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CircAGFG1 aggravates the progression of cervical cancer by downregulating p53

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Abstract. – OBJECTIVE: To explore the role of circAGFG1 in influencing the progression of cervical cancer (CC) and the underlying molecular mechanism.

PATIENTS AND METHODS: CircAGFG1 levels in CC tissues and paracancerous tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Its level in CC cell lines was detected as well. Meanwhile, circAGFG1 levels in CC patients with different tumor staging, metastatic statues, and tumor sizes were examined. The Kaplan-Meier method was introduced for assessing the prognostic potential of circAGFG1 in CC. The regulatory effects of circAGFG1 on the proliferative ability of CC cells were evaluated by performing the Cell Counting Kit-8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assay. The subcellular distribution of circAGFG1 in the CC cells was analyzed. Through chromatin immunoprecipitation (ChIP) and RNA immunoprecipitation (RIP) assay, the interaction between circAGFG1 and p53 was determined. Finally, the role of the circAGFG1/p53 axis in influencing the proliferation of CC cells was uncovered.

RESULTS: CircAGFG1 was upregulated in CC tissues and cell lines. Besides, the circAGFG1 level was closely related to worse tumor staging, a higher rate of metastasis, and larger tumor size in CC patients. Besides, CC patients with a high level of circAGFG1 presented worse prognosis. The knockdown of circAGFG1 attenuated the proliferative ability of SiHa and HeLa cells. CircAGFG1 was mainly distributed in the nucleus of the CC cells. The interaction between circAGFG1 and p53 was verified. The knockdown of p53 could partially reverse the regulatory effect of circAGFG1 on the proliferative ability of the CC cells.

CONCLUSIONS: CircAGFG1 is upregulated in CC. By recruiting EZH2, circAGFG1 downregulates p53 and thus exerts a carcinogenic role to accelerate the malignant progression of cervical cancer.

Key Words:

Cervical cancer, P53, CircAGFG1, EZH2, Proliferation.

Introduction

Cervical cancer (CC) is the second most common cancer in females worldwide. Globally, the incidence of CC is only second to breast cancer[1]. The number of newly diagnosed cases of CC has increased year by year. The long-term infection of human papillomavirus (HPV) is the major reason for the tumorigenesis of CC[2]. The inoculation with HPV vaccine can effectively prevent and control the occurrence of CC[3,4]. Previously, people considered that genetic mutations are the major reasons for the occurrence of CC. Recent epidemiological and molecular researches[5] have highlighted the role of the epigenetic changes in the occurrence and development of CC, except for HPV infection and genetic changes. In addition, the changes in the epigenetic markers could be utilized as diagnostic, therapeutic, and prognostic hallmarks for CC[6,7]. This study explored the diagnostic and therapeutic potentials of an abnormally expressed circRNA in CC.

Circular RNA (circRNA) is a special class of non-coding RNAs. Studies[8] have shown that circRNA exerts an important role in many biological activities. Epigenetics is the condition where heritable changes occur in gene expressions, while DNA sequences are not altered. It is essential for cellular behaviors and tumor development[9,10]. Epigenetics includes DNA methylation, histone modifications, and non-coding RNA dysregulation[11]. CircRNAs, alongside with miRNAs and lncRNAs, lack the ability to encode proteins[12,13]. However, they are significant during the epigenetic regulation[14].

The EZH2 gene encodes the histone lysine N-methyltransferase, which is involved in the DNA methylation to further inhibit the transcription of other genes[15]. Some certain genes are widely involved in many types of cancers[16]. The mutation or overexpression of EZH2 is associat-

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ed with multiple types of cancer, such as breast cancer, prostate cancer, melanoma, and bladder cancer[17]. Abnormally activated EZH2 can inhibit the normal expressions of tumor-suppressor genes. Hence, the inhibition of the EZH2 activity can slow down the tumor growth by activating the tumor-suppressor genes[18,19]. However, the role of EZH2 in the progression of CC is poorly understood.

