Research Article

IncRNA DLEU2 Accelerates Oral Cancer Progression via miR-30a-5p/RAP1B Axis to Regulate p38 MAPK Signaling Pathway

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1. Introduction

Oral cancer (OC) is common cancer in the world [1, 2], and the current treatment of OC is usually surgery combined with radiotherapy and chemotherapy [3], but the mortality rate of OC is still high [4], which is due to the degree of tumor invasion and progression affecting the survival of patients with OC. Moreover, once lymph node metastasis occurs, the risk to patients’ lives will rise dramatically [5]. Therefore, early detection of OC is very important. Therefore, OC tumor markers have important clinical value for identifying the mechanism of occurrence and metastasis of OC and finding new therapeutic methods.

Study have found that long noncoding RNAs (lncRNAs, >200 nt) do not encode proteins [6] but can directly participate in the regulation of gene expression [7] and can also interact with miRNAs as competing endogenous RNAs (ceRNAs) [8]. miRNAs play key roles in the posttranscriptional regulation of mRNAs by targeting their 3' untranslated regions (UTRs), leading to mRNA degradation or...
translational repression [9]. In recent years, many studies have found that lncRNAs are involved in the occurrence and development of OC as ceRNAs [10–12]. As a known oncogene, lncRNA deleted in lymphocytic leukemia 2 (DLEU2) plays a role in promoting cancer development in most cancers [13, 14], but the molecular mechanism in OC has not yet been reported.

In recent years, alterations in signaling pathways involved in OC have been shown to be important factors affecting OC development [15, 16]. Among them, the canonical mitogen-activated protein kinase (MAPK) signaling pathway is considered to be involved in the growth and development of most cells [17, 18]. As an important member of MAPK, phosphorylation of p38 MAPK mediates the migration and growth of OC cells [19].

In this article, we sought to examine the expression and molecular mechanism of lncRNA DLEU2 in OC and to evaluate its impact on the biological behavior of two OC cell lines. Furthermore, we revealed a novel mechanism by which lncRNA DLEU2 regulates OC cell proliferation and distal migration through miR-30a-5p/miRNA family (RAP1B)/MAPK signaling, which may provide new ideas for the discovery of OC therapeutic strategies.

2. Materials and Methods

2.1. Microarray Raw Data Analyses. Raw data are from National Center for Biotechnology Information Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). The expression profiles of lncRNA/mRNA in OC samples (GSE25099) were analyzed using GEO, which were divided into two groups: from 10 normal oral tissues (GSM616588-GSM616597) and 10 OC patient tissues (GSM616647-GSM616656). The expression profiles of miRNAs in samples (GSE98463) were divided into two groups: from 4 normal oral tissues (GSM2596879-GSM2596882) and 4 OC patient tissues (GSM2596874-GSM2596877). Differentially expressed lncRNAs/miRNAs/miRNAs were identified according to the following criteria: P < 0.05 and |fold change| ≥ 2. A heat map or volcano plot was constructed using differentially expressed lncRNA/mRNA/miRNA analysis results. KEGG pathway enrichment analyses were performed using the functional annotation tool of DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/summary.jsp). The miRNA-lncRNA interactions between lncRNA and miRNA were predicted using starBase (https://starbase.sysu.edu.cn/index.php). Putative targets of miRNA-mRNA were predicted using TargetScan 7.2 (https://www.targetscan.org/vert_72/).

2.2. Cell Lines and Culture and Transfection. Human oral cancer cell lines (CAL-27, Tca8113, and C4-2, 22RV1), and human embryonic kidney cells 293 (HEK 293T) were purchased from American Type Culture Collection (ATCC; VA, USA). All cells were maintained in DMEM medium (HyClone, UT, USA) with 10% fetal bovine serum (FBS, HyClone). RNAs was transfected into cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.).

