Original Article
LncRNA DLX6-AS1 promotes laryngeal squamous cell carcinoma growth and invasion through regulating miR-376c

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Abstract: Accumulating evidence showed that IncRNAs play important roles in tumour development. Recently, a novel IncRNA DLX6-AS1 was found to be overexpressed in some tumors such as lung adenocarcinoma, renal cell carcinoma and hepatocellular carcinoma. However, the functional roles of DLX6-AS1 in laryngeal squamous cell carcinoma (LSCC) are still unclear. In the study, we showed that the expression level of DLX6-AS1 was upregulated in the LSCC samples compared to the noncancerous tissues. By using CCK-8 analysis, we demonstrated that knockdown expression of DLX6-AS1 suppressed the Hep2 cell growth. DLX6-AS1 knockdown inhibited the Hep2 cell cycle and invasion. MiR-376c was identified to have the complementary binding sites with the DLX6-AS1. By luciferase reporter assay, we indicated that overexpression of miR-376c inhibited the luciferase activity of wild-type DLX6-AS1, but it failed to suppress luciferase activity of mutated one. DLX6-AS1 knockdown enhanced the expression of miR-376c in the Hep2 cell. Moreover, we showed that the expression level of miR-376c was lower in the LSCC samples than in the noncancerous tissues and the expression of miR-376c was negatively correlated with expression of DLX6-AS1 in LSCC tissues. Ectopic expression of miR-376c suppressed cell proliferation, cycle and invasion of LSCC cell. DLX6-AS1 knockdown suppressed cell proliferation, cycle and invasion via regulating miR-376c expression. These data proved that IncRNA DLX6-AS1 might play as an oncogene in LSCC development and tumorigenesis.

Keywords: Laryngeal squamous cell carcinoma, DLX6-AS1, miR-376c, IncRNA

Introduction
Laryngeal squamous cell carcinoma (LSCC) is 2nd most common neck and head squamous cell carcinoma [1-3]. Therapy options after original diagnosis include chemotherapy, radiation or surgery therapy [4-6]. Patients with LSCC at the early stage can be effectively treated with multi-modal or single treatment, but most cases diagnosed at the advanced stage die of metastasis and/or recurrence [7-10]. The survival and mortality rate of cases with LSCC has not significantly improved in recent twenty years and a variety of studies have been show to elucidate the mechanism of cancer metastasis and invasion [3, 7, 11-14]. Therefore, it is necessary to study the mechanism of occurrence and development of LSCC and identify risk factors for LSCC case mortality.

Long noncoding RNA (IncRNAs) are defined as a group of transcripts which are longer than 200 nucleotides without protein coding potential [15-19]. IncRNAs recently attracts more attention due to their important role in several cellular procedures, ranging from post-transcriptional and transcriptional modulation to the govern of subcellular localization, epigenetic modifications and cellular structural integrity [20-24]. IncRNAs has shown to play important roles in several biological processes including cell metastases, proliferation, cycle, apoptosis, invasion and migration [22, 25-27]. Recently, a large number of IncRNAs are deregulated in diverse tumors and the deregulation IncRNAs have been indicated to lead to aberrant expression of gene that contributes to development and progression of tumors including LSCC [28-31]. More recently, a novel IncRNA DLX6-AS1
was found to be overexpressed in some tumors such as lung adenocarcinoma, renal cell carcinoma and hepatocellular carcinoma [32-34]. For example, Li et al [32]. Firstly investigated the role of DLX6-AS1 in lung adenocarcinoma and demonstrated that DLX6-AS1 expression was upregulated in lung adenocarcinoma. Zeng et al [33]. demonstrated that the expression of DLX6-AS1 was upregulated in renal cell carcinoma and ectopic expression of DLX6-AS1 induced the renal cell carcinoma cell proliferation and tumorigenesis via regulating miR-26a/PTEN expression. Zhang and colleagues indicated that DLX6-AS1 expression was upregulated in the hepatocellular carcinoma tissues and knockdown expression of DLX6-AS1 suppressed cell invasion, migration and proliferation of hepatocellular carcinoma cell through regulating miR-203a/MMP-2 pathway [34]. However, the functional roles of DLX6-AS1 in LSCC are still unclear.

