Long Noncoding RNA LINC00265 Promotes Proliferation of Gastric Cancer by Acting as A Competing Endogenous RNA on microRNA-144-3p Thereby Upregulating CBX4

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Abstract

Background: The expression levels and detailed functions of LINC00265 in gastric cancer (GC) have not yet been explored. This study aimed to measure LINC00265 expression in GC tissues and cell lines, investigate its specific roles in the aggressive characteristics of GC cells in vitro and in vivo, and elucidate the regulatory mechanisms of LINC00265 action.

Materials and methods: The qRT-PCR was performed to test the RNA expression levels in GC tissues and cell lines. Cell proliferation was detected by CCK-8 and colony formation assays. Western blot assay was used to measure relevant protein expression. Luciferase reporter assays were performed to investigate the association between LINC00265 and microRNA-144-3p and CBX4.

Results: LINC00265 expression was high in GC tissue samples and cell lines; LINC00265 overexpression correlated with shorter overall survival of the patients. A LINC00265 knockdown inhibited GC cell proliferation in vitro and slowed tumor growth in vivo. Mechanism investigation revealed that LINC00265 acts as a competing endogenous RNA on microRNA-144-3p (miR- 144) in GC cells. Chromobox 4 (CBX4) mRNA was identified as a direct target of miR-144-3p in GC cells. The knockdown of miR-144-3p counteracted the reduction in the malignant characteristics of GC cells by the downregulation of LINC00265.

Conclusion: In conclusion, LINC00265 functions as a competing endogenous RNA targeting miR-144-3p and increases the malignancy of GC cells in vitro and in vivo by upregulating CBX4.

Background

Gastric cancer is one of the most common malignant cancer in the world and ranks the third most common causes of cancer mortality worldwide,with 1,000,000 new gastric cancer cases and 783,000 death predicted in 2018 [1]. Notably, the incidence rates are dramatically increased in Eastern Asia, which has the highest incidence level worldwide in both sexes [2, 3]. Risk factors contributing to the incidence of GC include Helicobacter pylori infection, dietary habits, tobacco use, and obesity [4]. Due to its non-specific symptoms, highly rate of metastasis and absence of sensitive and specific biomarkers, GC patients are always diagnosed at advanced stage with poor prognosis, especially in China [5, 6]. Moreover, current treatment options for this devastating disease are extremely limited [7]. Identification of novel therapeutic targets and biomarkers of GC is extremely urgent [8].

LncRNAs are a class of non-coding RNAs that transcript longer than 200 nucleotides and have been implicated in regulating cancer cell proliferation, metastatic cascades, and chemoradiotherapy resistance via participating in post-transcription regulatory processes such as sponging miRNAs [9–11]. Dysregulation of LncRNA is commonly detected in GC, which is associated with cancer progression and patients' prognosis [12] For example, LncRNA KCNQ1OT1 functions as a competing endogenous RNA to regulate LMX1A expression by sponging microRNA-9 (miR-9) regulates the malignant proliferation of GC [13]. Furthermore, lncRNA TRPM2-AS inhibits the expression of miR-612 by competitive endogenous RNA

action, thereby promoting promote gastric cancer progression and radioresistance [14]. LINC00265 is located on human chromosome 7p14.1 and there is no mouse ortholog gene. Recently, LINC00265 was shown to be upregulated in CRC and associated with poor prognosis. Its deficiency decreases cell viability and glycolysis through directly binding to and negatively regulating miR-216b-5p, while supplementation with ectopic miR-216b-5p significantly compromises the oncogenic activities of LINC00265 in CRC cell. AML patients with higher serum LINC00265 expression suffer poorer overall survival. LINC00265 contributes to AML migration and invasion via modulation of PI3K/AKT signaling [15, 16].

MicroRNAs (miRNAs) are noncoding RNAs 20–24 nt in length that are capable of modulating stability and translational efficiency of target mRNAs [17]. miRNAs regulate various biological processes such as cellular proliferation, cell differentiation, metabolic signaling, and apoptosis [18]. Previous studies have demonstrated that a variety of miRNAs are dysregulated in many types of cancer, including GC [19]. Faheim M. et al revealed that miR-144 was downregulated in GC cells, and miR-144 inhibited the proliferation and invasion of GC cells by directly targeting activating enhancer-binding protein 4 (AP4) [20]. Yao. Et al. indicated that cyclooxygenase-2 (COX-2) is a direct target of miR-144 and that miR-144 negatively regulated the expression of COX-2, which inhibits the viability of GC cells [21].

Chromobox family has eight members including CBX1-8, which is a subgroup of protein in the PcG family, and they have distinct biological functions in different tissues .Chromobox 4 (CBX4), also known as polycomb 2 (Pc2), is a special chromobox protein because it is not only a transcriptional repressor but also a SUMO E3 ligase [22]. Recently, CBX4 has been identified as an oncogene and therapeutic target in cervical cancer, breast cancer and osteosarcoma [23–25]. CBX4 enhances the proliferation and metastasis of lung cancer in vitro via up-regulating the expression of BMI-1, thereby increasing the expression and activity of P53, CDK2, Cyclin E, MMP2, MMP9 and CXCR4 [26]. In contrast, CBX4 suppresses metastasis of colorectal carcinoma via recruitment of HDAC3 to the runx2 Promoter [27]. However, the role of CBX4 in gastric cancer is still not clear.

To date, it is unclear whether LINC00265 has a role in GC carcinogenesis. In this study, the expression of LINC00265 and its clinical implication in gastric cancer were examined and revealed. The effect of LINC00265 on the cell growth and migration was determined. The underlying mechanism of LINC00265 biological function in gastric cancer was investigated. Our data suggest LINC00265 is overexpressed in gastric cancer and exerts oncogenic activities via the miR-144-5p/chromobox 4 (CBX4) signaling pathway.

Materials And Method

Ethics statement

Investigation has been conducted in compliance with the principles of the Declaration of Helsinki and according to national and international guidelines and has been approved by the authors' institutional

review board. The study protocol was approved by the Ethics Committee of The Second Affiliated Hospital of Nanchang University. Informed consent has been obtained from all patients.

Clinical tissues and cell lines

Primary GC tissue samples and adjacent normal tissues were obtained from 40 patients who underwent surgical resection at the Second Affiliated Hospital of Nanchang University. None of the patients had received preoperative chemotherapy, radiotherapy, or other anticancer modalities. After the resection, the tissue samples were immediately frozen in liquid nitrogen and, then, stored in liquid nitrogen until subsequent treatment and analysis. Eight GC cell lines—KATO-III, SGC-7901, BGC-823, HGC-27, AGS, NCI-N87, SNU-1, and SNU-16—as well as normal gastric cells (GES cells) were bought from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). All the cells were cultured at 37°C in a humidified atmosphere containing 5% of CO2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS),100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA).

Oligonucleotides, construction of plasmids, and cell transfection

The siRNA specifically decreasing the expression of LINC00265 (called si-LINC00265) and its negative control siRNA (si-NC) were designed and commercially synthesized by RiboBio Co., Ltd. (Guangzhou, China). AgomiR-144-3p, the corresponding agomir-NC, antagomiR-144-3p, and antagomir-NC were acquired from the GenePharma Co., Ltd. (Shanghai, China). The fulllength CBX4 sequence lacking its 3'-UTR was amplified by the GenePharma Co., Ltd., too, and subcloned into the pcDNA3.1 vector to generate the pcCBX4 plasmid. The empty pcDNA3.1 vector served as the control. Cells were seeded in 6-well plates and transfected with the agomir (50 nM), antagomir (50 nM), siRNA (100 pmol) or plasmid (4 μg) using the Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The transfected cells were processed for further in vitro experiments after incubation for different periods.

