The mechanism of \textit{LncRNA01977} in lung adenocarcinoma through the SDF-1/CXCR4 pathway

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\textbf{Background:} A significant correlation has been identified between lncRNA and tumor cell resistance, diagnosis, and prognosis. Although mRNA studies have dominated the field of non-coding RNA biology in tumorigenesis in recent years, long-chain non-coding RNA (the biological function) has also attracted increasing attention. However, the lncRNA associated with lung adenocarcinoma (LUAD) remains unexplored. This study used bioinformatics analysis to screen and identify \textit{LncRNA01977} as a key oncogenic driver of LUAD.

\textbf{Methods:} The experiment was divided into blank serum group (normal serum medium) and lung compound low, medium and high dose groups (5%, 10%, 15% and 15% lung compound drug serum medium, respectively). Transwell invasion ability of A549 cells was detected, and Western blot tested A549 cells SDF-1 specific receptor CXCR4, and CXCR4 gene expression in A549 cells were determined by reverse transcription–polymerase chain reaction (RT-PCR). In addition, western blotting, MTT proliferation test, colony formation test and apoptosis detection techniques were used to explore the mechanism of \textit{LncRNA01977}’s effects on LUAD.

\textbf{Results:} In vitro assays demonstrated that \textit{LncRNA01977} can significantly promote the progression of LUAD and that stromal cells in tumor microenvironment secrete chemokine CXCL12, also known as stromal derived factor-1 (SDF-1), and its receptor CXCR4 is low expressed in normal tissues and high expressed in LUAD tissues. Lung cancer patients with high expression of CXCR4 are more prone to metastasis.

\textbf{Conclusions:} \textit{LncRNA01977} can be used as a new prognostic indicator of LUAD, and can help patients to find more effective target treatment options for LUAD.

\textbf{Keywords:} Lung adenocarcinoma (LUAD); SDF-1/CXCR4; \textit{LncRNA01977}; LncRNA; tumor marker

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\section*{Introduction}

Studies have reported that lung adenocarcinoma (LUAD) has one of the highest mortality rates among all malignancies worldwide (1,2). Therefore, continuing research into the mechanisms of the malignant progression of LUAD is crucial, in order to discover more effective therapeutic targets for LUAD patients and develop more effective treatment strategies.
In cancer, LncRNA is generally highly expressed and is associated in many aspects of cancer development. LncRNA can promote the drug resistance of lung cancer cells or regulate miRNA to promote the apoptosis of lung cancer cells (3). Furthermore, recent studies (4,5) have suggested that LncRNA dysregulation promotes malignant cancer gene progression. MALAT can pass through the YAP1-MALAT-miR-126-5p axis, which promotes angiogenesis and epithelial-stromal transformation in colorectal cancer. Moreover, Du et al. (6) reported that LncRNA inhibits miRNA (MicroRNAs)-mediated CDK6 and FN1 degradation via adsorption of a microRNA let-7. Kikushige et al. (7) found that LncRNA is leading to the lung through the induction of autophagy activation Patients with adenocarcinoma are drug-resistant. There is increasing evidence that LncRNA01977 plays an important role in cell differentiation and development (8). Transcriptional profiling studies have found that LncRNA01977 is dysregulated in a variety of complex human diseases, highlighting the potential of LncRNA01977 as a biomarker for cancer diagnosis and prognosis. TCGA database includes gene expression, protein expression, DNA methylation, etc., which provides a good research basis for searching for tumor specific biomarkers. MiRNA usually affects gene expression processes (9). Since its discovery, miRNA has been shown to be involved in multiple pathological processes, including oncogenic effects (10). And it provide effective therapeutic targets for patients with LUAD (11,12). Chemokines and their receptors are considered to play an important role in tumor metastasis (13-15). Sdf-1 and its receptor CXCR4 have been widely studied. CXCR4 is highly expressed in a variety of highly metastatic tumors. Sdf-1/CXCR4 axis can activate a variety of signaling pathways and participate in tumor invasion and metastasis, improve cell motility, migration and invasion, and promote matrix degradation, intercellular adhesion and angiogenesis, suggesting that precise intervention of SDF-1/CXCR4 axis can inhibit tumor metastasis. It has potential clinical application prospect.

