Original Article

LncRNA *OXCT1-AS1* promotes the proliferation of non-small cell lung cancer cells by targeting the *miR-195/CCNE1* axis

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Background: Long non-coding RNAs (lncRNAs) are involved in various biological processes in non-small cell lung cancer (NSCLC). This study aimed to investigate the key lncRNA *OXCT1-AS1/miR-195/CCNE1* axis in the development of NSCLC and its potential molecular mechanism.

Methods: LncRNA *OXCT1-AS1* is considered to be a competitive endogenous RNA (ceRNA) and its potential targeting microRNAs (miRNAs) were predicted through LncBase predicted v.2. The expression of *OXCT1-AS1* and *miR-195* in NSCLC tissues and cells was detected by reverse transcription polymerase chain reaction (RT-PCR). The Cell Counting Kit-8 (CCK-8) and cell colony-forming test were used to detect the effect of cell proliferation. RT-PCR was used to detect the expression changes of *CCND1* and *CCNE1*. Western Blot was used to detect the changes of the *CCNE1* cell cycle protein. Dual luciferase activity was used to determine the potential mechanism of lncRNA *OXCT1-AS1*.

Results: LncRNA *OXCT1-AS1* was highly expressed and *miR-195* was lowly expressed in NSCLC tissues and cell lines. LncBase predicted v.2.0 reported a high-scoring binding between *OXCT1-AS1* and *miR-195*. The luciferase reporter assay defined the regulatory relationship between *OXTC1-AS1* and *miR-195*. In NSCLC cells, knockdown of *OXCT1-AS1* significantly increased the expression of *miR-195*, decreased the proliferation and colony formation number of cancer cells, and reduced the expression of *CCND1* and *CCNE1*. Meanwhile, overexpression of *miR-195* significantly inhibited the cell proliferation and colony formation number, and reduced the expression of *CCND1* and *CCNE1*. Furthermore, according to the results of the dual-luciferase activity assay, *miR-195* targeted the 3' untranslated regions (3' UTRs) of *CCNE1*, validating that *CCNE1* is a direct target of *miR-195*. Overexpression of *CCNE1* restored the role of *OXCR1-AS1* in NSCLC cells.

Conclusions: LncRNA *OXCT1-AS1* can regulate the proliferation of NSCLC cells via *miR-195/CCNE1* signaling. Therefore, *OXCT1-AS1* may act as a prospective biomarker and therapeutic target for patients with NSCLC.

Keywords: Non-small cell lung cancer (NSCLC); long non-coding RNA *OXCT1-AS1* (lncRNA *OXCT1-AS1*); *miR-195*; *CCNE1*; proliferation

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Introduction

Lung cancer (LC) is a common malignant tumor with high rates of morbidity and mortality. In fact, it has the highest mortality among various types of malignant tumors, which poses a great threat to human life and health (1). Among the traditional LC treatments, surgical treatment has been widely used. However, most patients with LC have atypical clinical manifestations in the early stage of the disease and clinical diagnostic techniques are limited at present, based on the fact that 75% of patients with LC are diagnosed with regional metastasis at the first visit. These patients' cancers spread to regional lymph nodes or directly infiltrate the surrounding normal tissues, resulting in a significant reduction in the 5-year survival rate (only 20.6%) (2,3). At present, the overall 5-year survival rate of LC patients is still at a low level (only about 15%), which is due to the fact that most patients are in the advanced stage when they are first diagnosed and have poor response to chemoradiotherapy (4).

Clinically, non-small cell lung cancer (NSCLC) accounts for 85% of all LC cases. The main causes of LC development are mutation or inactivation of tumor suppressor genes as well as activation of oncogenes (5). Other biological pathways may also have an important impact on the prognosis of NSCLC patients and the sensitivity of NSCLC therapeutics (6). Therefore, studying the incidence of NSCLC and its malignant mechanism contributes to a deeper understanding of the disease, which is of great importance for the formulation of individualized treatment, and also provides an effective criteria for prognosis.

Long non-coding RNAs (lncRNAs) are a series of transcripts with a length of more than 200 nucleotides (7). Recently, a large number of lncRNAs have been found to be aberrantly expressed in tumors (8). Although lncRNAs have been considered as "noise" in the genome, increasing evidence has suggested that they play an essential role in biological and pathological functions, such as cell proliferation, invasion, and apoptosis (9). A previous study has reported that lncRNAs act as competitive endogenous RNAs (ceRNAs) or microRNA (miRNA) "sponges" binding to miRNAs without degradation, thereby affecting downstream gene expression (10). It has been shown that lncRNA-*OXCT1-AS1* is lowly expressed in various tumors and is involved in the proliferation and metastasis of tumor cells (11). In glioma, lncRNA-*OXCT1-AS1* acts as a ceRNA against *miR-195* by increasing the expression of *CDC25A* and promoting the growth of cancer cells (12). The lncRNA, LINC00355, increases the expression of *CCNE1* by downregulating *miR-195*, and promotes the proliferation of lung adenocarcinoma (LUAD) cells (13). The lncRNA, *SNHG12*, affects the activity, apoptosis, and autophagy of prostate cancer cells by regulating *miR-195/CCNE1* (14). A study has shown that lncRNAs/miRNAs/target genes play an important role in NSCLC (15). The lncRNA-*OXCT1-AS1* promotes the metastasis of NSCLC *in vitro* and *in vivo* by stabilizing LEF1. Therefore, we constructed a presumptive lncRNA-*OXCT1-AS1/miR-195/CCNE1* network to elucidate the underlying mechanisms of the pathogenesis and progression of NSCLC. This study found that lnRNA *OXCT1-AS1* can regulate the proliferation ability of NSCLC through *miR-195/CCNE1* signaling, which aiming to provide clues for the identification of new diagnostic markers or therapeutic targets for NSCLC. We present the following article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-855/rc).

Methods

Research object

Tumor samples and adjacent tissues from eight NSCLC patients were collected for this study, none of whom had received radiotherapy or chemotherapy before surgery. The tissue specimens included primary NSCLC and adjacent tissues (>5 cm from the primary tumor, and pathologically confirmed no tumor cell infiltration). After resection, the specimens were flash frozen in liquid nitrogen and then transferred to a −80 ℃ freezer for further experimentation. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics board of The Third Affiliated Hospital of Qiqihar Medical University (No. 2021LL-9) and informed consent was taken from all the patients.

Cell lines and cell culture

Human LC cell lines (A549, NCI-H1299, and H1650) and normal human lung epithelial cells (BEAS-2B) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured with RPMI-1640 (HyClone, USA), containing 10% (v/v) fetal bovine serum (FBS, Gibco, USA), 1% penicillin/streptomycin (Sigma, Guangzhou, China), and placed in a cell incubator with 5% CO_2 at 37 ℃.

