Original Article
Long non-coding RNA LNC01133 promotes the tumorigenesis of ovarian cancer by sponging miR-126

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Abstract: Background: Ovarian cancer (OC) is the gynecologic malignancy with the highest mortality rate (70%), and it is urgent to find out a powerful prognostic marker for OC patients. LncRNAs are recently thought to be oncogenes in various cancers, and its expression levels are validated that can be inhibited by miRNAs. There are several studies indicating that sponging miRNAs will contribute to the tumorigenesis of cancers. Methods: In the present study, bioinformatics analysis is used to explore the potential oncogene and its target miRNAs; QRT-PCR is performed to count the expression level of several genes; Flow cytometric analysis is conducted to assess the apoptosis rate of several cell lines; Western blot assays are used to evaluate the expression levels of several proteins; Cells proliferation, migration and invasion abilities are detected by CCK-8 assay, Wound scratch assay and Transwell invasion assay, respectively. In vivo experiments are performed to assess the influence of LNC01133 on the formation of tumor. Results: We found LNC01133 was related to poor survival of OC patients, and identified that LNC01133 had significant influence on OC cells’ apoptosis, proliferation, migration and invasion abilities. Furthermore, we observed miR-126 could target LNC01133 and decreased the expression level of LNC01133 in OC cells. Therefore, we sponged miR-126 to further study the molecular mechanism of OC tumorigenesis, and found an elevation in proliferation, migration and invasion abilities of OC cells, which suggested that miR-126 could serve as a powerful prognostic marker for OC patients, and had great clinical significance on OC diagnosis and treatment. Conclusion: We found LNC01133 was an oncogene in OC, which is targeted by miR-126, miR-126 served as a powerful prognostic marker for OC patients because of its ability of promoting OC tumorigenesis after sponging.

Keywords: Long non-coding RNA, LNC01133, miR-126, tumorigenesis, ovarian cancer

Introduction

Ovarian cancer (OC) is a member of the frequent malignant tumors in the female reproductive system. Though its incidence rate is lower than that of cervical cancer and uterine cancer, the mortality rate is the highest among gynecologic malignancies. Because of the late clinical symptoms, the lack of effective early diagnosis, and the rapid disease progress, more than 70% of OC patients are diagnosed in advanced stage [1, 2]. In addition, chemotherapy resistance is another major reason of high ovarian cancer mortality [3, 4]. At present, the mechanism of occurrence, metastasis, relapse, and drug resistance of ovarian cancer is not completely clear. It is very important to improve the prognosis of ovarian cancer by finding the relevant target (i.e. prognosis marker) for early diagnosis and effective treatment.

Long non-coding RNAs (lncRNAs) are RNAs lacking protein-coding capacity, and whose length are about 200 nucleotides. Initially, lncRNAs were thought to be the spurious transcriptional noise and so that be rarely studied. But recently, lncRNAs have gotten attention because of its new role --- regulators of many biological processes, including post-transcriptional regulation, and RNA splicing. Growing evidence demonstrates that lncRNAs probably play a significant role in promoting the tumorigenesis of cancer [5-9]. Therefore, it is very necessary and significant to study how lncRNAs function in the tumorigenesis of various cancers.

MicroRNAs (miRNAs) are another type of non-coding RNA. They are single stranded RNAs comprised of 21-24 nucleotides, and are related to the post-transcriptional regulation of
mRNA as well [10, 11]. miRNAs can use the seed sequences to degrade or inhibit the translation of mRNAs by targeting their 3' UTRs [12]. Several reports had indicated that miRNAs had an ability to knock out IncRNAs, and served as powerful prognostic markers for cancer patients [9, 13-15].

In this study, we discovered the high LNC01133 expression level was related to poor survival of OC patients. Through a series of experiments, we verified that LNC01133 was connected with the apoptosis, proliferation, migration, and invasion of OC cells. Meanwhile, we found LNC01133 was negatively controlled by miR-126, and sponging miR-126 would lead to an elevation in proliferation, migration, and invasion of OC cells, which suggested that miR-126 could serve as a powerful prognostic marker for OC patients.