2.3. Transient DLEU2 Silencing. si-DLEU2-1, si-DLEU2-2, and si-DLEU2-3, and scrambled siRNA (si-NC) were designed and synthesized by Sigma-Aldrich. The siRNA sequences were as follows: si-DLEU2-1, 5′-GGTACTCTACTATAGT TTAdTdT-3′; si-DLEU2-2, 5′-GAATAACATCAATATG CAAAdTdT-3′; si-DLEU2-3, 5′-GTATGGAATAATTATA CTAAdTdT-3′; and si-NC, 5′-TTCTCCGAACGTGTCACG dTdT-3′. After the above siRNAs were transfected into Tca8113 and CAL-27 cells, the cells were harvested 24 hours later. The inhibitory efficiency of the three si-DLEU2 was evaluated by RT-qPCR, and the siRNA with the best inhibitory efficiency will be used as a DLEU2 antagonist for subsequent research and analysis.

2.4. RNA Isolation and Quantitative RT-qPCR Analysis. Total RNA was extracted from cells using TRIzol (Invitrogen, MA, USA) according to the manufacturer’s instructions. Real-time mRNA quantification for DLEU2, miR-30a-5p, RAP1B, U6, and 18sRNA was performed using SYBR Green qPCR SuperMix (Invitrogen) on a 7500 RT-qPCR System (Applied Biosystems, MA, USA). PCR experiments were carried out under the following conditions: 95°C for 10 min, 55°C for 2 min, and 72°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers for DLEU2, RAP1B, miR-30a-5p, 18sRNA (as internal normalization control for DLEU2 and RAP1B), and U6 (as internal normalization control for miR-30a-5p) were as follows: DLEU2 forward, 5′-TCTCTTCCCTGGAAG AGCAACA-3′, and DLEU2 reverse, 5′-TTGAGCCGTCT ATGCGTGTCA-3′; RAP1B forward, 5′-ACAGCGTGAG GTACTAGGT-3′, and RAP1B reverse, 5′-GTAAATTTGC TCCTTTCCTGC-3′; miR-30a-5p forward, 5′-ACACCT CAGCTGGGTGTAACATCCCTGCAC-3′, and miR-30a-5p reverse, 5′-CTCAACTGGGTGTCGTTGGA-3′; 18sRNA forward, 5′-CTCGGATACCGCACTAGAAG-3′, and 18sRNA reverse, 5′-GGGGGCGAATACGATGCCC-3′; and U6 forward, 5′-CTCGCTTCCGAGACGACA-3′, and U6 reverse, 5′-AACGCTTACGAATTTGCGT-3′. Relative DLEU2, RAP1B, and miR-30a-5p expression levels were calculated using the 2−ΔΔCt method [20]. All RT-PCR experiments were repeated three times.

2.5. Cell Proliferation Rate. Cell Counting Kit-8 (CCK8) reagent (Solarbio; 10 μL) was added to 96-well plates (containing Tca8113 and CAL-27 4 × 10^4 cells) at 0, 24, 48, and 72 h, respectively. The absorbance was measured at 450 nm using an enzyme-labeled instrument (Thermo Fisher Scientific) after 60 min of incubation in the dark at 37°C.

2.6. Transwell Migration and Invasion Assays. The migration and invasion of Tca8113 and CAL-27 cells were performed using 24-well Transwell inserts (BD Biosciences). For the migration assay, the cells (0.5 × 10^5) in serum-free medium were placed in the top chamber, whereas culture medium containing 10% FBS was added to the lower chamber. After incubating for 24 h, invasive cells were fixed using
Figure 1: Continued.
anhydrous ethanol for 30 min and stained with 0.1% crystal violet (Solarbio; 25°C) for 25 min. The cells were counted using a light microscope (Olympus Corporation) at ×200 magnification. For the invasion assay, inserts were first coated with Matrigel® (BD Biosciences) for 6 h at 37°C, then fixed, stained, and counted.

2.7. Dual-Luciferase Reporter Assay. The whole sequence of DLEU2 or RAP1B 3′ UTR was inserted into the psi-CHECK2 basic construct. We transfected HEK 293 T cells with a 0.5 μg reporter construct and 50 nM miRNA mimic per well using Lipofectamine 3000. Following 4 h of transfection, the transfection medium was replaced with complete culture medium. Following 48 h of culture, the cells were lysed with passive lysis buffer (Promega), and luciferase activity was measured at 490 nm using the dual-luciferase reporter assay system (Promega). The ratio of firefly to Renilla luciferase activity was used to normalize firefly luciferase values.

