Original Article Long noncoding RNA DLX6-AS1 targets miR-124-3p/CDK4 to accelerate Ewing's sarcoma

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Abstract: Ewing's sarcoma is one of leading cause of malignancy occurred in the children and adolescents worldwide. Given the emerging critical role of long noncoding RNA (IncRNA) in the human cancer, as well as Ewing's sarcoma, we aim to identify the biological role of DLX6-AS1 in the tumorigenesis. Results unveil that DLX6-AS1 expression was increased in the tissue sample and cells. Functionally, the silencing of DLX6-AS1 could repress the proliferation and accelerate the apoptosis of Ewing's sarcoma cells. Mechanically, DLX6-AS1 functioned as the sponge of miR-124-3p, and then miR-124-3p targeted the 3'-UTR of CDK4 mRNA, forming the DLX6-AS1/miR-124-3p/CDK4 regulatory pathway. In conclusion, the critical role of DLX6-AS1 might unveil a potential therapeutic target for Ewing's sarcoma.

Keywords: Ewing's sarcoma, DLX6-AS1, miR-124-3p, CDK4

Introduction

Ewing's sarcoma is a type of aggressive malignancy occurred in the children and adolescents, leading to increasing number of lethality worldwide [1, 2]. As regarding the clinic treatment, the surgery accompanied by chemotherapy or radiotherapy could play an important role [3]. However, there are still abundant patients with Ewing's sarcoma died of the disease. Therefore, the novel therapeutic strategies with high specificity are necessary [4].

Long noncoding RNAs (IncRNAs) are group of transcripts, ranging from hundreds nucleotide base to thousands nucleotide base, which is short of protein-coding potential [5-7]. Abnormal expression of IncRNAs has been identified to exist in multiple human cancers, and numerous IncRNAs regulate the tumorigenesis and development [8]. Being similar to micro RNA or circular RNA, IncRNAs could wildly regulate the tumorigenesis of human cancer [9]. For instance, IncRNA SBF2-AS1 is upregulated in lung adenocarcinoma and the SBF2-AS1-miR- 338-3p/362-3p-E2F1 axis promotes the tumorigenesis [10]. In human adipose-derived stem cells, IncRNA-PCAT1 is negatively correlated with miR-145-5p and positively regulated TLR4 to regulate osteogenic differentiation [11].

LncRNA DLX6-AS1 is a oncogene in the human cancer that identified in multiple human cancer, such as liver cancer, non-small cell lung cancer, pancreatic cancer, osteosarcoma and son on [12-15]. In this research, we identified that DLX6-AS1 was increased in the Ewing's sarcoma tissue and cells. Given the oncogenic role of DLX6-AS1 confirmed by our study, we discovered the biological function and mechanism of DLX6-AS1 on the tumorigenesis via miR-124-3p/CDK4.

Materials and methods

Clinical sample collection

Ewing's sarcoma tissues and the adjacent normal tissues were obtained from the patients who experienced surgery at Weinan Maternal and Child Health Hospital. Tumor samples were provisionally frozen in liquid nitrogen and then chronically stored at -80°C. Patients involved in this research signed the informed consent before study. This study obtained the permission from the ethical committee of Weinan Maternal and Child Health Hospital.

Ewing sarcoma cell lines

Ewing sarcoma cell lines (SK-ES-1, A673, RD-ES) and mesenchymal stem cells (MSCs) were provided by the American Type Culture Collection (ATCC). Cells were grown in DMEM (Corning) supplemented with 10% FBS (Omega Scientific) and 100 U/100 μ g, 2 mM glutamine (Invitrogen, USA).

Cellular transfection

The small interacting oligonucleotides targeting the DLX6-AS1 were designed by RiboBio (Guangzhou, China). The sequences were presented in the <u>Table S1</u>. The transfection was performed using the Lipofectamine 2000 (Invitrogen, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from Ewing's tissues and cells using TRIzol (Thermo, USA). Total RNA (1 ug) was conversely synthesized to form the first-strand cDNA using One-Step RT-PCR Kit (TaKaRa). The RT-PCR was performed an ABI-7500 RT-PCR system (Applied Biosystems). GAPDH acted as the housekeeping gene and U6 RNA acted as a miRNA internal control. The relative expression was detected with the $2^{-\Delta\Delta ct}$ method. The primers were presented in the Table S1.

Western blotting

All the primary antibodies were provided by Abcam, including rabbit anti-human antibody (1:1,000). Horseradish peroxidase-conjugated (HRP) anti-rabbit antibodies (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) acted as the secondary antibodies. Antibody dilution (1:1000) was used for anti-CDK4 (Abcam). The proteins were detected using enhanced chemiluminescence (ECL) system and exposed to X-ray film and quantified by Gel-Pro Analyzer (Media Cybernetics, Rockville, MD, USA) for the densitometry.

