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Functional characterization of the long noncoding RNA MIR22HG as a tumour suppressor in cervical cancer by targeting IGF2BP2

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Abstract. – OBJECTIVE: Cervical cancer is the most common malignant tumour in the female reproductive tract, ranking second in the global cause of female cancer and seriously endangering women's health. However, the underlying mechanisms leading to cervical cancer are unclear. Previous studies have reported the roles and general underlying mechanisms of the long noncoding RNA MIR22HG (MIR22HG) in multiple types of tumours.

PATIENTS AND METHODS: In this study, we describe the functional role of MIR22HG as a tumour suppressor lincRNA by regulating metastasis, growth and invasion by performing a series of *in vivo* and *in vitro* experiments.

RESULTS: Our data suggested that MIR22HG dramatically promoted cervical cancer apoptosis and inhibited invasion by targeting IGF2BP2.

CONCLUSIONS: The long noncoding RNA MIR22HG targets IGF2BP2 as a tumour suppressor in cervical cancer. Our findings will be helpful for developing potential therapeutics for cervical cancer.

Key Words:
MIR22HG, IGF2BP2, Tumour suppressor, Cervical cancer.

Introduction

Cervical cancer is a common tumour of the female reproductive system[1,2]. It ranks third among female malignant tumours and fourth in mortality, and 80% of cervical cancer cases occur in developing countries[2]. In recent years, cervical cancer screening has made great progress, which has greatly promoted the early diagnosis and treatment of cervical cancer, but the 5-year survival rate of patients in advanced stages is still not ideal. Moreover, the underlying mechanisms leading to cervical cancer are unclear. Therefore, a more comprehensive understanding of the molecular basis underlying invasion and proliferation is critical to identify efficacious therapeutic targets.

LncRNAs are a type of noncoding RNA molecule of more than 200 nucleotides in length[3,4]. Long noncoding RNAs (lncRNAs) critically participate in various physiological processes, and their dysregulation is associated with multiple human diseases, including cancers[5]. However, they do not encode proteins[6]. Previous studies have reported the roles and general underlying mechanisms of lncMIR22HG in hepatocellular carcinoma, lung cancer, and endometrial carcinoma. MIR22HG clearly exerts its function through different mechanisms depending on various contexts[7]. The specific mechanisms associated with cervical cancer initiation and progression still need elucidation. Here, we describe a functional role of MIR22HG as a tumour suppressor lincRNA by regulating metastasis, growth and invasion. Additionally, we identified IGF2BP2 as a target of MIR22HG in cervical cancer.

Patients and Methods

Cell Culture and Human Specimens

All cervical cancer cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with

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10% foetal bovine serum (FBS; HyClone, Logan, UT, USA). Human specimens were obtained from diagnostic biopsies. A total of 50 diagnostic patient specimens were used in this study (Table I). This research was approved by Chongqing University Cancer Hospital.

RNA Extraction and qRT-PCR

The primer sequences for the other genes used are detailed as follows: IGF2BP2 F: 5'-AGTGGAATTGCATGGGAAAATCA-3', R: 5'-CAACGGCGGTTTCTGTGTC-3'; GAPDH F: 5'-GTCGATGGCTAGTCGTAGCATCGAT-3', R: 5'-TGCTAGCTGGCATGCCCGATCGATC-3'; MIR22HG F: 5'-CGGACGCAGTGATTTGCT-3', R: 5'-GCTTTAGCTGGGTCAGGACA-3'; U6 F: 5'-GCTTCG GCAGCACATATACTAAAAT-3', R: 5'-CGCTTCACGAA TTTGCGTGTCAT-3'. RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR. MIR22HG and RNU6 expression was measured by qRT-PCR using the primer set from RiboBio (Guangzhou, China).

Transwell Assays

Cells were transfected with the indicated plasmids, 48 hours after transfection, the cells were stained with a 0.4% crystal violet solution. For this assay, 8.0 μm transwell Permeable Supports (Corning, Corning, NY, USA) were used. The transwell assay protocol was performed as described by Wu et al[7].

Cell Viability and Apoptosis Analysis

Cells were transfected with the indicated plasmids. Twenty-four hours after transfection, cell proliferation was evaluated using cell viability assays with a Cell Counting Kit-8 (CCK-8, Med Chem Express, Monmouth Junction, NJ, USA)[8]. SiHa cells were seeded in 6-well cell culture plates after 6 hours of transfection. Apoptosis assays were performed using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes NJ, USA). The stained cells were analysed by a PI/Annexin V-FITC kit (Calbiochem, Shanghai, China)[8].

Luciferase Reporter Assay

Luciferase activity was measured using the dual-luciferase assay system according to the manufacturer's protocol (Promega, Madison, WI, USA). The 3'-UTR segments of IGF2BP2 that were predicted to interact with MIR22HG were amplified by PCR from human genomic DNA and inserted into the Hind III and SacI sites of the lncRNA Expression Reporter Vector. For the luciferase reporter experiments, the indicated cells were seeded into 24-well cell culture plates and transfected with the indicated reporter plasmids and either the pre-MIR22HG or control (NC) oligonucleotides. Following 48 hours of incubation, the cells were subjected to a Luciferase reporter assay.

