DLEU2 promotes proliferation and glycolysis of oral squamous cell carcinoma by regulating SIX1

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Research Article

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Abstract

Objective
To look into the physiological functions of the IncRNA DLEU2 in the tumorigenesis of oral squamous cell carcinoma (OSCC), as well as whether it plays a role in the emergence and advancement of OSCC by governing SIX1.

Methods
The inhibitory role of DLEU2 on the proliferation of SCC-15 cells was examined by CCK8. Flow cytometry was used to study the influence of DLEU2 inhibitory activity on SCC-15 apoptotic cell death. In addition, trans-well assays were used to analyze the influence of DLEU2 suppression on SCC-15 cell differentiation and proliferation.

Results
The DLEU2 expression in OSCC cancerous specimens was considerably stronger than the corresponding healthy tissues; and DLEU2 was elevated in all four OSCC cells. The immunohistochemistry data also showed the level of DLEU2 was also greatly elevated in OSCC tissues than healthy specimens. After transfection of si-DLEU2, the viability of SCC-15 cells decreased significantly. Additionally, the number of apoptosis cells transfected with si-DLEU2 was significantly higher than controls. Using trans-well invasion assay, the data suggested the number of invasive cells formed by blocking DLEU2 of SCC-15 and SCC-25 cells was markedly lower than the controls. The results of ECAE and OCR also showed that DLEU2 could promote the glycolysis of OSCC cells while inhibit the oxidative phosphorylation progress of OSCC cells. Our subsequent analysis of the main enzymes affecting glycolysis, GLUT1 and HK2, showed that blocking expression of DLEU2 is able to obviously reduce the GLUT1 level, but not HK2. Subsequent ChIP experiments confirmed that SIX1 could bind to the promoter of GLUT1, and knocking down DLEU2 could reduce the binding ability of SIX1 to the promoter of GLUT1. Finally, we utilized luciferase assays to confirm that knockdown of DLEU2 expression could directly reduce GLUT1 transcript levels. The results of ECAR and OCR experiments also showed that overexpression of SIX1 could reverse the decreased glycolysis of OSCC cells brought down by knockdown of DLEU2.

Conclusion
DLEU2 is essential for OSCC tumorigenesis, migratory and glycogenolysis. The DLEU2/SIX1 role is implicated in OSCC cell invasion and aerobic glycolysis.

Introduction
Recent years have shown some progress in the oral squamous cell carcinoma (OSCC); however, no significant improvement is appeared in the prognosis of OSCC patients [1–2]. OSCC has high malignant potential, poor prognosis and high fatality [3–4]. With significant advancements in surgical intervention, pre-operative radiation therapy, and chemotherapy for OSCC recently, continued existence of patients has managed to enhance [5–6]. Unfortunately, the majority of OSCC cases are diagnosed in a late phase and cannot be surgically removed [7–8]. In addition, OSCC patients have worse prognosis due to malignant transformation, relapse and opposition to chemotherapy and radiation [9–10]. Therefore, there is a pressing need to identify diagnostic biochemical markers for the early screening and therapy of OSCC [11–12]. It is reported that many long-stranded non-coding RNA (lncRNAs) are contributed to tumorigenesis and development through transcriptional and post-transcriptional regulation of coding genes [13–14]. Among them, DLEU2 (Deleted in Lymphocytic Leukemia 2) is proved to serve mis-expressed in many tumors and is tightly link with tumorigenesis [15–16]. However, it has been confirmed that the DLEU2 level is increased in hepatocellular carcinoma (HCC), which improves the HCC processes by combining with EZH2[17–18]. DLEU2 is also enhanced in pancreatic cancer, and acts as the endogenous "sponge" of miR-455 to mediate the SMAD2 expression to promote proliferation and invasion of pancreatic carcinoma [19–22]. In conclusion, DLEU2 may play different roles in different tumors. However, very little widely recognized of DLEU2 in the onset and progression of OSCC. In addition, a number of studies have indicated that lncRNAs often uses the endogenous competitive RNA model as a "miRNA sponge" to adsorb miRNA, and then regulate the expression of target genes to involvement in the tumorigenesis processes [23]. Therefore, it is critical to thoroughly factors and targeted therapies for OSCC [24]. Our current study suggested DLEU2 played a vital part in the growth and glycolysis of OSCC. The role of DLEU2/SIX1 is implicated in OSCC cell invasion and aerobic glycolysis.

