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 hmgbl 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...
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 cell lines (SiHa, HeLa, GaSki 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% CO₂ 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). 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 were used for growth density.

 Colony formation
Cells (1 × 10⁵) 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 × 10⁵) 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 × 10⁵) 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 × 10⁵) 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 IncRNAs 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 IncRNAs with higher expression in CC tissues than that in adjacent non-tumor tissues (Fig. 1(A)). Additionally, IncRNAs (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 IncRNAs 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 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.
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)-(B), miR-3611 inhibitor or ZEB1 overexpression counteracted 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)). Furthermore, 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 hif2psi 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 tgf1 promotes prostate cancer progression via upregulating dgrc8 (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 rp4p 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-csp1 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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