Western Blot Analysis

The cells were lysed, and proteins were isolated. Then, 30 μg of protein was subjected to Western blotting with anti-IGF2BP2 (Proteintech, China, 1:3000), anti-Bax (Proteintech, China, 1:2000), anti-BCL2 (Abcam, Cambridge, MA, USA, 1:2000), anti-cleaved-caspase-3 (Abcam, Cambridge, MA, USA, 1:3000), and anti-β-actin (Invitrogen, USA, 1:10000). The membranes were blocked with 5% non-fat milk for 45 min and incubated overnight at 4°C with their corresponding primary antibodies, followed by incubation with secondary antibodies conjugated to horseradish peroxidase (HRP) for 2 hours at RT.

Table I. Characteristics of cervical cancer patients (n=50).

Characteristics	Variable	Patients N (%)
Age (years)	Range, (means ± SD)	36-87 (57 ± 5.6)
Family history of cancer	No.	42 (92.54)
	Yes	8 (7.46)
HPV	Positive	38 (53.7)
	Negative	12(46.3)
TNM stage	I	11 (11.2)
	II	17 (15.7)
	III	15 (38.8)
	IV	7 (34.3)
Differentiation grade	Moderate	27 (23.9)
	Poor	23 (76.1)

Immunohistochemistry and Immunofluorescence Staining

The IHC staining score was reviewed by an expert panel of pathologists. Xenograft specimen IHC assays were performed as described previously[8]. A human rabbit Ki-67 antibody (1:400; Cell Signaling Technology, Danvers, MA, USA, #9027) was used. The slides were reviewed using a light microscope (magnification, x100).

The IF-stained cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with a 5% blocking solution. The cells were then incubated with an anti-IGF2BP2 (1:100) antibody overnight at 4°C. Following a phosphate-buffered saline (PBS) wash, the cells were incubated with a goat anti-rabbit IgG-PE antibody for 1 hours. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured with a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Animal Experiments

Animal experiments were approved by the Laboratory Animal Welfare and the Ethics Committee of Chongqing University and they were conducted using six-week-old female athymic (nu/nu) mice, with nine mice in each group. The indicated cells were transfected with the indicated shMIR22HG, MIR22HG and IGF2BP2 expression lentiviruses. A total of 1×10^6 SiHa cells in 100 µl of serum-free medium were injected subcutaneously (s.c.) into each mouse (right back). The tumour dimensions were serially measured with electronic callipers, and the volumes were calculated with the following formula: a (the largest diameter) × b^2 (the perpendicular diameter). The method of euthanasia was CO_2 exposure for 10 min at a 20% fill rate of cage volume/min.

Statistical Analysis

Statistical significance was analysed by unpaired Student's *t*-tests or one-way ANOVA and Duncan's multiple range tests using Prism 8.0 statistical software, and *p*-values less than 0.05 were considered statistically significant. Kaplan-Meier survival analysis was used to calculate the overall survival rate of cervical cancer.

Results

MIR22HG Overexpression Is Correlated With Good Prognosis in Cervical Cancer Patients

Previous studies have shown that some lincRNAs are inversely correlated with cervical cancer progression, and MIR22HG expression was significantly decreased in the cervical cancer specimens compared to the paracancerous tissues from our hospital (Figure 1A). The TCGA data showed that MIR22HG expression was significantly decreased in cervical cancer specimens compared to normal tissues (Figure 1B). In addition, our clinical data showed that the