RNA isolation and RT-qPCR

The TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to isolate total RNA form tissue samples or cells. The miScript Reverse Transcription Kit was purchased from Qiagen GmbH (Hilden, Germany) and, then, used for the synthesis of cDNA from the total RNA. After reverse transcription, qPCR was performed using the miScript SYBR Green PCR Kit (Qiagen GmbH) to determine miR-144-3p expression. This expression was determined in relation to U6 small nuclear RNA. To quantitate LINC00265 and CBX4 expression, the synthesis of cDNA was performed using the PrimeScript RT-Reagent Kit (Takara Bio, Kusatsu, Japan), followed by qPCR with the SYBR Premix Ex Taq™ Kit (Takara Bio). Expression levels of LINC00265 and CBX4 were normalized to GAPDH. All the data were analyzed by the $2^{-\Delta\Delta Cq}$ method.

CCK-8 assay

In 96-well plates, after 24 h transfection, cells were maintained in 10% FBS-supplemented DMEM for 0, 24, 48, or 72 h. At every time point, 10 μl of the CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added into each well, and the cells were incubated further at 37°C for 2 h. Optical density was measured at 450 nm wavelength on a Sunrise™ microplate reader (Tecan Group, Ltd., Mannedorf, Switzerland).

Tumor xenograft experiment

A total of 10 six-week-old female BALB/c nude mice were purchased from Better Biotechnology Co., Ltd. (Nanjing, China). All the animal procedures were approved by the Nanchang University and carried out in compliance with the Animal Protection Law of the People's Republic of China-2009 for experimental animals. Si-LINC00265-transfected or siNC-transfected NCI-N87 cells (1×10^7) were resuspended in 100 μl of phosphate-buffered saline and inoculated subcutaneously into the flank of nude mice (n=5 for each group). The tumor size was recorded every 4 days, and tumor volume was calculated using the following formula: tumor volume = 1/2 × tumor length × tumor width2. After 4 weeks, all the mice were euthanized by cervical dislocation, and their tumor xenografts were excised and weighed.

Nuclear/cytoplasmic fractionation

The PARIS Kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to isolate the cytoplasmic and nuclearfractions.

Bioinformatics prediction and luciferase reporter assay

A target prediction tool, starBase 3.0 (http://starbase.sysu.edu.cn/), was used to search for potential miRNAs that could be inactivated by LINC00265. The target genes (mRNAs to be precise) of miR-144-3p were predicted by means of three miRNA target prediction databases: starBase 3.0, TargetScan (http://www.targetscan.org/). The CBX4 3'-UTRs containing either the wild-type (wt) binding sequence or the mutant (mut) binding sequence for miR-144-3p were synthesized by GenePharma Co., Ltd., and inserted into the pmirGLO luciferase reporter vector (Promega, Madison, WI, USA). The resultant plasmids are referred to as CBX4-wt and CBX4-mut, respectively. The LINC00265-wt and LINC00265-mut reporter plasmids were generated by similar experimental procedures. Either agomiR-144-3p or agomir-NC was cotransfected into cells with either a "wt" or "mut" reporter plasmid using the Lipofectamine® 2000 reagent. Luciferase activity was determined 48 h after the transfection using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). *Renilla* luciferase activity was normalized to that of firefly luciferase.

Western blotting analysis

Total protein was isolated by means of the RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China). The Bicinchoninic Acid Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to quantify the protein concentration. The protein samples were resolved by SDS-PAGE on a

10% gel and transferred onto polyvinylidene difluoride membranes, followed by 2 h blocking with 5% fat-free milk diluted in Tris-buffered saline containing 0.1% of Tween 20 (TBST). After incubation with a primary antibody against CBX4 (cat. 18544-1-AP; dilution 1:500; proteintech) or against GAPDH (cat. 60004-1-Ig; dilution 1:5000; proteintech), the membranes were washed thrice with TBST, probed with a goat anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase (cat. No. ab205718; dilution 1:5,000; Abcam) (secondary antibody) and, then, treated with the Pierce™ ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.) for visualization of the protein signals.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. The correlation between LINC00265 expression and clinical parameters among the patients with GC was examined with the $\chi 2$ test. Spearman's correlation analysis was conducted to determine the correlation between LINC00265 and miR-144-3p expression levels in GC tissue samples. The overall-survival curve was analyzed by the Kaplan–Meier method and compared between groups by the log-rank test. The comparisons between two groups were performed by Student's t test; one-way analysis of variance, followed by the Student–Newman–Keuls test, was applied to evaluate the differences among multiple groups. Data with $P < 0.05$ were considered statistically significant.

Results

Upregulation of *LINC00265* is associated with poor clinical outcomes among patients with GC

To determine the specific role of LINC00265 in GC, the expression profile of this lncRNA was examined in 32 pairs of GS tissue samples and adjacent normal tissue samples. LINC00265 was found to be overexpressed in the GC tissue samples relative to the adjacent normal gastric tissues, as revealed by reverse-transcription quantitative PCR (RT-qPCR; Figure 1A, $P < 0.05$). Additionally, the expression of LINC00265 was quantified in a panel of GC cell lines (KATO-III, SGC-7901, BGC-823, HGC-27, AGS, NCI-N87, SNU-1, and SNU-16) and in normal gastric cells (GES cells). The results showed that LINC00265 expression was higher in the eight tested GC cell lines than in GES cells (Figure 1B, $P < 0.05$). Notably, patients with GC overexpressing LINC00265 showed shorter overall survival and disease-free survival (Figure 1C, $P = 0.0354$; Figure 1D, $P=0.0120$) than the patients with GC underexpressing LINC00265. These results implied that LINC00265 may be closely associated with the pathogenesis of GC.

A reduction in *LINC00265* expression inhibits the malignant characteristics of GC cells in vitro

Having detected the aberrant upregulation of LINC00265 in GC, we next attempted to determine the functions of LINC00265 in GC progression. NCI-N87 and KATO III cells were chosen for subsequent experiments and were transfected with either a small interfering RNA [siRNA] against LINC00265 (si-LINC00265) or a negative control siRNA (si-NC). LINC00265 was successfully knocked down in NCI-N87 and KATO III cells after transfection of si-LINC00265 (Figure 2A, $P < 0.05$). A Cell Counting Kit-8 (CCK-8) assay was performed to evaluate the influence of si-LINC00265 on GC cell proliferation. The si-

LINC00265 transfection obviously reduced the proliferative ability of NCI-N87 and KATO III cells compared with that in the si-NC group (Figure 2B, $P < 0.05$). Then, colony formation assay was conducted to test whether si-LINC00265 introduction increases GC cell apoptosis. As expected, the number of si-LINC00265 group was lower among NCI-N87 and KATO III cells after transfection with si-LINC00265 (Figure 2C, $P < 0.05$). In addition, Transwell migration assays revealed that the LINC00265 knockdown notably reduced the migration (Figure 2D, $P < 0.05$) of NCI-N87 and KATO III cells. In general, these findings suggested that the LINC00265 downregulation slowed the malignant progression of GC in vitro.

LINC00265 acts as a competing endogenous RNA (ceRNA) on miR-144-3p in GC cells

To investigate the molecular events involved in LINC00265–mediated GC progression, a nuclear/cytoplasmic fractionation assay was conducted to determine the distribution of LINC00265 inside GC cells. The data indicated that LINC00265 is mainly located in the cytoplasm of GC cells (Figure 3A); this finding suggested that this lncRNA may serve as a ceRNA for some miRNA(s) [28]. Herein, the candidate miRNAs that could be inactivated by LINC00265 were predicted via starBase 3.0. The results indicated that LINC00265 contains one conserved binding site for miR-144-3p (Figure 3B). Luciferase reporter was performed to further characterize the relation between LINC00265 and miR-144-3p in GC cells. MiR-144-3p agomir (agomiR-144-3p) transfection-mediated upregulation of miR-144-3p (Figure 3C, $P < 0.05$) noticeably decreased the luciferase activity of the LINC00265-WT plasmid (the plasmid expressing LINC00265 containing the wild-type binding site for miR-144-3p; $P < 0.05$); however, the luciferase activity of LINC00265-MUT (the plasmid expressing LINC00265 containing a mutant binding site for miR-144-3p) was unaffected in both NCI-N87 and KATO III cells when miR-144-3p was overexpressed, as evidenced by the luciferase reporter assay (Figure 3D). This finding suggested that miR-144-3p is a target of LINC00265 in GC cells. MiR-144-3p expression was subsequently measured in the 40 pairs of GC tissue samples and adjacent normal gastric tissues by RT-qPCR. MiR-144-3p was found to be significantly underexpressed in the GC tissue samples (Figure 3E, $P < 0.05$). In addition, the expression of miR-144-3p was evaluated in LINC00265–deficient NCI-N87 and KATO III cells. The LINC00265 knockdown remarkably upregulated miR-144-3p in NCI-N87 and KATO III cells (Figure 3F, $P < 0.05$). Overall, these results meant that LINC00265 functions as a ceRNA (molecular sponge) for miR-144-3p in GC cells.