CCK-8 assay and colony formation assay

The proliferative ability of Ewing's sarcoma was detected using the CCK-8 assay and colony formation assay as previously described [16]. Ewing's sarcoma cells were seeded in a 96-well plate by the 2000 cells/well density. Then, 20 μ I of Cell Counting Kit-8 (Beyotime Institute of Biotechnology) was added to each well after 48 h. The absorbance was measured at 450 nm.

Flow cytometry analysis

The apoptosis and cycle analysis were performed using flow cytometry analysis. In brief, the cells were trypsinized and seeded in the 1 ml 2% FBS in PBS at the density of 1×10^4 cell per wells and washed with cold PBS. Cells were resuspended in binding buffer (100 µl) and stained with FITC Annexin V (5 µl) and propidium iodide (PI, 5 µl) for 15 min at room temperature in the dark. The apoptosis Kit (KeyGen, Nanjing, China) was administrated and subjected to flow cytometric analysis FACS Canto II flow cytometry (BD Biosciences). The cycle analysis was stained with 10 mg propidium iodide/ml and performed using analyzed using FACSCalibur flow cytometer (Becton Dickinson).

Subcellular fractionation location

The nuclear or cytosolic fraction was purified and extracted using the PARIS kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocols.

Dual-luciferase reporter assay

The sequences at the 3'-UTR of DLX6-AS1 and CDK4 were amplified and the products were cloned into the luciferase reporter vector pGL3-basic (Promega, USA). 48 h after transfection, the luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega) based on the Firefly/Renilla luciferase activity. Renilla luciferase vector was transfected as an internal control.

Statistical analysis

The data were determined by repeated analysis and presented as median and differences. The differences within different groups were analyzed by the student's t-test or one way ANOVA or Mann-Whitney U-test. Difference was regard-



Figure 1. LncRNA DLX6-AS1 is increased in Ewing's sarcoma. A. LncRNA DLX6-AS1 expression in Ewing sarcoma cell lines (SK-ES-1, A673, RD-ES) and mesenchymal stem cells (MSCs) by RT-PCR. B. IncRNA DLX6-AS1 expression in the clinical Ewing's sarcoma specimens. C. In the clinical dataset, the abundance of DLX6-AS1 in the Ewing's sarcoma. **Indicates the *p*-value less than 0.01.

ed as P<0.05. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

Results

LncRNA DLX6-AS1 is increased in Ewing's sarcoma

In the Ewing's sarcoma cells (SK-ES-1, A673, RD-ES), the expression of DLX6-AS1 was analyzed by the RT-PCR. As respected, the expression of DLX6-AS1 was remarkedly up-regulated compared to the normal cells (Figure 1A). In the clinical Ewing's sarcoma specimens, IncRNA DLX6-AS1 expression was up-regulated compared to the normal controls (Figure 1B). In the clinical dataset, the abundance of DLX6-AS1 was also highly expressed in the Ewing's sarcoma (Figure 1C). Therefore, IncRNA DLX6-AS1 is increased in Ewing's sarcoma.

The knockdown of DLX6-AS1 represses the proliferation and accelerates the apoptosis of Ewing's sarcoma cells

The over-expression of DLX6-AS1 in the Ewing's sarcoma tissue and cells indicated its potential oncogenic roles in the Ewing's sarcoma tumorigenesis. The independent small interfering RN-As were transfected into Ewing's sarcoma cells (SK-ES-1, A673) to silence the DLX6-AS1 expression (**Figure 2A**). The colony formation assay presented that DLX6-AS1 silencing re-

pressed the growth significantly in SK-ES-1 and A673 cells (Figure 2B). CCK-8 assay indicated that DLX6-AS1 silencing repressed the absorbance for these cells (Figure 2C). Flow cytometry demonstrated that DLX6-AS1 silencing accelerates the apoptosis of Ewing's sarcoma cells (SK-ES-1, A673) (Figure 2D). Moreover, the cycle analysis using flow cytometry illustrated that DLX6-AS1 silencing facilitated the G0/G1 phase arrest compared to the negative controls (Figure 2E, 2F). The evidence indicates that the knockdown of DLX6-AS1 represses the proliferation and accelerates the apoptosis of Ewing's sarcoma cells.

