LncRNA GClnc1 may contribute to the progression of ovarian cancer by regulating p53 signaling pathway

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ABSTRACT

Ovarian cancer (OC) is one of the most prevalent and deadly types of gynecological malignancy. Since current treatments are not effective against OC, it is imperative to develop novel potential therapeutic targets for managing OC. In this study, we aimed to uncover the underlying molecular mechanism of long non-coding RNA (lncRNA) GClnc1 related to p53 signaling pathway in OC. The expression of lncRNA H19 GClnc1 was markedly higher in OC samples than the related normal tissues. Next, we found that lncRNA GClnc1 inhibited p53. In addition, the lncRNA GClnc1 overexpression promoted the cell proliferation and migration *in vitro*. Subsequently, p53 silencing obligated the effect of lncRNA GCln1 knock down on cell proliferation and migration. To sum up, LncRNA GClnc1 contributes to the progression of OC by regulating p53 signaling pathway. Meanwhile, our findings also suggested that lncRNA GClnc1 may serve as a novel therapeutic target for OC patients.

Key words: lncRNA GClnc1; p53; ovarian cancer; target therapy

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Ethical Approval: All procedures with human subjects in this study were conducted in accordance with the Human Ethics Committee of the Sun Yat-sen Memorial Hospital of Sun Yat-sen University, China.

Patient consent for publication: All subjects gave their informed consent for inclusion before they participated in the study.

Introduction

Ovarian cancer (OC) is one of the common tumors of the female reproductive system and leading causes of death among females.[1,2] Current treatments are not effective against OC, the overall survival of OC patients is still unsatisfactory. The five year survival rate of around 30% with advances in surgery and chemotherapy.[3] Thus, it is imperative to develop novel potential therapeutic targets for managing OC.

Long non-coding RNAs (lncRNAs) belongs to the non-coding RNAs family with more than 200 nucleotides in length. Although lncRNAs do not template protein synthesis, they can regulate gene expression at transcriptional or post-transcriptional level.[4] LncRNAs have been reported to play vital roles in regulating cellular processes, such as signaling transduction, in various cancers.[5] Currently, it has been found that lncRNAs plays important roles in the progression of OC.[6] However, the specific influence of lncRNA GGlnc1 on the progression of OC remains largely unclear.

The transcription factor p53 is ~16-20 kb in length, which is a pair of alleles and acts as a tumor suppressor gene localized on human chromosome 17.[7] P53 functions as a suppressor of cell growth, and alterations in p53 lead to loss of this negative growth regulation and more rapid cell proliferation. The dysfunction of p53 contributes to the development of most human cancers.[8] Recently, lncRNAs have been shown to interact with p53 to play regulating roles in various cancers.[9] However, the interaction of lncRNA GGlnc1 and p53 on the progression of OC still remains unknown.

In this study, we aim to uncover whether lncRNA GClnc1 could regulate the progression of OC *via* p53 signaling pathway.

Materials and Methods

Clinical samples collection

Human OC tissue samples as well as related non-tumorous tissues were obtained from 42 patients, and all the specimens were reviewed and verified by pathologists and immediately frozen in liquid nitrogen. All subjects gave their informed consent for inclusion before they participated in the study. All experimental protocols were approved by the Ethics Committee.

RNA *in situ* hybridization

The RNA *in situ* hybridization (ISH) was performed as previously described.[9] The *in situ* detection of GClnc1 was performed on 6-μm formalin-fixed, paraffin-embedded sections using DIG-labeled miRCURYTM Detection probe (Exiqon, Woburn, MA, USA). Nikon 80i microscope with Nikon NIS-Elements F 2.3 software (Nikon, Shanghai, China) were used to analyze.

