LncRNALOXL1-AS1 Regulates the Proliferation and Apoptosis of Ovarian Cancer Cells by Targeting miR-761

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ABSTRACT

The aim of this study was to investigate the molecular mechanism of lncRNALOXL1-AS1 regulating the proliferation and apoptosis of ovarian cancer cells by targeting miR-761. Ovarian cancer cells SKOV-3 and normal ovarian cells FTE187 were divided into groups according to the experimental procedures, and were transfected negative, respectively. The expressions of lncRNALOXL1-AS1 and miR-761 in ovarian cancer cells were measured by qRT-PCR (quantitative real-time PCR). LncRNALOXL1-AS1 targeting miR-761 was detected by dual luciferase reporter gene assay. CCK8 and flow cytometry were used to detect the proliferation and apoptosis of ovarian cancer cells in each group. Compared with normal ovarian epithelial cell FTE187, the expression of LOXL1-AS1 in ovarian cancer SKOV-3 cells was significantly up-regulated and the expression of miR-761 was significantly down-regulated; compared with the miR-NC group, the expression level of miR-761 in SKOV3 cells was significantly increased in the miR-761 group; after LOXL1-AS1 interference, SKOV3 cell proliferation and colony formation were significantly decreased. miR-761 expression was up-regulated and apoptosis rate was increased. The biological behavior of the LOXL1-AS1+ miR-inhibitor NC group was similar to that of the LOXL1-AS1 siRNA group, and miR-inhibitor treatment could reduce the above effects of LOXL1-AS1 on cells. Further studies showed that the mechanism of LOXL1-AS1 action on ovarian cancer cells is related to the regulation by targeting miR-761. The regulation of LOXL1-AS1 may be an effective approach for the treatment and prevention of ovarian cancer.

INTRODUCTION

Ovarian cancer is the most fatal gynecological malignant tumor, with high morbidity and mortality around the world. It is also the fifth largest cancer-related death cause in the world (Shi et al., 2019). Due to the lack of typical symptoms, most cases were diagnosed as advanced diseases. Metastasis which already existed in the diagnosis of advanced diseases, resulted in poor prognosis (Dong et al., 2014). Unfortunately, despite encouraging progress in chemotherapy, surgery and sometimes radiotherapy, the 5-year survival rate of ovarian cancer in all stages is estimated to be 35-38%. Ovarian cancer accounts for more than 90% of all malignant ovarian tumors, which is mainly a disease of postmenopausal women. It is most common in the sixty or seventy years of life. About 13,850 women die of ovarian cancer every year, which is the most common cause of death of women with gynecological malignant tumors (Jelovac and Armstrong, 2011). Therefore, it is urgent to find new biomarkers to predict tumor occurrence and clinical diagnosis, have an in-depth understanding of the molecular mechanism of ovarian cancer pathogenesis, development and progression, and explore new therapeutic strategies and targets for ovarian cancer (Moric et al., 2019).

Non-coding RNA has complex functions, including microRNAs (miRNAs) and long-chain non-coding RNA (lncRNAs). LncRNA plays a key role in malignant tumor. Loxl1 antisense RNA 1 (LOXL1-AS1) of lncRNA has been proven to be a pre-oncogene in many tumor types. A large number of studies have shown that lncRNA can control the level of miRNA target by tricking miRNA into competing as endogenous non-coding RNA.
LncRNA as a kind of RNA with more than 200 nucleotides in length has no protein coding function. Studies have shown that the disorder of IncRNA expression is associated with the tumorigenesis (Qi and Du, 2013). The IncRNA shows various biological functions in various stages of cancer development, including cell development and immunity, cell proliferation and differentiation, apoptosis regulation, serving as a genomic imprinting marker as well as a competitive endogenous RNA (ceRNAs) sharing the adhesion site of miRNAs (Kogo et al., 2011; Sasaki et al., 2000). More and more researches on ovarian cancer focus on non-protein coding genes. The abnormal regulation of IncRNA leads to the malignant phenotype changes of ovarian cancer to a great extent (Wang et al., 2011; Mitra et al., 2017; Li et al., 2014). Therefore, IncRNA can be used as a biomarker for the treatment of ovarian cancer.

