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LncRNA DLEU2 Affects Nasopharyngeal Carcinoma Cells via the PI3K/Akt Pathway

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ABSTRACT The mechanisms in which down-regulating lncRNA deleted in lymphocytic leukemia 2 (DLEU2) expression affects the PI3K/Akt pathway as well as nasopharyngeal carcinoma (NPC) CNE2 cells were explored. CNE2 cells had notably increased DLEU2 expression compared with human nasal epithelial cells ($P<0.001$), and at 24, 48, and 72 h, si-DLEU2 group exhibited a remarkably decreased proliferation rate in comparison with Blank group plus NC group ($P<0.05$). There were evidently fewer invasive cells and *in vitro* tubes, as well as a higher apoptosis rate in NC and Blank groups than in si-DLEU2 group ($P<0.05$). In contrast to NC and Blank groups, si-DLEU2 group had markedly lowered p-p85, VEGF and p-Akt expressions ($P<0.05$). DLEU2 has high expression in NPC cells, which possibly activates the PI3K/Akt signal pathway to enhance the proliferation and invasion of cells, suppress the apoptosis and promote the angiogenesis. It is a potentially eligible therapeutic target in NPC.

INTRODUCTION

In Southeast Asia and southeastern China, the incidence rate of the clinically common head and neck malignancy nasopharyngeal carcinoma (NPC) is very high (Chang et al. 2017). NPC patients in China account for 80 percent of the total number worldwide (Wei et al. 2017). Currently, NPC patients in the early stage have a mean 5-year survival rate of about 50 percent, while that of patients with mid- and late-stage NPC is only 20-30 percent (Dwijayanti et al. 2020). About 70 percent of NPC patients have suffered from metastasis to the lymph nodes when diagnosed, and those with locally advanced NPC still have a treatment failure rate of 20-30 percent after receiving concurrent chemoradiotherapy, so distant metastasis plus local recurrence serve as the main problems (Parlak et al. 2019; Xu et al. 2019b). Hence, clarifying the molecular mechanism of malignant phenotype promoting NPC metastasis is an urgent need.

As high-throughput sequencing technique is advancing, increasing emphasis has been attached to non-coding ribonucleic acids (ncRNAs) in recent years (Tang and He 2021). NcRNAs are a class of RNAs working as regulators, and most of them do not code proteins. Besides, long ncRNAs (lncRNAs) also play crucial roles in NPC (Tian et al. 2020). According to reports, deleted in lymphocytic leukemia 2 (DLEU2), located on the chromosome 13, exerts oncogenic effects on laryngeal squamous cell carcinoma, liver cancer and so on (Li et al. 2020; Shi et al. 2021). DLEU2 has been widely involved in the regulation of invasion, proliferation, apoptosis as well as migration of cells by setting the genes associated with tumors as the targets (Ghafouri-Fard et al. 2021). For example, DLEU2 has a negative relation to patients' overall survival given its high expressions in non-small cell lung cancer tissues plus cells. In addition, it is capable of advancing non-small cell lung cancer from the aspect of metastasis in addition to growth (Zhou et al. 2019). Moreover, the cell lines together with tissues of glioma present high expressions of such an lncRNA. DLEU2 at an overexpressed level promotes glioma cells in terms of colony formation, invasion and migration (Xie et al. 2019). Nevertheless, how DLEU2 affects NPC cells through their propagation and apoptosis has rarely been studied so far.

Objectives

In view of this, small interference RNA (siRNA) targeting DLEU2 was transfected into NPC cells to down-regulate DLEU2 expression, and NPC cell apoptosis, proliferation, and invasion as well as the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway under the influences of DLEU2 expression were further assessed in

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this study, aiming to explore the promising prevention and treatment targets for NPC.

