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LncRNA LINC01772 promotes metastasis and EMT process in cervical cancer by sponging miR-3611 to relieve ZEB1

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Abstract: Cervical cancer (CC), has been identified as one of the most frequent malignant tumors all over the world, with high mortality in females. A growing number of investigations have confirmed that long noncoding RNAs (lncRNAs) play a crucial role in the progression of multiple cancers. Nonetheless, the biological function and regulatory mechanism of LINC01772 in CC haven't been explored so far. In this study, LINC01772 expression was found to be upregulated in tissues and cells of CC. Knocking down LINC01772 suppressed CC cell proliferation, migration and epithelial-mesenchymal transition (EMT) process. Through molecular mechanism assays, LINC01772 was verified to be bound with miR-3611 and LINC01772 acted as a sponge for miR-3611. Zinc finger E-box binding homeobox 1 (ZEB1) was a downstream target gene of miR-3611. ZEB1 was negatively regulated by miR-3611 but positively regulated by LINC01772. Rescue assays confirmed that miR-3611 inhibitor or ZEB1 overexpression offset the inhibitive effect of LINC01772 depletion on cell proliferation, migration and EMT process in CC. In a word, our study validated that LINC01772 promoted cell metastasis and EMT process in CC by sponging miR-3611 to upregulate ZEB1 expression, indicating that LINC01772 could serve as a new therapeutic target for patients with CC.

Introduction

Cervical cancer (CC), a malignant tumor, has been treated as one of the most leading causes of cancer-related mortality in women (Bray *et al*., 2018). The tumorigenesis and progression of CC is a complicated biological process involving many factors and steps. Human papillomavirus (HPV) infection has been reported to be a high-risk factor for CC (Chelimo *et al*., 2013). The main treatment methods for CC patients are surgery, radiotherapy, and chemotherapy owing to lack of efficient molecular targeted therapy (Li *et al*., 2016; Regalado Porras *et al*., 2018). Despite multiple efforts to improve the prognosis of CC patients, the outcome remains unsatisfactory (Diaz-Padilla *et al*., 2013). Thus, it is crucial to study the molecular regulatory mechanisms in CC so as to figure out better therapies for CC patients.

Long noncoding RNAs (lncRNAs), with no protein coding capacity, have been regarded as a member of noncoding RNAs > 200 nucleotides in length (Boon *et al*., 2016; Cao, 2014). A growing number of evidences have confirmed that lncRNAs are involved in the complex biological progression of many cancers, including the proliferation, migration and invasion of cells (Cao, 2014; Gong *et al*., 2014; Zhao *et al*., 2019). It has been reported that lncRNAs contribute a lot to the tumorigenesis and progression of CC. For example, lncRNA *pvt1* promotes CC development by sponging *mir-424* (Gao *et al*., 2017). LncRNA *anril* facilitates CC development by sponging *mir-186* (Zhang *et al*., 2018). LncRNA *tug1* upregulation enhances the proliferation and migration of CC cells (Hu *et al*., 2017). Nevertheless, the specific role of *linc01772* in CC remains to be explored.

MicroRNAs (miRNAs), a kind of short noncoding RNAs which is shorter than 25 nucleotides, have been validated to play a significant role in the development of multiple cancers (Acunzo *et al*., 2015; Bartel, 2004; Chen *et al*., 2018). Numerous investigations have verified that miRNAs are involved in the progression of CC. For example, *mir-101-5p* suppresses CC cell proliferation and metastasis by inhibiting *cxcl6* (Shen *et al*., 2019). *Mir-187* represses the development of CC by regulating *fgf9* expression (Liang *et al*., 2017). *Mir-142* suppresses the growth of CC cells by modulating *hmgb1* expression (Jiang *et al*., 2017). However, the underlying function of *mir-3611* in CC has not been figured out yet.

In this present study, we intended to investigate the biological function and regulatory mechanism of *linc01772*

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in CC. Based on the results of our study, *linc01772* could facilitate metastasis and EMT process in CC by sponging *mir-3611* to upregulate *zeb1* expression, presenting a potential and novel therapeutic target for patients with CC.

Material and Methods

Cell culture and transfection

Human CC cells lines (SiHa, HeLa, CaSki and C33A) and normal cervical epithelial cell line H8 were bought from Ruilu Biotechnology Co., Ltd. (Xuhui, Shanghai, China). These cells were then cultured in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C with 5% CO_2 in humid air.