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Table 1 The primer sequences in RT-PCR assay

Gene name	Forward 5'-3'	Reverse 5'-3'
CCNE1	5'-TGACCTAAGGGACTCCCACAA-3'	5'-TGATATGTGGAGAGGGCAGC-3'
CCND1	5'-TGGTGAACAAGCTCAAGTGGA-3'	5'-AGGGCGGTTGGAAATGAACT-3'
MiR-195	5'-ACACTCCAGCTGGGTAGCAGCACAGAAAT-3'	5'-TGGTGTCGTGGAGTCG-3'
U6	5'-CTCGCTTCGGGCAGCACA-3'	5'-AACGCTTCAGGAATTTGCGT-3'
GAPDH	5'-AAGTTCAACGGCACAGTCAA-3'	5'-TACTCACCAGCATCACC-3'

RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from NSCLC tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was amplified to complementary DNA (cDNA) using the TapMan-MiRNA Reverse Transcription Kit (Invitrogen, Carlsbad, USA) with reverse transcription at 16 ℃ for 30 min, 42 ℃ for 30 min, and 85 ℃ for 5 min under reaction conditions. qRT-PCR amplification was performed using SYBR Green (Qiagen, USA), under the following reaction conditions: 40 cycles of amplification at 95 ℃ for 5 min, 95 ℃ for 15 s, and 6 ℃ for 30 s. The resulting values were normalized to the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 for each replicate. The qRT-PCR primers are shown in *Table 1*.

For miRNA expression analysis, RNA (100 ng) was reverse transcribed using TaqMan Advanced MicroRNA Assay kit (Applied Biosystems, Thermo Fisher Scientific, China) and miRNA-specific primers. The primers were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The cycle threshold (Ct) value was recorded, and the relative expression level was calculated using the $2^{-\Delta\Delta Ct}$. All experiments were repeated three times.

Western blot

Cells from each group were collected, lysed by using RIPA buffer, and then centrifuged at 12,000 rpm for 10 minutes at 4 ℃. The supernatant was collected and the protein concentration in each group was quantified using the bicinchoninic acid (BCA) assay, and then separated by 12% polyacrylamide sodium dodecyl sulfate (SDS) gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. Blocking was then performed using 5% non-fat dry milk for 1 h, followed by subsequent incubation with the primary antibody, *CCNE1* (1:1,000, CST, Boston, USA), overnight at 4 ℃. The horseradish peroxidase-conjugated secondary antibody (1:1,000) was then incubated for 1 h. Signals were visualized by using enhanced chemiluminescence detection (BeyoECL Plus, Catalog No. P0018M; Beyotime Institute of Biotechnology). The ImageLab^{TM} software system (Bio-Rad, USA) used to conduct quantitative analysis of the proteins in each group.

Cell transfection

si-OXCT1-AS1, si-NC, and miR-195 mimic, NC-mimics, pcDNA3.1 (+) vector, and pcDNA3.1-*CCNE1* vector were designed and synthesized by Gene Pharma (Shanghai, China). The cells were seeded in six-well plates. Lipofectamine 2000 (Invitrogen, Carlsbad, USA) was used for transfection at 70% condensity according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were harvested for further *in vitro* experiments.

Luciferase reporter assay

OXCT1-AS1 and *CCNE1* 3' untranslated regions (3' UTRs) fragments containing the *miR-195* binding site were amplified and cloned into the psiCheck2 reporter vector (Promega, Shanghai, China). Next, the wild-type or mutant plasmid, pCheck2 plasmid, and an equal amount of negative control or *miR-195* mimic plasmid were co-transfected into the H1650 cells. Luciferase assays were performed using a dual luciferase reporter assay system according to the manufacturer's instructions (PROMEGA).

Cell proliferation assay

Cells at 10,000 cells/well were seeded in 96-well plates and

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placed in a cell incubator (Thermo Fisher Scientific, China) for culture. According to the manufacturer's instructions, cell proliferation was detected using a Cell Counting Kit-8 (CCK-8) at 0, 24, 48, and 72 h, respectively. Ten μL of CCK-8 reagent was added to each well, and after incubation for 1 h, the optical density (OD) value at 490 nm was record using a microplate reader (Shenzhen Mepco Technology Co., Ltd., Shenzhen, China). All experiments were repeated three times.

Colony forming assay

H16550 cells (100 cells/well) were used for the colony forming assay. One hundred cells were seeded in six-well culture plates. Visible colonies were fixed after 10–14 days and stained with 0.1% crystal violet in 20% methanol. All experiments were repeated three times

Target prediction

Bioinformatics analysis was performed by using the following bioinformatics tools: LncBase Predicted V2.0 (https://diana.e-ce.uth.gr/lncbasev3/expression), miRDB (http://mirdb.org/), TargetScan (https://www.targetscan.org/vert_71/), and miRTarBase (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php).

Survival analysis of CCNE1 in LUAD

In this study, high- and low-expression groups were cut off using the median expression value of *CCNE1*. Prognostic differences between different *CCNE1* expression groups were compared using Kaplan-Meier curves with the log-rank test. A time-dependent receiver operating characteristic (ROC) curve was used to evaluate the accuracy of *CCNE1* expression in predicting the prognosis of LUAD, which was performed by survival package in the R platform (https://cran.r-project.org/web/packages/ROCR/index.html) (12,16).

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Group comparisons were made using the Student's *t*-test or analysis of variance (ANOVA). P<0.05 was considered statistically significant. Statistical analyses were performed using Graph Pad Prism 8.0 (http://graphpadchina.com/).

Results

Different expression patterns of lncRNA OXCT1-AS1 and miR-195 in NSCLC

First, qRT-PCR was used to detect the mRNA expression levels of *OXCT1-AS1* and *miR-195* in eight pairs of NSCLC tissues and adjacent tissue samples. The results showed that the expression levels of *OXCT1-AS1* were significantly increased, whereas *miR-195* was significantly decreased in cancer tissues (as shown in *Figure 1A*,*1B*; P<0.001).

Furthermore, qRT-PCR was used to detect the mRNA expression levels of *OXCT1-AS1* and *miR-195* in BEAS-2B and NSCLC cell lines (A549, NCI-H1299, and H1650). Similarly, the results showed that *OXCT1-AS1* were significantly increased, coupled with markedly decreased levels of *miR-195* in NSCLC cell lines (*Figure 1C*,*1D*; P<0.001). The above results indicate that lncRNA *OXCT1-AS1* is highly expressed and *miR-195* is lowly expressed in NSCLC tissues and cell lines.