Cell culture

The human OC cell line, ES-2, was purchases from ATCC (Rockville, MD, USA). Cells were maintained in RPMI 1640 (Gibco, Grand Island, NY, USA). The normal immortalized human ovarian surface epithelial cell line T1074 was obtained from Abcam (Shanghai, China) and cultured in Prigrow 1 medium (Abcam, Shanghai, China). Medium was plus 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), and 1% streptomycin and penicillin (Sigma-Aldrich, St. Louis, MO, USA), and the cells were grown at 37°C with 5% CO_2.

qRT-PCR

TRIzol Plus RNA purification system was used to extract total RNA from specimens (Thermo Fisher Scientific, Beijing, China). Residual DNA was removed using DNA-free DNase (Ambion, Austin, TX). The cDNA was generated by reverse transcription using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Beijing, China). The SYBR Green method was performed to detect the expression of lncRNA GClnc1, p21, p53 and GAPDH. The primer sequences were listed in Table 1.

Overexpression and suppression constructs

The si-GClnc1, si-p53, pcDNA-GClnc1 and their related negative controls (si-NC and pcDNA-NC) were purchased from Suzhou Hongxun Biotechnologies (Suzhou, China). These constructs were transfected when cell density reached 60% according to the manufacturer's instructions of Lipofectamine 2000 (Invitrogen). The culture medium was replaced after 6 h.

Cell counting kit-8 (CCK-8) assay

CCK-8 assay kit was purchased from Dojindo (Shanghai, China) to monitor the cell viability. Cells were seeded in the 96-well plate (BD Biosciences, Shanghai, China) at a density of 5000 cells per well. After incubation for indicated time (0, 6, 24, 48, 72 and 96 h), we added 10 μL CCK-8 reagent into each well at indicated time point followed by incubation for 1 h at 37°C. Subsequently, the optical density (OD) was measured at 450 nm on a microplate reader.

Transwell assay

Cell migration was assayed by Boyden chamber assay with 24 well transwell permeable supports with 8 μm pores (Corning Coaster, Lowell, MA, USA). 200 μL of serum-free medium containing 0.1M cells for the migration assay were added to the filter. The bottom chamber was prepared with 750ul complete cell culture medium in which the FBS as a chemoattractant. After incubated for indicated time, the non-invasive cells were cleaned by scrubbing with a cotton swab. The cells that adhered to the outside of the membrane were fixed and dyed with crystal violet solution. The stained cells were dissolved in extraction buffer and solutions were transferred to a 96-well culture plates for colorimetric reading at OD 560 nm. The OD values reflected the cell ability of migration or invasion.[10]

Table 1. List of the primers used.

	Forward primer	Reverse primer
lncRNA GClnc1	TGGGGTAACTTAGCAGTTTCAAT	GGCAAGCAGTAATCTTACATGACAC
p53	CCCAAGCAATGGATGATTTGA	ATGAGGGTGCTGTCTTTGTAGG
p21	GGCAGACCAGCATGACAGATT	GCGGATTAGaGGCTTCCTCT
GAPDH	CGGAGTCAACGGATTTGGTCGT	GGGAAGGATCTGTCTCTGACC

Western blotting

The protein expression levels of p53, p21 and BAX after different treatment was detected by Western blotting as described previously.[11] The primary antibodies: anti-p53 (1:500, Millipore, Bedford, MA, USA), anti-p21 (1:500, Millipore), and GAPDH (Millipore). The secondary antibody was the goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:2,000; Bio-Rad, Philadelphia, PA, USA). All the results are from separate blots.

Dual luciferase reporter assay

Cells were inoculated into the 24-well plate (3 x 10^5 cells / well). Then, the cells were co-transfected with wild-type or mutant psiCHECK-2 p53 vector (Generay, Shanghai, China) and pcDNA-GClnc1 or pcDNA-NC with Lipofectamine 2000 (Thermo Fisher Scientific, Beijing, China) according with the manufacture's instruction. The luciferase activity of cells was measured by a Dual-Luciferase Reporter assay kit (Promega, Shanghai, China) after 24 h.

RNA-binding protein immunoprecipitation analysis

RNA immunoprecipitation (RIP) assay was used to detect the association of p53 and lncRNA GClnc1 according to the manufacturer's instructions of the Magna RIP RNA Binding Protein Immunoprecipitation Kit (Sigma-Aldrich). Finally, RNA was dissolved in 10 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China), and stored at -80°C. Subsequently, qRT-PCR was used to determine the expression of GClnc1 in co-p53 protein and IgG protein precipitate.