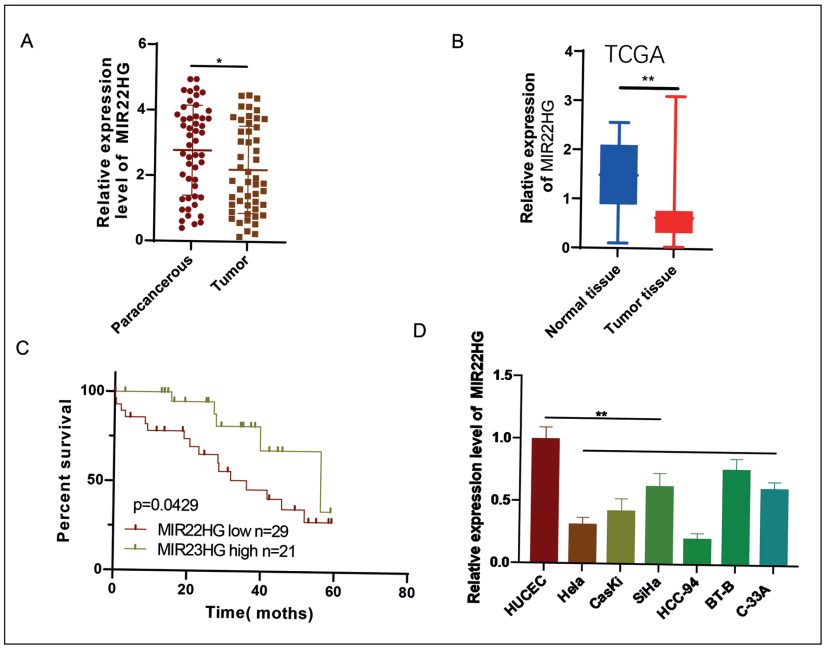

Figure 1. MIR22HG overexpression is corrected with good prognosis in cervical cancer patients. **A,** MIR22HG expression was significantly downregulated in cervical cancer specimens. The expression levels of MIR22HG were measured using qRT-PCR in paracancerous tissue (n=50) and tumor tissues (n=50). **B,** MIR22HG expression was significantly downregulated in cervical cancer specimens. The expression levels of MIR22HG were analysis from TCGA database. **C,** Survival rates for cervical cancer patients with low (n=29) and high (n=21) MIR22HG expression. **D,** cervical cancer cell lines showed lower expression level of MIR22HG compared to the HUCEC cells. *, $p<0.05$; **, $p<0.01$.

decreased expression of MIR22HG was significantly correlated with the overall survival of cervical cancer patients (Figure 1C). MIR22HG was downregulated in cervical cancer cell lines compared to the HUCEC cell line (Figure 1D). Taken together, these data suggest that MIR22HG may act as a tumour suppressor lincRNA in cervical cancer.

MIR22HG Suppresses Cervical Cancer Cell Growth and Invasion

To investigate the function of MIR22HG in the tumorigenesis of cervical cancer, we used SiHa cell lines, which exhibit an intermediate expression level of MIR22HG. We transfected siRNAs specific to MIR22HG in SiHa cells and detected MIR22HG expression at 72 h post transfection (Figure 2A). When MIR22HG was overexpressed, more cell apoptosis occurred in the MIR22HG-overexpressing group, whereas less cell apoptosis appeared in the siMIR22HG group (Figure 2B). As shown by Western blotting, the cell apoptosis-related protein further supported the above finding (Figure 2C). At the same time, CCK-8 assays showed that cell viability was significantly reduced in MIR22HG-overexpressing cells (Figure 2D). The transwell assay also showed a lower invasion rate when MIR22HG was overexpressed, but a higher invasion rate was observed when MIR22HG was knocked down (Figure 2E).

Transcriptome Sequencing Analysis of the Downstream Pathway of MIR22HG

We transfected MIR22HG or siMIR22HG into SiHa cells for 72 h and performed transcriptome sequencing analysis of the downstream pathway of MIR22HG. From the sequencing analysis, the Venn diagram showed that there were 98 genes that were significantly different in both the MIR22HG and siMIR22HG groups (Figure 3A). The heat map shows the expression of 98 genes,