LncRNA OXCT1-AS1 targets and inhibits the expression of miR-195 in NSCLC cell lines

Next, we explored the regulatory relationship between *OXCT1-AS1* and *miR-195*. As shown in *Figure 2A*, *OXCT1-AS1* obtained a high-scoring binding site with *miR-195* through the online database software, LncBaseV2.0 (https://starbase.sysu.edu.cn/starbase2/index.php). Given that *OXTC1-AS1* was expressed at the highest levels in H1650 cells, we then selected H1650 cells as the research objects in subsequent experiments. After transfection (si-NC, si-OXTC1-AS1), changes in miR-195 were detected using qRT-PCR. As shown in *Figure 2B*, the si-OXCT1-AS1-transfected H1650 cells successfully knocked down the expression of *OXCT1-AS1* (***P<0.001). Also, the detection of *miR-195* expression by qRT-PCR revealed that knockdown of *OXTC1-AS1* significantly increased the expression level of *miR-195* compared with the si-NC group (*Figure 2C*; ***P<0.001).

Furthermore, luciferase reporter analysis determined the regulatory relationship between *OXTC1-AS1* and *miR-195*. The experimental groups, GLO-OXTC1-AS1-WT and GLO-OXTC1-AS1-MUT, were co-transfected with NC-mimics or *miR-195* mimics, respectively, and the results showed that compared with NC mimics, luciferase activity was significantly reduced in the GLO-OXTC1-AS1-AS1-WT group after transfection with miR-195 mimics

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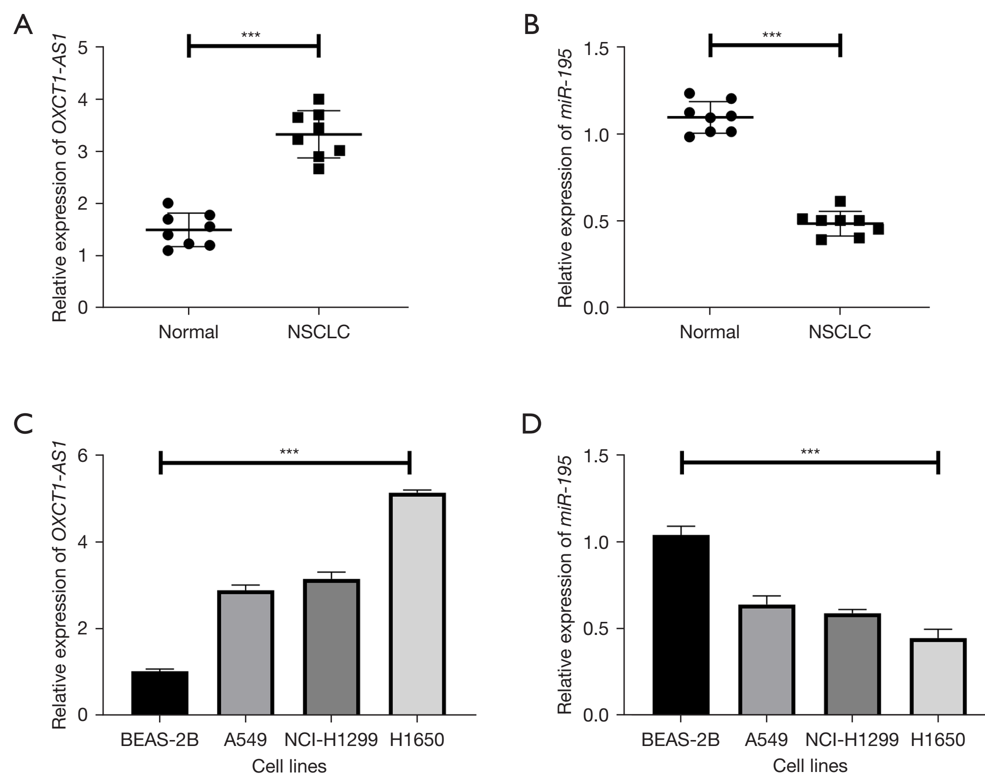

Figure 1 Expression levels of *OXCT1-AS1* and *miR-195* in NSCLC tissues and cell lines. (A) The expression levels of *OXCT1-AS1* in cancer tissues and adjacent tissues in eight patients with NSCLC were detected by qRT-PCR. (***P<0.001). (B) qRT-PCR was used to detect the expression levels of *miR-195* in cancer tissues and adjacent tissues in eight patients with NSCLC. (***P<0.001). (C) Expression of lncRNA *OXCT1-AS1* was detected in human NSCLC cell lines (A549, NCI-H1299, and H1650) and normal human lung epithelial cells (BEAS-2B) by qRT-PCR. (***P<0.001). (D) *MiR-195* expression was detected by qRT-PCR in human NSCLC cell lines (A549, NCI-H1299, and H1650) and normal human lung epithelial cells (BEAS-2B). (***P<0.001). NSCLC, non-small cell lung cancer; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

(*Figure 2D*). The above results indicated that lncRNA *OXCT1-AS1* targeted and inhibited the expression of *miR-195* in NSCLC cell lines.

LncRNA OXCT1-AS1 promotes the proliferation of NSCLC cell lines

To further explore the biological function of *OXCT1-AS1* in NSCLC, we transfected si-NC and si-OXTC1-AS1, respectively, into H1650 cells. Changes in cell growth curves were analyzed using the CCK-8 assay. Knockdown of *OXTC1-AS1* significantly inhibited the proliferative capacity of cells (*Figure 3A*). The results of colony forming assay and EdU assay showed that knockdown of *OXTC1-AS1* markedly suppressed the colony forming ability and proliferation ability of cells (*Figure 3B,3C*).

Cyclins also play an important role in the biological process of cell proliferation. Therefore, the effect of *OXCT1-AS1* on the expression levels of cyclins (*CCND1* and *CCNE1*) was examined by qRT-PCR. The results showed that knockdown of *OXTC-AS1* considerably downregulated the expression of *CCND1* and *CCNE1* compared with si-NC group, as shown in *Figure 3D,3E* (P<0.001). Western blotting results indicated that knockdown of *OXTC-AS1* significantly inhibited the protein expression of *CCNE1* compared with the si-NC group (*Figure 3F*). The above results indicated that knockdown of *OXCT1-A*S1 inhibited cell proliferation by inhibiting the expression of cyclins (*CCND1* and *CCNE1*) in NSCLC cells. In addition, a previous study found that knockdown of lncRNA *OXCT1-AS1* inhibited tumor growth in mice (16). This result can further support our findings at the cellular level *in vitro*.