Statistical analysis

Statistical Product and Service Solutions (SPSS, Chicago, IL, USA) 16.0 statistical software was used for all statistical analysis. Results are shown as means ± SE (standard errors). The unpaired student's t-test were used to compare the significance of differences between the mean of different groups. A value of $p<0.05$ indicated the statistical significance.

Results

LncRNA GClnc1 is enriched in OC tissues

LncRNA GClnc1 has been found to be upregulated and play important roles in bladder cancer and colorectal cancer.[12] ISH (Figure 1A) was used to establish the pathologic and clinical significance of GClnc1 expression in OC, which was carried out by the pathology department. To explore the relationship between the expression of GClnc1 and the development of OC, we also measured the expression levels of lncRNA GClnc1 in 18 OC tissue samples and related non-tumorous tissues using qRT-PCR. As shown in Figure 1B, the expression levels of lncRNA GClnc1 in OC cancer tissues were conspicuously higher than the related normal tissues. It indicated that GClnc1 was involved in the progression of OC.

LncRNA GClnc1 expression level alters cell proliferation and migration *in vitro*

To elucidate the underlying molecular mechanism of lncRNA GClnc1 regulating the progress of OC, we subsequently measured the expression of GClnc1 in ES-2 and T1074 cells. As shown in Figure 2A, GClnc1 expression in ES-2 cells was markedly higher than that of T1074 cells by qRT-PCR. In order to investigate the impact of GClnc1 on cell proliferation of OC cells, we knocked down GClnc1 by si-GClnc1. The expression level of GClnc1 was obviously decreased in ES-2 cells compared with si-NC, the negative control (Figure 2B). The cell proliferation and migration were significantly decreased after knockdown of GClnc1 expression by CCK-8 and transwell assays (Figure 2 C,D). Next, we overexpressed GClnc1 in T1074 cells. The expression level of GClnc1 was markedly increased in T1074 cells compared with pcDNA-NC (Figure 2E). Similarly, the cell proliferation and migration were significantly increased after overexpression of GClnc1 by CCK-8 and transwell assays (Figure 2 F,G).

LncRNA GClnc1 inhibits p53 activity

To illustrate the underlying mechanism of GClnc1 regulating the progression of OC, we further investigate the interaction

