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ORIGINAL ARTICLE

LINC00202 attenuates the progression of gastric cancer via suppressing expression level of KLF2

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Summary

Purpose: *We aimed to investigate the function of LINC00202 in influencing the malignant progression of gastric cancer (GC).*

Methods: *The relative level of LINC00202 in GC and adjacent normal tissues was examined by quantitative real-time polymerase chain reaction (qRT-PCR). The difference in LINC00202 level among GC patients with different TNM stages was compared. Subsequently, regulatory effects of LINC00202 on proliferative capacity of AGS and SGC-7901 cells were evaluated. Subcellular distribution of LINC00202 was analyzed. The interaction and correlation between LINC00202 and Krüppel-like factor 2 (KLF2) were analyzed by RNA immunoprecipitation (RIP), Chromatin immunoprecipitation (ChIP) and linear regression test. Finally, the involvement of KLF2 in LINC00202-mediated proliferative ability of GC cells was clarified. LINC00202 was upregulated in GC tissues compared with that in adjacent normal ones. Its level remained higher in GC patients with stage III-IV than those with stage I-II. Silencing LINC00202 markedly attenuated the proliferation of GC cells.*

Results: *LINC00202 was mainly enriched in nucleus. KLF2 level was negatively correlated to and interacted with LINC00202. Transfection of LINC00202 decreased the recruitment ability of EZH2 on KLF2. Importantly, KLF2 partially reversed the regulatory effect of LINC00202 on the proliferative ability of GC cells.*

Conclusions: *LINC00202 enhances the proliferative ability of GC cells via negatively regulating KLF2, thus aggravating the progression of GC.*

Key words: *gastric cancer (GC), LINC00202, KLF2*

Introduction

Gastric cancer (GC) is a severe malignancy that threatens human health globally and its mortality ranks second [1]. Great strides on the efficient diagnosis and treatment of GC have been achieved but the prognosis of advanced GC is unsatisfactory. It is reported that the mortality of GC is over 70% [2] therefore it is urgent to search for diagnostic and prognostic hallmarks for this disease. Body fluid biopsy is considered to be a promising non-invasive method. Nevertheless, positive rates for serum markers of GC, such as CEA, CA19-9 and CA72-4 are relatively low [3]. It is urgent to discover specific and sensitive hallmarks for GC.

Long non-coding RNAs (lncRNAs) are transcripts with over 200 nucleotides long distributed in cytoplasm or nucleus. They lack the ability to encode proteins [4]. The discovery of lncRNAs greatly promoted the research progress in non-coding RNAs. Functionally, lncRNAs participate in multiple important processes, including X chromosome silenc-

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ing, genomic imprinting, chromatin modification, transcriptional activation, intranuclear transport, etc. [5-7]. They are capable of affecting the disease progression through mediating cellular behaviors [8]. Recently, the biological function of lncRNAs in tumor progression has been well concerned. A relevant study suggested that LINC00202 is related to poor prognosis of retinoblastoma [9]. In addition, LINC00202 level is correlated to the overall survival of patients with renal cancer [10]. The function of LINC00202 in GC, however, is rarely reported.

The Krüppel-like factor (KLF) family contains 17 transcription factors with a zinc finger domain [11]. KLF family is able to affect various biological processes, including cell proliferation, differentiation and migration, and also exerts important functions in the development of various tumor types. KLF2 is a vital member in KLF family, involved in the tumorigenesis and progression of intestinal cancer, liver cancer and breast cancer [12,13]. LncRNA DLEU1 is reported to promote proliferative rate by epigenetic inhibition of KLF2 [14].

This study aimed to uncover the interaction between LINC00202 and KLF2 in GC, thus providing novel targets for GC treatment.

Methods

Sample collection

GC tissues and adjacent normal tissues were surgically harvested from 60 GC patients. TNM stage of enrolled GC patients was recorded. None of these patients was preoperatively treated. This study was approved by the Ethics Committee of Tongren People's Hospital. Signed written informed consents were obtained from all participants before the study entry.