DLX6-AS1 acts as a sponge of miR-124-3p

Bioinformatics tools indicated that there were series of miRNAs that could bind with DLX6-AS1 at the 3'-UTR (3'-untranslated regions) (Figure 3A). Besides, we found that the prior distribution of DLX6-AS1 in the SK-ES-1 was cytoplasm (Figure 3B). When the DLX6-AS1 plasmids (wild type or mutant) were co-transfected with the miR-124-3p (mimics or blank controls), the luciferase activities were measured using the luciferase reporter assay (Figure **3C**). This data suggested that DLX6-AS1 could bind with the miR-124-3p by molecular binding. In the Ewing's sarcoma specimens, miR-124-3p expression was decreased (Figure **3D**). In the Ewing's sarcoma cells, miR-124-3p expression was similarly down-regulated (Fi-



Figure 2. The knockdown of DLX6-AS1 represses the proliferation and accelerates the apoptosis of Ewing's sarcoma cells. A. The independent small interfering RNAs were transfected into Ewing's sarcoma cells (SK-ES-1, A673) to silence the DLX6-AS1 expression. B. The colony formation assay presented growth ability of Ewing's sarcoma cells. C. CCK-8 assay indicated the absorbance with or without DLX6-AS1 silencing. D. Flow cytometry demonstrated the apoptosis of Ewing's sarcoma cells (SK-ES-1, A673). E, F. Flow cytometry demonstrated the cycle analysis of the G0/G1 phase arrest compared to the negative controls. **Indicates the *p*-value less than 0.01.

gure 3E). When the DLX6-AS1 was silenced, miR-124-3p was inversely up-regulated (Figure 3F). In the clinical tissue samples, the interaction within DLX6-AS1 and miR-124-3p was negative (Figure 3G). Overall, DLX6-AS1 acts as a sponge of miR-124-3p in the Ewing's sarcoma cells.

DLX6-AS1/miR-124-3p conjointly regulates CDK4

For the target of the DLX6-AS1/miR-124-3p, we found that miR-124-3p could bind with the 3'-UTR of CDK4 (**Figure 4A**). Luciferase reporter assay indicated that CDK4 wild type could efficaciously target and conjugate with the miR-124-3p (**Figure 4B**). The mRNA level of CDK4 in the Ewing's sarcoma cells (SK-ES-1, A673) was

increased (Figure 4C). Furthermore, the miR-124-3p mimics transfection decreased the CDK4 mRNA, and the si-DLX6-AS1 transfection silence the CDK4 mRNA (Figure 4D). In the clinical landscape dataset, CDK4 expression was significantly up-regulated in the Ewing's sarcoma specimens (Figure 4E). Clinically, the higher expression of CDK4 in the Ewing's sarcoma cohort indicated the lower survival rate comparing with the normal specimens (Figure 4F). Overall, DLX6-AS1/miR-124-3p conjointly regulates CDK4 in the Ewing's sarcoma.

Discussion

The roles of IncRNAs in the human cancers were diversified and have caught more and more researcher's attention [17]. Ewing's sar-



Figure 3. DLX6-AS1 acts as a sponge of miR-124-3p. A. Bioinformatics tools indicated that miR-124-3p could bind with DLX6-AS1 at the 3'-UTR (3'-untranslated regions). B. The distribution of DLX6-AS1 in the SK-ES-1. C. The luciferase activities were measured using the luciferase reporter assay with the co-transfection of DLX6-AS1 plasmids (wild type or mutant) and miR-124-3p (mimics or blank controls). D. miR-124-3p expression in the Ewing's sarcoma specimens. E. miR-124-3p expression in Ewing's sarcoma cells. F. miR-124-3p expression when the DLX6-AS1 was silenced. G. The interaction within DLX6-AS1 and miR-124-3p in the clinical tissue samples. **indicates the *p*-value less than 0.01.

coma is an aggressive soft tissue malignancy of adolescents or children [18]. With the rapid development of high-throughput sequencing or next-generation sequencing technology, a large number of new identified IncRNAs have been discovered [19]. On this basis, the bioinformatics tools help the researchers to find the indepth interaction within the ncRNAs and human cancers, including micro RNAs, IncRNAs and circular RNA [9]. In the Ewing's sarcoma tissue and cells, we found that IncRNA DLX6-AS1 was significantly up-regulated compared with the normal tissue and cells. This data indicated that the DLX6-AS1 overexpression in this pathogenesis could act as the oncogene. The critical pathogenic roles of IncRNAs in the human cancers have been identified and reported. In multiple cancers, IncRNA DLX6-AS1 is reported to functions as the oncogene and onco-promoting element.



Figure 4. DLX6-AS1/miR-124-3p conjointly regulates CDK4. A. Bioinformatics tools showed that miR-124-3p could bind with the 3'-UTR of CDK4. B. Luciferase reporter assay indicated that CDK4 wild type could efficaciously target and conjugate with the miR-124-3p. C. The RT-PCR revealed the mRNA level of CDK4 in the Ewing's sarcoma cells (SK-ES-1, A673). D. The RT-PCR revealed the mRNA level of CDK4 in the transfection of miR-124-3p mimics and si-DLX6-AS1. E. CDK4 expression in the clinical landscape dataset for Ewing's sarcoma specimens. F. The survival rate of Ewing's sarcoma cohort with higher expression or lower expression of CDK4. **Indicates the *p*-value less than 0.01.