In this study, we used bioinformatics to screen and analyze LncRNA01977 with significant upregulation features in LUAD. we also evaluated tumor proliferation and invasion of LncRNA01977. Our results suggested that LncRNA01977 could promote LUAD progression by targeting the SDF-1/CXCR4 axis.

We present the following article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2903/rc).

Methods

Gene screening and identification

These data were processed by using the edge R package, and the parameters were set to FDR (False Discovery Rate) <0.05 and the absolute value log_2 >1. In addition, the TCGA pan-cancer of GTEx dataset was acquired from Xena (http://xena.ucsc.edu/). Moreover, we also mapped all the probes to the human genome by using the SeqMap method (GRCh38) to re-annotate the probe set of the GPL22755. All probes were mapped to a gene group. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell line selection and culture

A549 cells were cultured in RPMI medium containing 10% FBS in an incubator with 5% CO2 and 37 °C. A549 cells at logarithmic growth stage were selected and divided into blank serum group (normal serum medium) and experimental group. In addition, other LUAD cell lines such as HCC-78, KTA-7 and PC-9 were also cited for validation and study.

Plasmid construction

The DNA vector enables the target gene to be expressed in cells to achieve our research objectives. The artificially modified plasmid is one of the most commonly used vectors and usually has the following characteristics: (I) one or more restriction endonuclease cutting points for easy insertion of the target gene; (II) with marker genes (resistance genes, fluorescent genes, etc.) for easy screening; (III) suitable size (generally less than 10 KB) for transfection. Targeted LncRNA01977 short hairpin RNA (shRNA) sequence is as follows: 1,5'-TGTTCCTAATTTGGACACTGG TTTA-3'; and 2,5'-AACCAGATACCATGGGAAT-3'.

Cell proliferation test

In this study, a 3-(4,5-dimethylthiazole-2-group)-2,5-diphenyl tetrazole (MTT) determination was used to assess cell viability. Cell viability was determined according to the CCK-8 system (Dojindo, Japan). Cells were seeded in 96-well plates (1,104 cells per well). Cells were incubated in 37 °C dark, adding 10 μL CCK-8 solution at 0, 24, 48, 72 h, and incubated for another 4 h. The absorbance of each well was then assessed at 450 nm, using a microplate reader (Te-can, Switzerland).
Cells and tissue specimens

Human LUAD cell lines HCC-78, KTA-7 and PC-9, and human bronchial epithelial cell lines A549 were all purchased from the Cell Bank of Typical Culture Conservation Committee, Chinese Academy of Sciences. All cell lines were cultured in DMEM medium (Dulbecco’s Modified Eagle Medium, sigma, USA) containing 10% fetal bovine serum (Hyclone, General Life Sciences, USA), plus another 100 U/mL Streptomycin and 100 U/mL of penicillin (Thermo Fisher Scientific, USA). The culture conditions were obtained at 37 ℃ of 5% CO₂. Cells used in this study were all passed through 2 to 4 passages after resuscitation.

Cell culture

Human lung adenocarcinoma cells A549 were cultured using RPMI 1640 medium and Ham’s F-12K medium with fetal calf serum with volume fraction of 0.1, and human normal lung bronchial epithelial cells BEAS-2B using DMEM medium with volume fraction of 0.1, both in a 5% CO₂, 37 ℃ incubator. A549 group: conventional culture without any treatment, A549/NC group: transfer to overexpression control plasmid, A549/LncRNA01977 group: transfer to LncRNA01977 overexpression plasmid.