Cell culture and transfection

GC cell lines and gastric mucosal cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted at 80-90% confluence. Cells in logarithmic growth phase were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 24 h. Sequences of transfection vectors were as follows: sh-LINC00202: forward, 5'-GCTCTACCAAAGCGATCATTA-3' and reverse 5'-GTGCTCTCAAGTCCGAAGTTT-3', sh-NC: forward, 5'-CCGGTTTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAATTTTTG-3' and reverse, 5'-AATTCAAAAAGTTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAA-3'; sh-EZH2: forward, 5'-AGAGGTACCG-GACGAAGAATAATCATGG-3', reverse, 5'-TAGCTCGAGGGTAGCAGATGTAAGG-3'; sh-KLF2: forward, 5'-GATCCGCGCACCCACGACGACCTCAATTCAAGAGATTGAGGTCGTCGTCGGTGCCGTTTTTTC-3' reverse, 5'-AATTGAAAAAACGGCACCGACGACGACCTCAATCTCTTGAATTGAGGTCGTCGTCGGTGCCGCG-3'.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA extraction was performed using TRIzol method (Invitrogen, Carlsbad, CA, USA). The extracted RNA was quantified and reversely transcribed into complementary DNA (cDNA), followed by PCR using SYBR Green method (TaKaRa, Tokyo, Japan). Primer sequences were as follows: LINC00202: forward, 5'-TCAGTGGGTGTCCTCATTGGT-3' and reverse, 5'-GCACAGTTTCATCCTCCTTCC-3'; KLF2: forward, 5'-TTCGGTCTCTTCGACGACG-3' and reverse, 5'-TGCGAACTCTTGGTGTAGGTC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward, 5'-ACAACTTTGGTATCGTGGAAGG-3' and reverse, 5'-GCCATCACGCCACAGTTTC-3'.

Cell counting kit-8 (CCK-8)

Cells were inoculated in 96-well plates with 2×10^3 cells per well. Absorbance (A) at 450 nm was recorded at specified time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan), based on which the viability curve was plotted.

Colony formation

Cells were seeded in 6-well plates with 1×10^3 cells per well. After 2-week culture, cell colonies were fixed for 15 min, dyed with 0.05% crystal violet and captured for calculation.

5-Ethynyl-2'- deoxyuridine (EdU) assay

Cells were inoculated in 96-well plates (300 cells/well), and labeled with 50 μmol/L EdU at 37°C for 2 h. After 30-min fixation in 4% paraformaldehyde, cells were incubated with phosphate buffered saline (PBS) containing 0.5% Triton-100 for 20 min. After washing with PBS containing 3% bovine serum albumin (BSA), 100 μL of dying solution was applied per well for 1 h incubation in the dark and cells were counter-stained with 1×Hoechst 33342 for 30 min. EdU-positive cells, 4',6-diamidino-2-phenylindole (DAPI)-labeled nucleus and merged ones were captured under a microscope (magnification 100×).

Determination of subcellular distribution

Cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR. U6 was the internal reference of nucleus and GAPDH of cytoplasm.

RNA immunoprecipitation (RIP)

Cells were treated following the procedures of Millipore Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Cell lysate was incubated with anti-EZH2, or anti-IgG antibody at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/mL proteinase K containing 0.1% sodium dodecyl sulphate (SDS) to extract RNA. The magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. Finally, the extracted RNA was subjected to mRNA level determination using qRT-PCR.

Chromatin immunoprecipitation (ChIP)

Cells were subjected to 10-min cross-link with 1% formaldehyde at room temperature. Subsequently, cells were lysed using lysis buffer and sonicated for 30 min. Finally, the sonicated lysate was immuno-precipitated with anti-EZH2, anti-H3K27me3 or anti-IgG.