The inactivation of the p53 gene (a human tumor-suppressor gene) is crucial in the tumorigenesis[20]. Functionally, wild-type p53 stimulates the apoptosis of tumor cells and thus prevents tumorigenesis. Additionally, p53 is able to induce DNA repair[21]. Mutant p53 not only loses the effect of the wild-type p53 on tumor proliferation, but also the mutation itself turns into the carcinogenic effect[22]. An oligomeric protein formed by the interaction between mutant and wild-type p53 cannot bind to DNA, resulting in the transcriptional dysregulation of cancerous genes and thereafter tumorigenesis[23].

In this study, we found that upregulated circAGFG1 in CC tissues and cells inhibit the expression of p53 by recruiting EZH2, thus promoting the proliferation and progression of CC. Our results illustrated that circAGFG1 promoted DNA methylation to downregulate the tumor-suppressor gene, providing a theoretical basis for the clinical treatment of CC.

Patients and Methods

Sample Collection

Matched CC tissues and adjacent normal tissues were harvested from 39 CC patients undergoing a radical hysterectomy. None of these patients were preoperatively treated with anti-tumor therapy. The samples were postoperatively diagnosed. This study was approved by the Ethics Committee of the Second People's Hospital of Yunnan Province. The signed written informed consents were obtained from all participants before the study. This study was conducted in accordance with the Declaration of Helsinki.

Cell Culture and Transfection

Normal cervical epithelial cell line (HcerEpiC) and CC cell lines (C33A, HeLa, SiHa, and Caski) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C.

The cells were inoculated in a 6-well plate with 1×10^4 cells per well. At 75-85% confluence, the cells were cultured in 1.5 mL of serum-free medium and 500 μL of Lipofectamine 2000 containing the transfection vectors (Invitrogen, Carlsbad, CA, USA). At 4-6 h, the complete medium was replaced.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The cells and tissues were lysed using the TRIzol method (Invitrogen, Carlsbad, CA, USA) for harvesting the total RNAs. The RNA concentration and its qualification were detected using a spectrometer (Hitachi, Tokyo, Japan). Qualified RNAs were reversely transcribed into cDNA according to the instructions of the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was then performed following the recommendations of SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) at 94°C for 5 min, and 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The relative level was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: circAGFG1, F: 5'-GGCAGCACCGCTACTTCCTCG-3', R: 5'-GCATAGAGCGATCGTCGTGACGA-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

The cells were lysed using radioimmunoprecipitation assay (RIPA). The concentration of the extracted total protein was quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). An equal amount of each protein sample was loaded and separated by electrophoresis. Subsequently, the samples were transferred on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After 2-h blockage in 5% skimmed milk, the membranes were incubated with the primary antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody after rinsing with the buffer solution (TBST). Enhanced chemiluminescence (ECL) was carried out for band exposure, and the grey values of bands were analyzed by the Image Software (NIH, Bethesda, MD, USA).

Cell Counting Kit-8 (CCK-8) Assay

5.0×10^3 cells per well were inoculated in 96-well plates. At the appointed time points, 10 μL of CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) was applied in each well. The absorbance at 450 nm of each sample was recorded by a microplate reader (Bio-Rad, Hercules, CA, USA) for depicting the viability curves.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

The cells were inoculated into 96-well plates with 5×10^3 cells per well, and labeled with 50 μM EdU reagent (Thermo Fisher Scientific, Waltham, MA, USA) for 2 h. After being washed with Phosphate-Buffered Saline (PBS), the cells were fixed in 50 μL of fixation buffer, decolored with 2 mg/mL glycine, and permeated with 100 μL of penetrant. After PBS washing once, the cells were stained with 100 μL of 4',6-diamidino-2-phenylindole (DAPI) in the dark for 30 min. The EdU-positive cells were captured under a fluorescent microscope.

Determination of Subcellular Distribution

The cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR. U6 was served as the internal reference of the nucleus, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was that of cytoplasm.