Sh-*linc01772*#1/2/3, pcDNA3.1/*linc01772*, pcDNA3.1/*zeb1*, *mir-3611* mimics, *mir-3611* inhibitor, as well as the negative controls were bought from GenePharma (Shanghai, China). Afterwards, transfect all plasmids into SiHa and HeLa cells by using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RT-qPCR

Total RNAs were separated by using trizol reagent (Takara, Otsu, Japan). And the Omniscript RT Kit (HaoranBio, Xuhui, Shanghai, China) and TaqMan™ Advanced miRNA cDNA Synthesis Kit (Waltham, MA, USA) were then respectively applied to synthesize complementary DNA. Subsequently, the SYBR PrimeScript RT-PCR kit (TaKaRa, Dalian, China) was utilized to conduct the RT-qPCR on ABI 7500 System (Applied Biosystems, Carlsbad, California). GAPDH and U6 served as internal controls. Relative expression of RNAs was calculated by using the $2^{-\Delta\Delta Ct}$ method.

Colony formation

Cells (1×10^3) were seeded into six-well plates. Afterwards, they were maintained at 37°C. After 14 days, cells were fixed by 4% paraformaldehyde, and then stained by 0.1% crystal violet solution (BaoMan Biotechnology, Xuhui, Shanghai, China). A microscope (XSP-11CC; Caikon, Jiading, Shanghai, China) was used to capture the images of the colonies and the colonies were then calculated.

CCK-8

Cell counting kit-8 (CCK-8) reagent (Beyotime Institute of Biotechnology, Shanghai, China) was utilized to perform CCK-8 assay in accordance with the manufacturer's suggestions. Transfected cells (1×10^3) were plated in the 96-well plates and cultured for 0, 24, 48, 72 and 96 h. Then each well was added with CCK-8 reagent. After 4 h incubation, the absorbance at 450 nm was measured for growth density.

Transwell

Transwell chambers (Corning Incorporated, Corning, NY, USA) without matrigel (BD Biosciences, Bedford, MA, USA) were used for migration assay. Transfected cells (1×10^3) were cultured in the upper chambers of the serum-free RPMI 1640 medium and RPMI 1640 medium containing 10% FBS was put into the lower chambers. After 48 h incubation, migrated cells were fixed by methanol and stained by crystal violet (Amresco Co., Solon, OH, USA). Consequently, the number of the stained cells was counted under a light microscope (Olympus Corporation, Tokyo, Japan).

Western blot

After cells were lysed, a BestBio Protein Isolation kit (BestBio, Pudong, Shanghai, China) was used to extract total proteins. Bicinchoninic Acid Assay Kit (Biodragon Biotech, Haidian, Beijing, China) was used for quantification of the proteins. And then the proteins were isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were cultured with primary antibodies at 4°C for 24 h after blocking with defat milk. Afterwards, incubate them with corresponding secondary antibodies for an hour at 37°C. GAPDH was an internal control.

Subcellular fractionation

PARIS™ Kit (Ambion, Austin, TX, USA) was applied to isolate cytoplasmic and nuclear RNAs based on the manufacture's protocol. SiHa and Hela cells (1×10^3) were prepared, re-suspended in cell fraction buffer and then incubated on ice. After 10 min incubation, the upper solution was removed after centrifugation. The nuclear pellet was obtained and kept to separate RNAs by using cell disruption buffer. After that, isolated RNAs were measured by RT-qPCR. U6 served as nucleus control and GAPDH served as cytoplasm control.

Luciferase reporter assay

The wild-type *linc01772*, mutant *linc01772*, wild-type *zeb1* 3' -untranslated region (3'-UTR) or mutant *zeb1* 3'-UTR was separately subcloned into pGL3 empty vectors by Miaoling Bioscience & Technology Co., Ltd. (Wuhan, Hubei, China). Then these vectors were individually cotransfected with *mir-3611* mimics or NC mimics into SiHa and HeLa cells. The luciferase activity was measured by a dual-luciferase assay kit obtained from Bosunlife Biotechnology Co., Ltd.

RIP assay

RIP assay was conducted by a Merck Millipore RNA-Binding Protein Immunoprecipitation Kit (HaoranBio, Xuhui, Shanghai, China) according to the manufacture's protocol. Cells were lysed, and the cell lysis was cultured with magnetic beads and Ago2 antibody in RIP buffer. After 24 h, wash the beads and incubate them with proteinase K to isolate the protein. Then purify them using the phenol-chloroform-isoamyl alcohol reagent. Subsequently, relative expression of RNAs was detected by RT-qPCR. IgG was a negative control.

Statistical analysis

SPSS 20.0 software (SPSS, Chicago, IL, USA) was adopted for statistical analysis. Data has been displayed as the mean ± standard deviation (SD). The one-way ANOVA or student's *t*-test was utilized for the comparisons among groups. Each experiment of this study was performed in triplicate. Any value of $p < 0.05$ was thought to be of statistical significance.