MiR-144-3p acts as a tumor-suppressive miRNA in GC cells

To examine the manner in which miR-144-3p regulates the malignancy of GC, the NCI-N87 and KATO III cell lines were transfected with either agomiR-144-3p or agomir-NC and, then, subjected to functional experiments. Transfection with agomiR-144-3p appreciably decreased proliferation (Figure 4A, $P < 0.05$) of NCI-N87 and KATO III cells. Transwell migration assays showed that, when endogenous miR-144-3p was overexpressed, NCI-N87 and KATO III cells had weaker migratory (Figure 4C, $P < 0.05$). In summary, these data suggested that miR-144-3p may suppress GC progression.

CBX4 upregulation was correlated with poor prognosis of GC and enhances the malignant characteristics of gastric cancer

Again, to determine the specific role of CBX4 in GC, the expression profile of this lncRNA was examined in 40 pairs of GS tissue samples and adjacent normal tissue samples. CBX4 was found to be overexpressed in the GC tissue samples relative to the adjacent normal gastric tissues, as revealed by reverse-transcription quantitative PCR (RT-qPCR; Figure 5A, $P < 0.05$). Additionally, the expression of CBX4 was quantified in a panel of GC cell lines (KATO-III, SGC-7901, BGC-823, HGC-27, AGS, NCI-N87, SNU-1, and SNU-16) and in normal gastric cells (GES cells). The results showed that LINC00265 expression was higher in the eight tested GC cell lines than in GES cells (Figure 5B, $P < 0.05$). A cohort of 140 pair tissues samples were performed IHC assay, the IHC result showed that CBX4 was mainly expressed in the cytoplasm. Positive immmunoreactivity was depicted in 90.7% (127/140) of cancerous tissues and 27.1% (38/140) of noncancerous tissues. Elevated expression of CBX4 in cancer was found in 72.9% (102/140) of cases (Fig. 5C). Notably, patients with GC overexpressing CBX4 showed shorter overall survival (Fig. 5D, $P = 0.0184$) than the patients with GC underexpressing CBX4. To further reveal the influence of CBX4 on GC cells, CBX4 was successfully knocked down in NCI-N87 and KATO III cells after transfection of si-CBX4 (Figure 6A&B, $P < 0.05$). A Cell Counting Kit-8 (CCK-8) assay was performed to evaluate the influence of si- CBX4 on GC cell proliferation. The si-CBX4 transfection obviously reduced the proliferative ability of NCI-N87 and KATO III cells compared with that in the si-NC group (Figure 6C, $P < 0.05$). Then, colony formation assay was conducted to test whether si-CBX4 introduction increases GC cell apoptosis. As expected, the number of si-CBX4 group was lower among NCI-N87 and KATO III cells after transfection with si-CBX4 (Figure 6D, $P < 0.05$). In addition, Transwell migration assays revealed that the CBX4 knockdown notably reduced the migration (Figure 6E, $P < 0.05$) of NCI-N87 and KATO III cells. These results indicated that CBX4 may be closely associated with the pathogenesis of GC and could enhance the ability of proliferation and metastasis of gastric cancer cells.

CBX4 mRNA is a direct target of miR-144-3p in GC cells

By means of target prediction tools, including starBase 3.0 and TargetScan, CBX4 was predicted as a potential target gene of miR-144-3p (Figure 7A). To validate this prediction, a luciferase reporter assay was performed on NCI-N87 and KATO III cells after cotransfection with either agomiR-144-3p or agomir-NC and either plasmid CBX4-WT (a plasmid expressing luciferase mRNA containing the CBX4 3'-UTR harboring a wild-type binding site for miR-144-3p) or plasmid CBX4-MUT (a plasmid expressing luciferase mRNA containing the CBX4 3'-UTR harboring a mutated binding site for miR-144-3p). The ectopic expression of miR-144-3p significantly reduced the luciferase activity of CBX4-WT in NCI-N87 and KATO III cells ($P < 0.05$). By contrast, mutation of the binding site abrogated this phenomenon (Figure 7B). Next, the expression levels of CBX4 in miR-144-3p-overexpressing NCI-N87 and KATO III cells were determined to investigate whether CBX4 expression can be inhibited by miR-144-3p in GC. As expected, the protein levels (Figure7C, $P < 0.05$) and mRNA (Figure 7D, $P < 0.05$) of CBX4 in NCI-N87 and KATO III cells diminished in response to the agomiR-144-3p transfection. Collectively, the above findings identified CBX4 mRNA as a direct target of miR-144-3p in GC cells.

LINC00265 enhances the malignant characteristics of GC cells in vitro through the miR-144-3p/CBX4 axis

To test whether the oncogenic effects of LINC00265 on the malignancy of GC cells were mediated by its influence on the miR-144-3p/CBX4 pathway, rescue experiments were performed on LINC00265-deficient NCI-N87 and KATO III cells via transfection with miR-144-3p antagomir (antagomiR-144-3p). MiR-144-3p expression was found to be efficiently upregulated in NCI-N87 and KATO III cells after the co-transfection with si-LINC00265 and antagomiR-NC. But the miR-144-3p expression levels were relatively downregulated after co-transfection with si-LINC00265 and antagomiR-144-3p (Figure 7F, $P < 0.05$). On the contrary, protein expression levels of CBX4 were downregulated both in NCI-N87 and KATO III cells after the co-transfection with si-LINC00265 and antagomiR-NC, and regulated relatively co-transfection with si-LINC00265 and antagomiR-144-3p (Figure 7G, $P < 0.05$). These data revealed that the LINC00265 knockdown reduced the malignancy of GC cells in vitro by decreasing the sponging of miR-144-3p by LINC00265 and, thereby, reducing CBX4 expression.

The *LINC00265* knockdown inhibits the in vivo tumor growth of GC cells

Xenograft tumors were induced to test whether there is a similar influence of LINC00265 on tumor growth in vivo as in the above experiments in vitro. NCI-N87 cells transfected with either si-LINC00265 or si-NC were subcutaneously injected into a flank of nude mice. At 28 days postinoculation, the tumor growth curve indicated that the growth of tumor xenografts was much slower in the si-LINC00265 group than in the si-NC group (Figure 8A and 8B, $P < 0.05$). At the end of this experiment, all the mice were euthanized and the tumor xenografts were excised and weighed. The weight of the tumor xenografts derived from si-LINC00265–transfected NCI-N87 cells was obviously lower (Figure 8C, $P < 0.05$). Additionally, LINC00265 expression was still low in the tumor xenografts from the si-LINC00265 group (Figure 8D, $P < 0.05$). The expression of miR-144-3p was higher (Figure 8E, $P < 0.05$), whereas the protein expression of CBX4 was lower (Figure 8F, $P < 0.05$), in the si-LINC00265 group than in the si-NC group. These results indicated that the downregulation of LINC00265 retarded the in vivo tumor growth of GC cells by decreasing the output of the miR-144-3p–CBX4 axis.