Chromatin Immunoprecipitation (ChIP)

The cells were subjected to 10-min cross-link with 1% formaldehyde at room temperature. Subsequently, the cells were lysed using lysis buffer and sonicated for 30 min. Finally, the sonicated lysate was immuno-precipitated with anti-EZH2 or anti-IgG.

RNA Immunoprecipitation (RIP)

The cells were treated according to the procedures of Millipore Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). The cell lysate was incubated with anti-IgG, anti-EZH2, or anti-H3K27me3 at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/mL proteinase K containing 0.1% SDS for the purpose of the RNA extraction. The magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. Finally, the extracted RNA was quantified by performing the qRT-PCR.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 18.0 statistical software (SPSS Inc., Chicago, IL, USA) was applied for data analysis. The data were expressed as mean ± standard deviation ($\bar{x}\pm s$). The data between the two groups were compared using the *t*-test. The Kaplan-Meier method was used for survival analysis. $p<0.05$ was considered a statistically significant difference.

Results

CircAGFG1 Was Upregulated in CC, and Correlated to the Malignant Level

Compared with paracancerous tissues, circAGFG1 was upregulated in CC tissues (Figure 1A). Its level was identically higher in CC cell lines relative to cervical epithelial cell line (Figure 1B). We also determined the expression levels of circAGFG1 in CC patients with different tumor staging, metastatic statues, and tumor sizes. With the worsening of tumor staging, the circAGFG1 level gradually increased in CC patients with stage I, stage II, and stage III+IV (Figure 1C). Metastatic CC patients expressed a higher abundance of circAGFG1 than those without metastatic foci (Figure 1D). Moreover, the circAGFG1 level was higher in larger CC tissues (≥4 cm) relative to those smaller ones (<4 cm) (Figure 1E). The Kaplan-Meier curves indicated a worse prognosis in CC patients expressing a high level of circAGFG1 (Figure 1F). The above data demonstrated the involvement of circAGFG1 in the progression of CC.

Knockdown of CircAGFG1 Suppressed Proliferative Ability in CC

SiHa and HeLa cells were selected for the subsequent *in vitro* experiments. The transfection of sh-circAGFG1 markedly downregulated circAGFG1 in SiHa and HeLa cells (Figures 2A, 2B). The viability at day 2 and 3 was markedly reduced in the CC cells transfected with sh-circAGFG1 (Figures 2C, 2D). Similarly, the EdU-positive ratio decreased through the knockdown of circAGFG1 in SiHa and HeLa cells, suggesting the attenuated proliferative ability (Figures 2E, 2F).

CircAGFG1 Suppressed the Transcription of p53 by Recruiting EZH2

The subcellular distribution analysis showed that in SiHa and HeLa cells, circAGFG1 was