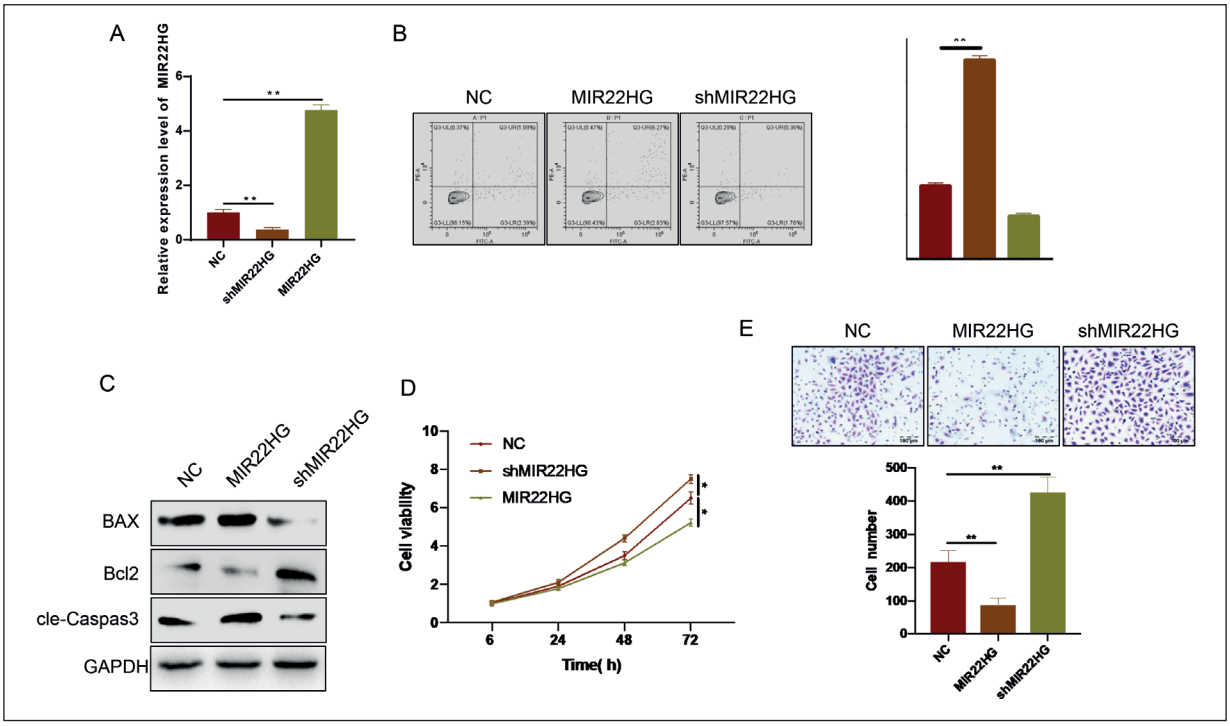

Figure 2. MIR22HG suppress cervical cancer cell apoptosis and invasion. **A,** SiHa cells were transfected with negative control oligonucleotide, MIR22HG or shMIR22HG. After 72 hours of transfection, isolated mRNAs were subjected to qRT-PCR. **B,** The MIR22HG overexpression enhanced apoptosis, consistently, knockdown MIR22HG inhibited apoptosis in SiHa cells. The SiHa cells were transfected with MIR22HG or shMIR22HG after 72 hours of transfection and analyzed with flow cytometry. **C,** MIR22HG stimulates the expression of cleaved-caspase3 and Bax but suppress the Bcl2 expression in SiHa cells. SiHa cells were transfected with the MIR22HG or shMIR22HG for 72 hours, and then subjected to Western blotting. **D,** Consistent with apoptosis, MIR22HG suppressed the proliferation of SiHa cells and shMIR22HG suppressed its proliferation. SiHa cells were transfected with the MIR22HG or shMIR22HG for 72 hours and then subjected to an CCK-8 assay. **E,** MIR22HG negatively regulates SiHa cell invasion. After 24 hours of transfection, cells were subjected to a transwell assay (100×). *, $p<0.05$; **, $p<0.01$.