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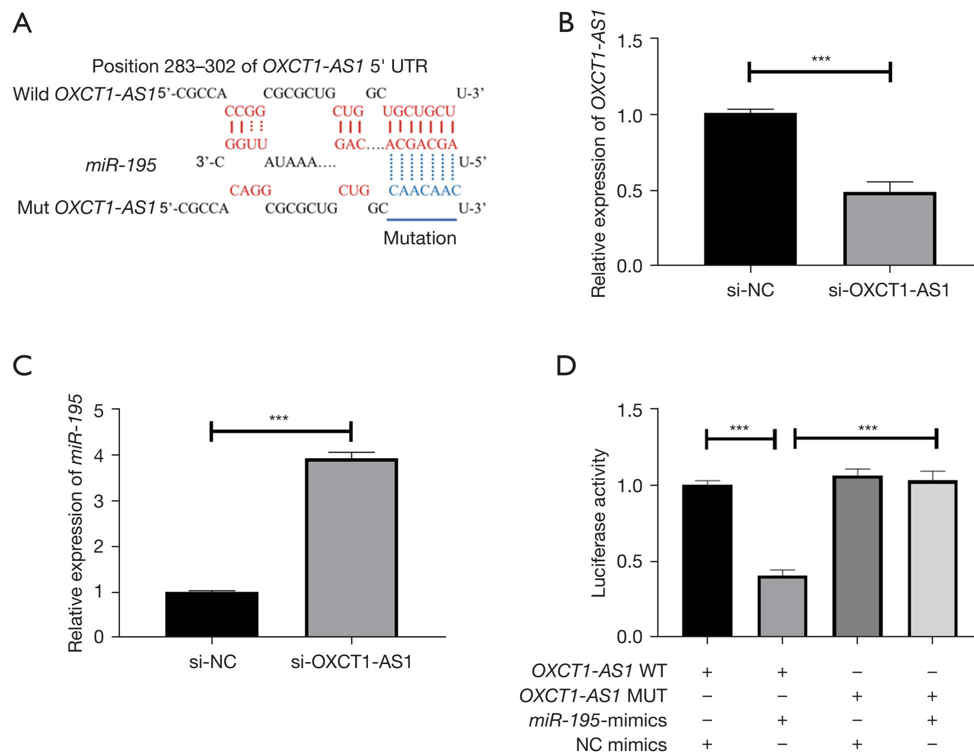

Figure 2 Regulatory relationship between *OXCT1-AS1* and *miR-195*. (A) The binding site of *OXCT1-AS1* and *miR-195*. (B) Transfection efficiency of si-OXCT1-AS1 was measured by qRT-PCR. (***P<0.001). (C) Effect on *miR-195* expression after knockdown of si-OXCT1-AS1 was measured by qRT-PCR. (***P<0.001). (D) Luciferase reporter gene assay was performed to validate the regulatory relationship between *OXCT1-AS1* and *miR-195*. +: added; –: not added. (***P<0.001). UTR, untranslated region; WT, wide type; MUT, mutant; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

In NSCLC cell lines, knockdown of lncRNA OXCT1-AS1 inhibited cell proliferation by upregulating miR-195

Next, we transfected si-NC, si-OXCT1-AS1, si-OXCT1-AS1 + miR-195 mimics, respectively, into H1650 cells, and the changes in cell growth curves were analyzed using CCK-8. The si-OXCT1-AS1 + miR-195 mimics group more significantly inhibited the proliferation of cells compared with the si-OXCT1-AS1 group (*Figure 4A*). The results of the colony forming assay also showed that the clome numbers were more significantly inhibited in the si-OXCT1-AS1 + miR-195 mimics group compared with the si-OXCT1-AS1 group (*Figure 4B*). Additionally, qRT-PCR assay indicated that the expressions of *CCND1* and *CCNE1* were more significantly inhibited in the si-OXCT1-AS1 + miR-195 mimics group compared with the si-OXCT1-AS1 group (*Figure 4C*). Western blotting results indicated that *CCNE1* protein expression was remarkably inhibited in the si-OXCT1-AS1 + miR-195 mimics group compared with the si-OXCT1-AS1 group (*Figure 4D,4E*). The above results indicated that knockdown of lncRNA *OXCT1-AS1* inhibited cell proliferation by upregulating *miR-195* in NSCLC cells.

MiR-195 targets and regulates CCNE1 expression in NSCLC cell lines

Generally, the biological function of miRNAs is achieved through their downstream target genes. Thus, the downstream target genes of *miR-195* were searched and analyzed. As shown in *Figure 5A*, *miR-195* binds to the 3'-UTR of *CCNE1*. To verify this binding, *miR-195* mimics was successfully transfected into H1650 cells to achieve its overexpression (*Figure 5B*; ***P<0.001). Detection of *CCNE1* expression by qRT-PCR revealed that the *miR-195* mimics group significantly inhibited the mRNA expression level of *CCNE1* compared with the NC mimics group

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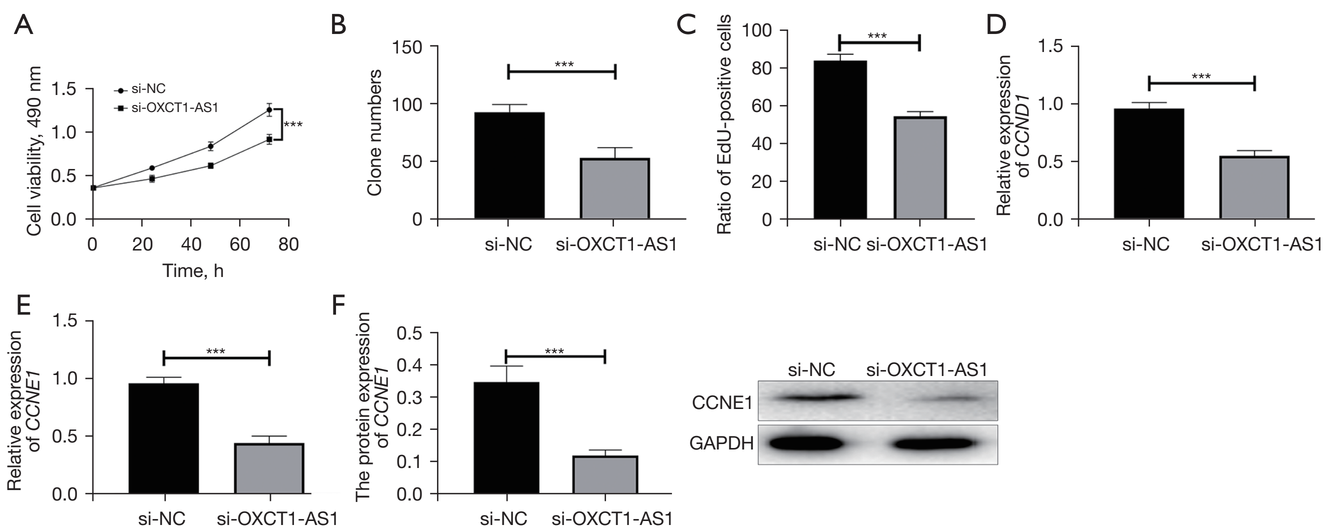

