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CircRNA 0025202 Induces Cervical Cancer Tumorigenesis by Sponging miR-556-5p To Activate HMGA2 Expression

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ABSTRACT

circRNAs are reported to demonstrate significant function in various cancers. Nevertheless, the detailed effects of circRNAs in cervical cancer development are seldom displayed. In our present research, we observed circ_0025202 was strongly overexpressed in cervical cancer tissues and cells. Meanwhile, circ_0025202 expression in the tissue samples of cervical cancer was positively associated with FIGO stage and lymphnode metastasis. Then, it was proved that knockdown of circ_0025202 obviously restrained cell growth while triggered cell apoptosis. In addition, we demonstrated that circ_0025202 acted as a sponge of miR-556-5p targeting HMGA2 in cervical cancer cells. Moreover, the increase of HMGA2 is a feature of malignant tumor. Increasing reports have suggested HMGA2 expression could be a biomarker in cervical cancer. Then, it was manifested miR-556-5p was decreased in cervical cancer. Up-regulation of miR-556-5p repressed cervical cancer growth significantly. Besides these, it was confirmed that loss of HMGA2 by shRNA-circ_0025202-mediated up-regulation of miR-556-5p enhanced cervical cancer cell growth. To sum up, our data indicated a mechanism that circ_0025202- miR-556-5p-HMGA2 network enhanced cervical cancer development.

INTRODUCTION

Cervical cancer is a prevalent type of cancer among women and is also a major cause of death worldwide Arbyn et al. (2011). Most patients with cervical cancer develop invasive cancer by the time of the disease is diagnosed. Patients with invasive cervical cancer may be at risk of developing secondary malignancies after definitive radiotherapy Jhamad et al. (2018). In the case of metastatic cervical cancer, the 5-year survival rate is almost 16.5%, and the 5-year survival rate of patients with stage III cancer or higher is less than 40% Li et al. (2016). Hence, it is important to study the pathogenesis of cervical cancer. Circular RNAs (circRNAs) are non-coding RNAs derived from RNA back-splicing events Chen et al. (2016). In recent years, circRNAs have emerged as crucial regulators of a wide variety of human diseases Han et al. (2018). The potential of oncogenic or tumor-suppressive circRNAs, which can function as biomarkers, has been widely recognized Chen et al. (2018), Meng et al. (2017), Kristensen et al. (2018). For example, circRNA_102171 induces papillary thyroid cancer development through the activation of β-catenin Bi et al. (2018). Circ_100395 regulates miR-1228 and TCF21 during lung cancer development Chen et al. (2018). Recent studies have found that various circRNAs are dysregulated in cervical cancer tissues Tornesello et al. (2020), Shi et al. (2020). Circ_0025202 regulates breast cancer progression via the regulation of miR-182-5p and FOXO3a Sang et al. (2019). Circ_0025202 represses breast cancer cell tumorigenesis and tamoxifen resistance by regulating the miR-197-3p and HIPK3 axis Li et al. (2021). Thus, targeting circRNAs can be promising for the development of novel therapies for patients with cervical cancer. The exact mechanism of the effect of circ_0025202 on cervical cancer progression is poorly understood. Some circRNAs have been demonstrated to serve as sponges of miRNAs to regulate mRNA expression Jeck et al. (2014), Arnaiz et al. (2019). MicroRNAs are short non-coding RNAs that have critical effects on cervical carcinogenesis Wilting et al (2013). For instance, miR-337 can inhibit cervical cancer progression by targeting specificity protein 1 Dong et al. (2017). miR-664 inhibits cervical cancer by targeting c-

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Kit Lv et al. (2019). In this study, we found that circ_0025202 can sponge miR-556-5p to increase HMGA2 expression, resulting in tumorigenesis during cervical cancer. We aimed to investigate the role of of circ_0025202 in cervical carcinogenesis and explore the underlying regulatory network.

MATERIALS AND METHODS

Tissue samples

Thirty pairs of cervical cancer tissues and corresponding adjacent normal tissues were collected from the Hospital of Chengdu University of Traditional Chinese Medicine. The tissues were maintained in liquid nitrogen at -80°C to extract RNA immediately. Informed consent was obtained from all the patients. The study was approved by the Hospital of Chengdu University of Traditional Chinese Medicine, and all the methods involving human participants were performed in accordance with the Declaration of Helsinki.