In this study, we showed that the expression level of DLX6-AS1 was upregulated in the LSCC samples compared to the noncancerous tissues. In addition, we demonstrated that knockdown expression of DLX6-AS1 suppressed the Hep2 cell growth, cell cycle and invasion.

Materials and methods

Human LSCC tissues and cell cultured and transfection

Human LSCC tissues and their pair noncancerous samples utilized in our study were collected from Zhoukou Central Hospital, Zhoukou, China under resections. No systemic or local therapies were performed in these cases before operation. All these samples were snap-frozen in the liquid nitrogen and then stored until RNA was extracted. Informed consent was collected from patients and our study was approved with clinical Ethics Committee of Zhoukou Central Hospital. Hep2 (LSCC cell line) was collected from Shanghai Chinese Academy of Science (Shanghai, China). Hep2 cell was cultured in the RPMI1640 (Gibco) supplemented with FBS, penicillin and streptomycin. miR-376c mimic and scramble, miR-376c inhibitor and control, si-DLX6-AS1 and si-control were synthesized from GenePharma (Shanghai, China) and then transfected into Hep2 cell with using Lipofectamine 3000 following to instruction.

RNA extraction and real-time PCR

Total RNA of cells or samples was separated by using TRizol kit (Invitrogen, CA, USA) following to standard protocol. qRT-PCR assay was done to analyze the expression of DLX6-AS1 and miR-376c by using SYBR Green mix (Applied Biosystems) on the iQ5 PCR Detection System (Bio-Rad, USA). The primers were shown as follows: DLX6-AS1, forward primers 5’-CAAATGCCATCCAGCC-3’ and reverse primers 5’-TCTGGCTTCCCTTAACCAA-3’; U6, forward primers 5’-CTCGCTTCGAGCGACA-3’ and reverse primers 5’-AACGTCTGGTCGACTGGAA-3’ and reverse primers 5’-GGTGCAAGCTCGGAGGTA-3’ and reverse primers 5’-GGTGCAAGCTCGGAGGTA-3’.

Cell proliferation, cycle and invasion assay

Cell Counting Kit-8 (CCK8, Dojindo, Kumamoto, Japan) was used to detect the cell growth. Cell was cultured in the 96 well plates and was incubated with 10 µl CCK8 for 3 hours at the set time points. The absorbance at 450 nm was determined by a microplate reader. For cell cycle, Hep2 cells were fixed with ethanol (70%) and treated with 50 mg/ml RNase A and then stained with 25 mg/ml PI (propidium iodide). Populations in the different phase were determined via flow cytometry (Beckman Coulter, CA, USA). For cell invasion assay, transwell chamber was used. The upper chamber was coated matrigel for filtering and the culture medium was added to lower chamber. The Hep2 cell was cultured in the medium (serum-free) and placed in the upper chamber. After 48 hours, no-invasive cell was removed and invasive cells were fixed and staining with crystal violet (0.1%).

Statistical analysis

Results were presented as mean values ± SD (standard deviation). Statistical assay was carried out by using Student’s t test for two groups and one-way ANOVA was utilized to analyze the differences between more than two groups. P less than 0.05 were considered to be statistically significant.

Results

DLX6-AS1 expression was upregulated in LSCC samples

To study the potential roles of IncRNA DLX6-AS1 in the development of LSCC, we firstly
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detected the DLX6-AS1 expression in LSCC samples and their pair noncancerous tissues. By using qRT-PCR assay, the expression level of DLX6-AS1 was higher in the LSCC samples than in the noncancerous tissues (Figure 1A). The DLX6-AS1 expression was upregulated in 23 patients (23/30, 77%) compared to adjacent noncancerous tissues (Figure 1B).

**DLX6-AS1 knockdown inhibited cell proliferation, cycle and invasion of LSCC cell**

Then, in LSCC cell line Hep2 transfected with si-DLX6-AS1, the expression level of DLX6-AS1 was downregulated compared to the si-control group (Figure 2A). By using CCK-8 analysis, knockdown expression of DLX6-AS1 suppressed the Hep2 cell growth (Figure 2B). Moreover, DLX6-AS1 knockdown decreased the S stage of Hep2 cell and increased the GO-G1 stage (Figure 2C). Furthermore, we showed that DLX6-AS1 knockdown suppressed the Hep2 cell invasion (Figure 2D and 2E).