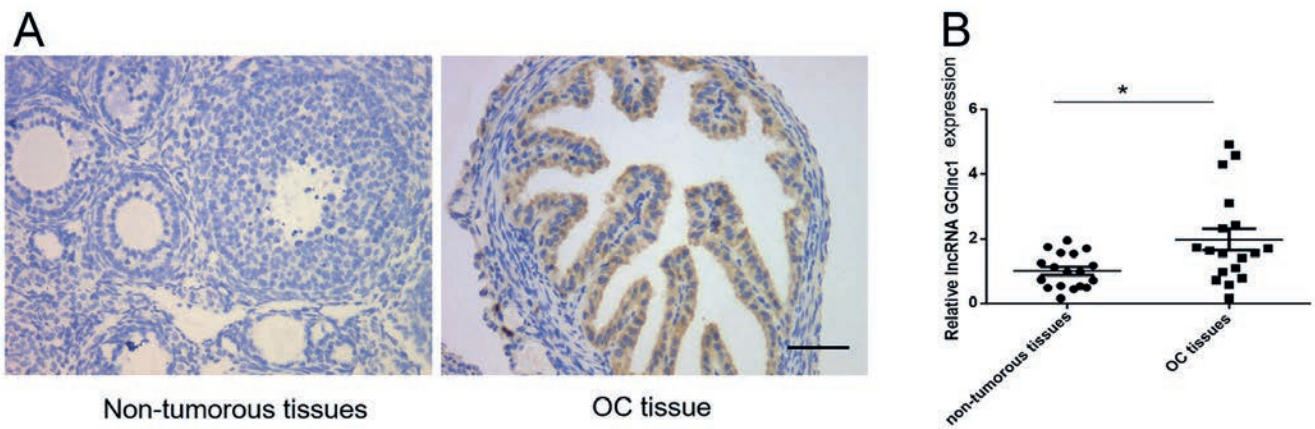

Figure 1. The expression of lncRNA GClnc1 in 18 OC patients was significantly higher than that of non-tumorous tissues. A) Representative images of GClnc1 expression in OC tissue and non-tumorous tissues were evaluated by ISH; scale bar: 100 μm). B) GClnc1 expression in OC tissue and non-tumorous tissues was measured by qRT-PCR. *p<0.05 *vs* non-tumorous tissues.