Cell culture

C-33A, HeLa, MS751, CaSki, and CerEpiC cells were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St Louis, MI, USA), 100 units/ml of penicillin, and 100 µg/ml of streptomycin (Sigma) at 37°C in a 5% CO2 incubator. All cells were routinely screened to confirm mycoplasma-negative status, and the cell lines were authenticated by examination of morphology, growth characteristics and STR genotype in 2021.

Cell transfection

shRNAs targeting circ_0025202 (sh-circRNA_0025202 #1/2) and the shRNA negative control (NC) (GenePharma, Shanghai, China) were used to silence circ_0025202. HMGA2 siRNA or NC was obtained from GenePharma (Shanghai, China). For overexpression of circ_0025202, circ_0025202 sequences were cloned into pcDNA3.1. miR-556-5p mimics, miR-556-5p inhibitors, and NCs were procured from GenePharma. Vectors were transfected using Lipofectamine 3000, according to the manufacturer's instructions.

Cell Counting Kit-8 (CCK-8) assay

Cell viability was evaluated using the CCK-8 assay. The cells were grown in 96-well plates and then incubated for 0, 1, 2, and 3 days prior to the addition of 10 µL CCK-8 solution. After 4 h, the absorbance was measured at 450 nm on a microplate reader.

Cell apoptosis

Apoptosis was assessed using an Annexin V-FITC Apoptosis Kit (KeyGEN, Nanjing, China). After washing twice with cold phosphate buffer saline (PBS), the cells were re-suspended in Annexin V-FITC for 30 min in the dark. Cell apoptosis was analyzed using a FACSAria Flow Cytometer.

Cell cycle analysis

Cells were stained with propidium iodide using a cell cycle detection kit (KeyGEN, Nanjing, China). Cell ratio analysis was carried out using a flow cytometer.

Wound healing assay

Cells were grown in 6-well plates. A pipette tip was used to create a scratch wound. The floating cells were then removed with PBS. After scratches were made at 0 and 48 h, images were captured using a microscope.

Transwell assay

After transfection, the cells were seeded into the upper chamber with a membrane without Matrigel solution. The full medium was added to the lower chamber. The migrated cells were fixed with 4% formaldehyde. Crystal violet (0.1%) was used to stain the cells. The cell invasion assay was performed in the same manner as given above, the only difference being that the membrane was precoated with Matrigel solution.

Western blotting

Cells were lysed using radioimmunoprecipitation assay (RIPA) lysate with protease inhibitors. The bicinchoninic acid protein assay kit (Nanjing KeyGen Biotech Co., Ltd.) was used to measure the protein concentration. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto polyvinylidene fluoride membranes. Skim milk (5%) was used to block the membranes for 1 h. The membranes were incubated with primary antibodies (rabbit polyclonal against HMGA2 and mouse monoclonal antibodies against GAPDH; Cell Signaling Technology, Danvers, MA, USA). The next day, the membranes were incubated with secondary antibodies. Subsequently, the immunoblots were visualized using an enhanced chemiluminescence reagent.

Quantitative reverse transcription-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA using the TIANScript II RT Kit (Tiangen, Beijing, China). To determine circ_0025202 and HMGA2 expression levels, we used the SYBR Green method (One Step SYBR Prime Script RT-PCR Kit

Perfect Real Time, Takara Biotechnology, Dalian, China). For miR-556-5p, a Bulge-LoopTM miRNA qRT-PCR Primer Set (RiboBio, Guangzhou, China) was used according to the manufacturer's instructions. Experiments were repeated thrice. All primer sequences are shown in Supplementary Table 1. Relative gene expression was evaluated using the 2−ΔΔCq method.

Dual-luciferase reporter analysis

The WT or MUT of circ_0025202 or HMGA2 was integrated into pmir-GLO to obtain the circ_0025202-WT, circ_0025202-MUT, HMGA2-WT, or HMGA2-MUT reporter vectors. The reporter vectors were transfected into cervical cancer cells, along with either the miR-556-5p mimic or NC mimic. After 48 h, the luciferase activity was evaluated using a dual-luciferase reporter assay system.