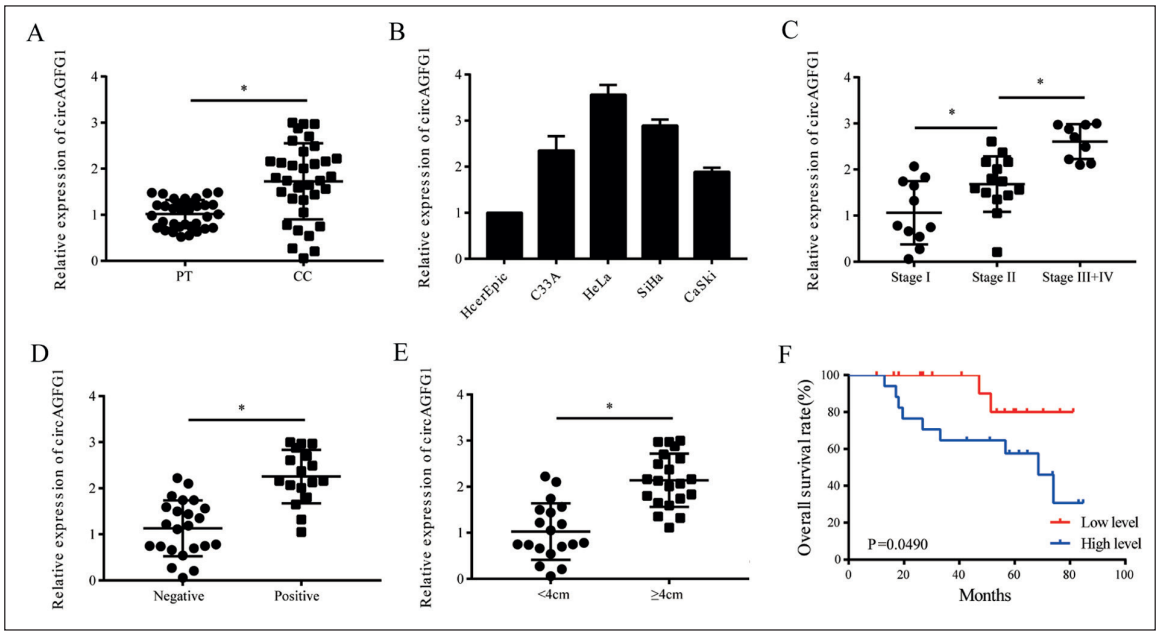

Figure 1. CircAGFG1 was upregulated in CC, and correlated to the malignant level. **A,** Relative level of circAGFG1 in cervical cancer tissues and paracancerous tissues. PT: paracancerous tissues; CC: cervical cancer. **B,** Relative level of circAGFG1 in cervical epithelial cell line (HcerEpiC) and CC cell lines (C33A, HeLa, SiHa and Caski). **C,** Relative level of circAGFG1 in cervical cancer patients with stage I, stage II, and stage III+IV. **D,** Relative level of circAGFG1 in cervical cancer patients either with metastasis or not. **E,** Relative level of circAGFG1 in cervical cancer patients with the tumor size ≥4 cm and <4 cm. **F,** The Kaplan-Meier curves showed the overall survival in cervical cancer patients either with high or low level of circAGFG1.