Materials And Methods

Cell culture

Oral squamous cell carcinoma cells were cultured in DMEM F12 medium (addition of 100 U/ml penicillin and 100 µG /ml streptomycin). OSCC SCC-15 cell line was grown in DMEM h medium with 10% fetal bovine serum (FBS). All cells were grown in the incubator at 37 °C with 5% CO2 and 95% relatively moisture. Cells in logarithmic growth phase were harvested and passaged every 48 h.

Cell viability

After the cells were dispersed by pipetting, the $1 \times 10^4$ cells were planted into a 96-well plate. After cellular adhesion, cells were collected at 0, 12, 24, and 48h. We supplied MTS reagent and 10µL of detection reagent into each 100µL of medium. Then, the plate was read using a microplate reader to read the OD490 data for 4 hours.

The Matrigel gel was dissolved at 4°C overnight, then diluted at a volume ratio of 1:3 with cool medium with serum. 40µL of the Matrigel gel was added to a cooled chamber, and incubate at 37°C for 2 hours to...
solidify the Matrigel gel. 100µL of serum-free medium was supplied to the top chamber, and 600µL of serum-free medium was put into the bottom chamber for overnight. 1 ×10^5 cells of each group were resuspended by medium without serum in the top chamber and 600µL of complete medium was supplied into the bottom chamber. After 48 hours, the cells in the top chamber were settled with 4% paraformaldehyde for fifteen minutes and soiled with dye solution for 10 minutes. Photographs were taken to calculate the number of cells crossing through the pore spaces.

Trans-well assay

Cells were counted for 1×10^5 cells using 100µl culture medium was resuspended and supplied to the top chamber, and 600µl was supplied to the bottom chamber with complete medium. After incubation for 48 h in culture incubator, the cells were fixed with 4% paraformaldehyde for 15 min followed by 1 wash in PBS buffer, stained with crystal violet for 10 min. The number of cells passing through small pores was photographed and counted.

Apoptosis

SCC-25 cells were selected and plated at a density of 4 × 10^5 cells were seeded into the plate with six wells, and the same 1.3. Cells from each transfection group were harvested synchronously 48 h after transfection, and 195µl Lannexinv FITC conjugate solution, gently resuspending the cells and subsequently adding 5µL Annexin V-FITC, 10µL propidium iodide, mix and then incubate in the dark at room temperature for 10 ~ 20min, flow cytometry analysis the apoptotic rate.

Statistical methods

Using Graphpad prism 8.0 performed the experimental data. The data are presented as mean ± standard deviation (x ± s). All of the experimental tests were carried out in triplicate. The compared data of various groups were performed using one-way ANOVA and corrected with the Tukey method. A number of subsequent analyses were carried out by LSD-t test and statistical significance was defined as P < 0.05.

Results

2.1 The level of DLEU2 expression in OSCC tissue and cells

We first searched the expression of a in OSCC patients using the TCGA database, and the results showed that the DLEU2 expression was significantly stronger in OSCC than that in healthy specimens (Fig. 1A and B) and the differences displayed greatly statistical significance. Meanwhile the DLEU2 level was considerably higher in OSCC specimens than adjacent noncancerous specimens with statistical differences. We subsequently examined the DLEU2 levels in four subtypes of OSCC cell lines. The elevated levels of DLEU2 were discovered among all four subtypes of OSCC cells than healthy NHOK cells (Fig. 1C). The results of immunohistochemistry also showed that the DLEU2 levels were also obviously elevated in OSCC tissues than healthy tissue (Fig. 1D).
2.2 Effect of DLEU2 knockdown on proliferation and apoptosis in OSCC cells

To explore the impact of DLEU2 expression in the growth of OSCC, we examined it by CCK8 assay. After transfection of si-DLEU2, the viability of SCC-15 cells decreased significantly. Similarly, after silencing DLEU2, the viability of SCC-25 was remarkably weaker than the controls (Fig. 2A). The apoptosis text suggested the amount of apoptosis cells transfected with si-DLEU2 was significantly higher compared to the control group (Fig. 2B), indicating that down-regulation of DLEU2 level was able to repress the OSCC ability to grow. Therefore, the inhibition of DLEU2 expression may limit the capability of OSCC cells to grow.

2.3 Effect of DLEU2 knockdown on invasion in OSCC cells

To examine the effect of SIX1 DLEU2 expression on the invasive ability of OSCC cells, trans-well assay was conducted. The trans-well test indicated that invasive cells formed by SCC-15 and SCC-25 cells transfected with si-DLEU2 was obviously fewer in comparison of the controls (Fig. 3A and B), indicating that down-regulation of DLEU2 expression could inhibit the invasion ability of OSCC cells.