MATERIAL AND METHODS

Cells and Main Reagents

Human nasal epithelial cells (HNEpCs) and NPC CNE2 cells offered by SIBCB, CAS (China) were utilized. Lipofectamine 2000 was sourced from Thermo Fisher (USA). Gibco (USA) supplied trypsin, fetal bovine serum (FBS) plus Roswell Park Memorial Institute (RPMI) 1640 medium. Penicillin-streptomycin dual antibody mixture was purchased from Sigma (USA). Methyl thiazolyl tetrazolium (MTT) was bought from Nanjing Manfute Biotechnology Co., Ltd. (China). Transwell kit and Annexin V-FITC/PI apoptosis assay kit for double staining offered by Nanjing Jiancheng Bioengineering Institute (China) were applied. The kit for total RNA extraction (TRIzol) was purchased from Invitrogen (USA). BCA kit, real-time quantitative polymerase chain reaction (qPCR) kit SYBR PCR Master Mix plus reverse transcription kit Prime Script RT Master Mix were supplied by TaKaRa Biotech (Dalian) Co., Ltd. (China). Rabbit polyclonal primary antibodies against GAPDH & vascular endothelial growth factor (VEGF) were manufactured by Abcam (USA). Moreover, rabbit monoclonal primary antibodies against phosphorylated PI3K regulatory subunits p85 (p-p85) and Akt (p-Akt) were bought from CST (USA). Goat anti-rabbit second antibodies conjugated with horseradish peroxidase originating in Wuhan Boster Biological Technology Co., Ltd. (China) were used. SiRNA fragment si-DLEU2 targeting DLEU2 (5'-CCTCAGCCTTCTCCTCTAA-3') and unrelated sequence si-negative control (NC) were purchased from Shanghai GenePharma Co., Ltd. (China). Primers for qPCR sourced from Sangon Biotech (Shanghai) Co., Ltd. (China) were adopted.

Cell Culture, Transfection and Grouping

The RPMI 1640 medium supplemented with penicillin-streptomycin (100 U/mL) and 10 percent FBS was used for routine CNE2 cell culture, followed by digestion with 0.25 percent trypsin and passage when 80-90 percent confluence was reached. Later, inoculation of the cells into a 6-well plate was performed with 1×10^5/well in density. When these cells grew to 60 percent, Lipofectamine 2000 was applied to transfect CNE2 cells using si-DLEU2 and si-NC, and three groups were set up, that is, blank control group (Blank group, untransfected), interference group (si-DLEU2 group, transfected with si-DLEU2), and negative control group (NC group, subjected to si-NC transfection).

Detection of mRNA Expressions by qRT-PCR

Extraction of total RNA in cells was accomplished through TRIzol reagent, and an RT kit and PCR were used for synthesis and amplification of cDNA, respectively. DLEU2 expression was determined *via* the SYBR PCR Master Mix kit with U6 being an internal reference gene. qRT-PCR was implemented under the conditions with 40 cycles of 10-min pre-denaturation (95°C), 50-s denaturation (95°C), 25-s annealing (60°C), and 15-s extension (72°C). $2^{-\Delta\Delta Ct}$ method was adopted to calculate DLEU2 expression level. The following sequences of primers were employed: DLEU2: F: 52 -GCCATCTTTCGAAGGCAAACAAGC-32 , R: 52 -GCCTTAAATGGTTGTCTCACAGAAC-32 , U6: F: 52 -CTCGCTTCGGCAGCACA-32 , R: 52 -AACGCTTCACGAATTTGCGT-32 .

Determination of Cell Proliferation After Transfection by MTT Assay

A plate with 96 wells was selected for cell inoculation, and each group was configured with 6 replicates. A complete medium was added to substitute the culture medium after transfection for 6 h, then at 24, 48, 72 and 96 h, each well was supplemented with MTT (5 mg/mL) in a volume of 20 ìL, and the upper medium was eliminated subsequent to 4 h of incubation at 37°C under 5 percent CO_2. Then every well was supplied with DMSO (150 ìL), and a microplate reader was utilized at 490 nm to obtain the optical density.