Western blot

Cells or tissues were digested through radioimmunoprecipitation assay (RIPA). The isolated total protein was qualified and subjected to electrophoresis (Beyotime, Shanghai, China). Protein sample was loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antibodies were inactivated through immersing membranes in PBS containing 5% skim milk. Two h later, they were reacted with primary antibodies at 4°C. The next day, 2-h secondary antibody reaction was carried out. Bands were exposed by electrochemiluminescence (ECL) (Pierce, Rockford, IL, USA) and analyzed by Image-Pro Plus (Media Cybernetics, Silver Springs, MD, USA).

Statistics

SPSS 20.0 software (SPSS IBM, Armonk, NY USA) was utilized for data analyses. Linear regression test was conducted for assessing the relationship between two genes. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the t-test. A significant difference was confirmed at the level of $p<0.05$.

Results

LINC00202 was upregulated in GC

QRT-PCR data showed higher abundance of LINC00202 in GC tissues relative to adjacent normal tissues (Figure 1A). GC patients in stage I-II presented a lower level of LINC00202 than those in stage III-IV (Figure 1B). Similarly, LINC00202 was upregulated in GC cell lines compared with that in gastric mucosal cell line (Figure 1C). We next constructed three transfection vectors targeting LINC00202, namely sh-LINC00202 1#, sh-LINC00202 2# and sh-LINC00202 3#. Transfection of sh-LINC00202 1# or sh-LINC00202 3# sufficiently downregulated LINC00202 level in AGS and SGC-7901 cells (Figure 1D).

Silencing of LINC00202 suppressed the proliferative ability of GC

CCK-8 assay showed reduced viability in AGS and SGC-7901 cells transfected with sh-LINC00202 1# or sh-LINC00202 3# compared with those of controls (Figure 2A, 2B). Colony formation assay revealed decreased number of colonies after transfection of sh-LINC00202 1# or sh-LINC00202 3# in GC cells (Figure 2C, 2D). Moreover, the number of