Tumor xenograft model

Animal were housed in a specific-pathogen-free environment at the animal facility of the Renmin Hospital of Wuhan UniversityUnless specified otherwise,the mice were provided with irradiated normal food and clean water.Briefly, 5-week-old BALB/c nude mice were injected with 5×106 HeLa cells stably transfected with the circ_0025202 shRNA or shRNA-NC (six in each group). Tumor volumes were monitored every 4 days. One month later, the mice were euthanized by cervical dislocation. At that time, the tumor weights were recorded.The study was approved by the Laboratory Animal Ethics Committee of the Renmin Hospital of Wuhan University in accordance with Basel Declaration (NO.WDRM-20230410D).

Immunohistochemical staining

After xylene dewaxing and dehydration, slides from the tumor samples were obtained. Tissue sections were treated with 3% hydrogen peroxide and incubated with primary antibodies against anti-Ki67 (Abcam) at 4°C overnight. The next day, incubation with secondary antibody was performed. The sections were then treated with 3,3'-diaminobenzidine and observed under a microscope.

Statistical analysis

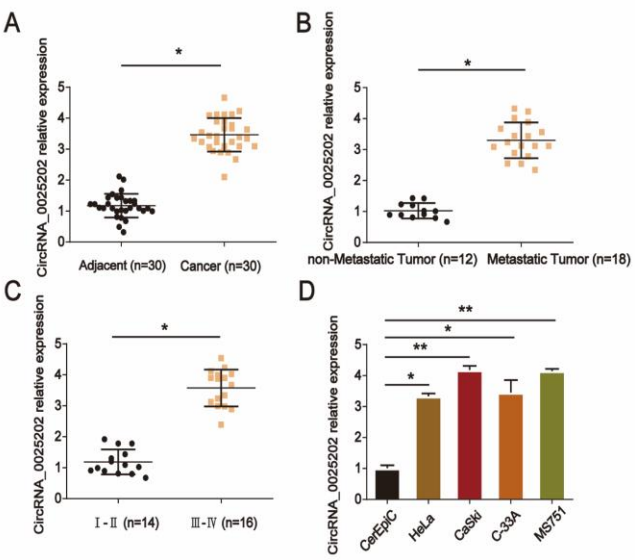
Statistical analysis was performed using GraphPad Prism version 5. Differences between two groups were determined using Student's t-test; differences among three groups were calculated using one-way ANOVA. $P < 0.05$ was considered as the criterion for statistical significance.

RESULTS

Circ_0025202 level increases in cervical cancer

First, we found that the circ_0025202 expression was higher than in adjacent normal tissues (Figure 1A). Next, the positive association between circ_0025202 levels and lung cancer metastases are shown in Figure 1B. We then observed that the circ_0025202 levels were higher in stage III/IV (n = 16) than in stage I/II (n = 14), as shown in Figure 1C. Circ_0025202 was higher in cervical cancer cells (C-33A, HeLa, MS751, and CaSki) than in HCerEpiC cells (Figure 1D). These results indicate that circ_0025202 is overexpressed in cervical cancer.

Figure 1: Circ_0025202 was elevated in cervical cancer.