Discussion

To date, numerous studies have revealed alterations in the expression of lncRNAs in GC [29]. The dysregulation of lncRNAs is closely related to tumorigenesis, metastasis, and prognosis or diagnosis.of GC by playing either a tumor-suppressive or oncogenic role [30]. Therefore, determination of the specific functions of lncRNA in GC is urgently needed for the identification and validation of novel diagnostic biomarkers of (and therapeutic targets in) GC. In this study, we first tested whether LINC00265 is dysregulated in GC and clarified the clinical value of LINC00265. Second, we used siRNA to knock down endogenous LINC00265 in GC cell lines, to study the influence of this LINC00265 knockdown on the malignant characteristics of GC cells in vitro and in vivo. Third, the mechanisms underlying the participation of LINC00265 in GC were elucidated in detail. LINC00265 is increased in colorectal cancer tissues. Elevated level of LINC00265 is correlated with lymph node metastases and advanced pathological stage. Knockdown of LINC00265 impaired cell proliferation and invasion, promoted cell cycle distribution and apoptosis in HT29 cells by suppressing the expression of EGFR [31]. LINC00265

was upregulated in CRC both in vivo and in vitro, which intimately associated with poorer prognosis. LINC00265-deficiency resulted into decreases in cell viability, glucose uptake, pyruvate production, and lactate production through regulating of miR-216b-5p/TRIM44 Axis [16]. LINC00265 is upregulated and predicts poor clinical outcome in human patients with CRC. LINC00265 promotes colorectal tumorigenesis via ZMIZ2 and USP7-mediated stabilization of β-Catenin [32]. Nevertheless, the expression of LINC00265 in GC has not yet been studied. In this work, we found that LINC00265 is upregulated in both GC tumors and cell lines. LINC00265 overexpression significantly correlated with the clinical stage and distant metastasis among the patients with GC. Notably, the patients with GC overexpressing LINC00265 showed substantially shorter overall survival and disease-free survival compared to the patients with GC underexpressing LINC00265. These observations suggest that LINC00265 may be an effective biomarker for the diagnosis and prognosis of GC. LINC00265 has been identified elsewhere as an oncogenic lncRNA during carcinogenesis and cancer progression in colorectal cancer, but studies on the detailed functions of LINC00265 in GC are rare. In this study, we found that the LINC00265 knockdown restricted the proliferation of GC cells in vitro and vivo.

Subsequently, we elucidated the molecular mechanisms underlying the oncogenic actions of LINC00265 in GC. In recent years, studies revealed that lncRNAs perform their functions by acting as ceRNAs or molecular sponges to inactivate miRNAs. Herein, our data provide reliable evidence that LINC00265 serves as a ceRNA for miR-144-3p. MiR-144-3p is frequently reported to be upregulated in oesophageal cancer [33] and downregulated in cervical cancer [34], ovarian cancer [35] and colorectal cancer [36]. MiR-144-3p is underexpressed in GC tumors and cell lines [37]. Its upregulation inhibits metastasis of gastric cancer by targeting MET expression [38]. In terms of the mechanism, cyclooxygenase-2 (COX-2) mRNA is proved to be a direct target of miR-144-3p in GC [21].

Our results on the expression and functions of miR-144-3p in GC are consistent with these observations. Another important finding of this study is that the tumor-suppressive activity of miR-144-3p in GC is due to CBX4 downregulation. CBX4, a key factor from Chromobox (CBX) family proteins are canonical components of PcG that regulate tumorigenesis and progression of many cancers including GC by inhibition of cell differentiation and self-renewal of cancer stem cells [39]. For example, CBX4 is highly expressed in lung tumors, which could promote proliferation and metastasis via regulating the expression of BMI-1 [26]. CBX4 maintained recruited histone deacetylase 3 (HDAC3) to the Runx2 promoter, which maintained a deacetylated histone H3K27 state to suppress Runx2 expression,suppressing metastasis of Colorectal Carcinoma [40]. As for GC, Ying Luo et al reported that CBX4 rs77447679 polymorphism was positively associated with GC, and individuals with CC genotype had less risk of GC [41]. Consequently, targeting the LINC00265/miR-144-3p/CBX4 pathway might be an innovative strategy to treat patients with GC.

In conclusion, we demonstrated that LINC00265 is overexpressed in GC and associated with poor clinical outcomes. Moreover, this study is the first to reveal that LINC00265 enhances the malignant characteristics of GC cells in vitro and in vivo. As for the mechanism, for the first time, LINC00265 is reported here to act as a ceRNA on miR-144-3p, thereby, upregulating CBX4 in GC. These findings may

offer a novel theoretical and experimental explanation for GC progression and should help to find attractive therapeutic targets in GC.

In conclusion, this study first analyzed the expression and molecular role of LINC00265 and CBX4 in gastric cancer; for the first time, we found the relationship between LINC00265 and miR-144-3p, miR-144-3p and CBX4 in gastric cancer cells; for the first time, we systematically proposed the important role of LINC00265 / miR-144-3p / CBX4 signal axis in gastric cancer progression. It provides a new theoretical basis for the mechanism of gastric cancer. However, there are still some deficiencies in this study. First, there is a lack of animal experiments to prove our preliminary results. Secondly, the case data of some patients in this study are not perfect, and the clinical data are not fully used to analyze the experimental results by mathematical model.

Conclusion

In this study, we found that LINC00265 is highly expressed in GC tissues and exerts its oncogenic effects via acting as a sponge of miR-144-3p, thereby up-regulating CBX4 expression.

The LINC00265 / miR-144-3p / CBX4 axis promotes GC progression and may serve as a potential promising therapeutic target of GC.

Declarations

Ethics approval and consent to participate

Every patient signed informed consent, based on the review of Ethics of committee of the Second Affiliated Hospital of Nanchang University.

Consent for publication

Not applicable.

Availability of data and materials

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

Conflicts of interests

The authors declare no conflicts of interest.

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Authors' contributions

LZD and WJL designed this study; ZXY, XOY and LZ conducted all experiments and performed data collection and analysis; ZXY and XOY contributed to manuscript preparation. All authors read and approved the final manuscript.