2.8. Western Blotting. Tca8113 and CAL-27 cells were lysed using RIPA lysis buffer (Solarbio) and estimated using a BCA protein assay kit (Solarbio). Denatured proteins were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Solarbio), and protein bands were transferred to a polyvinylidene fluoride membrane, blocked with 5% bovine serum albumin (Solarbio), and incubated overnight (4°C) with RAP1B (1:500; ab154756, Abcam, Cambridge, UK), p38 (1:1000; ab170099, Abcam), and p-p38 (1:1000; ab178867, Abcam) primary antibodies. Thereafter, the sections were rinsed with TBST buffer (Solarbio; containing 0.05% Tween 20) twice for 15 min and incubated with goat anti-rabbit antibody (1:20,000, ab205718; Abcam) for 2 h at 25°C. Anti-GAPDH antibody (1:5,000, ab181602; Abcam) was used as a loading control. Proteins were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Chemiluminescence signal acquisition was performed using an X-ray film (Kodak).

2.9. Statistical Analysis. Statistical analysis was performed using GraphPad Prism software (v8.3.0), and data were expressed as mean ± SD. To determine whether there was an overall statistically significant difference, a one-way ANOVA was performed with a Bonferroni post hoc test before Student’s t-test was performed to analyze the difference between any two groups. It was considered statistically significant if P > 0.05.

3. Results

3.1. DLEU2 Is Upregulated in OC Cells. Analysis of the results of OC patients and normal subjects based on lncRNA microarray data identified 20 differentially expressed transcripts of lncRNAs, including 15 upregulated and 5 downregulated (Figure 1(a)). Among them, DLEU2, as a known oncogene, expression was upregulated in the dataset and has not yet been studied on the molecular mechanism in OC. Furthermore, based on RT-qPCR assays, DLEU2 expression was significantly upregulated in 4 OC cells.
Therefore, we have reason to believe that DLEU2 may be closely related to the development of OC. Notably, DLEU2 was most expressed in Tca8113 and CAL-27 cells, so these two cell lines were selected to explore the functional role of DLEU2 in OC. RT-qPCR results showed that after transfec-ting the synthesized three kinds of siRNA-DLEU2 into Tca8113 and CAL-27 cells, siRNA-DLEU2-1 was found to have the best inhibitory effect. We used it as an antagonist of DLEU2 to study the molecular mechanism (Figure 1(c)). The results of CCK8 and Transwell showed that inhibiting the expression of DLEU2 slowed the proliferation of Tca8113 and CAL-27 cells and reduced the ability of migration and invasion (Figures 1(d)–1(g)). Furthermore, by RT-qPCR analysis, we observed that DLEU2 was mainly distributed in the cytoplasm of cells (Figure 1(h)), so the effect of DLEU2 on OC might be achieved through a ceRNA mechanism.
Enriched pathways

- PI3K-Akt signaling pathway
- Pathways in cancer
- Focal adhesion
- Cytokine-cytokine receptor interaction
- Regulation of actin cytoskeleton
- Rap1 signaling pathway
- Proteoglycans in cancer
- Ras signaling pathway
- Chemokine signaling pathway
- MAPK signaling pathway
- Amoebiasis
- Osteoclast differentiation

Count
- 30
- 40
- 50
- 60

Figure 3: Continued.
3.2. DLEU2 Directly Targets miR-30a-5p. According to the miRNA microarray data analysis results, using 4 OCs and 4 normal oral tissues, 209 differentially expressed hsa-miRNAs were identified, of which 88 were upregulated and 121 miRNAs including miR-30a-5p were downregulated (Figure 2(a)). Combined with the analysis of starBase database and miRNA microarray (Figure 2(b)), we observed that 3 miRNAs intersected, and miR-30a-5p was confirmed to be downregulated in OC [21], so we screened it as a potential target of DLEU2. The results showed that miR-30a-5p was downregulated in OC cells (Figure 2(c)), and inhibiting the expression of DLEU2 could increase the expression of miR-30a-5p (Figure 2(d)). The starBase database predicts that DLEU2 has a binding site for miR-30a-5p (Figure 2(e)); subsequent dual-luciferase experiments confirmed that DLEU2 directly targets miR-30a-5p (Figure 2(f)). Here, we can confirm that DLEU2 can sponge miR-30a-5p to regulate OC development.