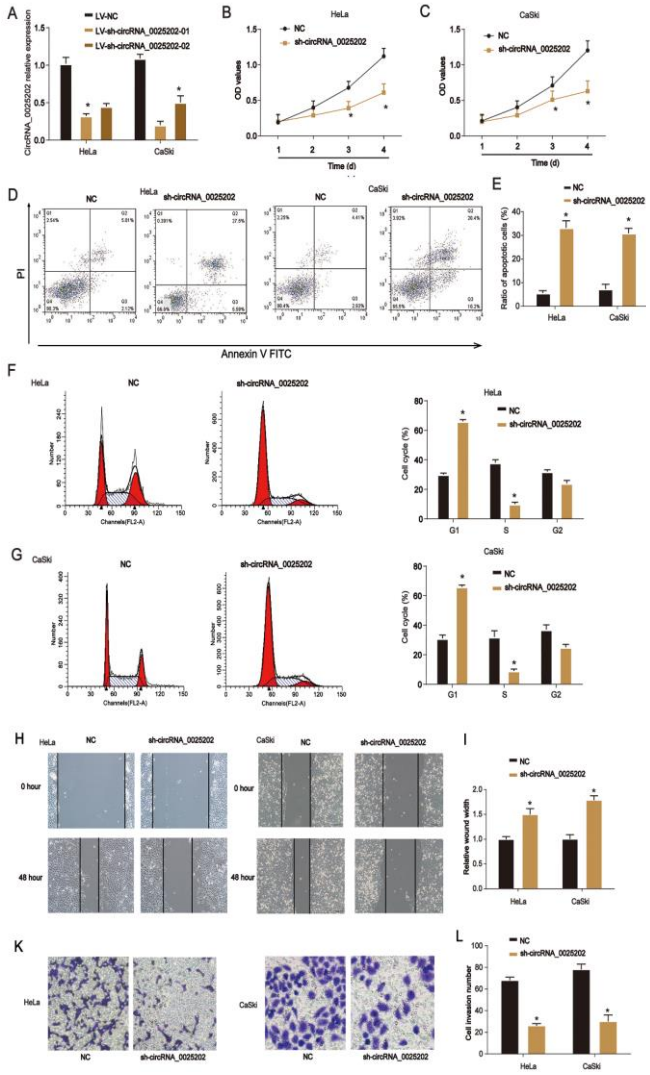
(A) Circ_0025202 expression level in 30 pairs of cervical cancer tissues. (B) Circ_0025202 expression in cervical cancer tissues with or without lymph node metastases. (C) Circ_0025202 expression in cervical cancer tissues in different FIGO stages. (D) Circ_0025202 expression in CerEpiC, C-33A, HeLa, MS751 and CaSki. *p <0.05; **p <0.01.

Circ_0025202 induces cervical cancer cell progression

To analyze how circ_0025202 enhanced cervical cancer development, function assays were performed. HeLa and CaSki cells were transfected with circ_0025202 shRNA. As shown in (Figure 2A), circ_0025202 shRNA successfully downregulated the circ_0025202 expression in vitro. CCK-8 proliferation assay showed that circ_0025202 knockdown greatly inhibited cervical cancer cell proliferation (Figure 2B and 2C). The flow cytometry results indicated that HeLa and CaSki cell apoptosis was induced by the loss of circ_0025202 (Figure 2D and 2E). Another cell cycle distribution analysis showed that knockdown of circ_0025202 repressed cell cycle progression (Figure 2F and 2G). It was shown that downregulation of circ_0025202 restrained the cell cycle, resulting in more cells stagnating in the G1 stage. These findings suggest that circ_0025202 promotes cell proliferation.

Furthermore, the migration ability of HeLa and CaSki cells after circ_0025202 knockdown was explored using a wound healing assay. Circ_0025202 shRNA dramatically reduced the migration of cervical cancer cells (Figure 2H and 2I). Meanwhile, decreased circ_0025202 level also strongly repressed HeLa and CaSki invasiveness (Figure 2K and 2L). These results demonstrate that circ_0025202 induces cervical cancer cell progression.

Figure 2: Loss of circ_0025202 impaired cervical cancer cell proliferation, migration and invasion, induced cell apoptosis and blocked cell cycle.