miRNAs are endogenous, small, non-coding RNA, which can regulate the instability and translation inhibition of miRNAs by combining with partial complementary sites of the target gene 3-UTR (Li et al., 2015). Growth studies have shown that miRNA is involved in many cellular processes, including development, differentiation, apoptosis, migration, invasion and proliferation (Zhao et al., 2012). There is also increasing evidence that miRNAs are abnormally regulated in various cancers such as gastric cancer, bladder cancer, breast cancer, osteosarcoma, lung cancer and hepatocellular carcinoma (Li et al., 2015). A large number of studies have shown that miRNA is not well expressed in cancer and can be used as both tumor suppressor and carcinogen. miR-761 has been proven to be closely related to the initiation and progression of several types of cancer. Although the study of miRNAs is still challenging due to the changes of downstream genes, the application of miRNAs in ovarian cancer management is vitally important (Tripathi et al., 2018). Therefore, the identification of new biomarkers will help us improve the living quality of cancer patients. miR/miRNAs are a kind of non-coding RNA, which can be combined by 3'-UTR, 5'-UTR or open reading frame to regulate the expression of target genes. A large number of studies have shown that miRNA is not well expressed in cancer and can be used as both tumor suppressor and carcinogen. miR-761 has been proved to be closely related to the initiation and progression of several types of cancer. It has been reported that the expression of miR-761 in ovarian cancer tissues is significantly lower than that in its paired non-cancerous tissues. Functional test has shown that miR-761 inhibits the proliferation and invasion of ovarian cancer by targeting MSI1 (Zhou et al., 2016). The aim of this study was to investigate the molecular mechanism of IncRNA LOXL1-AS1 regulating the proliferation and apoptosis of ovarian cancer cells by targeting miR-761.

MATERIALS AND METHODS

Cells and main reagents

Human ovarian cancer cells SKOV-3, immortalized normal human oviduct epithelial cell line (FTE187), trypsin, miR-761 mimics, negative control (NC), plasmid extraction kit, Trizol reagent, fluorescence quantitative kit, Annexin V-FITC/ PI apoptosis detection kit, fetal bovine serum, RPMI1640 culture medium, Lipofectamine 2000 transfection reagent, TRIzol reagent and RIPA lysis buffer were used in this study. SKOV-3 was preserved in McCoy 5A modified medium (Gibco, Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco).

Cell culture

An appropriate amount of ovarian cancer cells and normal ovarian cells suspension were added into RPMI1640 medium containing 10% fetal bovine serum. The mixture was cultured at 37℃, 5% CO₂ and 70% to 80% humidity in incubator. The culture medium was changed the next day. Cells were digested and collected with 0.25% trypsin when the cell density reached 80% to 90%

Experimental plan

According to the experimental requirements, four groups were set up (i) LOXL1-AS1 mimic, mimic-NC, (ii) LOXL1-AS1siRNA, siRNA-NC, (iii) miR-761 mimic, (iv) mimic NC. Lipofectamine 2000 transfection reagent was used to transfect cells with the above groups. After transfection, cells were digested with 0.25% trypsin, and 2x10⁵ cells in logarithmic growth phase were inoculated in 6-well plates. After 24h, the cell growth was observed under an inverted microscope until the cell density reached 30% to 50%. The effects of LOXL1-AS1 on ovarian cancer cells were analyzed by using LOXL1-AS1 overexpression vector and empty vector control (mimic-NC group), LOXL1-AS1 interference vector (LOXL1-AS1siRNA group) and control (siRNA-NC group). The effects of miR-761 on ovarian cancer cells were analyzed by miR-761 mimic (miR-761 mimic group) and mimic NC (NC group). LOXL1-AS1 interference vector (LOXL1-AS1siRNA group), control group (siRNA-NC group), LOXL1-AS1 interference vector (LOXL1-AS1siRNA group), control group (siRNA-NC group), LOXL1-AS1 interference +miR-761 inhibitor (LOXL1-AS1siRNA +miR inhibitor NC group) and LOXL1-AS1 interference + inhibitor NC (LOXL1-AS1siRNA+miR inhibitor NC group) were used to analyze whether LOXL1-AS1 acts through miR-761.

Quantitative PCR

The expression of IncRNA LOXL1-AS1 and miR-761
in ovarian cancer cells was measured by qRT-PCR, and the total RNA was extracted from ovarian cancer tissues by TRIzol reagent, taking the kit instructions as the standard. One Step SYBR PrimeScript TM qPCR Kit was used for detection on 7300 real-time fluorescence quantitative PCR instrument, and the detection was carried out 40 times at 95°C for 2 min, 95°C for 30s, 60°C for 30s and 72°C for 30s. The relative expression of LOXL1-AS1 and miR-761 was calculated by 2^(-△△CT) method. LOXL1-AS1 used β-actin as internal reference, and miR-761 used U6 as internal reference.