Results

Linc01772 expression is upregulated in tissues and cells of CC

Increasing investigations have suggested that lncRNAs participate in the progression of CC (Gao *et al.*, 2017; Zhang *et al.*, 2016; Zhang *et al.*, 2018). According to gene expression profile analysis, we found 500 lncRNAs with higher expression in CC tissues than that in adjacent non-tumor tissues (Fig. 1(A)). Additionally, lncRNAs (*linc01772*, *snhg22*, *far1-it1*, *muc20-ot1* and *mir600hg*), markedly upregulated in CC tissues were selected to be further studied. RT-qPCR assay depicted that *linc01772* expression was upregulated in comparison with other 4 lncRNAs in CC cells (Fig. 1(B)). Overall, the expression of *linc01772* is upregulated in tissues and cells of CC.

Linc01772 knockdown suppresses CC cell proliferation, migration and EMT process

To investigate the biological role of *linc01772* on CC progression, RT-qPCR assay was applied to examine the expression of *linc01772* in SiHa and HeLa cells transfected with sh-*linc01772*#1/2/3 or sh-NC as scramble control. The results suggested that *linc01772* expression was notably reduced by *linc01772* knockdown in SiHa and HeLa cells (Fig. 2(A)). CCK-8 and colony formation assays showed that *linc01772* knockdown inhibited the cell proliferation (Figs. 2(B)-2(C)). The capability of migration was remarkably decreased by *linc01772* depletion in SiHa and HeLa cells (Fig. 2(D)). In addition, western blot assay delineated that *linc01772* downregulation cut down the protein expression of N-cadherin, Vimentin and ZEB1 whereas increased the protein expression of E-cadherin in SiHa and HeLa cells, indicating that *linc01772* depletion inhibited EMT process in CC (Fig. 2(E)). Taken together, knockdown of *linc01772* represses CC cell proliferation, migration and EMT process.

Linc01772 acts as a sponge for mir-3611 in CC

Afterwards, we intended to explore the molecular mechanism of *linc01772* in CC. First, *linc01772* was mainly localized in cytoplasm based on the results of subcellular fractionation assay (Fig. 3(A)). Then, potential miRNAs (*mir-155-5p*, *mir-3611*, *mir-345-5p*, *mir-6512-3p*, *mir-6720-5p*, *mir-217*, *mir-6807-3p*, *mir-2467-3p*, *mir-3612*, *mir-650*, *mir-6884-5p*, *mir-485-5p* and *mir-1278*) which could possibly bind with *linc01772* were found from starBase. RT-qPCR assay was applied to examine the expression of these miRNAs in SiHa and HeLa cells transfected with sh-*linc01772*#1 or sh-NC as scramble control. The results demonstrated that the expression of *mir-3611*, *miR-6807-3p* and *miR-485-5p* was notably higher in sh-*linc01772*#1-transfected cells than that in sh-NC-transfected cells. Moreover, *mir-3611* expression was the most upregulated in comparison with other 12 miRNAs in sh-*linc01772*#1-transfected cells (Fig. 3(B)). Additionally, *linc01772* was found to have a binding site for *mir-3611* from starBase (Fig. 3(C)). As shown in Fig. 3(D), the luciferase activity of pGL3-*linc01772*-WT was decreased by *mir-3611* mimics while the luciferase activity of pGL3-*linc01772*-Mut showed no obvious change among different groups. RIP assay displayed that *linc01772* and *mir-3611* were enriched in Ago2 antibody group rather than in IgG antibody group (Fig. 3(E)). Collectively, *linc01772* sponges *mir-3611* in CC.