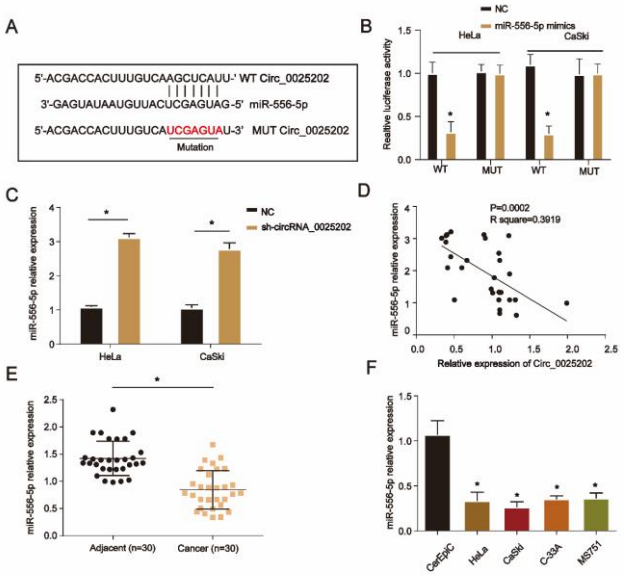


(A) Circ_0025202 expression in HeLa and CaSki cells infected with LV-shcirc_0025202 or LV-NC. (B and C) CCK8 assay was used to test HeLa and CaSki cell viability. (D and E) HeLa and CaSki cell apoptosis was assessed via flow cytometry analysis. (F and G) Flow cytometry analysis of HeLa and CaSki cell cycle. (H, and I) Wound healing assay was carried out to assess the effects of circ_0025202 on cervical cancer cell migration. (K and L) Transwell invasion assay was employed to assess the effects of circ_0025202 on cervical cancer cell invasion. *p <0.05.

Circ_0025202 sponges miR-556-5p

To explore the mechanism of circ_0025202 in cervical cancer progression, the potential binding partner of circ_0025202 was investigated. These data implied that circ_0025202 can bind to miR-556-5p (Figure 3A). Dual-luciferase assays confirmed that miR-556-5p reduced the luciferase activity of WT circ_0025202 (Figure 3B). Circ_0025202 knockdown in cervical cancer cells resulted in miR-556-5p overexpression (Figure 3C). These findings indicate that circ_0025202 sponges miR-556-5p in vitro. A negative correlation between them was observed in cervical cancer tissues, as shown in Figure 4D. miR-556-5p expression was downregulated in cervical cancer tumor tissues and cells (Figure 3E and 3F)

Figure 3: Circ_0025202 sponged miR-556-5p.

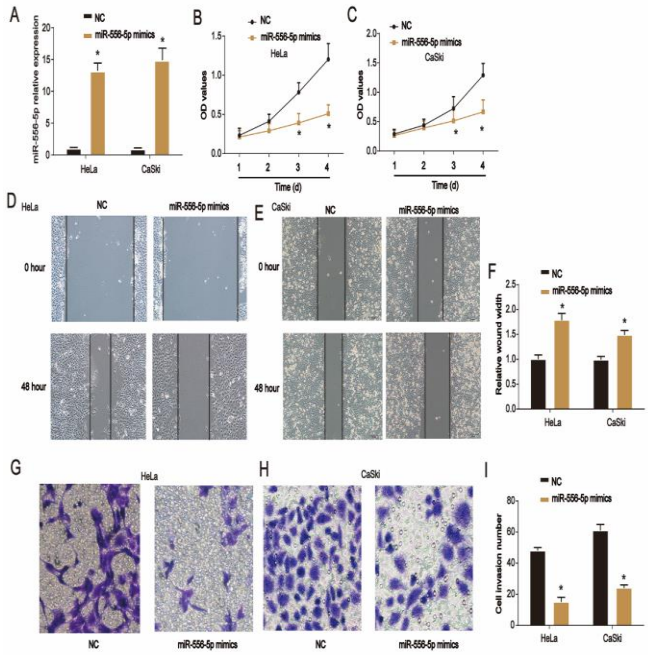


(A) Predicted binding sites of miR-556-5p within circ_0025202. (B) Luciferase reporter assays were carried out to assess the interaction between miR-556-5p and circ_002520. (C) Knockdown of circ_002520 elevated the levels of miR-556-5p in HeLa and CaSki cells. (D) Correlation analysis between miR-556-5p and circ_002520 expression. (E) Expression of miR-556-5p in cervical cancer tissues was determined. (F) Expression of miR-556-5p in cervical cancer cell lines and HCerEpiC cells. *p <0.05.

miR-556-5p represses cervical cancer cell growth

Because circ_0025202 enhanced cervical cancer development by sponging miR-556-5p, we investigated the effect of miR-556-5p. As shown in Figure 4A, cervical cancer cells were transfected with the miR-556-5p mimic. miR-556-5p reduced cervical cancer cell proliferation (Figure 4B and 4C), migration (Figure 4D, 4E, and 4F), and invasion (Figure 4G,4H, and 4I). These results indicate that miR-556-5p represses cervical cancer cell growth.

Figure 4: miR-556-5p repressed cervical cancer cells growth.