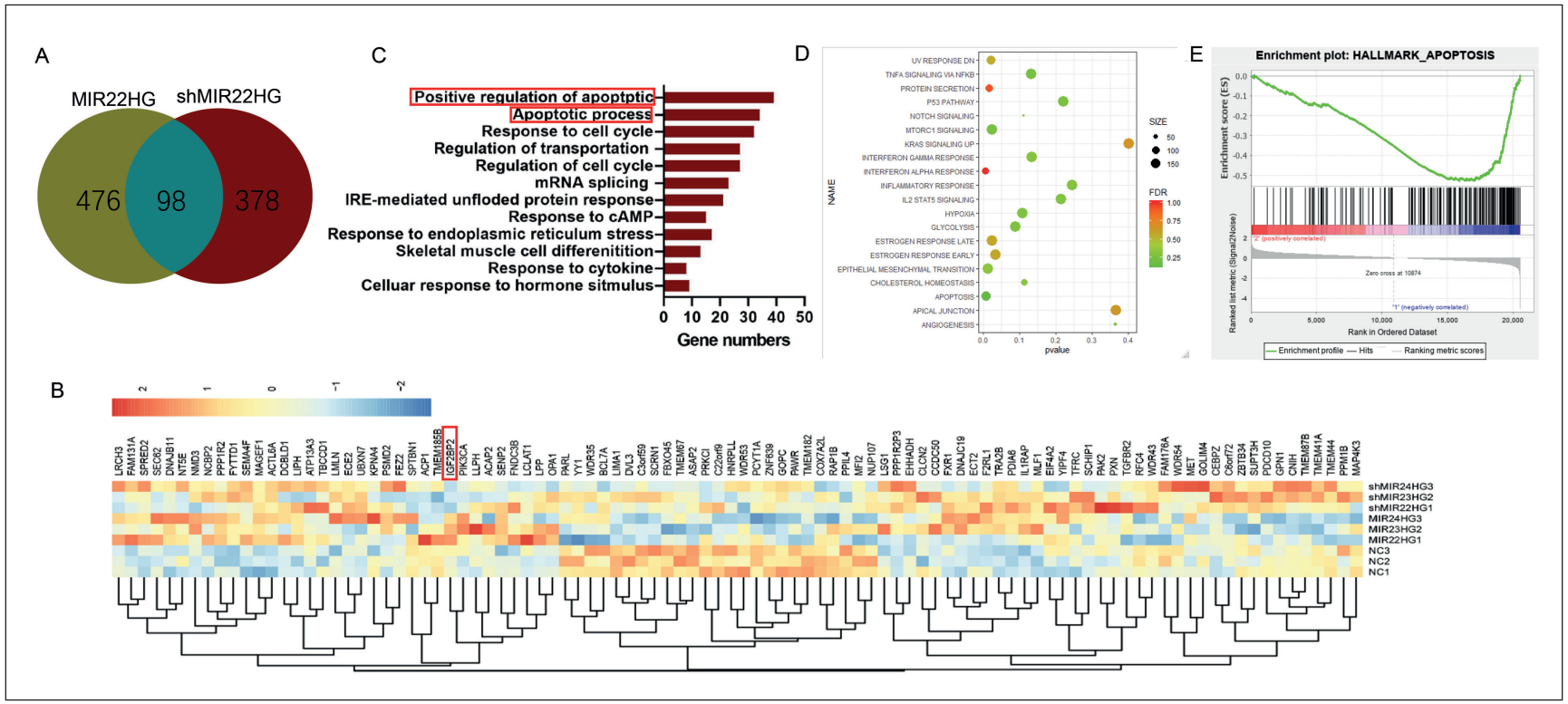

Figure 3. Transcriptome sequencing analysis of the downstream pathway of MIR22HG. SiHa cells was transfected MIR22HG or shMIR22HG 72h and detected the transcriptome sequencing analysis. **A,** The Venn diagram showed that there were total 98 genes were significantly different both in MIR22HG or siMIR22HG groups. **B,** The hot map showed the expression of 98 genes. **C,** The Go analyzed the downstream pathways, the apoptotic pathways significant differences. **D**, the TCGA database analyzed the IGF2BP2 high-expression and low-expression pathway difference by GASE analysis, the apoptosis pathway significant differences. **E**, The apoptosis pathway detailed display.

with a significant difference in the IGF2BP2 gene (Figure 3B). IGF2BP2 expression was upregulated in cervical cancer patients compared to normal controls in the analysis of the TCGA database (**Supplementary Figure 1A**). The GO analysis of the downstream pathways revealed significant differences in the apoptotic pathway (Figure 3C). Then, we used the TCGA database and analyzed the IGF2BP2 high-expression and low-expression pathway differences by GSEA, and the data showed that the apoptotic pathway was significantly different. This result is consistent with our sequencing results (Figure 3D, E).

IGF2BP2 Is a Target of MIR22HG

To investigate how MIR22HG regulates apoptosis and invasion, we searched for target gene candidates of MIR22HG (mirdb.org) and identified IGF2BP2 as a candidate of MIR22HG (Figure 4A). Thus, we further studied IGF2BP2, which has been reported to regulate the cell cycle and invasion in glioma[9]. To investigate whether MIR22HG is involved in IGF2BP2 regulation, SiHa cells were transfected with MIR22HG or siMIR22HG. After 72 hours of transfection, IGF2BP2 expression was measured by qRT-PCR and Western blotting. Our experimental results showed that IGF2BP2 expression was significantly downregulated or upregulated by the overexpression or inhibition of MIR22HG, respectively, at both the mRNA and protein levels (Figure 4B and 4C). We also confirmed this result using immunofluorescence (Figure 4D). Furthermore, we verified that MIR22HG directly targeted the 3' UTR of IGF2BP2 by a luciferase reporter assay (Figure 4E). Consistent with the *in vitro* results, the clinical sample analysis results also showed an inverse association between MIR22HG and IGF2BP2 in 47 cervical cancer specimens (Figure 4F). These findings indicated that MIR22HG inhibits IGF2BP2 mRNA and protein expression by directly targeting its 3' UTR.

MIR22HG Regulates Metastasis, Growth and Invasion Via IGF2BP2 in Cervical Cancer

Invasion and malignant proliferation are important factors in the progression and recurrence of cervical cancer. IGF2BP2 plays a key role in cancer proliferation, invasion and metastasis[10-12]. We investigated whether MIR22HG is involved in the proliferation and invasion regu-