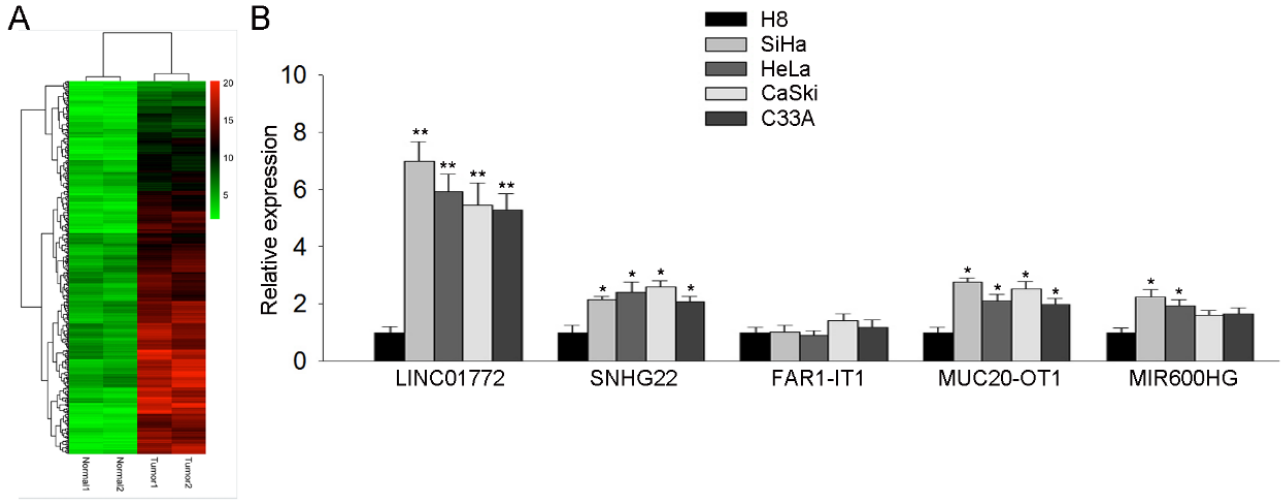

FIGURE 1. LINC01772 expression is upregulated in tissues and cells of CC. (A) The expression of 500 lncRNAs was found to be upregulated in CC tissues and adjacent non-tumor tissues by gene expression profile analysis. (B) RT-qPCR assay was conducted to detect the expression of 5 lncRNAs (LINC01772, SNHG22, FAR1-IT1, MUC20-OT1 and MIR600HG) in CC cells lines (SiHa, HeLa, CaSki and C33A) and normal cervical epithelial cell line H8. $^{*}p < 0.05$, $^{**}p < 0.01$.