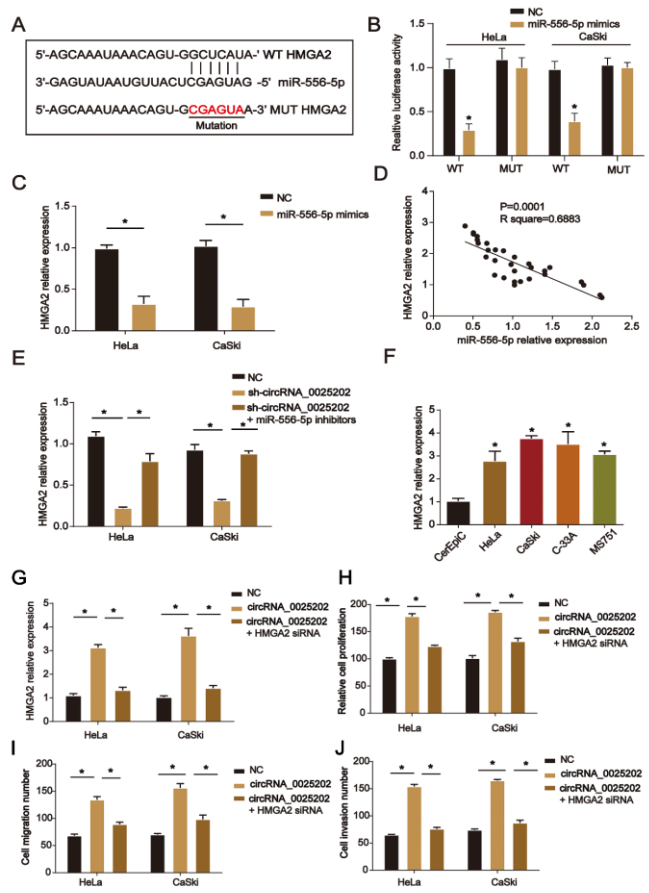
(A) Expression of miR-556-5p in HeLa and CaSki cells transfected with miR-NC or miR-556-5p mimics. (B and C) CCK8 assay for evaluation of cell proliferation. (D, E and F) Wound healing assay was used to detect the effects of miR-556-5p mimics on cervical cancer cell migration. (G, H and I) Transwell invasion assay was conducted to assess the effects of miR-556-5p mimics on cervical cancer cell invasion. *p <0.05.

Circ_0025202 promotes HMGA2 expression level via sponging of miR-556-5p

To concentrate on the mechanism of miR-556-5p in modulating cervical cancer, the binding partner of miR-556-5p was predicted. miR-556-5p could bind to the 3'-UTR of HMGA2 (Figure 5A). Dual-luciferase assays were performed, and the binding activity of miR-556-5p to HMGA2 was confirmed (Figure 5B).

In addition, the increase in miR-556-5p expression in cervical cells repressed HMGA2 expression (Figure 5C). The HMGA2 and miR-556-5p expression were negatively correlated in cervical cancer tissues (Figure 5D). Circ_0025202 regulated HMGA2 expression through miR-556-5p, as circ_0025202 knockdown repressed HMGA2 expression, while miR-556-5p inhibitors reversed this effect (Figure 5E). As shown in Figure 5F, HMGA2 expression was greatly induced in cervical cancer cells.

Figure 5: Circ_0025202 promoted HMGA2 expression through sponging miR-556-5p.