**DLX6-AS1 decreased expression of miR-376c in trophoblastic cells**

Bioinformatics analyse was analyzed to study molecular mechanism through which DLX6-AS1 modulates LSCC cell behavior. MiR-376c was identified to have the complementary binding sites with the DLX6-AS1 (Figure 3A). Then, in LSCC cell line Hep2 transfected with miR-376c mimic, the expression level of miR-376c was upregulated compared to the scramble group (Figure 3A). By luciferase reporter assay, we indicated that overexpression of miR-376c inhibited the luciferase activity of wild-type DLX6-AS1, but it failed to suppress luciferase activity of mutated one (Figure 3C). DLX6-AS1 knockdown enhanced the expression of miR-376c in the Hep2 cell (Figure 3D).

**miR-376c expression was downregulated in LSCC samples**

To study the potential roles of miR-376c in the development of LSCC, we then detected the miR-376c expression in LSCC samples and their pair noncancerous tissues. By using qRT-PCR assay, the expression level of miR-376c was lower in the LSCC samples than in the noncancerous tissues (Figure 4A). The miR-376c expression was downregulated in 23 patients (23/30, 77%) compared to adjacent noncancerous tissues (Figure 4B). In addition, we indicated that the expression of miR-376c was negatively correlated with expression of DLX6-AS1 in LSCC tissues (Figure 4C).

**Ectopic expression of miR-376c suppressed cell proliferation, cycle and invasion of LSCC cell**

By using CCK-8 analysis, ectopic expression of miR-376c inhibited the Hep2 cell growth (Figure 5A). Moreover, miR-376c overexpression decreased the S stage of Hep2 cell and increased the GO-G1 stage (Figure 5B). Furthermore, we showed that overexpression of miR-376c suppressed the Hep2 cell invasion (Figure 5C and 5D).

**DLX6-AS1 knockdown suppressed cell proliferation, cycle and invasion via regulating miR-376c expression**

Then, in LSCC cell line Hep2 transfected with miR-376c inhibitor, the expression level of miR-376c was downregulated compared to the control group (Figure 6A). The inhibition effect of DLX6-AS1 on cell proliferation was effectively recovered through miR-376c knockdown (Figure 6B). In addition, we showed that the inhibition effect of DLX6-AS1 on cell cycle was rescued by miR-376c knockdown (Figure 6C).
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Figure 2. DLX6-AS1 knockdown inhibited cell proliferation, cycle and invasion of LSCC cell. A. The expression of DLX6-AS1 was detected in the LSCC cell line Hep2 cell by using qRT-PCR analysis. B. By using CCK-8 analysis, knockdown expression of DLX6-AS1 suppressed the Hep2 cell growth. C. DLX6-AS1 knockdown decreased the S stage of Hep2 cell and increased the G0-G1 stage. D. DLX6-AS1 knockdown suppressed the Hep2 cell invasion. E. The relative invasive cells were shown. *P<0.05, **P<0.01 and ***P<0.001.

Figure 3. DLX6-AS1 decreased expression of miR-376c in trophoblastic cells. A. MiR-376c was identified to have the complementary binding sites with the DLX6-AS1 by using Bioinformatics analyse. B. The expression level of DLX6-AS1 was detected in Hep2 cell by using qRT-PCR analysis. C. Overexpression of miR-376c inhibited the luciferase activity of wild-type DLX6-AS1, but it failed to suppress luciferase activity of mutated one by using luciferase reporter assay. D. DLX6-AS1 knockdown enhanced the expression of miR-376c in the Hep2 cell. **P<0.01.

Moreover, the inhibition function of DLX6-AS1 on cell invasion was also recovered by miR-376c knockdown (Figure 6D).