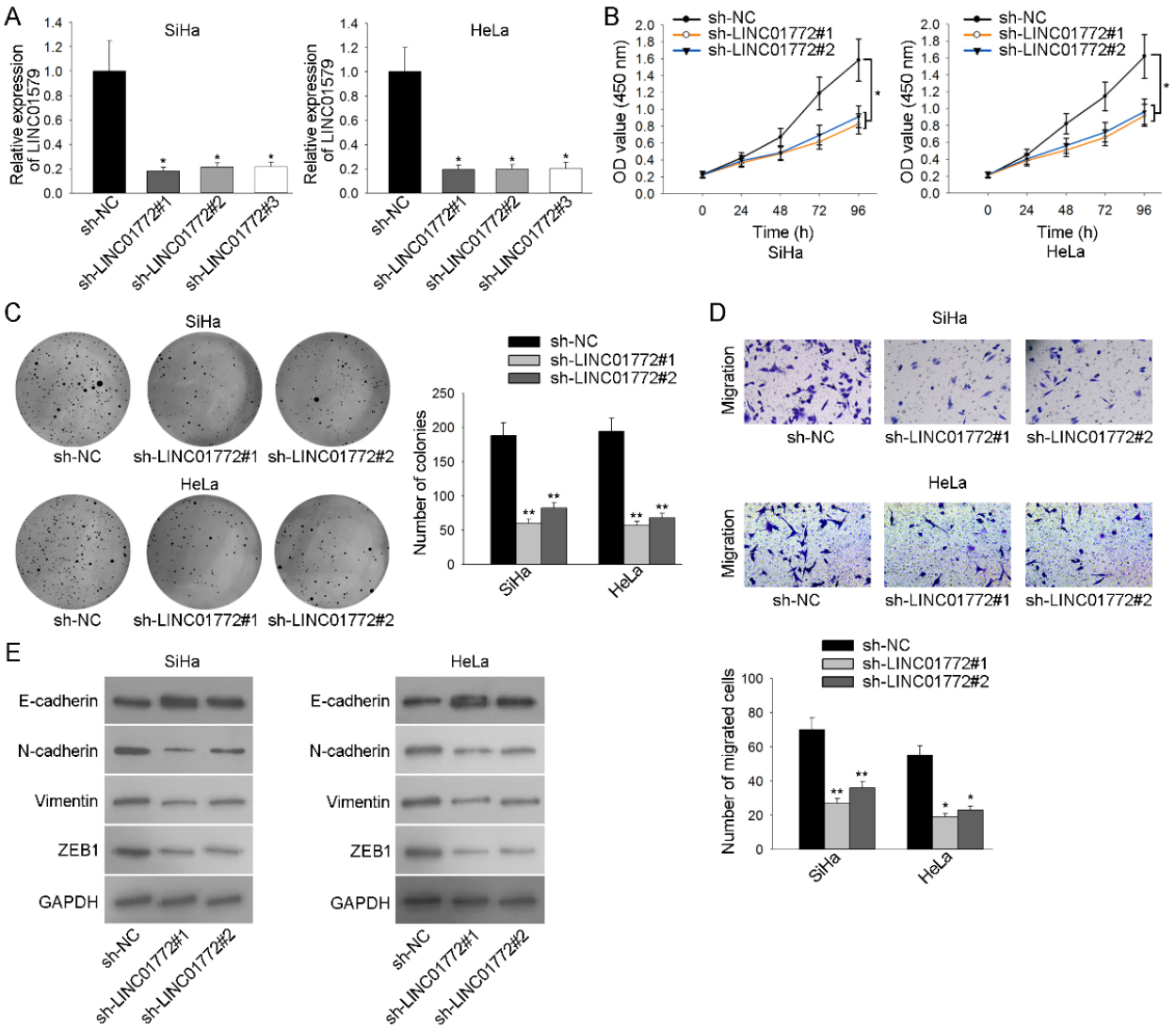

FIGURE 2. LINC01772 knockdown suppresses CC cell proliferation, migration and EMT process. (A) RT-qPCR assay was utilized to detect the expression of LINC01772 in SiHa and HeLa cells transfected with sh-LINC01772#1/2/3 or sh-NC. (B-C) CCK-8 and colony formation assays were performed to assess cell proliferation in SiHa and HeLa cells by transfection with sh-LINC01772#1/2 or sh-NC. (D) The migratory ability of transfected cells was measured by transwell assay. (E) Western blot assay was applied to examine the protein expression of E-cadherin, N-cadherin, Vimentin and ZEB1. GAPDH was an internal control. $^{*}p < 0.05$, $^{**}p < 0.01$.