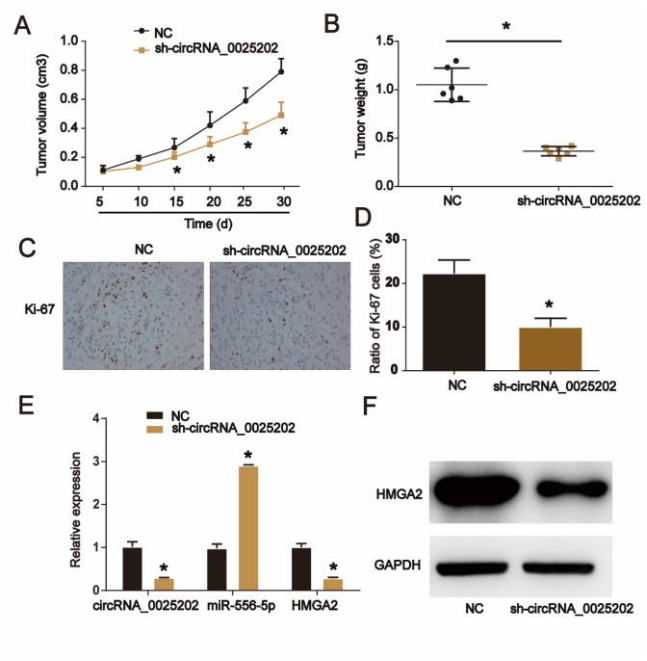
(A) Predicted binding sites of miR-556-5p within HMGA2 3'-UTR region. (B) The interaction between HMGA2 3'-UTR and miR-556-5p. (C) Overexpression of miR-556-5p reduced HMGA2 expression in HeLa and CaSki cells. (D) Correlation analysis between miR-556-5p and HMGA2 expression. (E) Knockdown of circ_0025202 inhibited HMGA2 expression while inhibitors of miR-556-5p reversed it. (F) Expression of HMGA2 in cervical cancer cells and HCerEpiC cells. (G) HMGA2 expression in cervical cancer cells transfected with circ_0025202 overexpression plasmid and HMGA2 siRNA. (H) CCK8 assay was utilized to evaluate the proliferation of HeLa and CaSki cells. (I and J) HeLa and CaSki cell migration and invasion. *p <0.05.

Depletion of circ_0025202 impairs tumor growth in vivo

Moreover, we focused on whether circ_0025202 suppressed cervical cancer growth in vivo. Then, nude mice were injected with HeLa cells infected with shRNA of circ_0025202 or shRNA control. Circ_0025202 knockdown decreased tumor size and weight, as was observed in Figure 6A and 6B. Ki-67 staining analysis is shown in Figure 6C and 6D. Loss of circ_0025202 reduced the Ki-67 positive cell ratio. In addition, we confirmed that the expression of circ_0025202 and HMGA2 in the tumor tissues was restrained by circ_0025202 shRNA, whereas that of miR-556-5p was

induced (Figure 6E and 6F). These results indicated that circ_0025202 regulated HMGA2 via sponging miR-556-5p in vivo in cervical cancer.

Figure 6: Depletion of circ_0025202 reduced tumor growth in vivo.



Dorsal flanks of nude mice were injected with 5×106 sh-circ_0025202 or NC HeLa cells. (A) Tumor volume. (B) Tumor weight. (C and D) Ki-67 positive cell ratio in the tumor tissues. (E and F) Circ_0025202/miR-56-5p/HMGA2 expression in the tumor tissues. *p <0.05.

DISCUSSION

Cervical cancer is a gynecological malignant tumor that is becoming a prominent public health problem Marth et al. (2017). Cervical cancer is the second most common gynecological malignancy and is a serious disease among women. To improve the efficacy of treatment, a better understanding of cervical cancer is required. In this study, we investigated the possible mechanism of action of circ_0025202 as a competing endogenous RNA (ceRNA) in the modulation of HMGA2 expression by sponging miR-556-5p. We observed that circ_0025202 mediated miR-556-5p and HMGA2, which regulate cervical cancer development. qRT-PCR results indicated that circ_0025202 levels were upregulated in tumor tissues and cancer cells. In addition, the loss of circ_0025202 repressed cervical cancer progression. Circ_0025202 sponges miR-556-5p and indirectly regulates HMGA2 expression.

To date, circRNAs have been shown to act as tumor regulators under different circumstances. The circRNA BCRC-3 inhibits bladder cancer proliferation by regulating miR-182-5p and p27 Xie et al. (2018). Circ_0000285 can induce cervical cancer development by regulating FUS Chen et al. (2019). CircSLC26A4 can contribute to cervical cancer by modulating miR-1287-5p and HOXA7 Ji et al. (2020). In addition, circ_0000069 can induce cervical cancer progression by modulating miR-873-5p and TUSC3 Zhang et al. (2020). Our in vitro assays clearly showed that circ_0025202 acts as an oncogene in cervical cancer.