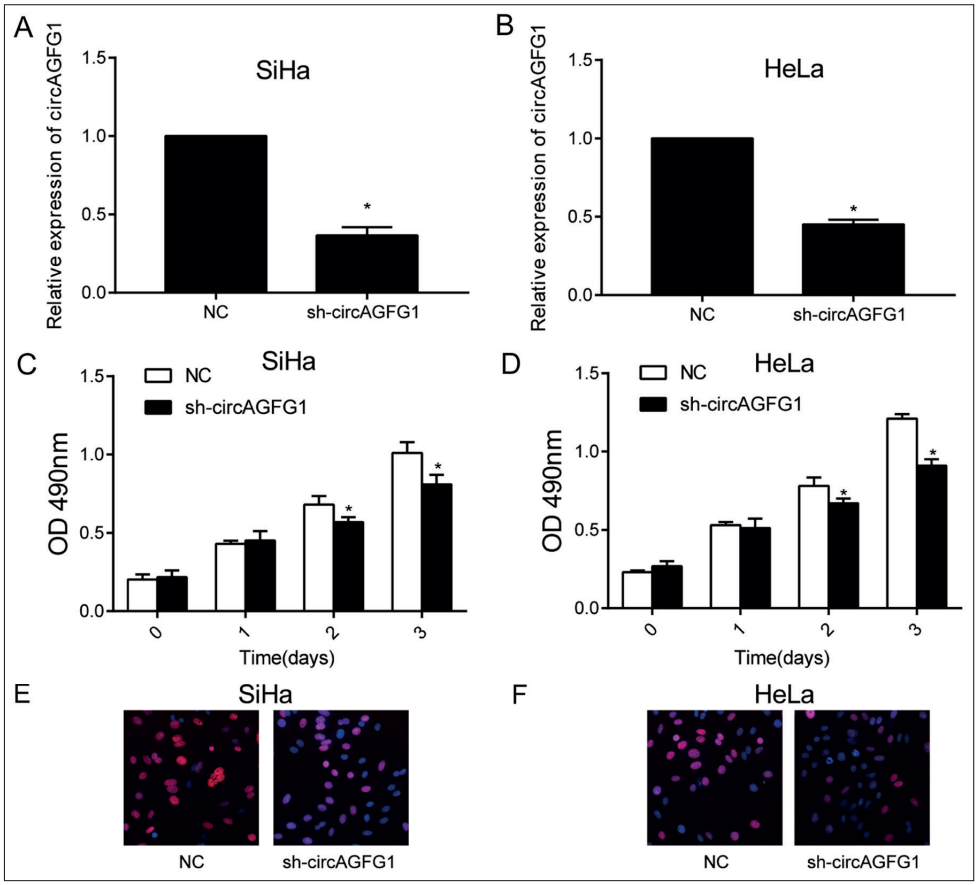

Figure 2. Knockdown of circAGFG1 suppressed the proliferative ability in CC. **A,** Transfection efficacy of sh-circAGFG1 in SiHa cells. **B,** Transfection efficacy of sh-circAGFG1 in HeLa cells. **C,** Viability in SiHa cells transfected with NC or sh-circAGFG1. **D,** Viability in HeLa cells transfected with NC or sh-circAGFG1. **E,** EdU-positive cells in SiHa cells transfected with NC or sh-circAGFG1 (magnification × 40). **F,** EdU-positive cells in HeLa cells transfected with NC or sh-circAGFG1 (magnification × 40).

mainly distributed in the nucleus (Figures 3A, 3B). Subsequently, the involvement of EZH2 in the circAGFG1-mediated progression of CC was elucidated. RIP assay identified a much higher enrichment of circAGFG1 in anti-EZH2 relative to anti-IgG, verifying the interaction between circAGFG1 and EZH2 (Figures 3C, 3D). In CC cells, the transfection of sh-circAGFG1 markedly upregulated the protein level of p53 (Figures 3E, 3F). The transfection of sh-EZH2 identically upregulated the p53 level in SiHa and HeLa cells (Figures 3G, 3H). At last, the ChIP assay revealed the lower immunoprecipitation of circAGFG1 in anti-EZH2 and anti-H3K27me3 after the knockdown of circAGFG1 (Figures 3I, 3J). Collectively, EZH2 could bind to the promoter region of p53. CircAGFG1 suppressed the transcription of p53 by recruiting EZH2.

Knockdown of p53 Stimulated Proliferative Ability in CC

To uncover the role of p53 in the progression of CC, sh-p53 was constructed. The transfection of sh-p53 sufficiently downregulated the p53 level