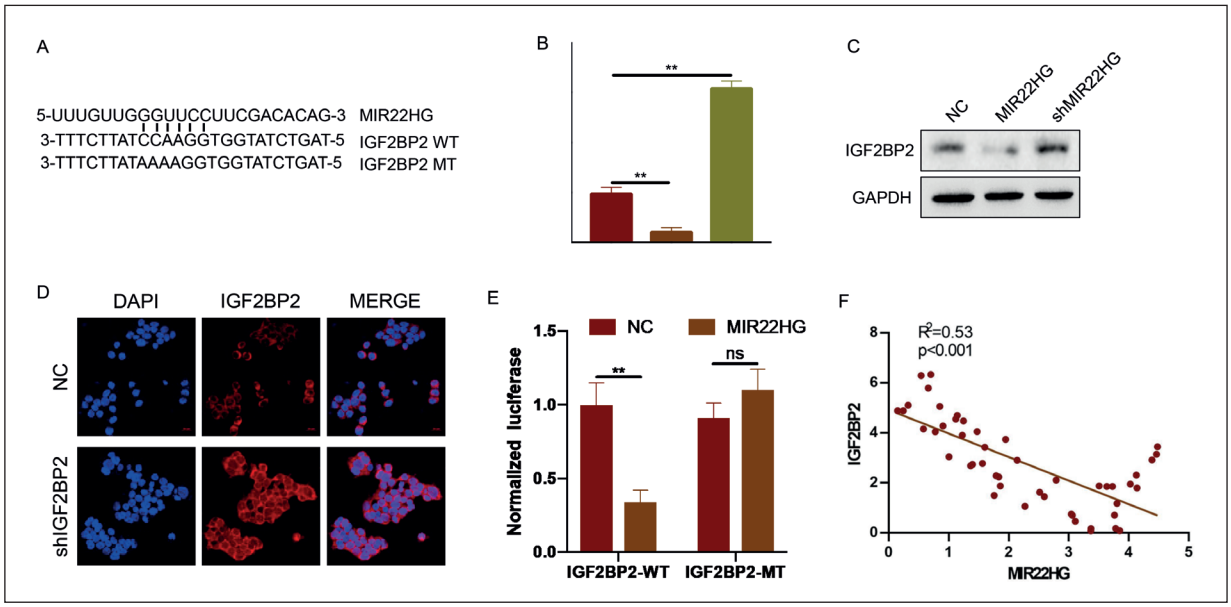

Figure 4. MIR22HG could target IGF2BP2. **A,** The MIR22HG seed sequence is complementary to the 3' UTR of IGF2BP2. **B,** and **C,** MIR22HG inhibited IGF2BP2 mRNA and protein expression. After 72 hours of transfection of MIR22HG and shMIR22HG, using qRT-PCR and Western blot measure. **D,** The immunofluorescence analyzes the IGF2BP2 expression, After 72 hours of transfection of MIR22HG and shMIR22HG (200×). **E,** Activity of the luciferase gene linked to the 3′ UTR of IGF2BP2. The luciferase reporter plasmids of wildtype (WT) or mutated 3′ UTR sequences of IGF2BP2 (MT) were transfected into HEK-293 cells with or without the MIR22HG. **F,** The expression levels of MIR22HG and IGF2BP2 showed a negative correlation in 47 patients with cervical cancer. Which were measured by RT-qPCR. NC, negative control. shMIR22HG, MIR22HG shRNA; NS: No significant; **, $p<0.01$.

lation of cervical cancer by IGF2BP2. The cell viability analysis results showed that the overexpression of IGF2BP2 restored the cell growth inhibited by MIR22HG (Figure 5A). Consistent with these results, the apoptosis analysis showed that the overexpression of IGF2BP2 attenuated MIR22HG-induced cell apoptosis (Figure 5B) and IGF2BP2 blocked the apoptosis protein regulated by MIR22HG when subjected to Western blotting (Figure 5C). In addition, transwell experiments showed that the overexpression of IGF2BP2 blocked the MIR22HG-induced invasion effect (Figure 5D). Overall, these results suggest that MIR22HG regulates the growth and invasion due to IGF2BP2 in cervical cancer cells.

MIR22HG Inhibited Cervical Cancer Growth by IGF2BP2 In Vivo

As shown in the animal experiment, the tumour volume (Figure 6A) and tumour weight (Figure 6B) were significantly decreased when MIR22HG was overexpressed. When we overexpressed both MIR22HG and IGF2BP2, IGF2BP2 reversed the inhibition of tumour growth by MIR22HG. The IHC assay showed that the proliferation-related protein Ki-67 was overexpressed in the shMIR22HG group but suppressed in the MIR22HG group (Figure 6C). The co-transfection of MIR22HG and IGF2BP2 reversed the inhibition of Ki-67 expression by MIR22HG compared to MIR22HG alone. Taken together, these data suggest that MIR22HG is essential to block tumour growth by targeting IGF2BP2 *in vivo* (Figure 6D). The efficiency of plasmid and shRNA transfection is shown in Supplementary Figure 1B, C.

Discussion

The occurrence of malignant growth and invasion in cervical cancer patients indicates a poor prognosis[13,14]. MIR22HG has previously been identified as a prognostic marker in hepatocellular carcinoma[7]. Cui et al[15] reported that MIR22HG inhibits endometrial carcinoma cell