Detection of Cell Invasive Ability by Transwell Assay

CNE2 cells were trypsinized and then prepared into a 5×10^5/mL suspension using the RPMI 1640 medium with no serum. Then they were inoculated into a Transwell chamber upper layer with 1×10^5/

mL in density, where 3 replicates were set for every group. After culture of cells in serum-free medium, the chamber lower layer was added with complete medium. Subsequently, aseptic cotton swabs were used to wipe the chamber upper layer to remove the cells cultured for 24 h. Afterward, twice PBS washing of cells infiltrating the chamber lower layer, 10 min of fixation in methanol, air-drying and 5 min of crystal violet staining were carried out. With the staining solution removed, the cells underwent twice rinsing in double-distilled water and observation by virtue of the inverted microscope (magnification: 400×). Finally, 5 visual fields were randomly selected for counting.

Determination of Cell Apoptosis by Annexin V-fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Staining and Flow Cytometry

The cells transfected for 48 h received EDTA-free trypsin (0.25%) digestion, PBS washing, and three times of centrifugation, for the purpose of clearing the serum. Next, binding buffer (500 μL) was supplemented to resuspend the cells, and 10 μL of PI plus 5 μL of Annexin V-FITC were added for blending. Finally, after 15 min of room-temperature reaction away from light, FACSCalibur flow cytometry was executed to measure the apoptosis rate.

Detection of In Vitro Tube Formation

The mixed culture medium was added to a 96-well plate with cells inoculated for overnight culture in an incubator. During culture, the cell integrity as well as tubular arrangement was surveyed. 24 h later, the culture was finished, and a clear visual field was selected under the microscope for counting.

Measurement of VEGF, p-p85 and p-Akt Expressions by Western Blotting

After the same cell grouping as mentioned above, total cell protein was extracted and quantified. Next, sample buffer was added to react with the protein, followed by 10-min denaturation at 95°C or above. Later, the sample was subjected to SDS-PAGE (80 V for 2 h), and the product was transferred to a membrane (90 V for 60 min). Subsequently, 2 percent BSA was applied to overnight block the membrane, Tris-buffered saline-Tween 20 (TBST) was used for 4 times of washing, and primary antibodies were added for overnight incubation at 4°C. Later, following 4 times of TBST washing, the membrane and secondary antibodies were incubated together for 1 h at 37°C. Then 4 times of membrane rinsing in TBST, 30 s of reaction, as well as exposure detection and image acquisition *via* the imaging system were accomplished. With β-actin as the internal reference, calculation of relative protein expression level was conducted.

Statistical Analysis

Statistical analysis was performed by means of SPSS 16.0 software, while plotting was finished through GraphPad Prism 5.01 software. Comparison among groups was realized using *t*-test. A difference of statistical significance was presented as P<0.05.

RESULTS

DLEU2 Expressions in HNEpCs and NPC CNE2 Cells

CNE2 cells displayed a significantly elevated DLEU2 expression level compared with HNEpCs (P<0.001), and in contrast to NC and Blank groups, si-DLEU2 group had a notably raised DLEU2 expression level (P<0.001) (Table 1), suggesting successful construction of NPC cell line with stably down-regulated DLEU2 expression.

Table 1: DLEU2 expressions detected by qPCR

Group	*DLEU2 expression*	*F/T*	*P*
HNEpC	0.23±0.03	7.823	<0.001
CNE2	4.78±0.67		
NC	4.75±0.78	37.275	<0.001
Blank	4.81±0.84		
si-DLEU2	0.14±0.058$^{*,\#}$		

DLEU2: Deleted in lymphocytic leukemia 2; HNEpC: human nasal epithelial cell; NC: negative control; qPCR: quantitative polymerase chain reaction; si: small interference

Effect of down-regulating DLEU2 Expression on CNE2 Cell Proliferation

An evidently lower proliferation rate was observed in si-DLEU2 group than in NC plus Blank

groups at 24, 48 and 72 h (P<0.05), but no significant difference in NC group from Blank group was detected (P>0.05) (Table 2).