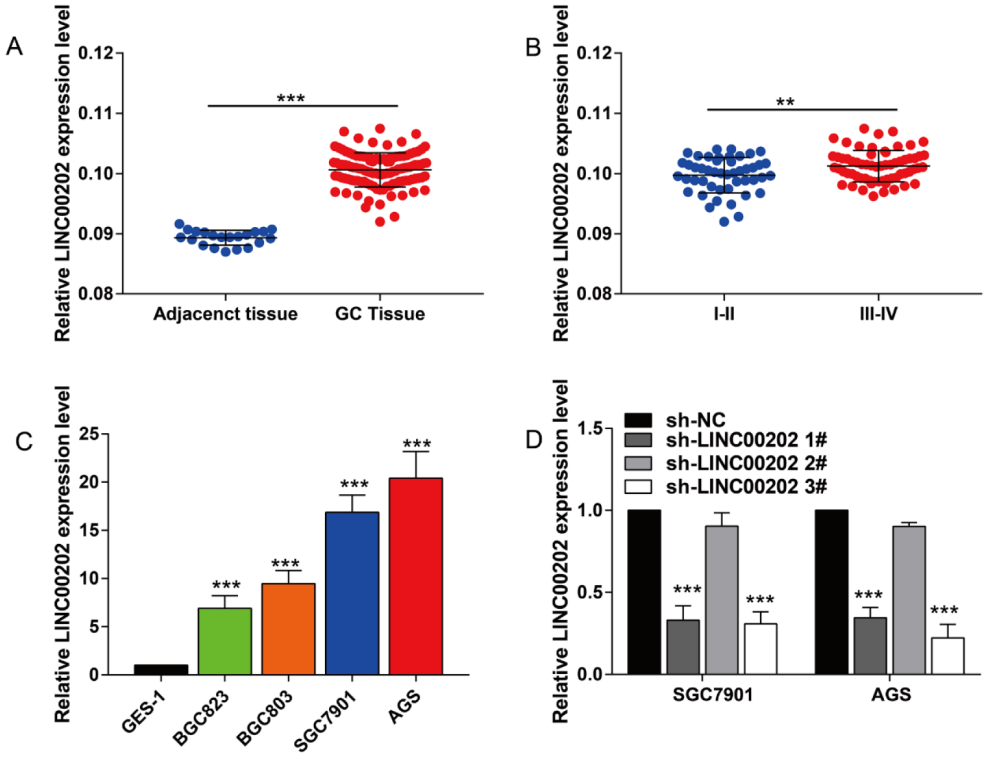

Figure 1. LINC00202 was upregulated in GC. **A:** Relative level of LINC00202 in adjacent normal tissues and GC tissues. **B:** Relative level of LINC00202 in GC patients with stage III-IV and stage I-II. **C:** Relative level of LINC00202 in GC cell lines and gastric mucosal cell line. **D:** Transfection efficacy of sh-LINC00202 1#, sh-LINC00202 2# and sh-LINC00202 3# in AGS and SGC-7901 cells. (**p<0.01, ***p<0.001).

EdU-positive cells markedly decreased after transfection of sh-LINC00202 1# or sh-LINC00202 3# (Figure 2E, 2F). All these data demonstrated the promotive effect of LINC00202 on the proliferative ability of GC cells.

LINC00202 downregulated KLF2 by recruiting EZH2

Subcellular distribution analysis illustrated that LINC00202 was mainly distributed in the nucleus of AGS cells (Figure 3A). A negative correlation was observed between expression levels of LINC00202 and KLF2 (Figure 3B). Transfection of sh-LINC00202 1# or sh-LINC00202 3# remarkably upregulated protein level of KLF2 in AGS and SGC-7901 cells (Figure 3C). RIP assay revealed higher enrichment of LINC00202 in anti-EZH2 relative to anti-IgG, suggesting the interaction between LINC00202 and EZH2 (Figure 3D). Notably, protein level of KLF2 was markedly downregulated by transfection of sh-EZH2 (Figure 3E). ChIP assay further showed