Figure 3 Effect of *OXCT1-AS1* on the proliferation of NSCLC cells. (A) After transfection of si-NC or si-OXCT1-AS1 in H1650 cells, cell viability was analyzed at 0, 24, 48, and 72 h by using CCK-8 to plot changes in the cell growth curves. (***P<0.001). (B) After transfection of si-NC or si-OXCT1-AS1 in H1650 cells, changes in the number of cell colony formations were detected using the colony forming assay. (***P<0.001). (C) After transfection of si-NC or si-OXCT1-AS1 in H1650 cells, changes of cell proliferation ability were detected using EdU assay. (***P<0.001). (D,E) qRT-PCR was performed to detect the expression changes of *CCND1* and *CCNE1* after transfection of si-NC or si-OXCT1-AS1 in H1650 cells. (***P<0.001). (F) Western blotting was used to detect protein expression changes of *CCNE1* after transfection of si-NC or si-OXCT1-AS1 in H1650 cells, and the statistical analysis of protein expression. (***P<0.001). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CCK-8, Cell Counting Kit-8; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

(*Figure 5C*; ***P<0.001).

Also, luciferase reporter assay was performed to determine the regulatory relationship between *miR-195* and *CCNE1*. The experimental groups, CCNE1-WT and CCNE1-MUT, were co-transfected with NC-mimics or *miR-195* mimics, respectively. The results showed that in CCNE1-WT group, luciferase activity was significantly reduced after transfection with miR-195 mimics compared with NC (*Figure 5D*). The above findings indicated that *miR-195* directly targeted the expression of *CCNE1* in NSCLC cell lines.

Survival analysis of CCNE1 in LUAD

Survival analysis was performed in different *CCNE1* expression groups for LUAD in The Cancer Genome Atlas (TCGA) datasets. Patients with high *CCNE1* expression had a shorter median survival time compared with patients with low *CCNE1* expression (high *CCNE1 vs.* low *CCNE1* =580 *vs.* 645.5 days; *Figure 6A*). We observed that high *CCNE1* expression was notably associated with an unfavorable prognosis in LUAD as well as an increased risk of cancer-related death of LUAD patients {P<0.01; hazard ratio (HR) =1.48 [95% confidence interval (CI): 1.1–1.99]; *Figure 6B*}. The time-dependent ROC curve indicated that the expression level of *CCNE1* gene had a certain prognostic value in the long-term survival of patients with LUAD. The area under the ROC curve for the 1-, 3-, and 5-year survival were 0.592, 0.59, and 0.62, respectively (*Figure 6C*).

We then transfected pcDNA3.1 vector or pcDNA3.1-CCNE1, respectively, into H1650 cells, and the transfection efficiency was verified by qRT-PCR. The experimental results showed a successful overexpression of *CCNE1* in H1650 cells (*Figure 7A*). Changes in cell growth curves and colony formation were analyzed, and were found to be significantly increased in the si-OXCT1-AS1 + pcDNA3.1-CCNE1 group compared with si-OXCT1-AS1 + pcDNA3.1 vector group (*Figure 7B,7C*). Overexpression of *CCNE1* restored the cell proliferation against the knockdown of *OXTC1-AS1*. Indeed, the expression level of CCNE1 was significantly increased in si-OXCT1-AS1 + pcDNA3.1-CCNE1 group compared with si-OXCT1-AS1 group (*Figure 7D-7F*). Therefore, we surmised that overexpression of *CCNE1* could reverse the proliferation

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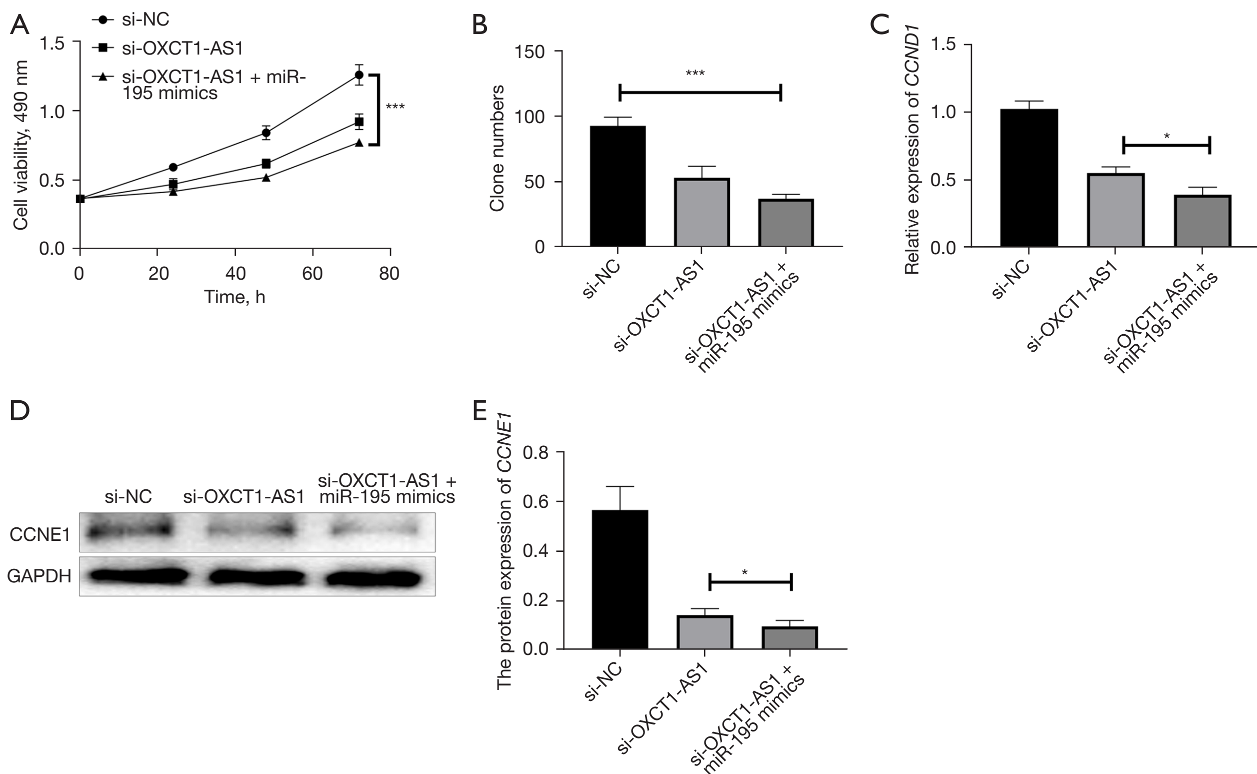