**Dual luciferase analysis**

Bioinformatics software Target Scan predicted that miR-761 was the target gene of lncRNA LOXL1-AS1. In this study, the wild 3’ UTR (WT-lncRNA LOXL1-AS1) and the mutant 3’UTR (MUT-lncRNA LOXL1-AS1) of lncRNA LOXL1-AS1 were connected with the dual fluorescein vector (pMIR-Report Luciferase vector). They were co-transfected with miR-NC and miR-761-mimics, respectively. The luciferase activity was detected by dual luciferase detection kit and GloMax 20/20 luminescence detector.

**Clone molding**

Cells in logarithmic growth phase were digested with 0.25% trypsin and then blown into single cells. Then, the cells were suspended in complete medium and diluted by gradient dilution method. Each group of cells was inoculated in a 37°C pre-temperature culture dish with a cell density of 200. Cells were cultured at 37°C and 5% CO2 for 2 weeks. After that, they were fixed with 4% paraformaldehyde for 15 min, dyed with crystal violet solution (RBG1019- 1, role-bio) for 30 min, and washed with ultrapure water twice. After air drying, images were taken.

**Determination of cell count**

Cell proliferation was measured by CCK8 method. The cell density was 1x10^5 cells/ml, and each well was 100 µL. The cells were placed in a 96-well plate with three parallel wells. After 24h, 48h, 72h and 96h, CCK-8 solution (PA137267, Pierce) was added to each well. And after culture for 4h, the absorbance (OD) was measured by enzyme-linked immunosorbent assay at 450 nm.

**Assay of apoptosis**

The Annexin V/PI apoptosis kit was used to detect apoptosis according to the instructions. SKOV-3 cells were inoculated into a 6-well plate (2x10^5 cells/well), cultured in complete medium for 48h, digested and collected with trypsin, washed with frozen PBS, and resuspended with 100µl binding buffer. Then 5µl annexin V-FITC and 5µl PI working solution (100 µg/ml) were added into 100µl cell suspension. The cell suspension was cultured at room temperature in the dark for 15 min. Then, 400µl of binding buffer was added to the cell suspension. The samples were analyzed by flow cytometry. Each sample was tested three times.

**Western blotting experiment**

RIPA lysis buffer (pH=7.5) was used to extract the total protein and generate the whole lysate. The same amount of 40µg protein was loaded into 12% SDS-PAGE gel to separate the protein by electrophoresis. After electrophoresis, protein was transferred to polyvinylidene fluoride (PVDF) membrane. After blocking the nonspecific antigen with 5% skim milk, the primary antibody was incubated at 4°C overnight. Then the secondary antibody which can recognize the primary antibody was added, and the immunoactivity was determined by enhanced chemiluminescence autoradiography (Thermo Scientific, PA, USA). GAPDH was developed synchronously for load control. The sequence of primers is shown in Table 1.

**Table 1. Sequence of primers used.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOXL1-AS1</td>
<td>U 5′-GATATGTTGGATGATGGA-3′</td>
</tr>
<tr>
<td></td>
<td>D 5′-GATATGTTGGATGATGGA-3′</td>
</tr>
<tr>
<td>mir-76</td>
<td>U 5′-ACAGCAGGCCACAGAC-3′</td>
</tr>
<tr>
<td></td>
<td>D 5′-GAGCAGGCTGGAGAA-3′</td>
</tr>
<tr>
<td>U6</td>
<td>U 5′-CTCAGTCCAGCCACCATAT-3′</td>
</tr>
<tr>
<td></td>
<td>D 5′-ACGGTTCACAGGATCTGTC-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>U 5′-TTTTGACAGTGCCGCGC-3′</td>
</tr>
<tr>
<td></td>
<td>D 5′-GGTGTCTGAGGATGTCG-3′</td>
</tr>
</tbody>
</table>

U, upstream; D, downstream.

**Statistical analysis**

SPSS 19.0 statistical analysis software was used for data processing, and the data analysis results were expressed by mean standard deviation (SD). One-way ANOVA was used to analyze the data among multiple groups. Tukey test was used for subsequent analysis. p<0.05 indicated statistically significant difference between the two groups.

**RESULTS AND DISCUSSION**

RT-qPCR results showed that compared with those in normal ovarian epithelial cells FTE187, LOXL1-AS1 expression in ovarian cancer cells SKOV-3 was significantly up-regulated and miR-761 expression was significantly down-regulated (p < 0.05, Table II).
Table II. Expression of LOXL1-AS1 and miR-761 in SKOV-3 cells and FTE187 cells (x±s, n = 3).