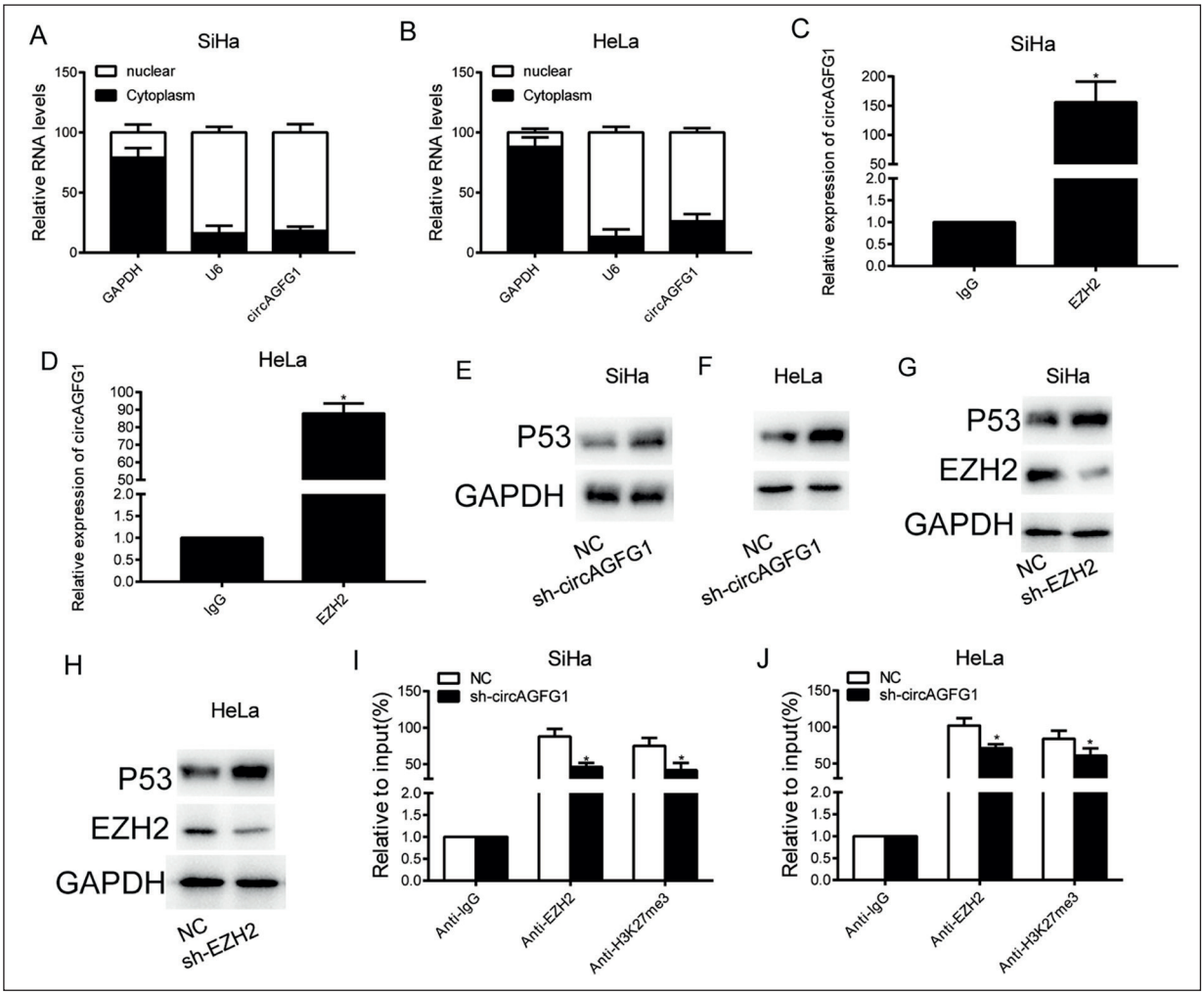

Figure 3. CircAGFG1 suppressed the transcription of p53 by recruiting EZH2. **A,** The subcellular distribution of circAGFG1 in nuclear and cytoplasmic fraction of SiHa cells. U6 and GAPDH were served as nuclear and cytoplasmic internal references, respectively. **B,** The subcellular distribution of circAGFG1 in nuclear and cytoplasmic fraction of HeLa cells. U6 and GAPDH were served as nuclear and cytoplasmic internal references, respectively. **C,** Enrichment of circAGFG1 in anti-IgG and anti-EZH2 of SiHa cells. **D,** Enrichment of circAGFG1 in anti-IgG and anti-EZH2 of HeLa cells. **E,** The protein level of p53 in SiHa cells transfected with NC or sh-circAGFG1. **F,** The protein level of p53 in HeLa cells transfected with NC or sh-circAGFG1. **G,** The protein levels of p53 and EZH2 in SiHa cells transfected with NC or sh-EZH2. **H,** The protein levels of p53 and EZH2 in HeLa cells transfected with NC or sh-EZH2. **I,** Immunoprecipitant of circAGFG1 in anti-IgG, anti-EZH2, and anti-H3K27me3 in SiHa cells transfected with NC or sh-circAGFG1. **J,** Immunoprecipitant of circAGFG1 in anti-IgG, anti-EZH2, and anti-H3K27me3 in HeLa cells transfected with NC or sh-circAGFG1.

in SiHa and HeLa cells (Figures 4A, 4B). Notably, the knockdown of p53 could partially reverse the decreased viability in CC cells with a circAGFG1 knockdown at day 2 and 3 (Figures 4C, 4D). The reduced EdU-positive ratio in CC cells transfected with sh-circAGFG1 was elevated to some extent by the co-transfection of sh-p53 (Figures 4E, 4F). As a result, circAGFG1 aggravated the progression of CC by downregulating p53.