2.4 The blocking of DLEU2 suppresses aerobic glycolysis in OSCC cells

Aerobic gluconeogenesis is considered as a crucial indicator of human cancer. Thought the measurements of consumed glucose and lactic acid generations, the down-regulated DLEU2 was observed to cause a great decline in glucose consumption and lactic acid production in SCC-15 and SCC-25 cells (Fig. 4A B and C). In addition, the results of ECAE and OCR also showed that DLEU2 could promote the glycolysis of OSCC cells while inhibit the oxidative phosphorylation progress of OSCC cells (Fig. 4D and E). As a result, we hypothesize that DLEU2 enhances aerobic glycolysis in OSCC cells, which will provide a competitive system for OSCC cell transformation.

2.5 DLEU2 promotes GLUT1 expression and glycolysis in OSCC cells by regulating transcription factor SIX1

To explore the possible mechanism of DLEU2, we first analyzed the cellular localization of a by using the IncRNA ATLAS database suggesting DLEU2 was mainly distributed in the nucleus (Fig. 5A), which was similarly confirmed by our experimental results (Fig. 5B). This study revealed that a could be participated in genomic transcriptional regulation. Our subsequent analysis of the main enzymes affecting glycolysis, GLUT1 and HK2, showed silencing DLEU2 expression is capability of obviously reduction the level of GLUT1, but not HK2 (Fig. 5C). Subsequent ChIP experiments confirmed that SIX1 could bind to the promoter of GLUT1, and knocking down the expression of DLEU2 could reduce the binding ability of SIX1 to the promoter of GLUT1 (Fig. 5D and E). Finally, we utilized luciferase assays to confirm that knockdown of DLEU2 expression could directly reduce GLUT1 transcript levels (Fig. 5F). Taken together, our results show that DLEU2 promotes GLUT1 expression and glycolysis in OSCC cells by regulating transcription factor SIX1.

2.6 DLEU2 promotes proliferation and glycolysis of OSCC via SIX1
To verify whether DLEU2 promotes the proliferation and glycolysis of OSCC via SIX1, we performed rescue experiments. The findings revealed that abnormally high SIX1 expression could reverse the decreased proliferation of OSCC cells brought down by knockdown of DLEU2 (Fig. 6A and B). Meanwhile, the results of ECAR and OCR experiments also showed that overexpression of SIX1 could reverse the decreased glycolysis of OSCC cells brought down by knockdown of DLEU2 (Fig. 6C and D). The above facts suggest that, DLEU2 promotes proliferation and glycolysis of OSCC via SIX1.

**Discussion**

The OSCC patients have worse outcomes due to metastatic disease, relapse and barrier properties to chemotherapy and radiotherapy [25–26]. A huge amount of lncRNAs have been considered to have a significant role in tumorigenesis, development and drug resistance, and can be used as potential tumor diagnosis and prognosis biomarkers and molecular therapy targets [27]. In hepatocellular carcinoma and pancreatic cancer, the expression of DLEU2 is up-regulated, which plays the role of oncogene [16, 28]. However, it is not clear whether DLEU2 is mis-expressed in OSCC and whether it performs a role in the onset and progression of OSCC. In this study, OSCC cell lines SCC-15 and SCC-25 were used as experimental objects to report the biological mechanism of DLEU2 and DLEU2/SIX1 in the growth and metastasis of OSCC.

In this study, various functional deficit tests were conducted explore biological function of DLEU2 in the occurrence and development of OSCC. By synthesizing the siRNA sequence targeting DLEU2 and transfecting SCC-15 and SCC-25 cells, we successfully established the cells expressing DLEU2 silently. Subsequently, through CCK8, it was found that silencing DLEU2 expression could reduce the proliferation ability of SCC-15 and SCC-25; through cell scratch test and trans-well experiment, it was found that silencing DLEU2 expression could reduce the migration and invasion ability of SCC-15 and SCC-25. These results suggest that DLEU2, as an oncogene, affects the growth and metastatic ability of OSCC. In hepatocellular carcinoma and pancreatic cancer, silencing DLEU2 could inhibit the invasive ability and growth of tumor cells [17, 29–30]. Our results are consistent with the above studies.