Figure 4 Key role of *miR-195* in lncRNA *OXCT1-AS1*-regulated the proliferation of NSCLC cells. (A) After transfection of si-NC, si-OXCT1-AS1, and si-OXCT1-AS1 + miR-195 mimics in H1650 cells, cell viability was analyzed at 0, 24, 48, and 72 h using the CCK-8 to plot changes in cell growth curves. (***P<0.001). (B) After transfection of si-NC, si-OXCT1-AS1, si-OXCT1-AS1 + miR-195 mimics in H1650 cells, changes in the number of cell colony formations were detected using the colony forming assay. (***P<0.001). (C) qRT-PCR was performed to detect the expression changes of *CCND1* after transfection of si-NC, si-OXCT1-AS1, and si-OXCT1-AS1 + miR-195 mimics in H1650 cells. (*P<0.05). (D) The protein expression changes of *CCNE1* after transfection of si-NC, si-OXCT1-AS1, and si-OXCT1-AS1 + miR-195 mimics in H1650 cells were detected by western blotting. (E) Statistical analysis of protein expression for (D). (*P<0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; lncRNA, long non-coding RNA; CCK-8, Cell Counting Kit-8; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

against lncRNA *OXCT1-AS1* knockdown in NSCLC cells.

Discussion

LC is a common malignant tumor, with the highest rates of morbidity and mortality worldwide. In China, the peak morbidity and mortality has never declined. In 2012, there were about 326,600 new cases of LC and 56,400 deaths (17). A previous study has shown that NSCLC accounts for 85% of all LC cases. The biological characteristics of NSCLC are invasion and metastasis, which is also the leading cause of treatment failure and the high mortality rate (18). Due to the lack of effective therapeutic targets, the survival rate of cell LC is still decreasing year by year (19). Therefore, it is essential to find novel and effective therapeutic targets.

LncRNAs have been found to be aberrantly expressed during tumor development and progression, and are expected to be potential biomarkers and therapeutic targets. A recent study has found that aberrantly-expressed lncRNAs play important biological roles in various tumors, and are expected to be potential biomarkers and therapeutic targets (20). For example, lncRNA LINC00899 blocks the progression of breast cancer by inhibiting miR-425 (21), and lncRNA ZFAS1 regulates the development of esophageal squamous cell carcinoma via the miR124/STAT3 axis (22). Also, the lncRNA, MALAT1, promotes glioblastoma multiforme (GBM) proliferation and progression by targeting the miR-199a/ZHX1 axis (23). A recent study has shown that

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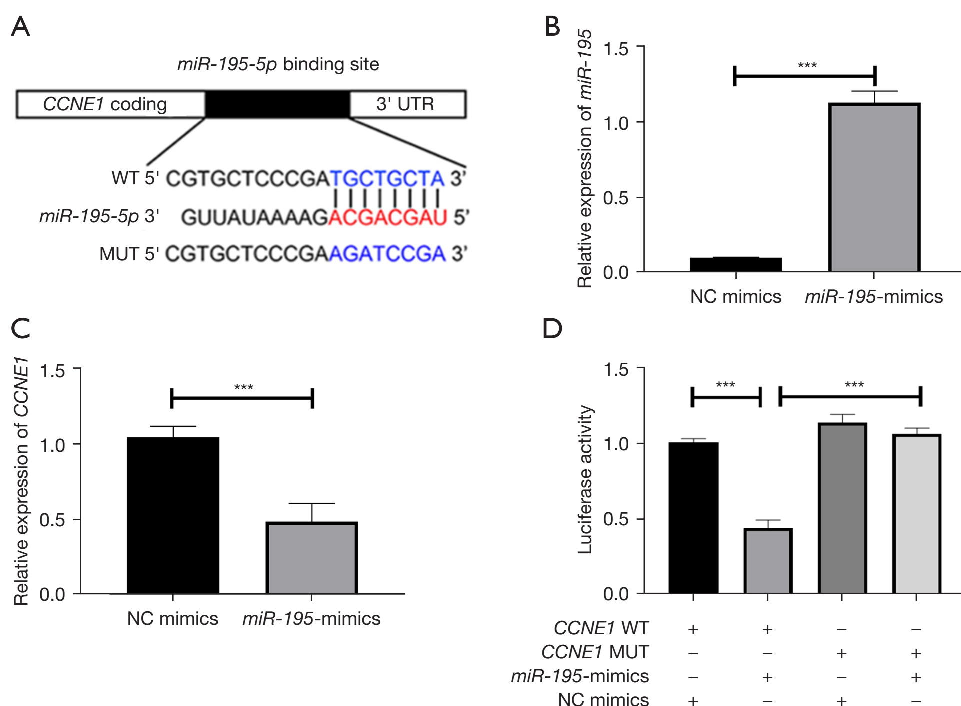

Figure 5 *miR-195* and *CCNE1* targeting relationship. (A) The binding site of *CCNE1* mRNA and *miR-195*. (B) Transfection efficiency of *miR-195* mimic was measured by qRT-PCR. (***P<0.001). (C) Effect of *miR-195* overexpression on *CCNE1* expression was measured by qRT-PCR. (***P<0.001). (D) Luciferase reporter assay was performed to validate the regulatory relationship between *miR-195* and CCNE1. +: added; –: not added. (***P<0.001). UTR, untranslated region; WT, wide type; MUT, mutant; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

the lncRNA, AC079630, is associated with prognosis as a tumor suppressor in LC (24). Furthermore, m6A transferase METTL3-induced lncRNA ABHD11-AS1 promotes the Warburg effect in NSCLC (25). The above studies highlight the crucial role played by lncRNAs in the diagnosis and treatment of human cancers.

At present, it has been demonstrated that lncRNA *OXCT1-AS1* (antisense RNA1 of *OXCT1*) is a ceRNA in human glioblastoma; it is upregulated in tumor tissues, and its high expression is associated with poor prognosis (11). Inhibition of *OXCT1-AS1* expression significantly inhibits cell proliferation, migration, and invasion, and its mechanism of action potentially involves *OXCT1-AS1* acting as a ceRNA of *miR-195* to enhance the expression of *CDC25A*, thereby promoting the progression of glioma cells (11). It has also been shown that lncRNA *OXCT1-AS1* expression is significantly increased in bladder cancer patients with lymph nodes metastasis, and the lncRNA, *OXCT1-AS*, reduces binding to the target gene, *JAK1*, by inhibiting *miR-455-5p*. This in turn upregulates the expression of *JAK1*, which promotes the proliferation and metastasis of bladder cancer (11). Thus, the lncRNA, *OXCT1-AS1*, can be used as a potential biomarker and therapeutic target in patients with human glioblastoma and bladder cancer.