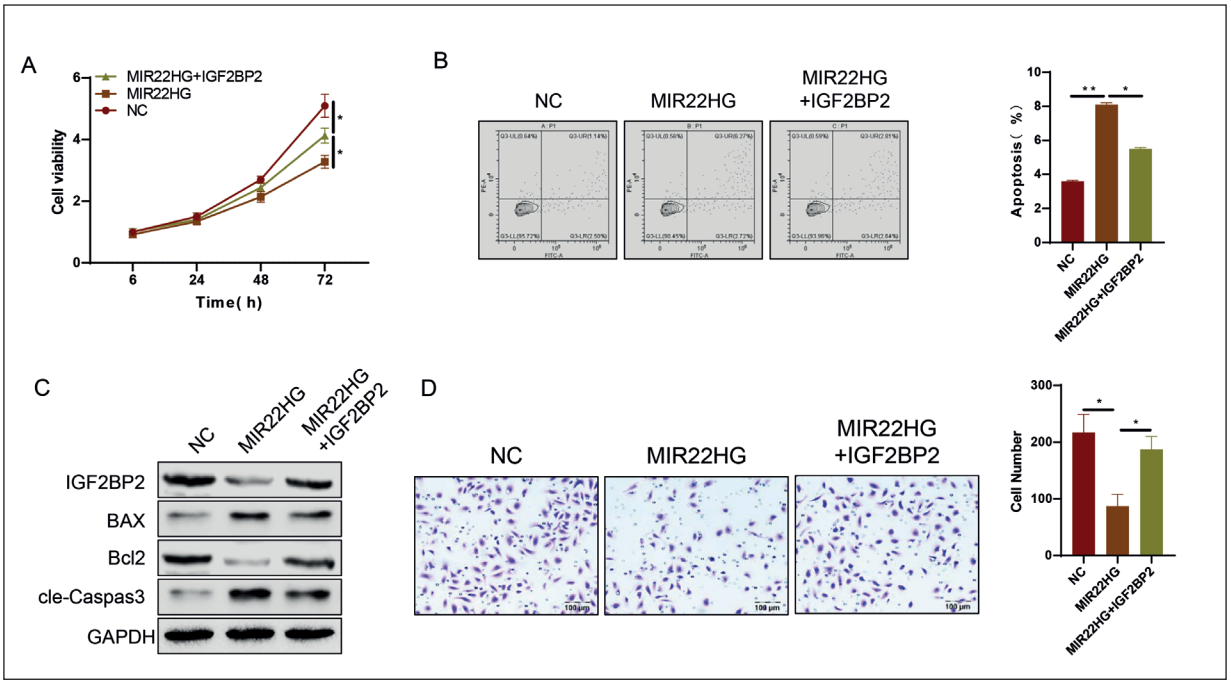

Figure 5. MIR22HG regulating growth and invasion *via* IGF2BP2 in cervical cancer cells. A, The cell viability analysis results show that the overexpression of IGF2BP2 restored the cells viability inhibited by MIR22HG. SiHa cells were transfected with the MIR22HG and IGF2BP2 plasmid for 72 hours and then subjected to an CCK-8 assay. **B,** The apoptosis analysis IGF2BP2 overexpression attenuated the MIR22HG induced cell apoptosis. SiHa cells were transfected with the MIR22HG and IGF2BP2 plasmid for 72 hours and analyzed with flow cytometry. **C,** IGF2BP2 blocked the apoptosis protein regulated by MIR22HG. SiHa cells were transfected with the MIR22HG and IGF2BP2 plasmid for 72 hours, and then subjected to Western blotting. **D,** The transwell assay showed that the overexpression of IGF2BP2 attenuated the MIR22HG-induced metastasis effect (200×). NC, negative control oligonucleotides; MIR22HG, MIR22HG mimics; IGF2BP2: IGF2BP2 plasmid. *, $p<0.05$; **, $p<0.01$.