Materials and methods

Bioinformatics analysis

The information about expression levels of LNC01133 and miRNA in OC patients from GEO were obtained from the exon expression dataset. This dataset was downloaded from the UCSC Cancer Browser, a web-based tools that could participate in the visualization, integration and analysis of cancer genomics and their correlated clinical data. The expression level of LINC01133 was detected by averaging the exons expression level. MiRanda was used to predict the potential target miRNAs of LINC01133.

Cell lines

Five human ovarian cancer cell lines (A2780, Caov3, HO-8910, OVCAR3, and SKOV3) and a human ovarian epithelial cell line (HOEpiC) acquired from Chinese Academy of Sciences were used in this study. Their culture medium and conditions were listed as follows: RPMI 1640 medium (Invitrogen), 100 U/ml penicillin, 10% fetal bovine serum (HyClone), 100 mg/ml streptomycin sulfate, humid air environment with 5% CO2 under 37°C. Cells used in the following experiments were collected in their logarithmic growth phase.

Total RNA extraction and qRT-PCR

Total RNA was isolated from cell lines by Trizol reagent (Invitrogen) and reversed by Prime Script™ RT reagent Kit (TaKaRa) following manufacturer's introductions. QRT-PCR was used to detect the expression level of LNC01133 through SYBR®Green (TaKaRa) on Potable qPCR instrument, and the internal control was GAPDH. Correlated primer information was shown as follow: LINC01133-primer-F sequence was CCTAATCTCACCACAGCCTGG, LINC01133-primer-R sequence was TCAGAGGCACTGATGTTGGG, GAPDH-primer-F sequence was ACCACGCTATGCCCATCAC, and GAPDH-primer-R sequence was TACACACCCTGTGTCCTGTA. The quantification method of transcripts was comparative Ct method. The method 2-DDCT was used to count the target genes’ fold-change after internal control normalization.

Transfection assays

GenePharma was used to design and synthesis the negative control of various siRNA and their mimics and inhibitors. The over-expression vector of LNC01133 was recombined with pcDNA3.1 (+) by Genscript. Transfection assay of each element was performed using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions.

Flow cytometric analysis

FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) was used to conduct the apoptosis assay in this study. Cells were collected using trypsin, washed with ice-cold PBS for twice, re-suspended with 1*Binding Buffer and at the concentration of 1*10^6 cells/ml. Transferring 100 μL solution obtained from above operations to 5 mL culture tube, was followed by adding 5 μL PI and 5 μL FITC Annexin V. After incubating at 25°C in the dark for 15 min, adding 400 μL 1*Binding Buffer to each tube, then using FACSCalibur Flow Cytometer (BD Biosciences) to analyze the stained cells.

Western blot assays

Cleaved PARP (dilution ratio 1:500), pro-Caspase (1:500), PARP (1:500), PCNA (1:500), MMP2 (1:500), MMP9 (1:500), β-catenin (1:1000), Snail (1:500), and GAPDH (1:1000) primary antibodies were used in western blot conducted step by step as below: Lysing the stimulated cells by RIPA buffer, which was consisted by 50 mM Tris-Cl at pH 7.5, 1% Triton X-100, 150 mM NaCl, 0.5% Na-deoxycholate and protease inhibitors; Separating 20-100 μg
lysates above on SDS PAGE gels with concentration of 8%-12%; Transferring to a PVDF membrane; Incubating the membrane with various primary antibodies at 4°C for overnight; Incubating with HRP-conjugated secondary antibodies; Detecting the bound antibodies by ECL substrate.

CCK-8 assay

CCK-8 assay (Boster) was used to assess the cell proliferation. Briefly introducing, plating about 2*10^3 cells into a 96-well plate at first. Adding 10 μL CCK-8 solution into every well after cells’ adhering, and then incubating for 1 h at 37°C. Measuring the absorbance of such solution obtained from above operations on 450 nm at every indicated time points, and the curves of cell proliferation would be plotted. These experiments were conducted in triplicates.