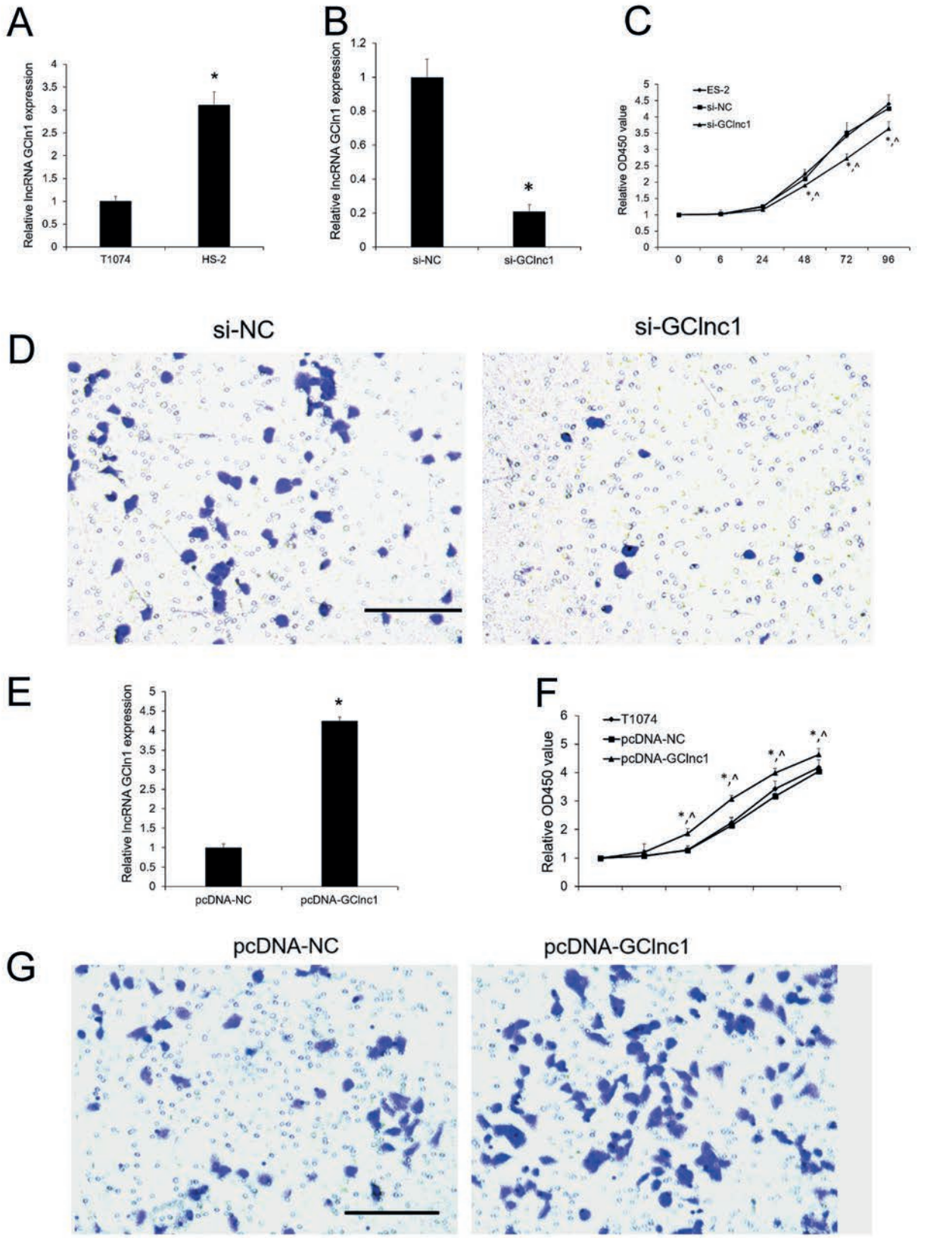

Figure 2. **LncRNA GClnc1 expression altered the cell proliferation and migration *in vitro*. A) GClnc1 expression in ES-2 cells was significantly higher compared with T1074 cell by qRT-PCR. B) GClnc1 expression was remarkedly decreased in ES-2 cells after knock down. C) GClnc1 knockdown significantly suppressed the proliferation of ES-2 cells. D) GClnc1 knockdown significantly decreased the migration of ES-2 cells; scale bar: 10 μm. E) GClnc1 expression was markedly increased in ES-2 cells after overexpression. F) GClnc1 overexpression significantly promoted the proliferation of T1074 cells. G) GClnc1 overexpression significantly increased the migration of T1074 cells; scale bar: 10 μm. *p<0.05 *vs* si-NC or pcDNA-NC; ^p<0.05 *vs* ES-2 or T1074 (n=3).**

between GClnc1 and p53. The level of GClnc1 in the p53 antibody precipitation complex was found to be significantly higher than that of the IgG control group *via* RIP experiment in ES-2 cells (Figure 3A). Next, the luciferase reporter gene assay proved that overexpression of GClnc1 inhibited the luciferase activity of p53 in T1074 cells (Figure 3B). Furthermore, the mRNA and protein expression of p53, as well as p21, were markedly decreased after GClnc1 overexpression (Figure 3 C,D). Those data suggested that GClnc1 inhibited the activity of p53.

LncRNA GClnc1 promotes cell proliferation and migration *via* p53

To verify that GClnc1 promoted the proliferation of OC cells by altering p53 activity, silenced GClnc1 was knocked down in ES-2 cells. At the same time, p53 was simultaneously silenced in cells as well (Figure 4A). The CCK-8 and transwell experiments showed that p53 silencing markedly reversed the inhibition in cell proliferation and migration caused by GClnc1 knockdown (Figure 4 B,C).

Discussion

Molecular mechanisms underlying the progression of OC still remain complex and largely unknown. In this study, we mainly investigated the biological function of lncRNA GClnc1 and p53 interaction in the progression of OC.

Numerous studies have reported that lncRNAs are regulators in a wide range of biological functions and play complex and extensive roles in cancer development and progression.[13,14] Among those widely studied lncRNAs, lncRNA GClnc1 has attracted a lot of attention with targeting multiple genes, such as MYC. LncRNA GClnc1 has been reported to promote proliferation and invasion of bladder cancer through activation of MYC.[12] Meanwhile, LncRNA GClnc1 has been identified to promote gastric carcinogenesis and may act as a modular scaffold of WDR5 and KAT2A complexes to specify the histone modification pattern.[15] In our work, GClnc1 was found to be remarkedly upregulated in OC tissue samples compared with related normal tissues. What's more, the overex-