Transwell trial

Two small interfering RNA species (small interfering RNA, siRNA, Oligobio, China) targeting LncRNA01977 were designed and synthesized, and the negative scrambled control sequence siRNA (si-NC) was set. The TAF15 overexpression plasmid vector was constructed based on the pcDNA3.1 plasmid (Invitrogen, USA). Cells were seeded in six-well plates with a density of 1.8×10⁵/well and placed at 37 ℃ culture conditions were used for 24 h. Subsequently, cells were transfected with the corresponding vector for DMEM containing 10% FBS without antibiotics for 48 h, and cells were collected for the relevant experiments.

Construction of SDF-1 lung adenocarcinoma cells

The wild-type LncRNA01977 construct and SDF-1 mimics were co-transfected into LUAD cells. After 24 h of incubation, the transfected cells were seeded in 96-well plates. 48 h after transfection, lung adenocarcinoma cells were detected using the dual luciferase reporter gene assay system (Promega).

Real-time PCR analysis

At 24 h after transfection, total RNA from cells in each group was extracted by TRizol and transcribed by polymerase-chain reaction after reverse transcription. The primer sequence is as follows: LncRNA01977 upstream primer: 5-CAGTGG ACTTGCGACGGTATG-3, downstream primer: 5-CCGCCCTCGCCTAG TCTGGTC TC-3; GAPDH upstream primer: 5-GTTGGAG GTTGGAGTC AACGG-3, the downstream primer: 5-GAG GGATCTCGCTCCTGGAGGA-3. GAPDH was used as an internal reference in 3 independently repeated experiments.

Western blotting analysis

Total protein from transfected cells in each group were extracted and used for SDS-PAGE gel electrophoresis. Primary antibodies were prepared in 5% blocking buffer according to the dilution of the corresponding antibodies. 4 ℃ was incubated overnight and washed at room temperature and incubated with secondary antibodies for 1 h. After washing, they were visualized using an ECL chemiluminescent solution-actin was used as an internal reference for 3 independent repeated experiments.

Weighted gene co-expression network analysis

To obtain more accurate results, 1,000 permutation tests were performed. The Z-summary score was used to determine which modules are low-saving modules, with a Z-score <10 indicating that the module was weakly preserved.

Statistical analysis

All experiments in this study were repeated at least three times, and the experimental data were expressed as the mean ± standard deviation (SD). In addition, we performed statistical analysis using GraphPad Prism 8 (Graphpad, USA).

Results

LncRNA01977 predicts a poor prognosis in patients with LUAD

We performed different analyses in the TCGA and GSE155478 datasets, respectively based on the limma..
In order to analysis of correlation of SDF-1, we transfed the SDF-1 simulants into HCC-78, KTA-7 and PC-9 cells. We found that LincRNA01977 promotes the growth and proliferation of lung adenocarcinoma cells through the SDF-1 pathway, and chemical resistance to DOX in HCC-78, KTA-7 and PC-9 10 cells (Figure 5A-5C). Target Scan Human 7.2 (http://www.targetscan.org/vert_72/) was used to speculate that LncRNA01977 expression could partially rescue this effect (Figure 5D,5E).

LncRNA01977 inhibits CXCR4 expression level

In order to analysis of correlation of SDF-1, we transfed the SDF-1 simulants into HCC-78, KTA-7 and PC-9 cells. We found that LncRNA01977 promotes the growth and proliferation of lung adenocarcinoma cells through the SDF-1 pathway, and chemical resistance to DOX in HCC-78, KTA-7 and PC-9 10 cells (Figure 5A-5C). Target Scan Human 7.2 (http://www.targetscan.org/vert_72/) was used to speculate that LncRNA01977 expression could partially rescue this effect (Figure 5D,5E).

LncRNA01977 Inhibit the development of LUAD

Decreased number of colony formations (Figure 2A–2C). Cytotoxicity assays indicated that downregulation of LncRNA01977 reduced the DOX sensitivity of LUAD cells, with reduced IC50 values compared to the controls (Figure 2D,2E). The effect of LncRNA01977 on cell proliferation was assessed by EdU cell staining assay and CCK-8 cell proliferation assay. After inhibition of LncRNA01977 expression in A549, Calu-3, the number of EdU stained cells was significantly reduced, while the increase of EdU stained cells increased significantly after LncRNA01977 overexpression (P<0.05) (Figure 2F,2G).