Wound scratch assay

The migration ability of cells was detected by wound scratch assay. The detail steps were listed as follow: Seeding cells with concentration of 3*10^5 cells/well in a 6-well plate; Exposing the plate to various treatments accordingly; Using a sterilized pipette tip to make a straight scratch in each well after cells reached 100% confluence; Capturing images at every indicated time points by digital microscopy. The migration ability was determined by the wound closure areas.

Transwell invasion assay

The invasion ability of cells was detected by Transwell invasion assay. The detail steps were listed as follow: Collecting cells 24 h post transfection; Suspending 5*10^4 cells into 100 μL of serum-free medium; Seeding the medium above to the upper chamber which was pre-coated with Matrigel Matrix (BD Biosciences); Adding 600 μL medium which contained 10% FBS into the lower chamber, incubating for 24 h; Removing the cells that didn’t invade through the membrane using a cotton swab; Fixing the cells on the bottom surface of the membrane by 4% paraformaldehyde for 10 min; Staining the cells with 0.4% crystal violet solution; Imaging the invaded cells by digital microscopy. The invasion ability was determined by the number of invaded cells.

In vivo experiments

Caov3 cell lines that were stably transfected with LNC01133-shRNA and negative control vector all were constructed by our laboratory. All the transfected Caov3 cells were labeled with pHIV-Luciferase. 8 NOD/SCID mice were divided as two groups, and tail vein injecting 1*10^6/0.1 mL LNC01133-shRNA or shRNA (sh-nc) transfected Caov3 cells, respectively. The bioluminescent flux was determined every 2 weeks, and the tumor foci in ovarian was assessed by IVIS spectrum imaging system with the software of Living Image. Mice were sacrificed 35 days after injection.
LNC01133 promotes OC through miR-126
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Figure 2. The influence of LNC01133 on OC cells’ apoptosis, proliferation, migration and invasion abilities. A. The efficiency of siRNA knocking-down in Caov3 cell line counted by qRT-PCR. B. The efficiency of siRNA knocking-down in A2780 cell line counted by qRT-PCR. C. The validation of pcDNA3.1-LNC01133 over-expression vector in HOEpiC cell line counted by qRT-PCR. D. The cell apoptosis rate of Caov3 cell line transfection with si-NC or si-L2 determined by flow cytometric analysis. E. The PARP and pro-Caspase 3 expression level in si-NC or si-L2 transfecting Caov3 cell line counted by western blot. F. The cell proliferation of Caov3 cell line transfect with si-NC or si-L2/si-L5 determined by CCK-8 proliferation assays. G. The migration abilities of si-NC or si-L2/si-L5 transfecting Caov3 cell line and pcDNA3.1-LNC01133 transfecting HOEpiC cell line counted by wound scratch assays. H. The invasion abilities of si-NC or si-L2/si-L5 transfecting Caov3 cell line and pcDNA3.1-LNC01133 transfecting HOEpiC cell line counted by Transwell invasion assays. I. The tumor growth rate of NOD/SCID mice after injected with LNC01133-shRNA or sh-nc transfecting Caov3 cell line. Error bars equal the mean ± Standard Deviation. * stands for $P < 0.05$, ** stands for $P < 0.01$, *** stands for $P < 0.005$. 
Results

**LNC01133 is related to poor survival rate of OC patients**

After screening in the GEO database for OC specific LncRNAs, as shown in **Figure 1A** the high expression level of LNC01133 was thought to be related to the poor overall survival rate of OC patients, which indicated that LNC01133 might be an oncogene for OC. **Figure 1B** showed the expression levels of LNC01133 in 5 OC cell lines (Caov3, SKOV3, HO-8910, OVCAR3 and A2780) and 1 human ovarian epithelial cell line (HOEpiC) detected by the qRT-PCR method. Finally, we discovered Caov3 possessed the highest expression level of LNC01133, while HOEpiC possessed the lowest expression level of LNC01133.