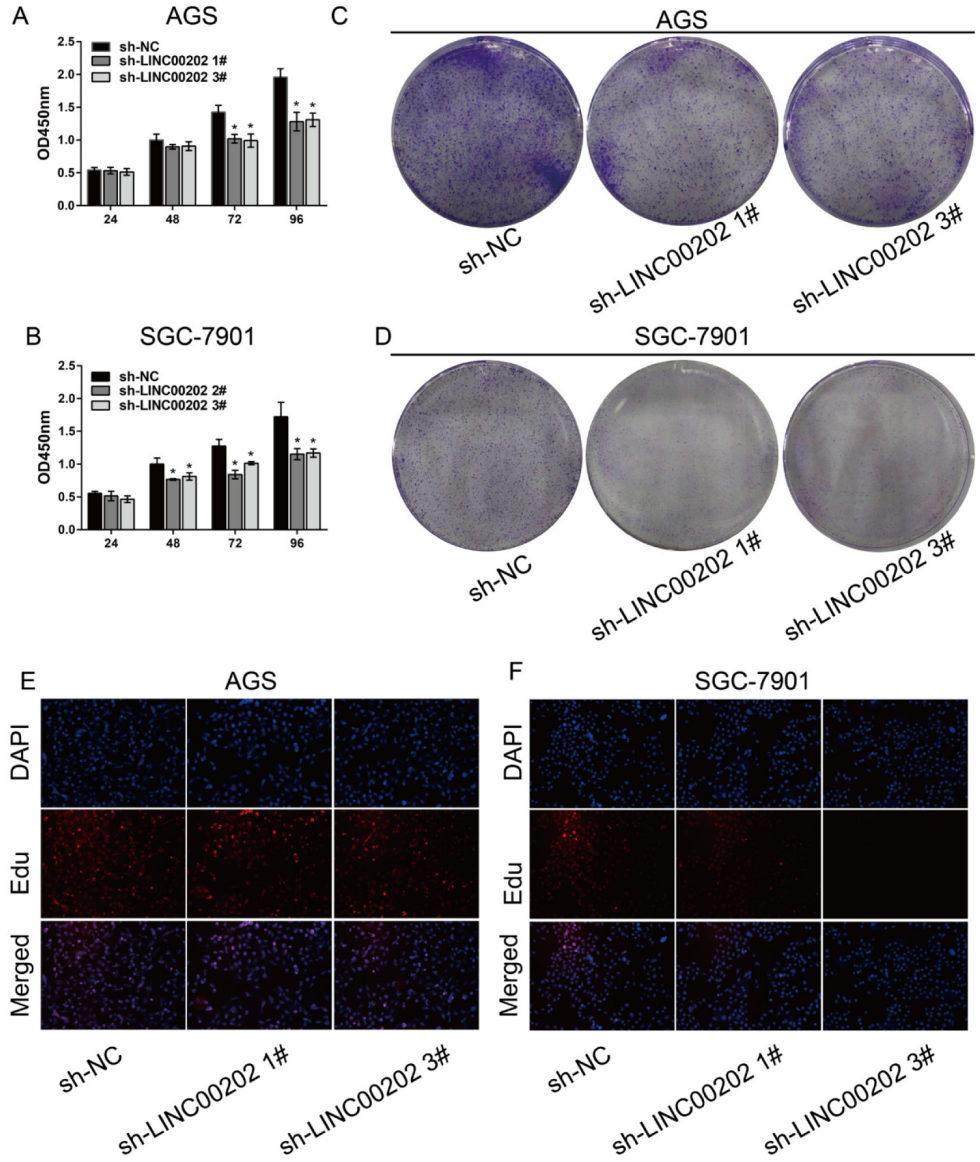

Figure 2. Silence of LINC00202 suppressed the proliferative ability of GC. **A:** CCK-8 assay showed the viability in AGS cells transfected with sh-NC, sh-LINC00202 1# or sh-LINC00202 3#. **B:** CCK-8 assay showed the viability in SGC-7901 cells transfected with sh-NC, sh-LINC00202 1# or sh-LINC00202 3# (*p<0.05). **C:** Colony formation assay showed the formed colonies in AGS cells transfected with sh-NC, sh-LINC00202 1# or sh-LINC00202 3#. **D:** Colony formation assay showed the formed colonies in SGC-7901 cells transfected with sh-NC, sh-LINC00202 1# or sh-LINC00202 3#. **E:** EdU assay showed DAPI-labeled (blue), EdU-labeled (red) and merged cells in AGS cells transfected with sh-NC, sh-LINC00202 1# or sh-LINC00202 3#. **F:** EdU assay showed DAPI-labeled (blue), EdU-labeled (red) and merged cells in SGC-7901 cells transfected with sh-NC, sh-LINC00202 1# or sh-LINC00202 3#.

that the immunoprecipitants of anti-EZH2 and anti-H3K27me3 were reduced after transfection of sh-LINC00202 1#, indicating that EZH2 was recruited by LINC00202 (Figure 3F). To sum up, KLF2 level was mediated by LINC00202-induced EZH2 recruitment.

Silencing of KLF2 partially reversed the regulatory role of LINC00202 in GC

We constructed sh-KLF2 to further explore the role of KLF2 in GC progression. Transfection of sh-KLF2 greatly downregulated mRNA level of KLF2, indicating the great transfection efficacy