A recent study has shown that the lncRNA, *OXCT1-AS1*, promotes the metastasis of NSCLC *in vitro* and *in vivo* by stabilizing *LEF1* (26). In this study, we found that lncRNA *OXCT1-AS1* expression was elevated and *miR-195* expression was decreased in NSCLC tissues and cells. In NSCLC cell lines, knockdown of *OXCT1-AS1* expression significantly inhibited cell viability and proliferation, together with the reduction of *CCND1* and *CCNE1*. In contrast, overexpression of *miR-195* significantly decreased cell viability and proliferation, and inhibited the expression of cyclins, *CCND1* and *CCNE1*. Meanwhile, we found that knockdown of *OXCT1-AS1* could significantly inhibit *miR-195* expression, and the luciferase reporter assay further verified that *OXCT1-AS1* targets and regulates *miR-195* expression. It has been reported that reduced *miR-*

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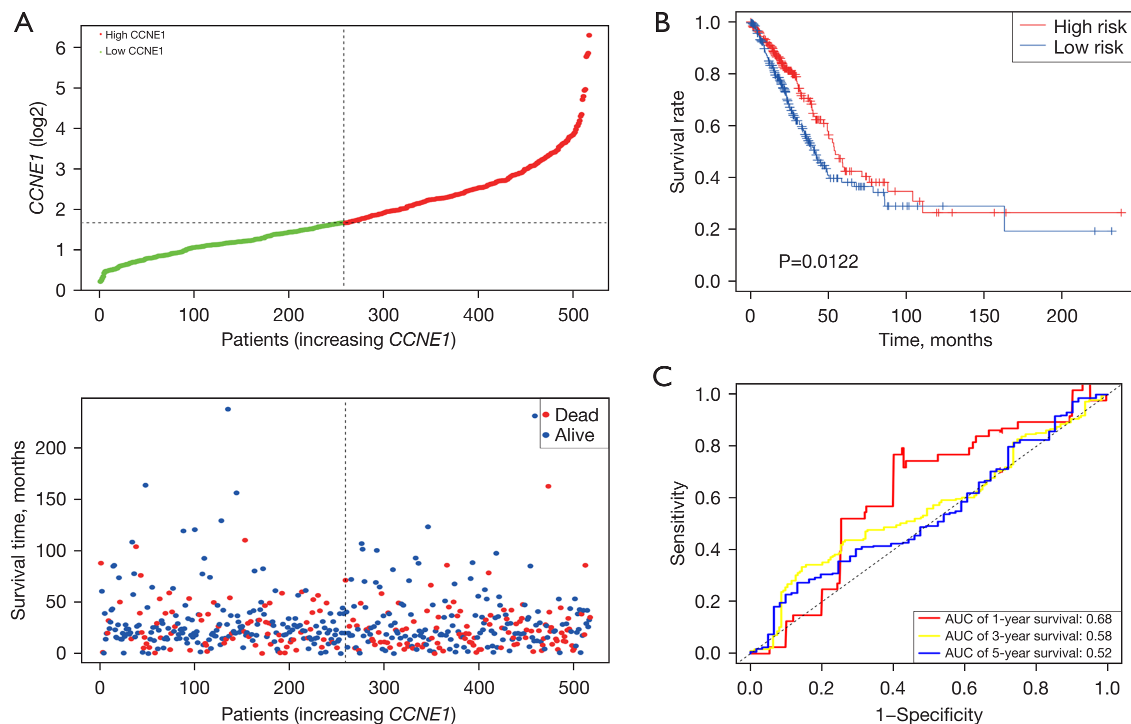

Figure 6 OS analysis between high- and low-*CCNE1* expression groups in LUAD. (A) Expression of *CCNE1* and LUAD patients' survival time scatter gram. (B) The survival curves between the high- and low-*CCNE1* expression groups. (C) The time-dependent ROC curve of *CCNE1* expression in predicting LUAD OS. AUC, area under the curve; OS, overall survival; LUAD, lung adenocarcinoma; ROC, receiver operating characteristic.

195 expression in NSCLC promotes aspects of cancer progression, such as cancer cell proliferation, which is consistent with our findings. Taken together, our data suggested that *OXCT1-AS1* acts as an oncogene in NSCLC.

A study has reported that *miR-195* is abnormally expressed in a variety of malignancies, including NSCLC (25). As a tumor suppressor, *miR-195* is involved in various cell functions such as cell cycle regulation, DNA damage and repair, gene transcription, migration, invasion, and tumor cell apoptosis (26,27). According to a previous study, the downstream target genes of *miR-195* include *CCNE1*, *BIRC5*, *IGF1R*, and *CCND3* (28). In addition, increased *miR-195* expression levels were reportedly associated with decreased survival rates in NSCLC (29). In our study, we hypothesized that *miR-195* inhibits the growth of NSCLC cells by targeting downstream genes. To further investigate the underlying mechanism, potential targets of *miR-195* were explored and *CCNE1* was selected due to its tight association with cell proliferation. We found that *CCNE1* served as a direct target of *miR-195*.

Cyclins are involved in cell cycle regulation, and interact with cyclin-dependent kinases (CDKs) and cell cycle-dependent kinase inhibitors (CKIs) (30,31). *CCNE1* is an isoform of cyclin E. The *CCNE1*-CKD2 complex induces hyperphosphorylated cyclin D-CDK4/6 to trigger Rb protein phosphorylation (32), releasing E2F, which allows cell cycle progression G1 to S phase and DNA synthesis (33). Some studies have illustrated the abnormal expression of *CCNE1* in a variety of malignant cells (34,35). It has been shown that overexpression of *CCNE1* is associated with poor prognosis in NSCLC (36). The lncRNA, SENP3-EIF4A1, acts as a sponge for *miR-195-5p* and drives the progression of triple-negative breast cancer by overexpressing *CCNE1* (37). Also, the lncRNA, LINC00355, increases the expression of *CCNE1* by downregulating *miR-195*, thereby promoting the proliferation of LUAD cells (12). Additionally, the lncRNA, SNHG12, affects the activity, apoptosis, and autophagy of prostate cancer cells by regulating *miR-195/CCNE1* (13). An increasing number of studies have shown that lncRNAs/miRNAs/target genes play an important role in NSCLC