Discussion

CC is the most common gynecological malignancy. *In situ* carcinoma of CC mainly affects the females in 30-35 years, and invasive CC is highly prevalent in 45-55-year-old women. The onset age of CC has become younger in recent years[24]. Persistent infection of high-risk HPV, premature sexual behavior, and an excessive number of deliveries are considered to be the pathogenic factors for CC[25]. Although HPV infection is found in most of CC cases, the genetic and epigenetic changes are required for the disease progression[26]. Previous studies[27] have discovered the vital role of genetic mutations in the development of CC. With the progression in biological researches, the role of epigenetic changes has been concerned. The epigenetic changes in CC caused by DNA methylation abnormalities and histone modifications have been extensively studied[28].

Unlike traditional linear RNAs (containing 5' and 3' ends), circRNAs have a closed-loop structure, which is the reason for their resistance to RNA exonuclease. CircRNAs are stably expressed and hardly degradable[29]. CircRNAs could sponge miRNAs to abolish the inhibitory effect of miRNAs on the target genes, that is, the ceRNA theory[30,31]. Like, circRNA_100269 is downregulated in gastric cancer and inhibits tumor cell growth by targeting miR-630[32]. CircRNA_100290 influences the progression of oral cancer by acting as a sponge for miR-29[33]. CircRNA_102171 promotes the progression of the papillary thyroid carcinoma by modulating CTNNBIP1-dependent activation of the β-catenin pathway[34]. In pancreatic cancer, circRNA_100782 regulates the proliferation of cancer cells *via* the IL6-STAT3 pathway[35]. By interacting with the functional pathways, circRNAs are important in tumor diseases.

Abnormal expression of EZH2 is frequently observed in many types of cancer. EZH2 promotes tumorigenesis by inhibiting the expressions of many tumor-suppressor genes[36]. In