Overexpression of LncRNA01977

We reached a similar conclusion in the CCK-8 trial. Upon inhibition of LncRNA01977, cell proliferation decreased and increased after overexpression of LncRNA01977 (Figure 3A–3D). Based on the knockdown results, LncRNA01977 overexpression increased HCC-78, as did the IC50 values of DOX in KTA-7 and PC-9 cells (Figure 3E). LncRNA01977 overexpression group showed stronger DOX resistance (Figure 3F–3H).

LncRNA01977 and SDF-1 correlation analysis

Cell grade analysis revealed that assays to investigate whether LncRNA01977 and SDF-1 were present in the RISC, and its main component is Argonaute2 (AGO2) (Figure 4A,4B). Furthermore, transfection of the SDF-1 mimic reversed LncRNA01977 overexpression, while the transfection of LncRNA01977 SDF-1 expression was inhibited by the overexpression vector (Figure 4C–4E). To further investigate the relationship between LncRNA01977 and SDF-1 (Figure 4F,4G).

Discussion

The chemokine SDF-1, also known as CXCL12 PBSF and its specific receptor, CXCR4, are widely expressed in various tissues and organs (16-20). SDF-1/CXCR4 signaling pathway have significant influence in the multiple tumors as well as metastasis, which may function through the MAPK or AKT pathway (21). The SDF-1/CXCR4 biological axis refers to the coupled molecule with close relationship to cell signal transduction and the interaction of CDF and its specific receptor CXCR4 (22-24). Cell migration lies in the high affinity and absolute specificity of CXCR4 for its ligand SDF, namely, CXCR4 is the only receptor for SDF. Studies show that the impact of the SDF-1/CXCR4 biological axis on tumors is multifaceted and playing an important role in the occurrence, development and metastasis of multiple tumors (25-30).