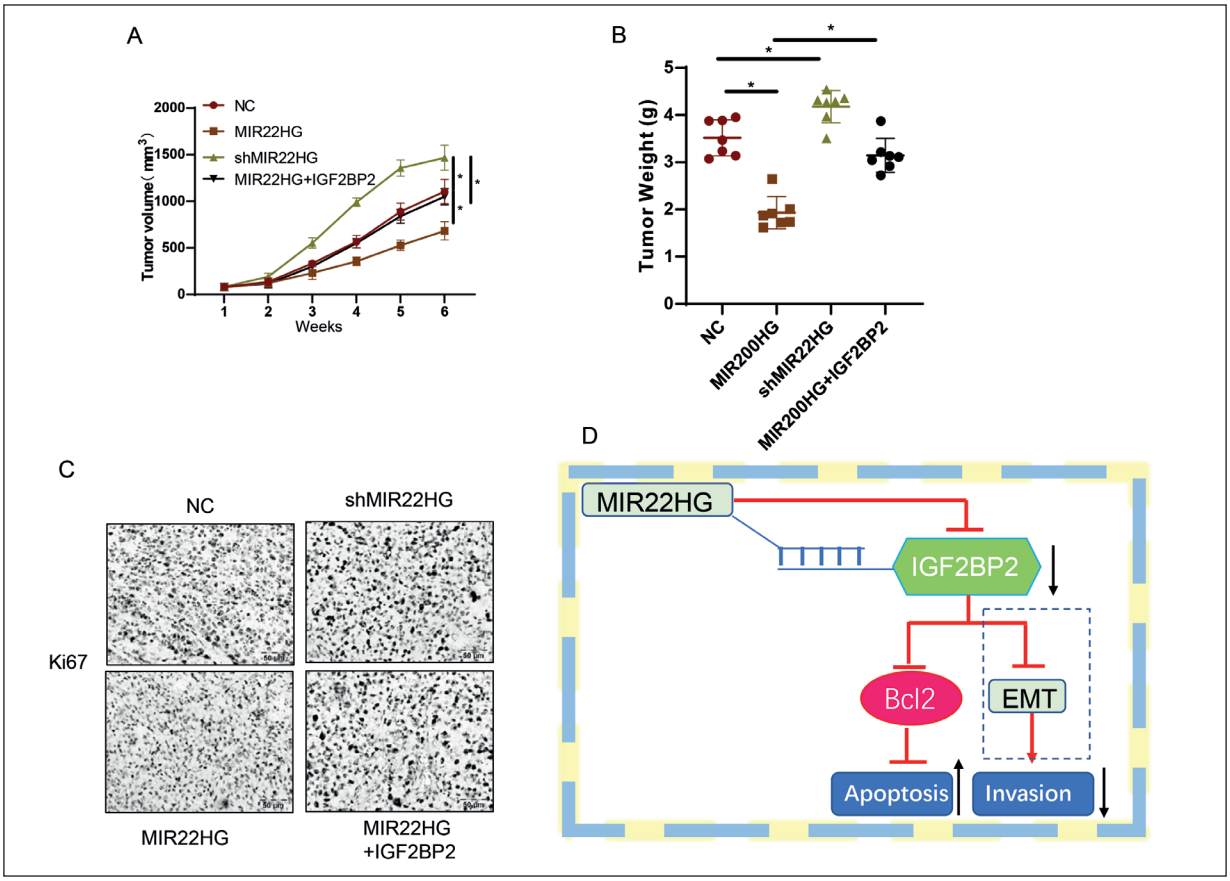

Figure 6. MIR22HG target IGF2BP2 inhibited cervical cancer growth *in vivo*. **A,** Tumor volume in xenografts, siHa cells were transfected with the MIR22HG or IGF2BP2 plasmid. These cells and injected s.c. into nude mice (n = 7/group, 1×10^5 cells/mouse). MIR22HG could suppress the tumor growth, but overexpression IGF2BP2 could attenuate MIR22HG-induced inhibition of tumor growth. **B,** The xenografts weight. **C**, The Ki-67 immunohistochemistry assay show that MIR22HG could suppress the tumor proliferation and knockdown MIR22HG could promote the tumor proliferation, but overexpression IGF2BP2 could attenuate MIR22HG-induced inhibition of tumor proliferation (100×). **D**, A schematic model of MIR22HG target IGF2BP2 inhibited cervical cancer growth. **, $p<0.01$.

proliferation. MIR22HG has an inhibitory effect on thyroid papillary carcinomas growth[16]. However, the inhibitory effect of MIR22HG on cervical cancer has not been reported. Here, we found that increased expression of MIR22HG was significantly correlated with good clinical outcomes in cervical cancer patients. This result is consistent with Su et al[17] study on lung cancer. In addition, our *in vitro* study showed that the overexpression of MIR22HG enhanced the apoptosis of cervical cancer cells and inhibited invasion.

We further clarified the mechanism of the effect of MIR22HG on cervical cancer. We used a series of experiments to identify IGF2BP2 as a target gene of MIR22HG in cervical cancer. IGF2BP2 has been described to be oncogenic in several types of cancer[7,18,19]. However, little is known about its role in cervical cancer progression. Our data showed that IGF2BP2 expression was increased or downregulated in cervical cancer cells by the inhibition or ectopic expression of MIR22HG, respectively. Previous scholars[20] have shown that the expression of IGF2BP2 is highly positively correlated with Bcl2 expression in pancreatic cancer. MIR22HG may regulate cell apoptosis by targeting IGF2BP2, so we investigated the apoptotic regulatory mechanism of MIR22HG in cervical cancer. Our findings showed that the restoration of IGF2BP2 blocked the MIR22HG overexpression-induced apoptosis and inhibition of cancer invasion. In general, these data suggest that MIR22HG promotes apoptosis in cervical cancer and inhibits invasion by targeting IGF2BP2. IGF2BP2 could promote EMT and invasion *via* the IGF2/PI3K/Akt pathway in glioblastoma[9]. Therefore, specific regula-

tory mechanisms in cervical cancer need to be further studied.

Conclusions

In summary, we combined clinical and experimental studies to establish the role of MIR22HG in cervical cancer apoptosis. Overexpression of MIR22HG dramatically promoted cervical cancer apoptosis and inhibited invasion by IGF2BP2. Our findings will be helpful for developing potential therapeutics for cervical cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgement

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Authors' Contribution

Jin Shu was responsible for doing the main experimental. Jin Shu and Dong Wang were jointly involving in extracting data and writing the manuscript. Dong Wang conceived and designed this study..

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