**The influence of LINC01133 on OC cells’ apoptosis, proliferation, migration, and invasion abilities**

In order to find out the role of LNC01133 in the tumorigenesis of OC, Caov3, A2780 and HOEpiC cell lines were chosen for the following experiments. **Figure 2A, 2B** showed the expression level of LNC01133 two days post transfection of siRNA that was specific for LNC01133 in Caov3 and A2780 cell lines, respectively, and we found that si-L2 and si-L5 had the best knockdown efficiency for LNC01133, therefore were used in the following experiments to reduce the possibility of off-target effects. Meanwhile, **Figure 2C** showed an extremely elevated expression level of LNC01133 in HOEpiC cell line when transfected with pc-DNA3.1-LNC01133 over-expression vector. **Figure 2D** showed that LNC01133 knock-down via si-L2 transfection would increase the apoptosis rate a little in the Caov3 cell line. **Figure 2E** showed an increased expression of cleaved PARP while a decreased expression of pro-Caspase 3 in Caov3 cell line, which could be more evidence for slightly elevated apoptosis rate. **Figure 2F** showed the result of CCK-8 proliferation assay, namely, the proliferation ability of cells transfected with si-L2 and si-L5 were both remarkably decreased.

Metastasis and invasion of tumor, the abilities of which could detected through wound scratch assays and transwell invasion assays, respectively, make OC lethal. **Figure 2G** showed a larger wound closure area of Caov3 cell line when LNC01133 was knocked down, but a smaller wound closure area of HOEpiC cell line was observed when over-expressed LIN-C01133. **Figure 2H** showed a sharply declined number of Caov3 cell line after transfecting si-L2 and si-L5 while a significantly increased number of HOEpiC cell line after over-expressing LNC01133. In vivo experiment was performed as well, and **Figure 2I** showed the significant inhibition of tumor formation after knocking-down the LNC01133 completely. The results mentioned above suggested that
LNC01133 promotes OC through miR-126

**Figure 4.** Molecular mechanism of OC tumorigenesis promoting via sponging miR-126. A. The expression level of LNC01133 in miR-126 inhibitors and si-L2 transfecting Caov3 cell line counted by qRT-PCR. B. The expression level of LNC01133 downstream genes in miR-126 inhibitors and si-L2 transfecting Caov3 cell line counted by western blot. C. The migration ability of miR-126 inhibitors and si-L2 transfecting Caov3 cell line counted by wound scratch assay. D. The invasion ability of miR-126 inhibitors and si-L2 transfecting Caov3 cell line counted by Transwell invasion assay.
LNC01133 had a great influence on apoptosis, viability, migration, invasion abilities of OC cells.

Negative correlation between LNC01133 and miR-126

We had predicted which miRNAs could target LNC01133 using miRanda (a prediction tool of human miRNA targets) integrated with the data of GEO database. Several miRNAs were selected, but only the miR-126 got the highest score. In order to verify this prediction, following experiments were conducted. First of all, we detected the expression level of miR-126 in multiple cell lines mentioned before. Figure 3A showed a contrary result to the expression level of LNC01133 in Caov3 and HOEpiC cell lines, i.e higher in HOEpiC cell line but lower in Caov3 cell line. Then, we detected the expression level of LNC01133 in Caov3 and A2780 cell lines transfected with miR-126 mimics. As Figure 3B, 3C show, the expression level of LNC01133 in the two miR-126 mimics transfecting cell lines decreased significantly comparing to the cell line transfected with NC mimics, which further proved that miR-126 could target LNC01133. Those results mentioned above suggested that LNC01133 was negatively related to miR-126.