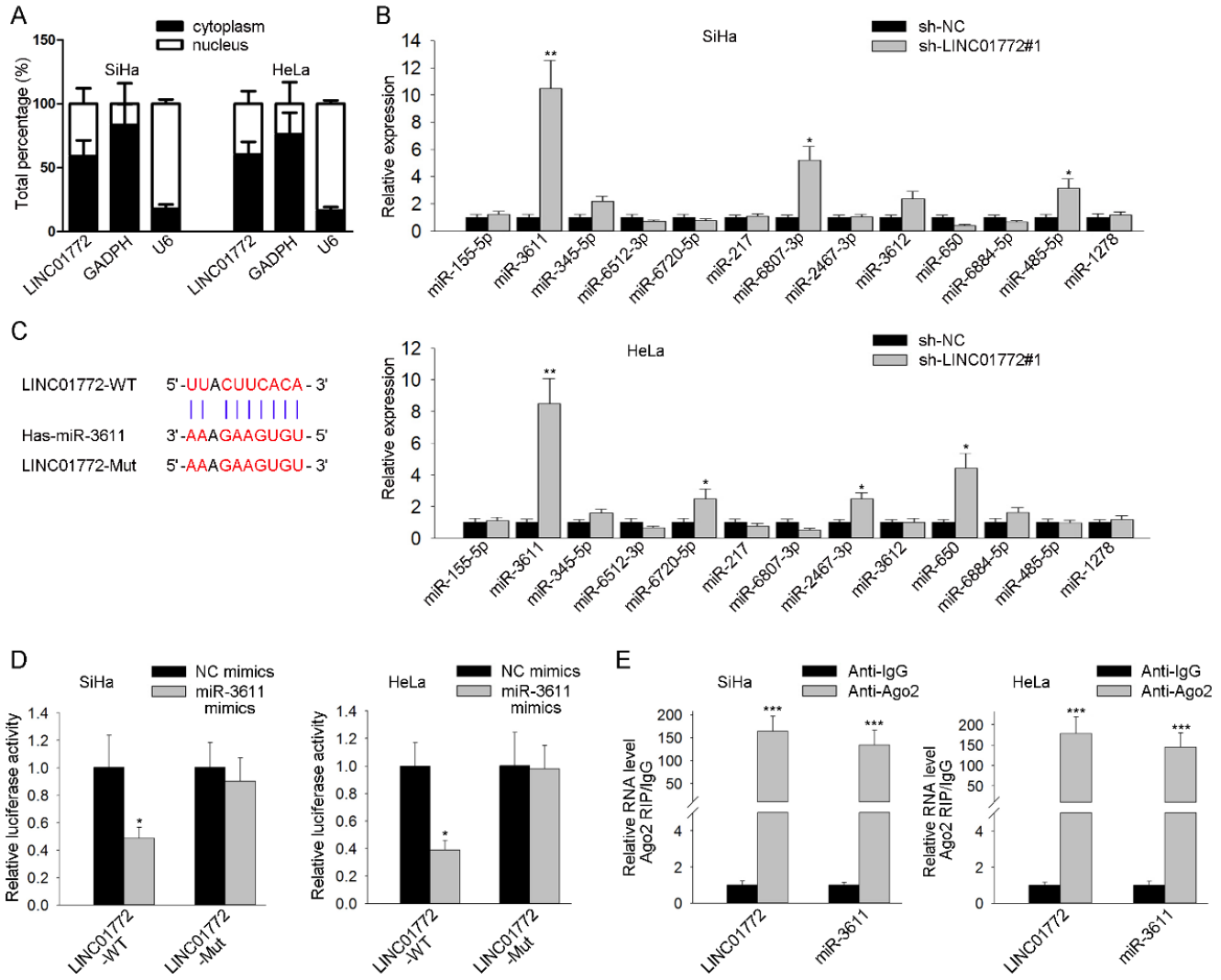

FIGURE 3. LINC01772 acts as a sponge for miR-3611 in CC. (A) The distribution of LINC01772 was probed by subcellular fractionation assay. (B) 13 predicted miRNAs that could bind with LINC01772 were chosen from starBase. The expression of these miRNAs was detected by RT-qPCR in SiHa and HeLa cells transfected with sh-LINC01772#1 or sh-NC. (C) LINC01772 had a binding site for miR-3611. (D-E) Luciferase reporter and RIP assays testified the interaction between LINC01772 and miR-3611. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Zeb1 is a target gene of mir-3611 in CC

Increasing evidences have suggested that miRNAs contributes to the development of CC by targeting specific genes (Liang *et al.*, 2017; Shen *et al.*, 2019). *Zeb1* was found to have a binding site for *mir-3611* through searching starBase (Fig. 4(A)). In addition, the luciferase activity of pGL3-*zeb1*-WT was observably decreased by *mir-3611* overexpression whereas the luciferase activity of pGL3-*zeb1*-Mut showed no evident change in transfected cells (Fig. 4(B)). Furthermore, *linc01772* overexpression reversed the luciferase activity of pGL3-*zeb1*-WT caused by *mir-3611* mimics while the luciferase activity of pGL3-*zeb1*-Mut showed no distinct change among different groups (Fig. 4(C)). In addition, *linc01772*, *mir-3611* and *zeb1* were aggregated in anti-Ago2 group but not in anti-IgG group (Fig. 4(D)). As illustrated in Fig. 4(E), the mRNA and protein expression of ZEB1 were conspicuously declined by *mir-3611* upregulation in SiHa and HeLa cells. Furtherly, RT-qPCR assay delineated that *zeb1* expression was dramatically cut down by *linc01772* deficiency in SiHa and HeLa cells (Fig. 4(F)). All the findings above validate that *zeb1* is a target gene of *mir-3611* in CC.