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Figures

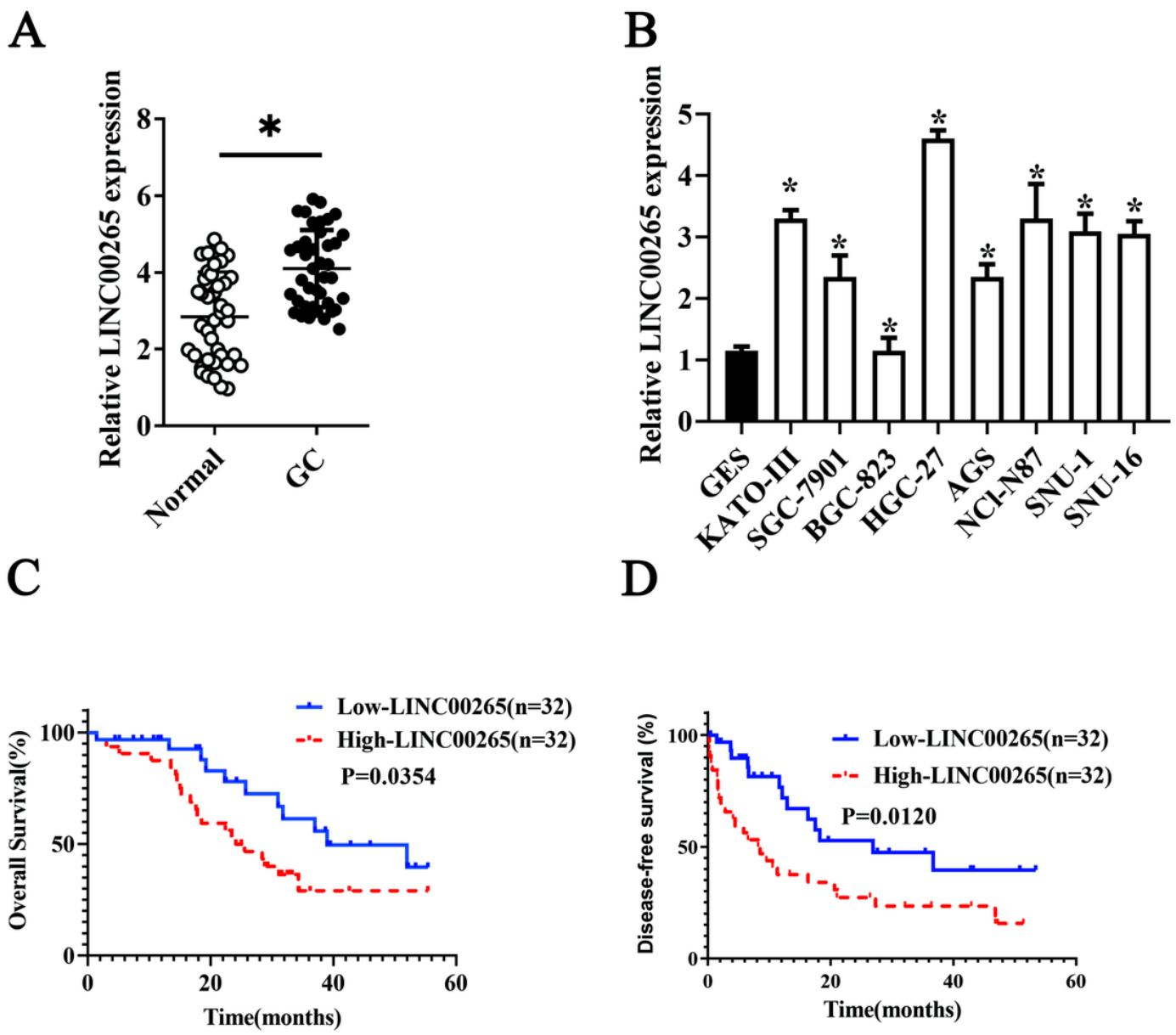

Figure 1

LINC00265 is overexpressed in GC tissue samples and cell lines. (A) The expression of LINC00265 was analyzed in 32 pairs of GC tissue samples and adjacent normal gastric tissues using RT-qPCR. *P < 0.05 vs. the normal gastric tissues. (B) RT-qPCR was performed to determine LINC00265 expression in eight GC cell lines (KATO-III, SGC-7901, BGC-823, HGC-27, AGS, NCI-N87, SNU-1, and SNU-16) and normal gastric cells (GES cells). *P < 0.05 vs. GES cells. (C, D) The Kaplan–Meier survival analysis and log-rank test were applied to assess the relation between LINC00265 levels and the overall survival (P=0.0354) and disease-free survival (P=0.0120) of patients with GC. The median value of LINC00265 expression among the GC tissue samples was chosen as a cutoff.

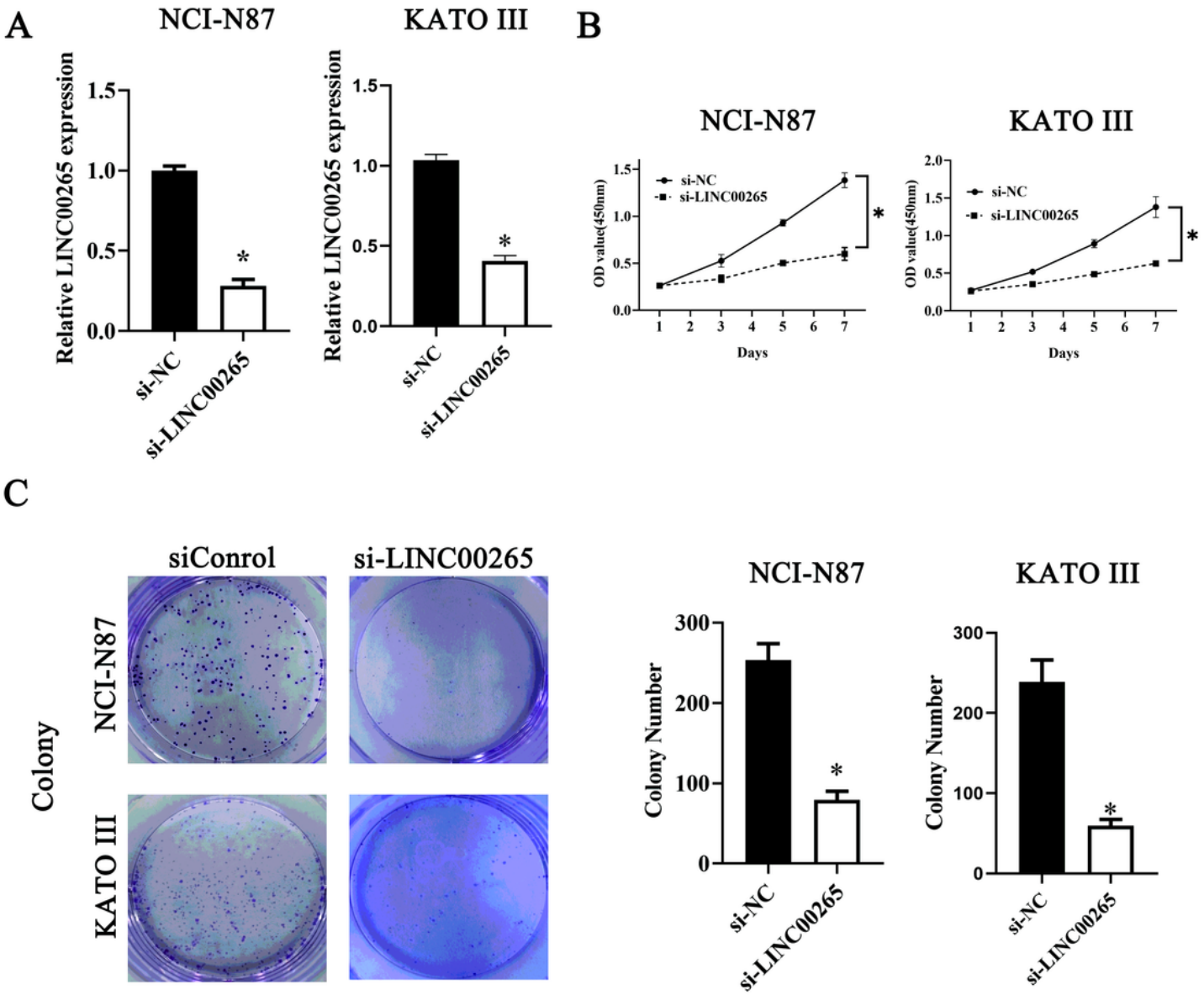

Figure 2

The LINC00265 knockdown suppresses the proliferation of NCI-N87 and KATO III cells. (A) NCI-N87 and KATO III cells were transfected with either si-LINC00265 or si-NC. At 48 h post-transfection, the cells were collected and, then, subjected to RT-qPCR analysis for transfection efficiency evaluation. *P < 0.05 vs. the si-NC group. (B) The CCK-8 assay was conducted to assess cellular proliferation after 0, 3, 5, and 7 day of cultivation of si-LINC00265-transfected or si-NC-transfected NCI-N87 and KATO III cells. *P < 0.05 vs.

group si-NC. (C) Colony formation assay was performed to determine the proliferation ability of NCI-N87 and KATO III cells after transfection with either si-LINC00265 or si-NC. *P < 0.05 vs. the si-NC group.