Molecular mechanism of OC tumorigenesis promoting via sponging miR-126

Following experiments were performed to further research the molecular mechanism of OC tumorigenesis promoting. Figure 4A showed the expression level of LNC01133 was increased when miR-126 inhibitors transfecting but was decreased when LNC01133 was knocked down in the Caov3 cell line, which meant miR-126 could target LNC01133 and sponging it would lead to an elevated expression level of LNC01133. Figure 4B showed a significant down-regulation of PCNA (related to cell growth), MMP-2, 9 (related to metastasis), β-catenin, and Snail (related to EMT) when LNC01133 was knocked down, but their expression levels were almost rescued when transfected with miR-126 inhibitors in Caov3 cell line. Figure 4C, 4D indicated that transfected with miR-126 inhibitors in Caov3 cell line would enhance its migration and invasion abilities. These evidences mentioned above suggested that sponging miR-126 could enhance the abilities of proliferation, migration and invasion of OC cells.

Discussion

Ovarian cancer (OC) is one of the malignant tumors in women worldwide and whose five-year survival rate is lower than 30%. Powerful prognostic markers are the base of patients’ classification for suitable adjuvant therapies and precisely survival outcomes prediction, and there are plenty of markers including BTF4/GCS [16], Galectins-1/-3/-7 [17], MAGE-A family [18], miR-100 and miR-203 [19] have already been chosen as the prognostic markers for OC patients in the previous studies. In this study, we found LNC01133 was an oncogene in OC patients. Simultaneously, we investigated miR-126 could regulate the expression level of LNC01133 in OC cells, and sponging it would lead to an increased proliferation, migration, and invasion abilities of OC cells, which suggested that miR-126 could serve as a powerful prognostic marker for OC patients as well.

miR-126 gene is located on epidermal growth factor like 7 (EGFL7) intron of humans, and was primarily discovered in human breast cancer tissue [20, 21]. There have been many reports that claim that miR-126 is involved in cancer tumorigenesis, such as colorectal cancer [22-24], pancreatic cancer [25], breast cancer [26], stomach cancer [27], prostate cancer [28], liver cancer [29], lung cancer [30] and so on.

miR-126 has a low expression level in most tumor tissues and is negatively correlated to the tumorigenesis and development of cancer. Its role for cancer inhibition is achieved by down-regulating the expression level of several target genes and participating in the regulation of multiple signal pathways. For example, Guo et al. found that up-regulating the expression level of miR-126 would significantly decrease the level of p85β protein and phosphorylated serine/threonine protein kinase (Akt), and they concluded that miR-126 could target p85β for the negatively regulation of PI3K/Akt signal pathway, and could inhibit the growth of colorectal cancer cells [22]. There were other studies indicated that miR-126 was negatively corrected with RhoA/ROCK signal pathway in colorectal cancer cells as well [23, 24]. However, several surveys reported that miR-126 was an oncogene in cancer cell. Otsubo et
al found that miR-126 could target SOX2 and positively regulate the PLAC1 gene, and over-expressing miR-126 might contribute to gastric carcinogenesis [27]. Barshack et al. had found a higher expression level of miR-126 in hepatocellular carcinomas as well [31]. These results suggested that miR-126 had various target genes and heterogeneous effects in cancer cells, which indicated the complex function mechanism of miR-126.

As a research focus of targeted therapy, miRNAs have immeasurable values in diagnosis, drug efficacy evaluation, prognosis, and treatment of various cancers. miR-126 is such a miRNA that is abnormally expressed in many tumors and has significantly influence on the growth, proliferation, metastasis and invasion processes of tumor cells, which makes it important for the diagnosis, treatment and prognosis of tumor. However, the role of miR-126 plays in many tumors and its mechanism are still unclear, resulted a limited clinical application. It is believed that with the further study of miR-126, its regulatory mechanism in different tumors will be cleared and is expected to serve as a new method of tumor therapy in the future.

**Conclusion**

We found LNC01133 was an oncogene in OC, as for its positive influence on OC cells’ proliferation, migration and invasion processes. MiR-126 could target LNC01133 and down-regulate its expression level in OC cells, so that could serve as a powerful prognostic marker for OC patients. This study advanced our understanding of miR-126 in OC cells, and had great clinical significance on the diagnosis and treatment of OC patients.

**Disclosure of conflict of interest**

None.

**Abbreviations**

OC, Ovarian cancer; lncRNA, long non-coding RNA; miR-126, microRNA 126.

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**References**


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