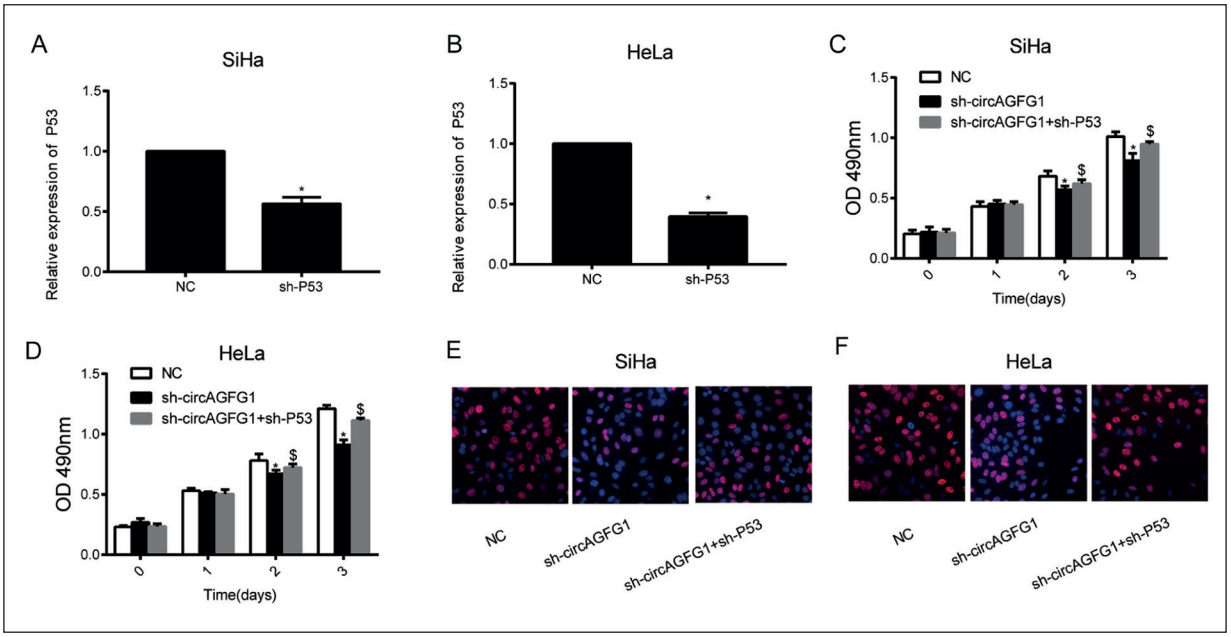

Figure 4. Knockdown of p53 stimulated the proliferative ability in CC. **A,** Transfection efficacy of sh-p53 in SiHa cells. **B,** The transfection efficacy of sh-p53 in HeLa cells. **C,** The viability in SiHa cells transfected with NC, sh-circAGFG1, or sh-circAGFG1+sh-p53. *$p<0.05$, *vs.* NC group; $^{\$}p<0.05$, *vs.* sh-circAGFG1 group. **D,** The viability in HeLa cells transfected with NC, sh-circAGFG1, or sh-circAGFG1+sh-p53. *$p<0.05$, *vs.* NC group; $^{\$}p<0.05$, *vs.* sh-circAGFG1 group. **E,** EdU-positive cells in SiHa cells transfected with NC, sh-circAGFG1, or sh-circAGFG1+sh-p53 (magnification × 40). **F,** EdU-positive cells in HeLa cells transfected with NC, sh-circAGFG1 or sh-circAGFG1+sh-p53 (magnification × 40).

bladder cancer, the overexpression of circRNA BCRC4 regulates the apoptosis of tumor cells by targeting miRNA-101/EZH2 axis[37]. During the tumorigenesis of skin cancer, circ100284 mediates the arsenite-induced cell cycle progression in human keratinocytes by absorbing miR-217[38]. The newly discovered hsa_circ_0020123 exerts a carcinogenic role in NSCLC by downregulating miR-144[39]. This study explored the role of circAGFG1 in regulating the progression of CC by interacting with EZH2.

P53 is a well-known tumor-suppressor gene. P53 mutation occurs in over 50% of malignancies[40]. P53-encoded transcriptional factors are able to control the initiation of cell cycle[41]. Under the normal circumstance, p53 slows down or monitors cell division[42]. According to the degree of DNA variation, p53 triggers cell self-repair at a small variation; otherwise, it induces cell apoptosis[43]. LncRNA MEG3 inhibits the proliferation and metastasis of gastric cancer through the p53 signaling pathway[44]. P53 induces tumor cell metastasis by targeting lncRNA PICART1[45]. The upregulated lncRNA in liver cancer activates the p53-p21 pathway to accelerate the growth of nasopharyngeal carcinoma[46].

In this paper, circAGFG1 was upregulated in CC tissues and cell lines. The circAGFG1 level was positively related to tumor staging, metastasis, and tumor size. CC patients with a high level of circAGFG1 presented worse prognosis. The knockdown of circAGFG1 attenuated the proliferative ability of SiHa and HeLa cells. CircAGFG1 was mainly distributed in the nucleus of CC cells. By recruiting EZH2, circAGFG1 suppressed p53 transcription. Notably, the knockdown of p53 could partially reverse the regulatory effect of circAGFG1 on the proliferative ability of the CC cells. It is concluded that circAGFG1 downregulated p53 by recruiting EZH2, thus aggravating the progression of CC.

Conclusions

For the first time, we indicated that circAGFG1 is upregulated in cervical cancer. It downregulates p53 by recruiting EZH2, thus exerting a carcinogenic role to accelerate the malignant progression of cervical cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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