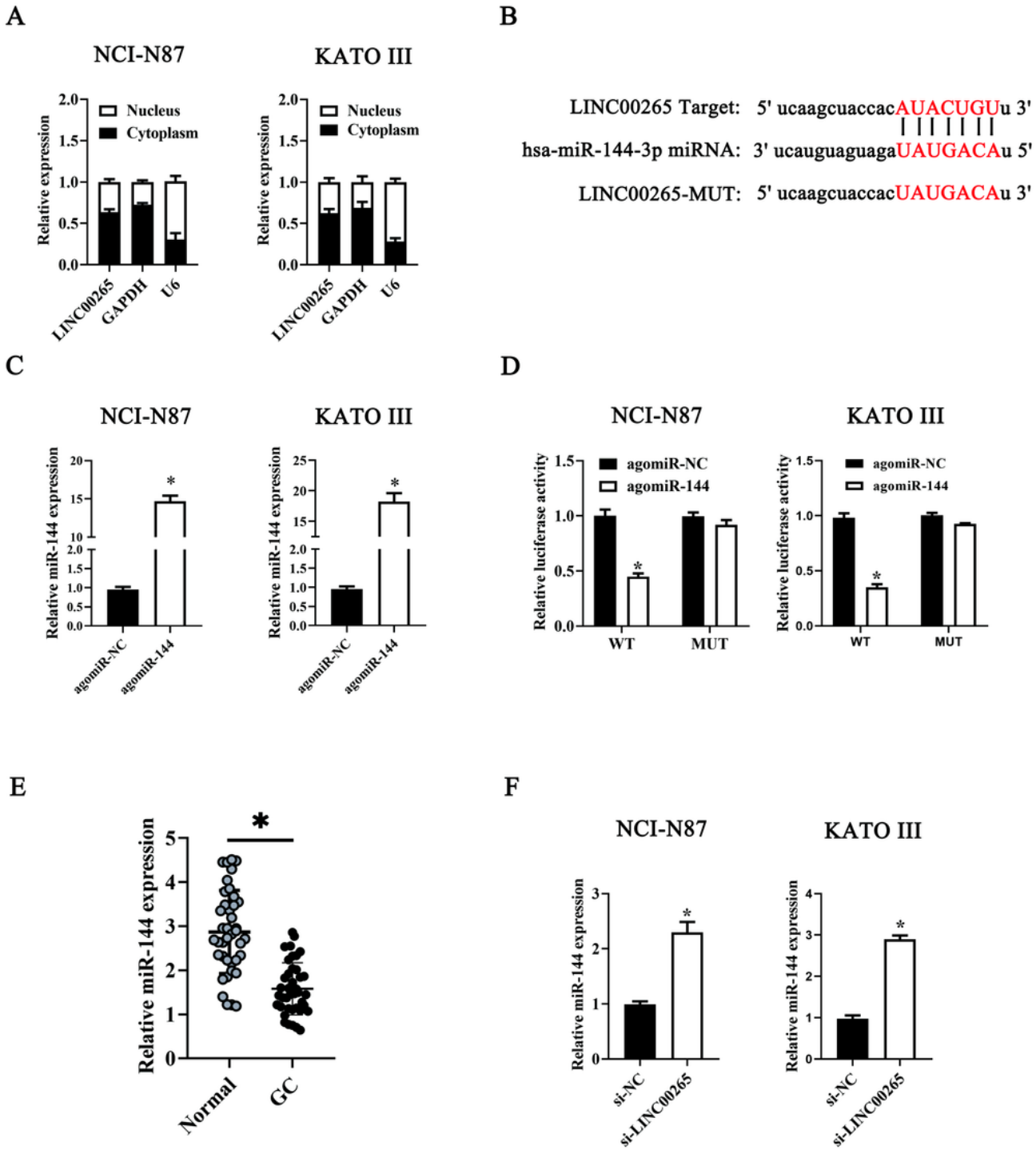

Figure 3

LINC00265 functions as a ceRNA for miR-144 in GC cells. (A) The distribution of LINC00265 within GC cells was determined by the nuclear/cytoplasmic fractionation assay. (B) The wild-type miR-144-binding sequences in LINC00265, as predicted by starBase 3.0. The mutations in the LINC00265 sequence that

disrupt the interaction between LINC00265 and miR-144 are shown too. (C) NCI-N87 and KATO III cells that were transfected with either agomiR-144 or agomir-NC were harvested and analyzed for miR-144 expression by RT-qPCR. *P < 0.05 vs. the agomir-NC group. (D) Luciferase reporter assays were performed on NCI-N87 and KATO III cells that were transfected with either agomiR-144 or agomir-NC and either LINC00265-wt or LINC00265-mut. *P < 0.05 vs. group agomir-NC. (E) The expression profile of miR-144 in the 40 pairs of GC tissues and adjacent-normal-gastric tissue samples was analyzed by RT-qPCR. *P < 0.05 vs. the normal tissues. (F) Expression of miR-144 in NCI-N87 and KATO III cells transfected with either si-LINC00265 or si-NC was determined by RT-qPCR. *P < 0.05 vs. the si-NC group.