Such as hepatocellular carcinoma tissue and cells, IncRNA DLX6-AS1 expression is up-regulated compared with adjacent normal tissue. Moreover, bioinformatics analysis revealed the targeting within miR-203a and DLX6-AS1 3'-UTR and MMP-2 mRNA 3'-UTR [20]. In renal cell carcinoma, DLX6-AS1 is upregulated and DL-X6-AS1 promotes renal cell carcinoma development via regulation of miR-26a/PTEN axis [21].

There are numerous IncRNAs, oncogenic or anti-cancer, in the disease and tumorigenesis [22]. In the glioma carcinogenesis, enhanced DLX6-AS1 expression in plasmids and silenced DLX6-AS1 expression by siRNAs inhibited the proliferation, invasion and tumor growth in vitro and in vivo [23]. In the pancreatic cancer, the high expression of DLX6-AS1 is positively correlated with lymph node metastasis, advanced TNM stage and larger tumor size [24]. Therefore, these researches support the conclusion that DLX6-AS1 might function as the oncogenic factor in the human cancer, as well as the Ewing's sarcoma tumorigenesis identified in present study.

The subcellular location of DLX6-AS1 in the Ewing's sarcoma is cytoplasm, suggesting the potential post-transcriptional regulation [25]. In other hand, if the lncRNAs are mainly located in the nuclear, the regulatory mechanism for them might be transcriptional regulation occurred in the nuclear [26]. In this study, we found that DLX6-AS1 could act as the sponge of miR-124-3p, and then miR-124-3p targets the CDK4 mRNA. Therefore, this research identified the DLX6-AS1/miR-124-3p/CDK4 axis in the Ewing's sarcoma. In the Ewing sarcoma, long non-coding RNA-277 (EWSAT1) is a downstream target of EWS-FLI1, by which facilitating the progression of Ewing sarcoma [27].

In Ewing's sarcoma, it has been emerged that CDK4 4/6 pathway acts as a dependency [28, 29]. Therefore, the specific single agent inhibitor target CDK4 4/6 was discovered to explore novel drugs for Ewing sarcoma. Moreover, the high-expression of CDK4 is closely correlated with the poor prognosis and indicates the bad outcome for Ewing sarcoma [30, 31]. In present study, we found that CDK4 acts as the target of DLX6-AS1/miR-124-3p, constructing a regulation in the oncogenesis. The cell cycle and proliferation are the critical elements of Ewing's sarcoma. CDK4 acts as the essential factor in the cycle. Moreover, the cell-cycle inhibition might be a therapeutic target for Ewing's sarcoma. Clinically, a small-molecule CDK4 and CDK6 (CDK4 and 6) inhibitor is currently under clinical investigation [32].

Overall, this research presents a finding that DLX6-AS1 promotes the Ewing's sarcoma tumorigenesis via regulating miR-124-3p/CD-K4, constructing a cascade regulation. Given the critical role of CDK4 in the Ewing's sarcoma, the role of DLX6-AS1 might unveil a potential therapeutic target for Ewing's sarcoma.

Disclosure of conflict of interest

None.

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DLX6-AS1/miR-124-3p/CDK4 promotes Ewing's sarcoma

	Sequences
DLX6-AS1	Forward, 5'-TCTGACCGCGTATGCGCGG-3'
	Reverse, 5'-TTCGCGAGGCTGCGTTAAG-3'
miR-124-3p	Forward, 5'-GAAGTGGACGTTAGTCTG-3'
	Reverse, 5'-AATTACGATTGATTACA-3'
si-DLX6-AS1-1	5'-GGCATGCCTGTCCCCCGT-3'
si-DLX6-AS1-2	5'-ACGCCAGCTTACAGTTCCGT-3'
si-DLX6-AS1-3	5'-TCATACGCACATTAAGTC-3'
CDK4	Forward, 5'-ATGGCTACCTCTCGATATGAGC-3'
	Reverse, 5'-CATTGGGGACTCTCACACTCT-3'
miR-124-3p inhibitor	5'-ATUUAAGGGGUGUUAAGGAC-3';
miR-124-3p mimics	5'-GTAAAUGGACACCAATGGGCT-3';
GAPDH	Forward, 5'-AGAAGGCTGGGGCTCATTTG-3'
	Reverse, 5'-AGGGGCCATCCACAGTCTTC-3'

 Table S1. Primers sequences for qRT-PCR and sequences of siRNA