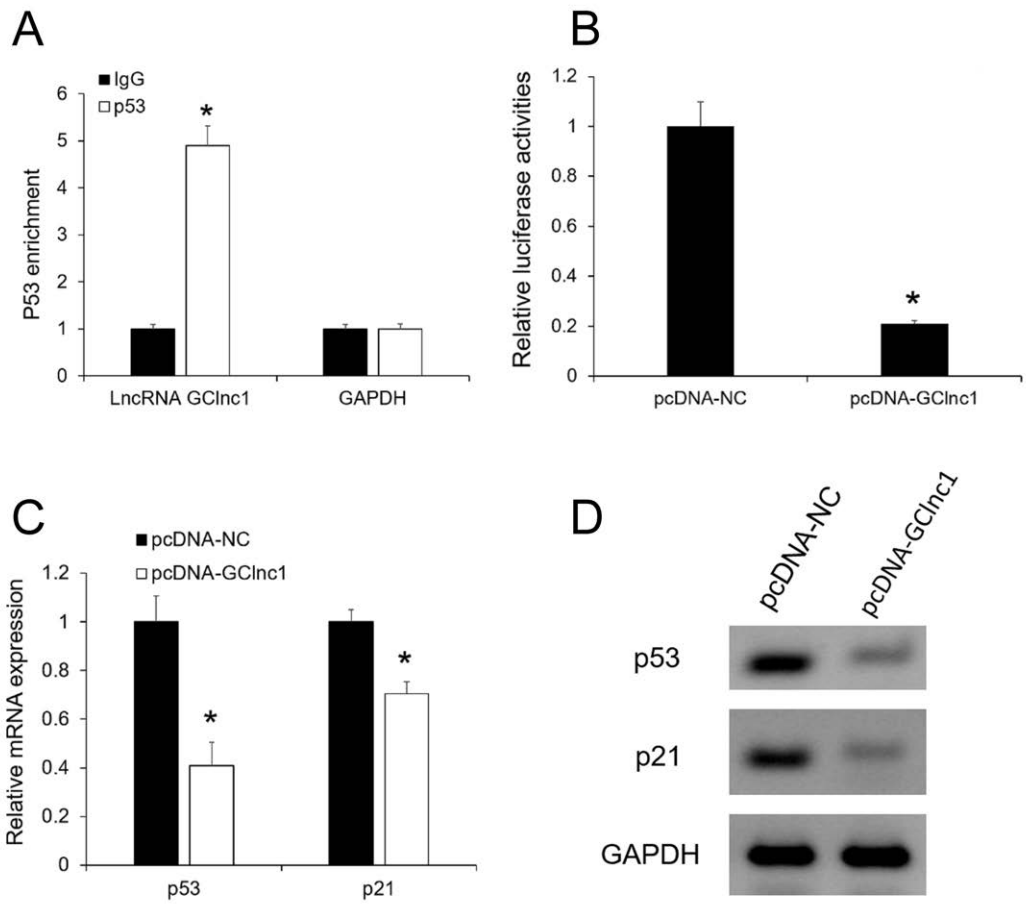

Figure 3. LncRNA GClnc1 interacted with p53. A) The correlation of GClnc1 and p53 was detected by RIP and qRT-PCR. B) Luciferase reporter assay was used to investigate the activity of p53 after GClnc1 overexpression. C) p53 and p21 expression levels were analyzed by qRT-PCR. D) Western blotting analysis was performed to determine the protein expression levels of p53 and p21. *p<0.05 *vs* negative control (n=3).