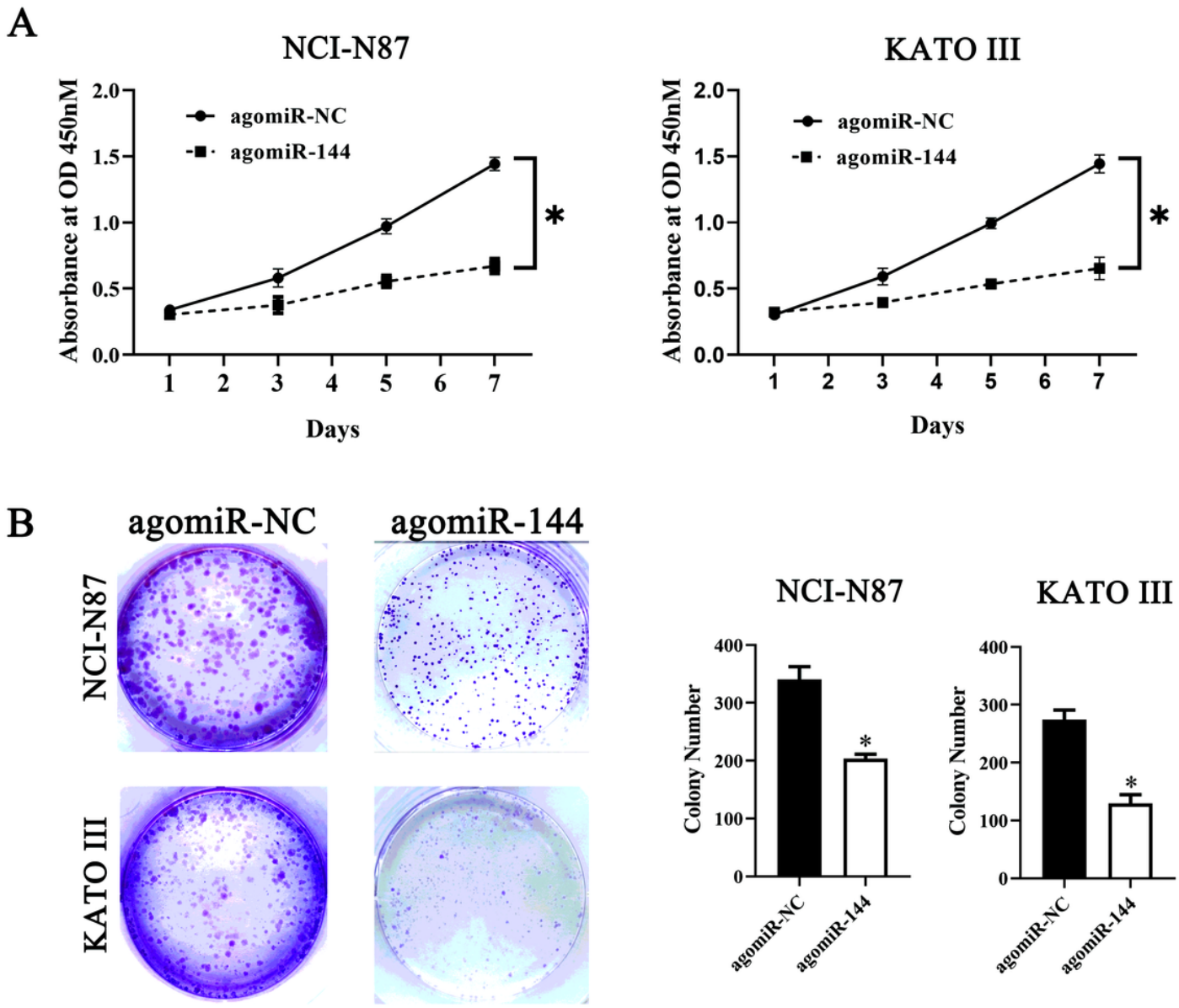

Figure 4

MiR-144 exerts a tumor-suppressive action on the growth of NCI-N87 and KATO III cells. (A) The CCK-8 assay uncovered a change in proliferation of miR-144-overexpressing NCI-N87 and KATO III cells. *P < 0.05 vs. group agomir-NC. (B) NCI-N87 and KATO III cells were treated with either agomiR-144 or agomir-NC. After the transfection, Colony formation assays were carried out. *P < 0.05 vs. group agomir-NC.

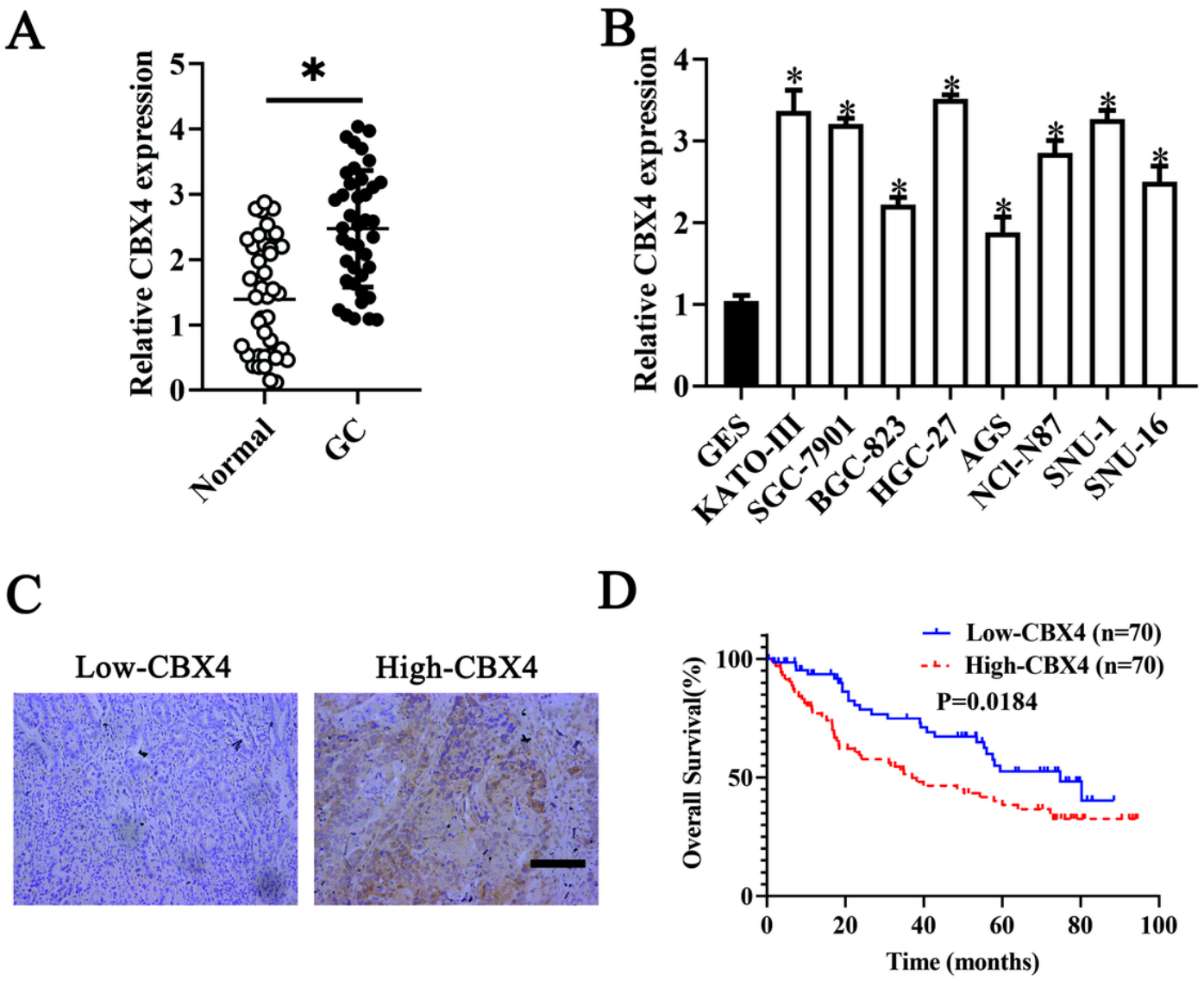

Figure 5

CBX4 upregulation was correlated with poor prognosis of GC. (A) The expression of CBX4 was analyzed in 32 pairs of GC tissue samples and adjacent normal gastric tissues using RT-qPCR. *P < 0.05 vs. the normal gastric tissues. (B) RT-qPCR was performed to test CBX4 expression in eight GC cell lines (KATO-III, SGC-7901, BGC-823, HGC-27, AGS, NCI-N87, SNU-1, and SNU-16) and normal gastric cells (GES cells). *P < 0.05 vs. GES cells. (C) The protein expression was determined using immunohistochemistry (scale

bar=200um). (D) The Kaplan–Meier survival analysis and log-rank test were applied to assess the relation between CBX4 levels and the overall survival (P=0.0184) of patients with GC. The median value of CBX4 expression among the GC tissue samples was chosen as a cutoff.