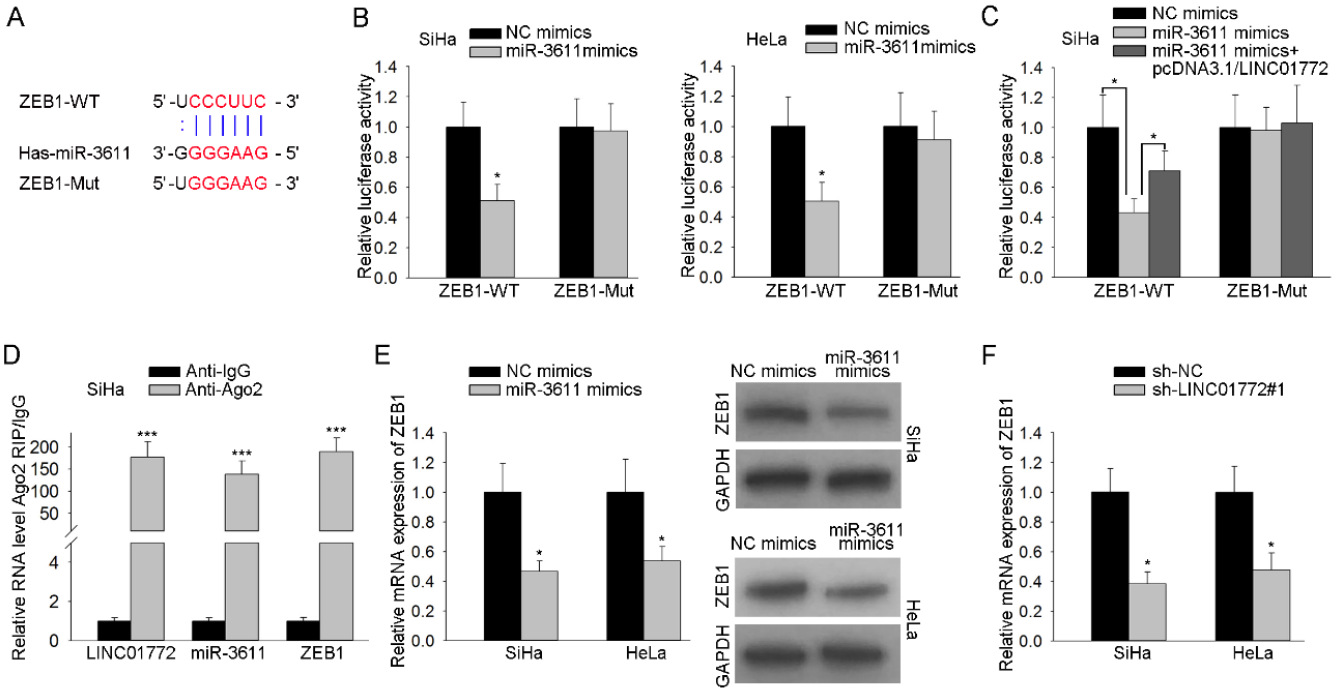

FIGURE 4. ZEB1 is a target gene of miR-3611 in CC. (A) ZEB1 had a binding site for miR-3611. (B-D) The interaction among LINC01772, miR-3611 and ZEB1 was confirmed by luciferase reporter and RIP assays. (E) The mRNA and protein expression of ZEB1 in transfected cells were analyzed by RT-qPCR and western blot assays. (F) A notably decrease of ZEB1 expression was observed by RT-qPCR in sh-LINC01772#1-transfected cells. *$p < 0.05$, ***$p < 0.001$.