Lung cancer is the highest incidence and fatality malignancy worldwide (31). Despite the certain success of conventional therapies and targeted therapies, the 5-year overall survival rate of lung cancer is still only 10% to 15% (32-35). Recent immune-related targeted therapies such as cytotoxic T lymphocyte-associated antigens 4, the programmed death receptor 1 (PD-1), and the programmed death receptor Antibody-targeted therapy with body-ligand programmed death receptor 1/CXCR4 axis is a long chain non-coding RNA of 200 nucleotides with
Figure 1 LncRNA01977 is an oncogenic driver and predicts poor prognosis in patients with LUAD. (A) Paired normal samples of 112 tumors obtained from TCGA lung adenocarcinoma data; (B) different analysis of DOX-sensitive and DOX-resistant cell lines from GSE155478. (C) DOX IC50 estimated drug response in TCGA LUAD patients based on GDSC drug response data, with three subgroups representing the LncRNA01977 expression levels. (D) TCGA LUAD patients Kaplan-Meier survival analysis. (E) Expression profile of LncRNA01977 in LUAD progression cell line models. (F) Case of the expression of LncRNA01977 in the pan-cancer dataset. LUAD, lung adenocarcinoma. *, P<0.05, **, P<0.01, ***, P<0.001, ****, P<0.0001; ns, meaningless; Student’s t-test. LUAD, lung adenocarcinoma; DOX, Docetaxel; TCGA, The Cancer Genome Atlas; GDSC, Genomics of Drug Sensitivity in Cancer.
Figure 2 LncRNA01977 knockdown decreased the proliferation, metastasis, and chemochemical resistance to DOX in LUAD cells. (A) The efficiency of shRNA knockdown was assessed by real-time PCR in HCC-78, KTA-7 and PC-9 cells. (B) The MTT assay was used to assess the cell viability of HCC-78, KTA-7 and PC-9 transfected with a control or knockdown plasmid. (C) The colony formation assay showed the effect of LncRNA01977 knockdown on LUAD cell proliferation. (D) DOX IC50 in the control plasmid or HCC-78, KTA-7 and PC-9 transfected with the knockdown plasmid. (E) The cytotoxicity assay was used to test the inhibition of DOX resistance caused by LncRNA01977 knockdown in HCC-78, KTA-7 and PC-9 cells. DOX concentration: 0.1 M in HCC-78, KTA-7 and PC-9 cells, 0.5 M in MCF-7 cells. (F) Cell migration and invasion assay [Cell HE staining]. (G) Cell scratch assay (*, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001). LUAD, lung adenocarcinoma; DOX, Docetaxel; TCGA, The Cancer Genome Atlas; PCR, polymerase chain reaction; MTT, Methyl tetrazolium; HE, hematoxylin-Eosin; NC, normal contras; ns, meaningless. (Observational method: High power microscope ×45).
Figure 3 LncRNA01977 overexpression promotes proliferation, metastasis and chemoresistance to DOX in LUAD and carcinoma cells. (A) The efficiency of LncRNA01977 expression was assessed by real-time PCR in HCC-78, KTA-7 and PC-9 cells. (B) MTT assay. (C) Colony formation assay. (D) EDU assay. (E) IC50. (F) Cytotoxicity assay. (G) Apoptosis assay (*, P<0.05; **, P<0.01; ***, P<0.001). (H) Transwell assay (Staining method: Cell HE staining). MTT, Methyl tetrazolium; PCDH: Protocadherin; PCR, Polymerase Chain Reaction; EDU, Ethynyl-2'-deoxyuridine; HE, Hematoxylin-eosin; ns, meaningless; LUAD, lung adenocarcinoma; DOX, Docetaxel; TCGA, The Cancer Genome Atlas.
no or low translational potential, regulates gene expression at epigenetic, transcriptional and posttranscriptional levels and is widely involved in the organism pathophysiological process (39). For example, \textit{LncRNA01977} can act as a target for a transcription factor or RNA polymerase, disrupting their binding to target gene promoters, thereby promoting or repressing gene expression, which contain immune-related genes (40). Currently, the immune-related lncRNA has been less frequently reported in lung adenocarcinoma. The bioinformatics analysis of the immune-related lncRNA and its relationship with the prognosis of lung adenocarcinoma patients is expected to provide a
reference for the prognosis judgment and treatment of lung adenocarcinoma (41). The SDF-1/CXCR4 biological axis plays an important role in the dissemination and organ-specific metastasis of a wide variety of tumors. It is suggested that intervention of SDF-1/CXCR4 biological axis can be used to treat tumors (42-44).

In conclusion, LncRNA01977 plays a very important regulatory role in the occurrence and development of lung adenocarcinoma, and its maladjustment will promote the rapid growth and proliferation of lung adenocarcinoma cells, and has a certain correlation with the metastasis of tumor cells. In addition, LncRNA01977 also has a proportional
Figure 6 LncRNA01977 promotes LUAD progression and chemoresistance to DOX by targeting the SDF-1/CXCR4 axis. (A) WGCNA, the module preservation analysis in each module is represented by its color code and name. The left panel shows the save median level. The right figure shows the saved Z-summary value. A Z-summary value < 10 represents the low-retention module. (B) Gene ontology analysis. (C) The expression efficiency of CXCR4 was verified by the protein blotting assay. (D) MTT assay. (E) Cytotoxicity Assay. (F) Cell migration array (staining method: Cell HE staining). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, meaningless; Student’s t-test; PCMV, number of a plasmid. LUAD, lung adenocarcinoma. DOX, Docetaxel; TCGA, The Cancer Genome Atlas; MTT, Methyl tetrazolium; HE, hematoxylin-eosin; WGCNA, weighted gene co-expression network analysis.
relationship with docetaxel resistance in patients with lung adenocarcinoma, and may play its biological effect by targeting sdF-1/CXCR4 axis, which broadens our understanding of post-transcriptional regulatory mechanisms, and helps to provide new prognostic indicators and therapeutic targets for patients with LUAD.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2903/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2903/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2903/coif). The authors have no conflicts of interest to declare.

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).

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