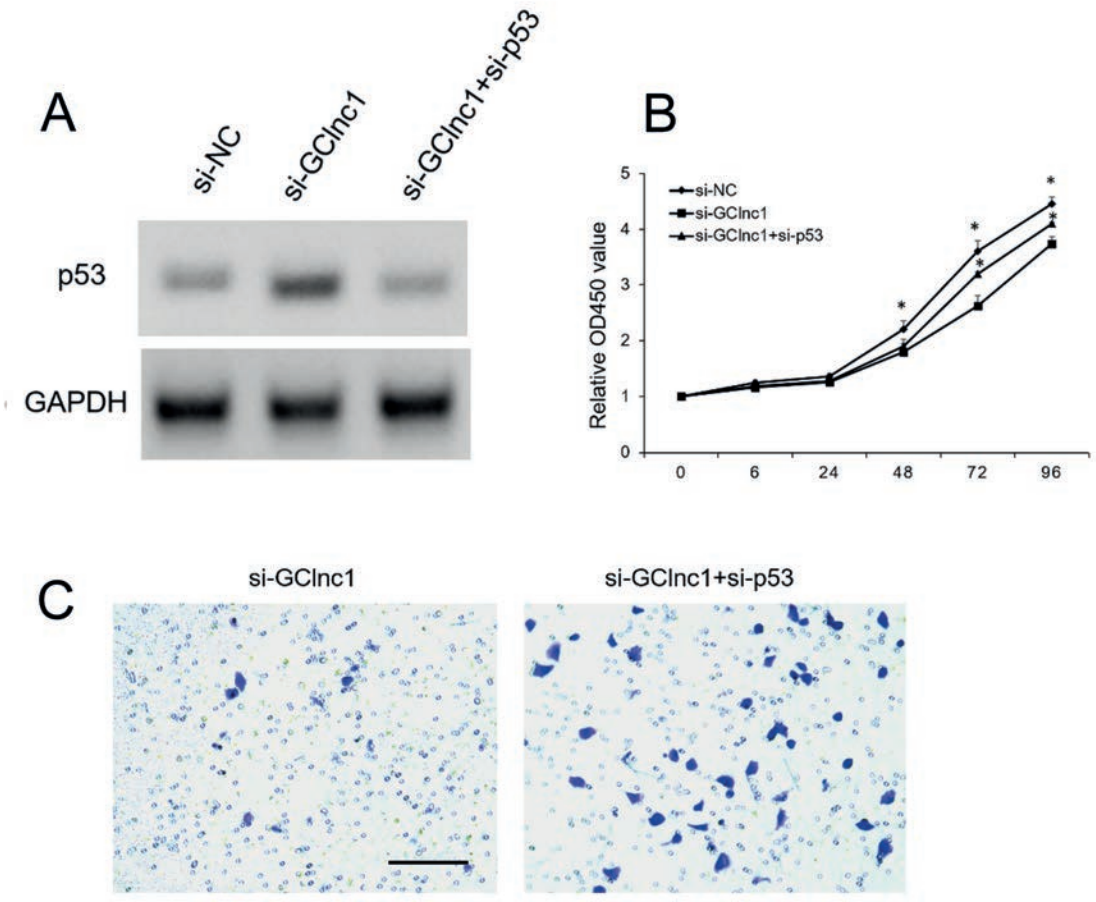

Figure 4. LncRNA GClnc1 altered cell proliferation and migration through p53. A) Western blotting was carried out to determine the protein expression level of p53 in cells with p53 silencing after knockdown of GClnc1. B) p53 silencing obligated the suppression of cell proliferation caused by knockdown of GClnc1. C) p53 silencing reversed the inhibition of cell migration caused by knockdown of GClnc1; scale bar: 10 μm. *p<0.05 *vs* si-GClnc1 (n=3).

pression of GClnc1 was also confirmed to promote the cell proliferation and migration, while the knockdown of GClnc1 was verified to suppress the cell proliferation and migration *in vitro*.

Recently, accumulated studies have shown that p53 signaling pathway interacted with lncRNA GClnc1 to play important roles in various cancers. LncRNA GClnc1 has been confirmed to promote the progression of colorectal cancer by inhibiting p53 signaling pathway.[9] In addition, lncRNA GClnc1 has also been reported to promote tumorigenesis in osteosarcoma by inhibiting p53 signaling.[16] In our work, we found that lncRNA GCln1 inhibit p53, as well as p21, activity in OC. Moreover, the silencing of p53 reversed the effect of GClnc1 knockdown on the proliferation of the human OC cell line, ES-2. These results allow suggesting that lncRNA GClnc1 may contribute to the progression of OC by regulating p53 signaling pathway. However, to confirm this hypothesis, further studies should be performed *in vivo* and on cell models *in vitro* to directly assess whether lncRNA GClnc1 might induce possible alternation in the proliferation related biomarkers and the dysfunction of p53 downstream genes in OC cells. Once its role in modulating the p53 pathway had been confirmed, GClnc1 would become a novel therapeutic target for OC patients.

Based on our study results, we conclude the lncRNA GClnc1 contributes to the progression of OC by regulating p53 signaling pathway. Our findings suggested that GClnc1 may serve as a novel therapeutic target for OC patients.

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