We then defined how circ_0025202 exerts its function. More recently, an increasing number of circRNAs have been recognized as miRNA sponges in many cancers; however, the reports of this model in cervical cancer are scarce. In this study, miR-556-5p showed a sequence complementary to circ_0025202 based on bioinformatic analysis. Previously, it was shown that circRPPH1 induces breast cancer progression by regulating miR-556-5p and YAP1 Zhou et al. (2020). Meanwhile, we found that mimics of miR-556-5p reduced cervical cancer progression. miR-556-5p can serve as a tumor suppressor in cervical cancer. Thus, circ_0025202 promoted cervical cancer development by sponging miR-556-5p. Eventually, miR-556-5p was identified as an endogenous competing RNA using a dual-luciferase reporter assay.

HMGA2 is an architectural transcription factor and modulates transcription by inducing structural changes in the chromatin, which can enable the transcriptional machinery to regulate the expression of many mammalian genes Sgarra et al. (2004). HMGA2 has been shown to be associated with many cancers. HMGA2 expression is increased in many human tumor tissues Shi et al. (2016), Tan et al. (2016). For example, miR-302a-5p/367-3p modulates endometrial cancer by regulating HMGA2 expression Ma et al. (2018). HMGA2 promotes gastric cancer progression by regulating CD44 expression Sun et al. (2017). In addition, HMGA2 gene loss reduces cervical cancer development by targeting ATR/Chk1 Wang et al. (2018). Circ_0025202 promoted cervical cancer progression via HMGA2. HMGA2 has been shown to be a target of miR-556-5p. Silencing of HMGA2 rescued cancer progression resulting from overexpression of circ_0025202. Thus, circ_002520, miR-556-5p, and HMGA2 form a network that modulates cervical cancer.

Currently, we have validated that circ_0025202 exerts regulatory effects via a sponge mechanism. Another probable mechanism of miR-556 binding sites modulated by circ_0025202 could be investigated in future studies. In addition, circ_0025202 has been shown to regulate miR-182/FOXO3a and miR-197-3p/HIPK3 in breast cancer Sang et al. (2019), Li et al. (2021). We confirmed the relationship between circ_0025202 and miR-556 in cervical cancer. Our studies indicate that circ_0025202 can control several microRNAs, and we would like to focus on other miRNAs in future. miR-556 can also control other mRNA targets besides HMGA2. In our future studies, other target genes of miR-556 could be explored in the context of cervical cancer.

CONCLUSION

In conclusions, Circ_0025202 acts as a potential oncogene in cervical cancer that sponged miR-556-5p expression, which led to the overexpression of HMGA2. We identified a critical circ_0025202–miR-556-5p–HMGA2 network in cervical cancer development.

ABBREVIATIONS

circRNAs: Circular RNAs, HMGA2: High mobility group AT-hook 2, CCK-8: Cell Counting Kit-8, qRT-PCR: Quantitative reverse transcription-polymerase chain reaction, cDNA: complementary DNA, IHC: Immunohistochemistry

DECLARATIONS

Authors' contributions

Simeng Yuan: Formal analysis, Investigation, Methodology, Validation, Writing-original draft. **Lin Li:** Formal analysis, Investigation, Methodology, Validation, Data curation. Writing-review & editing. **Jinting Zhou:** Investigation, Validation, Methodology. **Huabing Lv:** Formal analysis, Investigation, Validation. **Min Zhou:** Formal analysis, Investigation, Methodology. **Xiaogang Mao:** Formal analysis, Investigation, Validation. **Xiaomin Qin:** Formal analysis, Investigation, Methdology. **Jiang Yang:** Conceptualization, Data curation, Funding acquisition, Investigation, Resources, Supervision, Validation, Writing-original draft, Writing-review & editing. **Hui Xing:** Formal analysis, Investigation, Methodology, Validation, Writing - review & editing, Funding acquisition.

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Institutional Review Board Statement

Our study obtained the approval by the Xiangyang Central Hospital (Approval Code: 2023-071), and all methods involving human participants were performed in accordance with the Declaration of Helsinki.

We confirmed that each human participant signed an informed consent statement. Besides, Procedures of animal assays were performed with the approval of the Animal Care and Use Committee in Renmin Hospital of Wuhan University (NO. WDRM-20230410D). And The study was carried out in compliance with the ARRIVE guidelines.

Informed Consent Statement

Not applicable

Data Availability Statement

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

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

Not Applicable

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