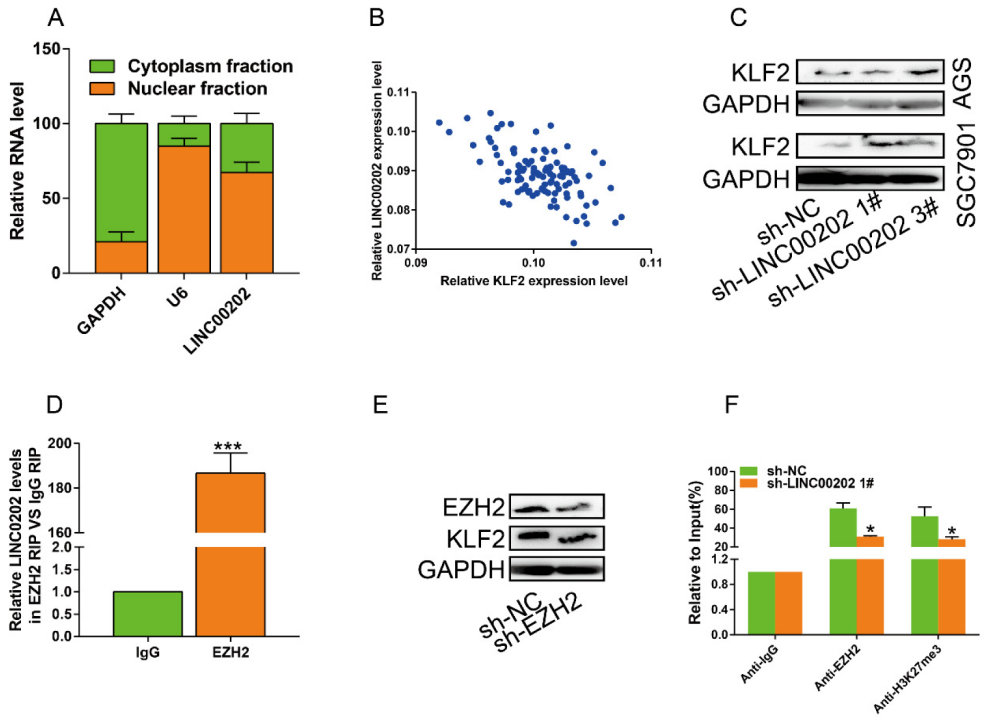

Figure 3. LINC00202 downregulated KLF2 by recruiting EZH2. **A:** Subcellular distribution of LINC00202 in cytoplasm fraction and nuclear fraction. GAPDH and U6 were served as cytoplasmic and nuclear internal reference, respectively. **B:** A negative correlation between LINC00202 and KLF2. **C:** Protein level of KLF2 in AGS and SGC-7901 cells transfected with sh-NC, sh-LINC00202 1# or sh-LINC00202 3#. **D:** RIP assay showed enrichment of LINC00202 in anti-IgG and anti-EZH2 (***p<0.001). **E:** Protein levels of EZH2 and KLF2 in AGS cells transfected with sh-NC or sh-EZH2. **F:** ChIP assay showed relative immunoprecipitant of AGS cells transfected with sh-NC or sh-LINC00202 1# in anti-IgG, anti-EZH2 and anti-H3K27me3 (*p<0.05).