3.3. RAP1B Directly Targets miR-30a-5p. According to the mRNA microarray data analysis results, a total of 1496 upregulated mRNAs including RAP1B were identified (Figure 3(a)). KEGG can analyze and predict that different mRNAs affect different signaling pathways. We found that the classical MAPK involved in cell growth and development is one of the key signaling pathways that affect the progression of OC (Figure 3(b)), and 44 mRNAs are predicted to be involved. Combined with the 4117 potential target genes of miR-30a-5p predicted by the starBase database, we observed that 8 mRNAs intersected (Figure 3(c)). The study has confirmed that RAP1B is upregulated in OC [22]; therefore, we screened the RAP1B gene as a potential target for validation. The QRT-PCR results showed that RAP1B was highly expressed in OC cells (Figure 3(d)). The TargetScan database predicts that RAP1B has a binding site for miR-30a-5p (Figure 3(e)); subsequent dual-luciferase assays confirmed that RAP1B directly targets miR-30a-5p (Figure 3(f)).
Figure 4: Continued.
After transfection of si-DLEU2, RAP1B expression was downregulated in Tca8113 and CAL-27 cells (Figure 3(g)).

3.4. DLEU2 Is Positively Correlated with RAP1B. To confirm the association between DLEU2 and RAP1B, we constructed a RAP1B overexpression plasmid (ov-RAP1B). The QRT-PCR and western blot results indicated that the gene or protein levels of RAP1B was upregulated in ov-RAP1B-transfected Tca8113 and CAL-27 cells, confirming the effectiveness of the RAP1B overexpression plasmid (Figures 3(h) and 3(i)). After cotransfection with the addition of si-DLEU2, the inhibition of DLEU2 expression was reversed by ov-RAP1B (Figure 3(j)). Thus, miR-30a-5p directly targets DLEU2 and RAP1B, and DLEU2 positively correlates with RAP1B.

3.5. The DLEU2/miR-30a-5p/RAP1B Axis Affects OC Development through MAPK Signaling. The above results suggest that MAPK is one of the key signaling pathways affecting OC development, and p38 MAPK is the core protein of MAPK. Based on western blot results, we found that knockdown of DLEU2 significantly reduced p-p38 MAPK protein levels in Tca8113 and CAL-27 cells (Figure 4(a)). However, the presence of ov-RAP1B reversed the effect of si-DLEU2 (Figure 4(b)). In addition, the results of cell function experiments showed that si-DLEU2 inhibited the proliferation of Tca8113 and CAL-27 cells (Figures 4(c) and 4(d)), and the effects of reducing migration and invasion ability (Figures 4(e) and 4(f)) were also reversed by ov-RAP1B. In conclusion, the DLEU2 sponge adsorbs miR-30a-5p and regulates the transcription and translation of RAP1B, thereby affecting downstream MAPK signaling and intervening in OC development.