DLEU2 performed an important part in majority of malignant tumors [31]. It encourages growth, sustenance, angiogenesis and metastatic spread while also inhibiting cell death and immunodeficiency. Yang and colleagues demonstrated that DLEU2 promotes lung cancer cell proliferation and inhibits apoptosis [32]. Moreover, DLEU2 participates in the progresses of mammy malignancy underneath the control of MALAT1[33]. We discovered that the blocking of DLEU2 restrained the invasion of OSCC cells with consistency of previous findings. DLEU2 mainly affects transcription by translocation into the nucleus and engaging to particular DNA binders. The molecular mechanism of Tyr705 is required for nuclear accumulation, but some observations have suggested that DLEU2 could indeed access the nucleus without being phosphorylated [34]. Additionally, level of DLEU2 in ESCC implied pSTAT3 at Tyr705 indicated a significantly greater proportion and frequency level in hypo fractionated tumors having nothing to do with tumor differentiation [35]. The trans-well invasion assay displayed invasive
cells formed by SCC-15 and SCC-25 cells transfected with si-DLEU2 was considerably lower than the control group.

Notwithstanding their genetic variability, cancerous cells share a range of features, including regularly increased glucose absorption and higher aerobic glycolysis levels than healthy cells to maintain the rising energy demands of operations like development [36]. We discovered that DLEU2 boosts OSCC cell aerobic glycolysis by growing glucose utilization and lactate generation, as well as aiding movement, invasion and EMT. The increased level of GRIM-19 can cause an increase in exogenous utilization, reduce aerobic glycolysis as well as cell proliferation reducing the expression of DLEU2. In addition, DLEU2 can improve HCC aerobic glycolysis by activation of hexokinase [37]. However, DLEU2 can inhibit glycogen synthesis of HeLa and MCF-7 via enhancing PSA-mediated cellular death. It is probably because various cancerous microenvironments and DLEU2 in the regulation of glycogen synthesis of OSCC need further investigations.

By aiming the JAK2 mRNA gene encoding, miR-145 blocks STAT3 phosphorylation at Tyr705 in bladder cancer cells, boosting SIX1 promoter translation and restricting tumor progression [34]. Trastuzumab combined with MPA significantly reduced the constitutive activation of DLEU2 and led to stronger pro-apoptotic regulators like p27 and SIX1[38]. The data are consistent in present results, which is the suppression of DLEU2 leads to the increase of SIX1 transcriptional activity. However, a previous study indicated that DLEU2 can booster T cell and IL-2 generations by directly interacting with the SIX1 promoter and great level of SIX1. The difference in this interaction may be due to different types of cell populations. SIX1 has many functions, such as promoting cell proliferation and regulation of immune reactions. In liver SIX1 knock downed mice, the whole glycogen subtype changed into the decrease of gluconeogenesis and the increase of glycolysis. SIX1 inhibits MYC-mediated gluconeogenesis and mitochondrial biogenesis in vascular endothelium, slowing metabolic reactions. MYC is a powerful driving force of synthetic metabolism. The metabolic role of SIX1 in OSCC cellular environment has yet to be discovered. The subsequent analysis of the main enzymes affecting glycolysis, GLUT1 and HK2, showed that blocking of DLEU2 expression can obviously reduce GLUT1 level, but not HK2. Subsequent ChIP experiments confirmed that SIX1 could bind to the promoter of GLUT1, and knocking down the expression of DLEU2 could reduce the binding ability of SIX1 to the promoter of GLUT1. The results of ECAR and OCR experiments also showed that overexpression of SIX1 could reverse the decreased glycolysis of OSCC cells brought down by knockdown of DLEU2.

In conclusion, we elucidate that DLEU2 promotes the migration and invasive phenotype and OSCC cells gluconeogenesis by SIX1. Given that tumors are typically characterized by subtype variability, it is necessary to clarify metabolic heterogeneity and flexibility in the future, which will help DLEU2/SIX1 targeted therapy to better treat OSCC.

References


Figures
Figure 1

The level of DLEU2 Expression in OSCC tissue and cells
Figure 2

Effect of DLEU2 knockdown on proliferation and apoptosis in OSCC cells
Figure 3

Effect of DLEU2 knockdown on invasion in OSCC cells
Figure 4

The blocking of DLEU2 suppresses aerobic glycolysis in OSCC cells
**Figure 5**

DLEU2 promotes GLUT1 expression and glycolysis in OSCC cells by regulating transcription factor SIX1.
Figure 6

DLEU2 promotes proliferation and glycolysis of OSCC via SIX1