<table>
<thead>
<tr>
<th>Groups (cells)</th>
<th>LOXL1-AS1</th>
<th>miR-761</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTE187</td>
<td>1.00±0.13</td>
<td>1.00±0.12</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>2.84±0.32</td>
<td>0.40±0.03</td>
</tr>
</tbody>
</table>

Compared with miR-NC group, the expression level of miR-761 in SKOV3 cells of miR-761 group was significantly higher. The expression levels of cyclinD1, MMP2 and MMP9 decreased significantly, and the cell survival rate decreased significantly. The number of migrating cells decreased significantly (P <0.05, Fig. 1, Table III).

Table III. Effect of high expression of miR-761 on proliferation and apoptosis of SKOV3 cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>miR-761</th>
<th>CyclinD1</th>
<th>MMP9</th>
<th>MMP2</th>
<th>Cell survival rate</th>
<th>Apoptosis rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR- NC</td>
<td>1.00±0.11</td>
<td>1.15±0.12</td>
<td>0.98±0.10</td>
<td>0.86±0.09</td>
<td>100.86±10.08</td>
<td>6.78±0.69</td>
</tr>
<tr>
<td>miR-761</td>
<td>2.89±0.30*</td>
<td>0.38±0.04*</td>
<td>0.45±0.05*</td>
<td>0.37±0.04*</td>
<td>61.27±6.13*</td>
<td>18.2±1.81*</td>
</tr>
</tbody>
</table>

At first, LOXL1-AS1 interfering cell lines need to be constructed. qRT-PCR results showed that the expression of miR-761 in LOXL1-AS1 siRNA group was significantly higher than that in si-NC group (p < 0.05). The results of MTT assay showed that the proliferation activity of ovarian cancer cells in si-LOXL1-AS1 group was significantly lower than that in si-NC group (p < 0.05).

It can be seen from Figure 2 that the expression of LOXL1-AS1 was significantly down-regulated in LOXL1-AS1 siRNA group and significantly up-regulated in LOXL1-AS1 simulation group (p<0.05). The proliferation ability and colony formation of SKOV3 cells significantly decreased after LOXL1-AS1 interference (Fig. 2b, c, p<0.05). The results of flow cytometry showed that LOXL1-AS1 interference promoted apoptosis (Fig. 2d, p<0.05). In contrast, LOXL1-AS1 simulation group showed completely opposite results. The results indicated that LOXL1-AS1 could regulate the proliferation and apoptosis of ovarian cancer cells.

Table III. Effect of high expression of miR-761 on proliferation and apoptosis of SKOV3 cells.
LncRNA LOXL1-AS1 Regulates the Proliferation and Apoptosis of Ovarian Cancer Cells

Table IV. Effect of LOXL1-AS1 inhibition on proliferation of ovarian cancer cells SKOV3 (x±s, n=3).

<table>
<thead>
<tr>
<th>Group</th>
<th>LOXL1-AS1</th>
<th>Cell viability (OD 490 nm)</th>
<th>Culture for 24 h</th>
<th>Culture 48 h</th>
<th>Culture 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si-NC</td>
<td>1.00±0.07</td>
<td>0.38±0.03</td>
<td>0.70±0.05</td>
<td>1.15±0.09</td>
<td></td>
</tr>
<tr>
<td>si-LOXL1-AS</td>
<td>0.43±0.05b</td>
<td>0.35±0.03a</td>
<td>0.42±0.04b</td>
<td>0.47±0.05b</td>
<td></td>
</tr>
</tbody>
</table>

Table V. Dual luciferase report experiment (x±s, n=9).

<table>
<thead>
<tr>
<th>Group</th>
<th>WT-LOXL1-AS1</th>
<th>MUT-LOXL1-AS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-NC</td>
<td>1.00±0.08</td>
<td>0.97±0.09</td>
</tr>
<tr>
<td>miR-NC</td>
<td>0.43±0.04a</td>
<td>0.99±0.09a</td>
</tr>
</tbody>
</table>

Fig. 3. LOXL1-AS1 negatively regulated the expression of miR-761.

LOXL1-AS1 siRNA group, siRNA-NC group, LOXL1-AS1 siRNA+miR inhibitor group and LOXL1-AS1 siRNA+miR inhibitor NC group were constructed with siRNA, and their proliferation ability (Table VI), colony formation (Fig. 4a) and apoptosis (Fig. 4b) and other biological behavior were detected. The results showed that after LOXL1-AS1 interference, cell proliferation and colony formation were inhibited, but apoptosis rate increased. The biological behavior of LOXL1-AS1+miR inhibitor NC group was similar to that of LOXL1-AS1 siRNA group. Treatment with miR inhibitor could weaken the above-mentioned effects of LOXL1-AS1 on cells. The above results suggested that LOXL1-AS1 regulated the proliferation and apoptosis of ovarian cancer cells by regulating miR-71.

