates the effect of miR-138-5p. (A) HeLa and SiHa cells were cotransfected with miR-138-5p mimics and pcDNA-SIRT1 or the vector. CCK-8 assay was used to measure the proliferation. Cell apoptosis (B) and cell cycle (C) assays of HeLa and SiHa cells cotransfected with miR-138-5p mimics and pcDNA-SIRT1 or the vector are shown. (D) SIRT1 expression was detected by qRT-PCR in HeLa and SiHa cells transfected with miR-138-5p mimics and pcDNA-SIRT1 or the vector. $^{*}p<0.05$.

SIRT1 was a potential target of miR-138-5p. Luciferase reporter assay showed that miR-138-5p significantly suppressed the luciferase activity of the Wt 3′-UTR but not the Mut 3′-UTR of SIRT1 in HeLa cells. Moreover, we found that the ectopic expression of miR-138-5p suppressed the SIRT1 mRNA and protein level in both HeLa and SiHa cells. In addition, our data showed that SIRT1 overexpression attenuated the suppressive effect of miR-138-5p. The data suggested that SIRT1 was a direct target of miR-138-5p in cervical cancer.

Recent studies showed that deregulation of lncRNAs profoundly influenced the expression of miRNAs[20]. For example, Li et al. found that through the action of miR-140-5p, the lncRNA maternally expressed 3 (MEG3) reduced adipogenesis and promoted osteogenesis of human adipose-derived mesenchymal stem cells[21]. Sun et al.

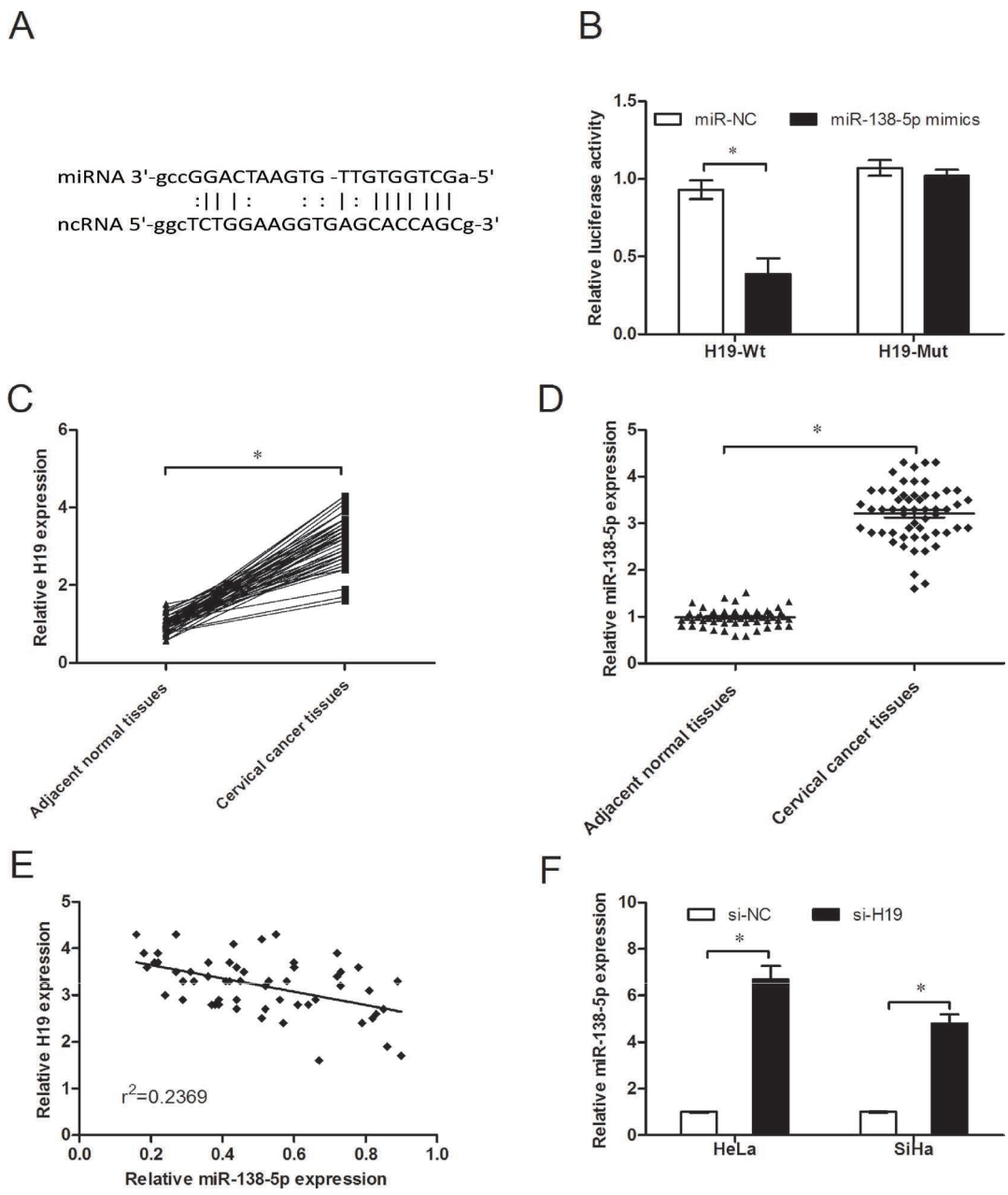

Figure 5. Long noncoding RNA (lncRNA) H19 negatively regulates miR-138-5p expression in cervical cancer. (A) The predicted miR-138-5p binding sites on lncRNA H19. (B) Dual-luciferase reporter assay showed that miR-138-5p mimics reduced the intensity of fluorescence in HeLa cells transfected with H19-Wt but had no effect on the H19-Mut vector. (C, D) Expression of lncRNA H19 was increased in cervical cancer tissues compared to adjacent normal tissues as determined by qRT-PCR. (E) A negative association was verified between H19 and miR-138-5p in a cohort with 58 cervical cancer patients ($r^2 = -0.2369$). (F) Deceased expression of H19 upregulated miR-138-5p expression in HeLa and SiHa cells. *$p < 0.05$.

revealed that in human nasopharyngeal carcinoma, lncRNA LOC100129148 targets miR-539-5p to function as an oncogene[22]. In our study, bioinformatics showed that lncRNA H19 had a complementary sequence of miR-138-5p. H19 expression was upregulated in cervical cancer tissues and has a negative relationship with miR-138-5p expression in cervical cancer. In addition, we showed that decreased expression of H19 significantly increased miR-138-5p in cervical cancer cells. These data indicated that lncRNA H19 might serve as an endogenous sponge of miR-138-5p in the progression of cervical cancer.

In conclusion, the present study identified that decreased expression of miR-138-5p in cervical cancer tissues was related to malignant clinical features and poor prognosis. Function assays showed that miR-138-5p could act as a novel inhibitor for tumor growth in cervical cancer. The anticancer functions of miR-138-5p might be due to the inhibition of SIRT1. Together, we indicated that miR-138-5p could become a novel prognostic biomarker and potential therapeutic target in the treatment of cervical cancer.

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