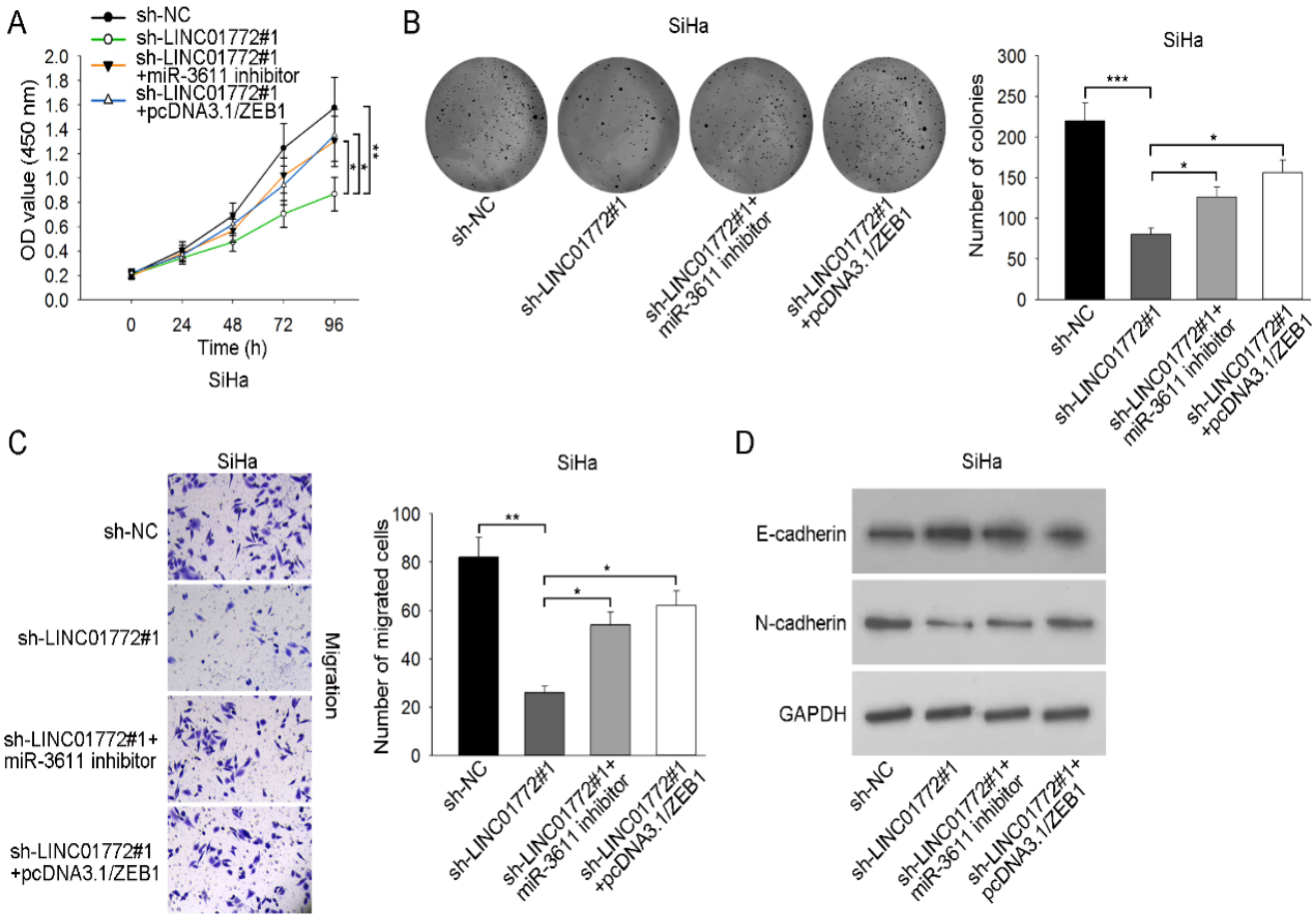

FIGURE 5. LINC01772 facilitates cell proliferation, migration and EMT process in CC by sponging miR-3611 to upregulate ZEB1 expression. (A-B) The proliferative capability of transfected cells was evaluated by CCK-8 and colony formation assays. (C) Transwell assay was adopted to examine the migratory ability of transfected cells. (D) The EMT process in transfected cells was measured by Western blot assay. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Linc01772 facilitates cell proliferation, migration and EMT process in CC by sponging mir-3611 to upregulate zeb1 expression

To proof whether *linc01772* contributed to CC progression by targeting *mir-3611*/*zeb1* axis, rescue assays were performed. As displayed in Figs. 5(A)-5(B), *mir-3611* inhibitor or *zeb1* overexpression countervailed the inhibitory function of *linc01772* knockdown on cell proliferation in CC. Similarly, *mir-3611* inhibitor or *zeb1* overexpression offset the inhibitory function on the migration of CC cells caused by *linc01772* depletion (Fig. 5(C)). Furtherly, *mir-3611* inhibitor or *zeb1* overexpression recovered the protein expression of E-cadherin and N-cadherin caused by *linc01772* deficiency, suggesting that *mir-3611* suppression or *zeb1* upregulation reversed the *linc01772* knockdown-mediated inhibitory function on EMT progression in CC (Fig. 5(D)). In summary, *linc01772* promotes cell proliferation, migration and EMT process in CC by sponging *mir-3611* to relieve *zeb1*.

Discussion

Deemed as a malignant tumor, CC has been reported to take a large proportion in cancer-related mortality in females (Bray *et al*., 2018). Many researchers have indicated that lncRNAs exert their function on the progression of various cancers. For instance, lncRNA *hif2put* inhibits the progression of osteosarcoma stem cells by regulating *hif2* expression (Zhao *et al*., 2019). LncRNA *snhg14* promotes the development of breast cancer by sponging *mir-193a-3p* (Xie *et al*., 2019). LncRNA *tug1* promotes prostate cancer progression via upregulating *dgcr8* (Yang *et al*., 2019). LncRNA *snhg12* promotes the progression of ovarian cancer by sponging *mir-129* to upregulate *sox4* expression (Sun *et al*., 2019). In this study, *linc01772* expression was tested to be upregulated in tissues and cells of CC. Knockdown of *linc01772* inhibited CC cell proliferation, migration and EMT process.

Former investigations have elucidated that lncRNAs induce the progression of cancers by sponging miRNAs. For illustration, lncRNA *rp4* promotes colorectal cancer progression by sponging *mir-7-5p* (Liu *et al*., 2018). LncRNA *peg10* facilitates cell growth in human bladder cancer by regulating *mir-134* expression (Jiang *et al*., 2019). LncRNA *snhg7* promotes cell proliferation of pancreatic cancer through *id4* by sponging *mir-342-3p* (Cheng *et al*., 2019). In this work, *linc01772* had a binding site for *mir-3611* and *linc01772* could bind with *mir-3611* in CC.

Zeb1 has been reported to elicit an oncogenic impact on the tumorigenesis and development of cancers. For example, *lbx2-as1*, activated by *zeb1*, accelerates esophageal squamous cell carcinoma progression by modulating *hnrnpc* to improve the stability of *zeb1* and *zeb2* (Zhang *et al*., 2019). *Circ-cspp1* promotes cell development in ovarian cancer by sponging *mir-1236-3p* (Li *et al*., 2019). *Siah1*/*zeb1*/*il-6* axis was associated with the doxorubicin resistance of cells in osteosarcoma (Han *et al*., 2019). Nevertheless, the specific role of *zeb1* in CC still needs to be investigated. Our study proved that *zeb1* was a downstream target for *mir-3611*. Molecular mechanism assays demonstrated that *zeb1* was negatively regulated by *mir-3611* but positively regulated by *linc01772*. Rescue assays verified that *mir-3611* suppression or *zeb1* upregulation reversed the *linc01772* knockdown-mediated inhibitory effect on cell proliferation, migration and EMT progression in CC.

To sum up, this study confirmed that *linc01772* contributed to metastasis and EMT progression in CC via *mir-3611*/*zeb1* axis, which provided a new insight for researchers to figure out better treatments for CC patients.

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Conflicts of Interest

No conflicts of interest exist.

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