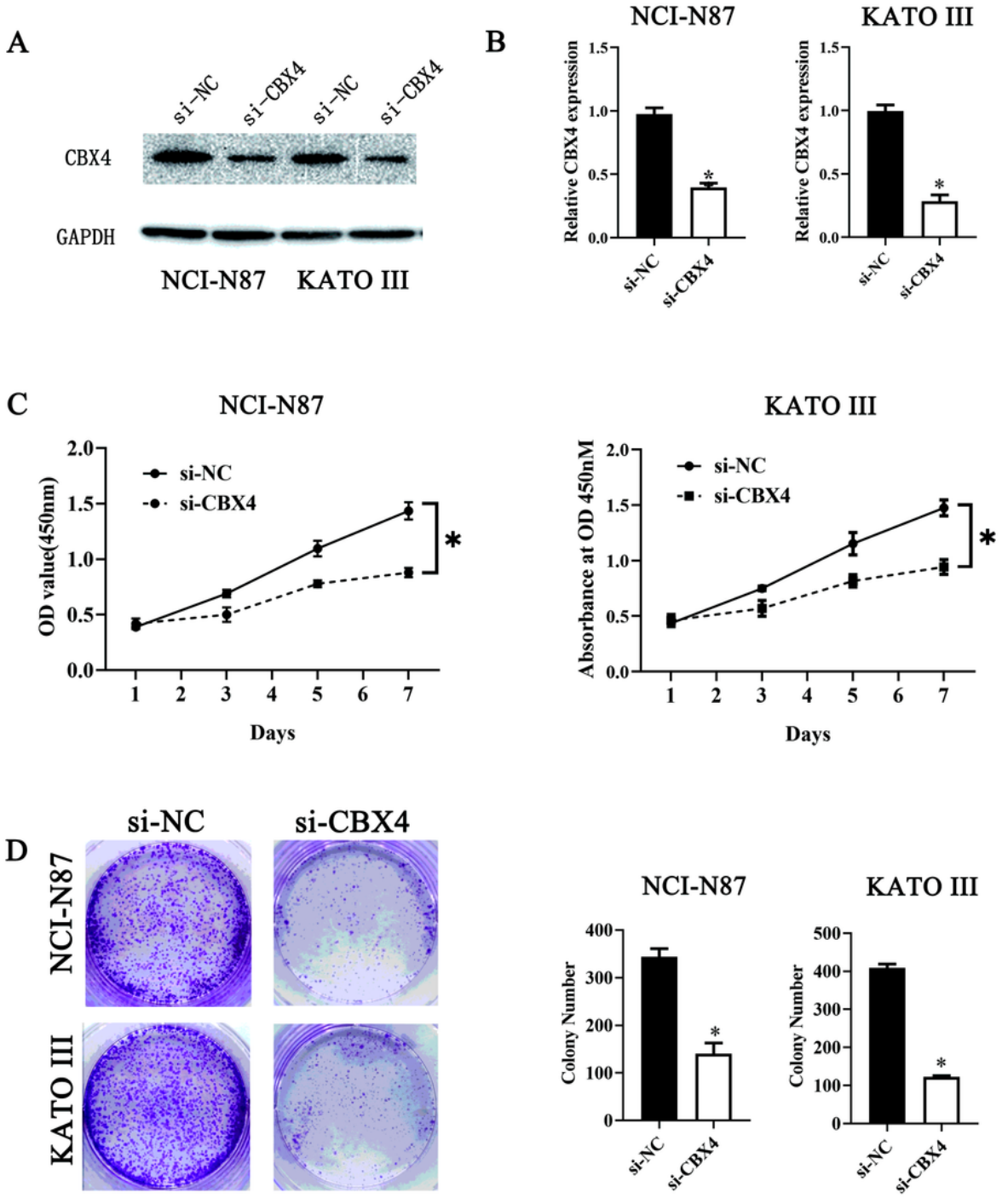

Figure 6

CBX4 upregulation enhances the malignant characteristics of gastric cancer. (A, B) NCI-N87 and KATO III cells were transfected with either si-LINC00265 or si-NC. At 48 h post-transfection, the cells were collected

and, then, subjected to western blotting and RT-qPCR analysis for transfection efficiency evaluation. (C) The CCK-8 assay showed a change in proliferation of CBX4-knockdown NCI-N87 and KATO III cells. *P < 0.05 vs. group agomir-NC. (D) NCI-N87 and KATO III cells were treated with either agomiR-144 or agomir-NC. After the transfection, Colony formation assays were carried out. *P < 0.05 vs. group agomir-NC.

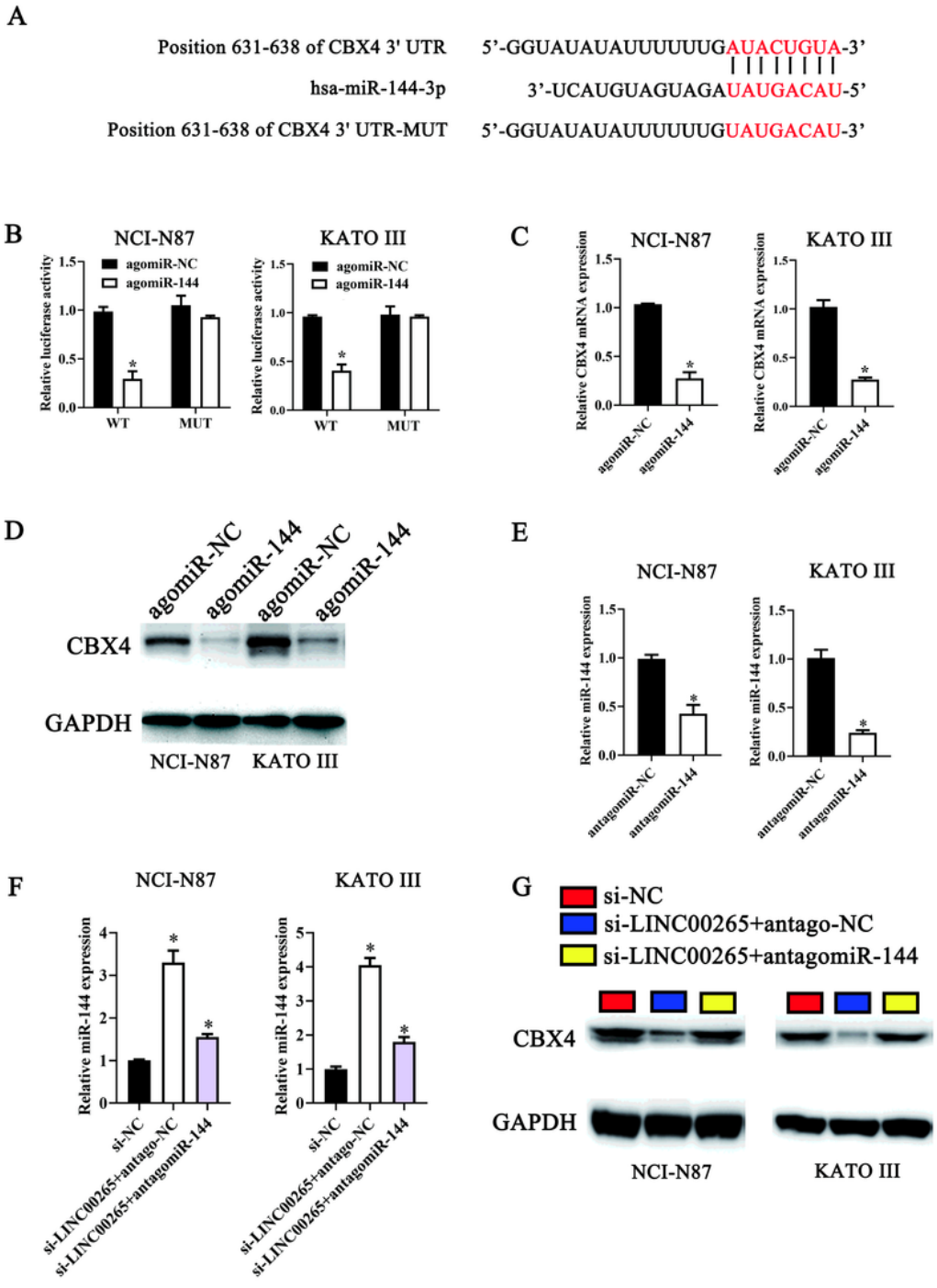

Figure 7

LINC00265 enhances the malignant characteristics of GC cells in vitro through the miR-144-3p/CBX4 axis (A) MiR-144 and its wild-type binding site in the 3'-UTR of CBX4 mRNA. The mutations were introduced into the site complementary to the seed region of miR-144. (B) The luciferase reporter assay was performed to test whether the 3'-UTR of CBX4 mRNA could be directly targeted by miR-144 in GC cells. NCI-N87 and KATO III cells were cotransfected with either agomiR-144 or agomir-NC and either the CBX4-wt or CBX4-mut plasmid. After 48 h of cultivation, the transfected cells were assayed with the Dual-Luciferase Reporter Assay System to measure the luciferase activity. *P < 0.05 vs. the agomir-NC group. (C, D) Expression levels of CBX4 mRNA and protein in miR-144-overexpressing NCI-N87 and KATO III cells were respectively determined by RT-qPCR and western blotting. *P < 0.05 vs. the agomir-NC group. (E) Expression levels of miR-144 in NCI-N87 and KATO III cells transfected with antagomiR-144 or antagomir-NC. (F, G) Si-LINC00265 in combination with either antagomiR-144 or antagomir-NC was transfected into NCI-N87 and KATO III cells. After 48 h transfection, expression levels of the CBX4 protein and miR-144 were determined respectively by western blotting and RT-qPCR. *P < 0.05 vs. group si-NC. #P < 0.05 vs. group si-LINC00265+antagomir-NC.