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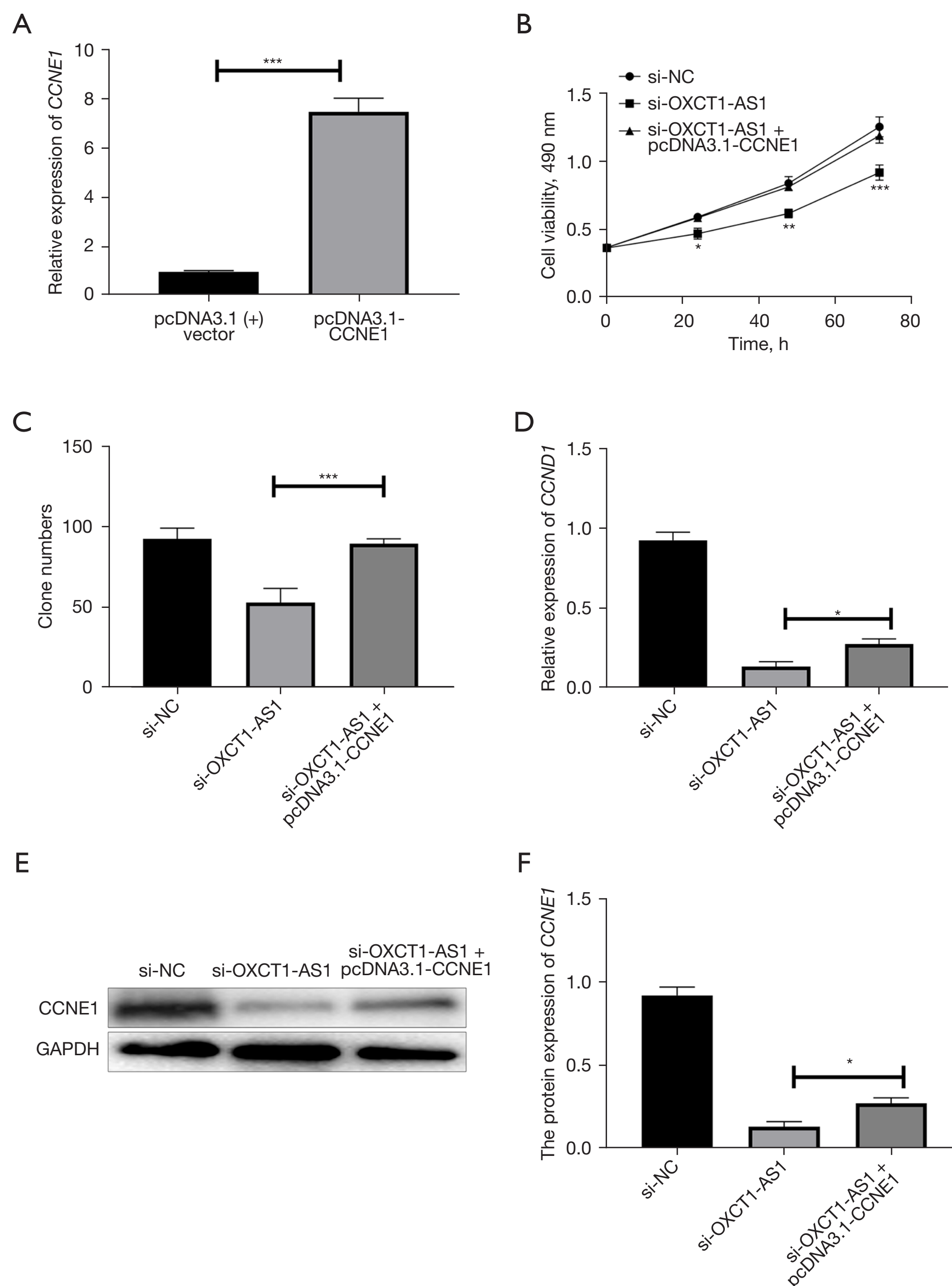

Figure 7 *CCNE1* affects the proliferation in *OXCT1-AS1* knock-down NSCLC cells. (A) Transfection efficiency of *CCNE1* was verified by qRT-PCR. (***P<0.001). (B) After transfection of si-NC, si-OXCT1-AS1, and si-OXCT1-AS1 + pcDNA3.1-CCNE1 in H1650 cells, cell viability was analyzed at 0, 24, 48, and 72 h using the CCK-8 to plot changes in cell growth curves. (*P<0.05; **P<0.01; ***P<0.001). (C) After transfection of si-NC, si-OXCT1-AS1, and si-OXCT1-AS1 + pcDNA3.1-CCNE1 in H1650 cells, changes in the number of cell colony formations were detected using the colony forming assay. (***P<0.001). (D) qRT-PCR was performed to detect the expression changes of CCND1 after transfection of si-NC, si-OXCT1-AS1, and si-OXCT1-AS1 + pcDNA3.1-CCNE1 in H1650 cells. (*P<0.05). (E) A western blotting assay was performed to detect the expression changes of *CCNE1* after transfection of si-NC, si-OXCT1-AS1, and si-OXCT1-AS1 + pcDNA3.1-CCNE1 in H1650 cells. (F) Statistical analysis of protein expression for (E). (*P<0.05). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NSCLC, non-small cell lung cancer; qRT-PCR, quantitative reverse transcription polymerase chain reaction; CCK-8, Cell Counting Kit-8.

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(14,15). In our study, it was found that knockdown of lncRNA *OXCT1-AS1* inhibited cell proliferation via *miR-195/CCNE1* in NSCLC cell lines.

In future studies, we will continue to explore whether lncRNA *OXCT1-AS1* can serve as a potential biomarker for patient risk stratification and loco regional metastasis in NSCLC. We are collecting more clinical information and gene expression data of NSCLC patients to provide a basis for future studies on the application of lncRNA *OXCT1-AS1* as a clinical marker in patient risk stratification and loco regional metastasis of NSCLC. However, the clinical application of lnRNA *OXCT1-AS1* still faces huge challenges. From the perspective of sensitivity, specificity, and reproducibility of lnRNA *OXCT1-AS1*, we still need more in-depth research.

In conclusion, there were significant differences in the expression of lncRNA *OXCT1-AS1* and *miR-195* in NSCLC tissues and cells. LncRNA *OXCT1-AS1* was significantly upregulated and *miR-195* was markedly downregulated in NSCLC tissues and cells. LncRNA *OXCT1-AS1* can regulate the proliferative ability of NSCLC cells via the *miR-195/CCNE1* signaling axis. Therefore, *OXCT1-AS1* may be a prospective biomarker and therapeutic target for patients with NSCLC.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics board of The Third Affiliated Hospital of Qiqihar Medical University (No. 2021LL-9) and informed consent was taken from all the patients.

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