4. Discussion

Our goal was to identify a novel IncRNA as a potential therapeutic target for OC therapy; therefore, a comprehensive understanding of the regulatory mechanisms of IncRNAs may help to develop new and promising therapeutic strategies for OC. Previous reports indicated that IncRNAs are important players in the development of OC [23]. For example, Zhang et al. [24] found that the IncRNA LINC01296 promoted the development of oral squamous cell carcinoma by binding to SRSF1. Lu et al. [25] demonstrated that IncRNA HOTAIR inhibited cancer stemness and metastasis in oral cancer stem cells. In our study, the oncogenic roles of DLEU2 and RAP1B in OC and the tumor suppressor role of miR-30a-5p were first determined. Mechanistically, we found that DLEU2 sponges absorb miR-30a-5p, RAP1B is a target of miR-30a-5p, and DLEU2 is upregulated synergistically with RAP1B in OC progression. In addition, si-DLEU2 also inhibited the p38 MAPK pathway. MAPK family members mediate a variety of cellular behaviors in response to extracellular stimuli [26]. As one of the major members of MAPKs, p38 MAPKs function in a cell environment-specific and cell type-specific manner to integrate signals affecting proliferation and migration [18]. Taken together, our results suggest that the DLEU2/miR-30a-5p/RAP1B axis can regulate the progression of OC through the p38 MAPK pathway, providing a new therapeutic strategy for OC.
In various cancers, DLEU2 plays a promoting regulatory role in a variety of cancers. For example, He et al. found that DLEU2 promotes cervical cancer progression [27] and promotes the proliferation and invasive capacity of colorectal cancer cells [28]. Likewise, our results showed that DLEU2 was upregulated in OC cells, its high expression significantly shortened the disease-free survival of OC patients, and si-DLEU2 inhibited OC cell proliferation, migration, and invasion by reducing the phosphorylation of p38 MAPK. It is suggested that DLEU2, as an oncogene, will block the development of OC when its expression is suppressed. In addition, DLEU2, as a ceRNA, plays a role in phagocytosing miRNAs in cancer. For example, Wu et al. found that DLEU2 accelerated tumorigenesis and invasion of non-small-cell lung cancer by sponging miR-30a-5p [29]. Li et al. [30] found that DLEU2 promotes gastric cancer progression through sponge adsorption of miR-23b-3p. In this study, inhibiting the expression of DLEU2 promoted the expression of miR-30a-5p in OC cells, so that the transcription and translation of RAP1B directly bound to miR-30a-5p were also simultaneously degraded. This result indicates that miR-30a-5p as a tumor suppressor gene was confirmed in this study, which is consistent with the previous studies on the role of miR-30a-5p in suppressing cancer such as breast cancer [31] and lung adenocarcinoma [32].

The bioinformatics database predicted that miR-30a-5p targets the 3’UTR of RAP1B, and we confirmed its targeting mechanism by dual-luciferase reporter gene experiments. Furthermore, in OC cells, RAP1B expression was negatively correlated with miR-30a-5p expression. Notably, our study shows that RAP1B promotes OC progression. Furthermore, in the ceRNA axis, DLEU2 was coordinately upregulated with RAP1B, a target gene of miR-30a-5p, in OC cells, and DLEU2 decreased the expression of RAP1B, whereas overexpression of RAP1B reversed si-DLEU2-mediated degradation of p38 MAPK phosphorylation. These results suggest that si-DLEU2 exerts a tumor suppressor effect through the interaction with miR-30a-5p and RAP1B. Furthermore, the tumor suppressor effect of RAP1B on OC is consistent with its role in other cancers such as thyroid cancer [33] and colorectal cancer [34].

The MAPK pathway directly regulates OC development [35, 36]. In the present study, when the phosphorylation level of p38 MAPK was decreased, the proliferation inhibition, migration, and invasion abilities of OC cells were decreased. si-DLEU2 inhibited OC cell growth and decreased p38 MAPK phosphorylation levels. Excessive RAP1B promotes cancer cell development, elevates p38 MAPK phosphorylation levels, and reverses the effects of si-DLEU2 on OC cells. Therefore, the DLEU2-miR-30a-5p-RAP1B axis controls the p38 MAPK signaling pathway, thereby regulating OC progression.

In conclusion, this study confirms that DLEU2 is a potential therapeutic target and provides more directions and theoretical basis for the treatment of OC.

**Abbreviations**

OC: Oral cancer

IncRNA: Long noncoding RNA

miRNA, miR: microRNA

ceRNAs: Competing endogenous RNAs

UTR: Untranslated region

MAPK: Mitogen-activated protein kinase

GEO: Gene Expression Omnibus

NOKs: Normal oral keratinocytes

DLEU2: Deleted in lymphocytic leukemia 2

RAP1B: Member of RAS oncogene family.

**Data Availability**

The data used to support the findings of this study are included within the article.

**Conflicts of Interest**

The authors declare that they have no competing interests.

**Authors’ Contributions**

Wenbo Zhang, Yanchun Wang, and Pu Xu conceived and designed the study and developed the methodology. Wenbo Zhang, Yongxiu Du, and Weiwei Guan performed the experiments and collected the data. Yanchun Wang, Pu Xu, and Weiwei Guan analyzed and interpreted the data. Wenbo Zhang drafted the manuscript. All authors read and approved the final manuscript.

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**References**