Discussion

Increasing evidences have shown to that IncRNAs act essential roles in tumour development and have been proved to be important regulators of signaling pathways underlying the carcinogenesis [18, 35-37]. In the study, we showed that the expression level of DLX6-AS1 was upregulated in the LSCC samples compared to the noncancerous tissues. By using CCK-8 analysis, we demonstrated that knockdown expression of DLX6-AS1 suppressed the Hep2 cell growth. DLX6-AS1 knockdown inhibited the Hep2 cell cycle and invasion. MiR-
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Figure 4. miR-376c expression was downregulated in LSCC samples. A. By using qRT-PCR assay, the expression level of miR-376c was lower in the LSCC samples than in the noncancerous tissues. B. The miR-376c expression was downregulated in 23 patients (23/30, 77%) compared to adjacent noncancerous tissues. C. The expression of miR-376c was negatively correlated with expression of DLX6-AS1 in LSCC tissues.

Figure 5. Ectopic expression of miR-376c suppressed cell proliferation, cycle and invasion of LSCC cell. A. Ectopic expression of miR-376c inhibited the Hep2 cell growth by using CCK-8 analysis. B. miR-376c overexpression decreased the S stage of Hep2 cell and increased the G0-G1 stage. C. Overexpression of miR-376c suppressed the Hep2 cell invasion. D. The relative invasive cells were shown. *P<0.05, **P<0.01 and ***P<0.001.
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For instance, Li and colleagues first investigated the role of DLX6-AS1 in lung adenocarcinoma and found that the expression of DLX6-AS1 was upregulated in lung adenocarcinoma [32]. Zeng et al [33] demonstrated that DLX6-AS1 expression was upregulated in renal cell carcinoma and ectopic expression of DLX6-AS1 induced the renal cell carcinoma cell proliferation and tumorigenesis through regulating miR-26a/PTEN expression. Zhang and colleagues demonstrated that DLX6-AS1 expression was upregulated in hepatocellular carcinoma samples and knockdown expression of DLX6-AS1 decreased cell invasion, migration and proliferation of hepatocellular carcinoma cell via regulating miR-203a/MMP-2 pathway [34]. However, the functional roles of DLX6-AS1 in LSCC are still unclear. In our study, we firstly detected the DLX6-AS1 expression in LSCC samples and their pair noncancerous tissues. Our data showed that the expression level of DLX6-AS1 was higher in the LSCC samples than in the noncancerous tissues. The DLX6-AS1 expression was upregulated in 23 patients (23/30, 77%) compared to adjacent noncancerous tissues. DLX6-AS1 knockdown inhibited cell proliferation, cycle and invasion of LSCC cell.

Recently, accumulating references indicated that one potential mechanism of IncRNA was to interact with miRNAs as the sponge and modulate their activity and expression [24, 38]. For example, Yang and their colleagues showed that LOC554202 promoted LSCC cell proliferation, cell cycle and cell invasion via suppressing the miR-31 expression [31]. Shen and their colleagues indicated that lncRNA AC026166.2-001 suppressed that LSCC cell growth and migration through modulating miR-24-3p/p27 expression [39]. Wang and colleagues demonstrated that lncRNA NEAT1 enhanced the LSCC progression via modulating miR-107/CDK6.
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pathway expression [40]. Moreover, Yan and their colleagues indicated that DLX6-AS1 expression was upregulated in the preeclampsia and regulated trophoblasts invasion and migration via modulating miR-376c/GADD45A expression [41]. In our study, we used Bioinformatics analyse to find that miR-376c was identified to have the complementary binding sites with the DLX6-AS1. By using qRT-PCR assay, the expression level of miR-376c was lower in the LSCC samples than in the noncancerous tissues. Ectopic expression of miR-376c suppressed cell proliferation, cycle and invasion of LSCC cell. Furthermore, we showed that DLX6-AS1 knockdown suppressed cell proliferation, cycle and invasion via regulating miR-376c expression.

In conclusion, we indicated that the expression level of DLX6-AS1 was upregulated in the LSCC samples compared to the noncancerous tissues. DLX6-AS1 knockdown suppressed cell proliferation, cycle and invasion via regulating miR-376c expression. These data suggested that IncRNA DLX6-AS1 might play as an oncogene in LSCC development and tumorigenesis.

Disclosure of conflict of interest

None.

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