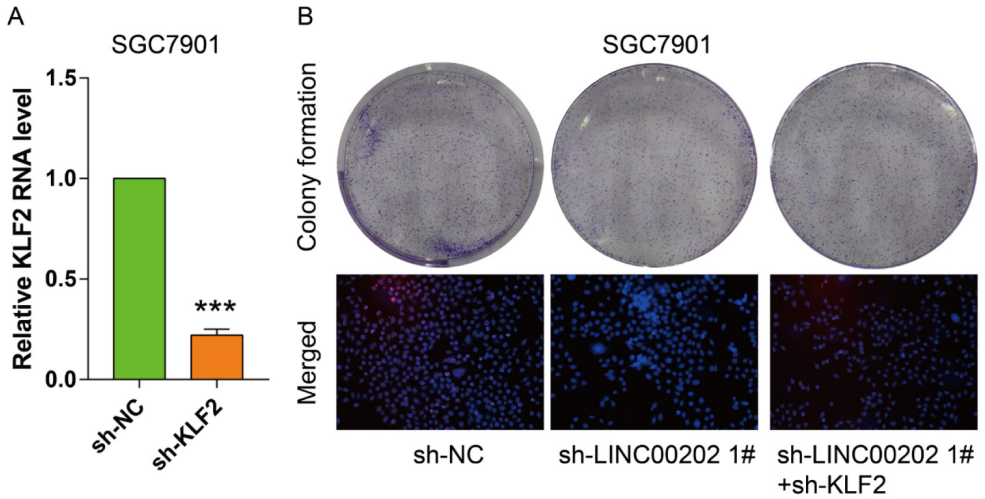

Figure 4. Silence of KLF2 partially reversed the regulatory role of LINC00202 in GC. **A:** Transfection efficacy of sh-KLF2 in SGC-7901 cells (***p<0.001). **B:** Colony formation assay showed the formed colonies in SGC-7901 cells transfected with sh-NC, sh-LINC00202 1# or sh-LINC00202 1#+sh-KLF2 (upper panel). EdU-assay showed the merged image of SGC-7901 cells transfected with sh-NC, sh-LINC00202 1# or sh-LINC00202 1#+sh-KLF2 (bottom panel).

(Figure 4A). Decreased numbers of colonies and EdU-positive cells in SGC7901 cells transfected with sh-LINC00202 1# were partially reversed by co-transfection of KLF2 (Figure 4B). Hence, it is believed that LINC00202 influenced GC proliferation by downregulating KLF2.

Discussion

As the most common malignant tumor of the gastrointestinal tract, GC is characterized by high morbidity and mortality. Owing to the strong invasiveness and metastasis, as well as lack of specific tumor hallmarks, the detection rate of early-stage GC is unsatisfactory [15]. Therapeutic strategies in GC, including surgical procedures, chemotherapy and radiotherapy have greatly progressed [16]. However, the prognosis of advanced GC patients is relatively poor. Therefore, it is necessary to clarify the mechanism underlying the tumorigenesis of GC. Differentially expressed lncRNAs in GC can be utilized to develop targeted therapies and enhance therapeutic efficacy.

LncRNAs are well-known molecules able to affect tumor occurrence and progression [17]. Several lncRNAs abnormally expressed in GC have been discovered. For example, Mo et al [18] illustrated that upregulated lncRNA XIST in GC tissues and cell lines stimulates tumor cell growth. Yuan et al [19] reported that lncRNA DANCR suppresses the growth of GC via inhibiting CT-NNB1 level. A recent study demonstrated that LINC00202 is capable of accelerating the progression of retinoblastoma through downregulating miR-3619-5p, thereby stimulating the upregulation of the RIN1 oncogene [9]. In this article, LINC00202 was upregulated in GC tissues and cell lines. Silencing LINC00202 remarkably attenuated the proliferative ability of GC cells, indicating the key role of LINC00202 during the progression of GC.

KLF2, a well-known tumor suppressor, is dysregulated in various types of tumors [20-22]. Ma et al [23] suggested that lncRNA SNHG15 accelerates the proliferative ability of pancreatic cancer cells by downregulating KLF2. GHET1 is proved to epigenetically inhibit KLF2 transcription in hepatocellular carcinoma cells via interacting with EZH2 [24]. Moreover, upregulation of GHET1 is negatively correlated with KLF2 downregulation in prostate cancer patients [25]. In the present study, we found that LINC00202 level was negatively correlated with KLF2. Besides, KLF2 level was influenced by LINC00202-induced recruitment of EZH2. Collectively, LINC00202 was confirmed to be able to accelerate proliferation of GC cells by downregulating KLF2. Our conclusions lay a solid foundation for lncRNA-targeted therapy of GC.

Conclusions

LINC00202 enhances the proliferative ability of GC cells by negatively regulating KLF2, thus aggravating the progression of GC.

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Conflict of interests

The authors declare no conflict of interests.

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