Fig. 4. LOXL1-as1 regulates proliferation and apoptosis of ovarian cancer cells by regulating mir-761 (a. Proliferation of ovarian cancer cells; b. Apoptosis map of ovarian cancer cells).

Table VI. Cell proliferation.

<table>
<thead>
<tr>
<th>Group</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
<th>96h</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA-NC</td>
<td>0.31</td>
<td>0.52</td>
<td>0.63</td>
<td>0.65</td>
</tr>
<tr>
<td>LOXL1-AS1 siRNA</td>
<td>0.24</td>
<td>0.37</td>
<td>0.39</td>
<td>0.42</td>
</tr>
<tr>
<td>LOXL1-AS1 siRNA+inhibitor NC</td>
<td>0.3</td>
<td>0.36</td>
<td>0.39</td>
<td>0.41</td>
</tr>
<tr>
<td>LOXL1-AS1 siRNA+miR inhibitor</td>
<td>0.31</td>
<td>0.45</td>
<td>0.60</td>
<td>0.63</td>
</tr>
</tbody>
</table>

DISCUSSION

Epithelial ovarian cancer is the most fatal gynecological malignant tumor, largely because most patients are diagnosed at advanced stage. Screening strategies using ultrasound and cancer antigen (CA) 125 tumor markers are currently under study, which may reduce the diagnosis stage. But there is no evidence to improve the survival rate. Although most ovarian cancer patients respond to initial chemotherapy, most of them will eventually relapse. Chemotherapy for recurrent diseases includes: platinum-based multi-drug treatment for women who have relapsed for more than 6-12 months after the end of the initial treatment; for women with early recurrence, new targeted biological agents, especially those involving vascular endothelial growth factor pathway and those targeting poly (ADP-ribose) polymerase (PARP), are highly possible to improved the prognosis of ovarian cancer (Wu et al., 2020). Targeted therapy is an innovative treatment for malignant tumors. At present, the research is to inhibit or kill cancer cells by specific factors. For ovarian cancer, scholars pay more attention to the effect of molecular mechanism on molecular targeted therapy.

The signal network of lncRNA regulating cancer development is complex and needs further study. Studies
have shown that IncRNA can regulate the expression of genes through complementary binding with miRNA. In this study, we found that LOXL1-AS1 could complement miR-761 and regulate the proliferation and apoptosis of ovarian cancer cells by targeting miR-761. LOXL1-AS1 is a newly discovered cancer-promoting IncRNA, which is involved in the occurrence of various cancers (Wang et al., 2012; Sun et al., 2018). We studied several basic characteristics of ovarian cancer and found that LOXL1-AS1 could regulate the proliferation and apoptosis of ovarian cancer cells in vivo and in vitro. miR-761 is used as a tumor suppressor gene in some cancers such as lung cancer and gastric cancer (Sasaki et al., 2000). Database analysis and dual luciferase analysis shows that LOXL1-AS1 can complement miR-761. In vitro and in vivo experiments further show that LOXL1-AS1 regulates the proliferation and apoptosis of ovarian cancer cells by regulating miR-761.

CONCLUSION

To sum up, IncRNA LOXL1-AS1 was up-regulated in ovarian cancer tissues, interfering with the expression of ovarian cancer significantly, effectively inhibiting the rapid reproduction of ovarian cancer cells and accelerating the apoptosis of cancer cells. Its mechanism is related to the targeted regulation of miR-761 expression. In addition, the expression of miR-761 was down-regulated in ovarian cancer tissues, and the overexpression of miR-761 had a certain anti-ovarian cancer effect. Many experimental data prove that IncRNA LOXL1-AS1 may promote the biological function of ovarian cancer cells. As the experimental data proved, the expression of IncRNA LOXL1-AS1 significantly decreased the proliferation of SKOV3 cells and the expression of CyclinD1 and Bcl-2 protein at 24h, 48h and 72 h, and greatly improved the apoptosis rate and the expression of p21 and Bax protein. And these data show that IncRNA LOXL1-AS1 and oncogene play a role in ovarian cancer. This study provided a valuable biomarker and therapeutic target for ovarian cancer. Human beings have made more and more in-depth research on the non-coding region of genome. Many studies have shown that IncRNA and miRNA have mutual regulation in the development of tumor, which is of great significance to the early detection and treatment of tumor.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES


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