Table 2: Effect of down-regulating DLEU2 expression on proliferation of CNE2 cells

Group	*OD*			
	24h	*48h*	*72h*	*96h*
NC	1.78±0.11	2.73±0.17	3.68±0.34	4.02±0.11
Blank	1.82±0.09	2.77±0.21	3.72±0.31	4.06±0.09
si-DLEU2	1.83±0.12	2.31±0.24$^{*,\#}$	2.61±0.28$^{*,\#}$	2.54±0.12$^{*,\#}$

*P<0.05 vs. NC group, $^{\#}$P<0.05 vs. Blank group

Effect of Down-regulating DLEU2 Expression on Invasion of CNE2 Cells

The Si-DLEU2 group had significantly fewer invasive cells than those of NC and Blank groups (P<0.05). Between NC group and Blank group, there were no significantly different numbers of invasive cells (P>0.05) (Table 3).

Table 3: Effect of down-regulating DLEU2 expression on invasion of CNE2 cells

Group	*Number of invasive cells*
NC	154±11
Blank	151±8
si-DLEU2	43±9$^{*,\#}$

*P<0.05 vs. NC group, $^{\#}$P<0.05 vs. Blank group

Effect of down-regulating DLEU2 expression on apoptosis of CNE2 cells

NC plus Blank groups in comparison with si-DLEU2 group had a prominently lowered apoptosis rate (P<0.05), but the two groups exhibited insignificant variations (P>0.05) (Table 4).

Table 4: Effect of down-regulating DLEU2 expression on apoptosis of CNE2 cells

Group	*Apoptosis rate (%)*
NC	15.32±1.02
Blank	17.83±1.32
si-DLEU2	25.42±2.39$^{*,\#}$

*P<0.05 vs. NC group, $^{\#}$P<0.05 vs. Blank group

Effect of Down-regulating DLEU2 Expression on *in vitro* Tube Formation of CNE2 Cells

Compared with NC and Blank groups, si-DLEU2 group presented a distinctly reduced number of tubes (P<0.05), whereas the difference between NC and Blank groups was not significant (P>0.05) (Table 5).

Table 5: Effect of down-regulating DLEU2 expression on *in vitro* tube formation of CNE2 cells

Group	*Tube number*
NC	25±5
Blank	24±7
si-DLEU2	13±3$^{*,\#}$

*P<0.05 vs. NC group, $^{\#}$P<0.05 vs. Blank group

Effect of Down-regulating DLEU2 Expression in CNE2 Cells on Expressions of p-Akt, VEGF, and p-p85

According to Western blotting results, VEGF, p-p85 and p-Akt expressions obviously declined in si-DLEU2 group compared with those in NC plus Blank groups (P<0.05), but they were not markedly different in NC group from those in Blank group (P>0.05) (Table 6).

Table 6: Effect of down-regulating DLEU2 expression on expressions of VEGF, p-p85 and p-Akt in CNE2 cells

Group	*p-Akt*	*p-p85*	*VEGF*
NC	0.89±0.08	1.26±0.25	0.83±0.09
Blank	0.88±0.07	1.18±0.19	0.92±0.05
si-DLEU2	0.34±0.03$^{*,\#}$	0.41±0.05$^{*,\#}$	0.26±0.04$^{*,\#}$

*P<0.05 vs. NC group, $^{\#}$P<0.05 vs. Blank group.

DISCUSSION

Being crucial players in tumorigenesis and other numerous biological processes (Fu et al. 2017; Li et al. 2017), several lncRNAs have correlations with the diagnosis, treatment and prognostic prediction of cancer patients, as new markers for various malignant tumors (Chen et al. 2016). Both cell lines and tissues of NPC have remarkably highly expressed lncRNAs PVT1 (Cui et al. 2019), HCG18 (Li et al. 2019), AK027294 (Zhang et al. 2020), MINCR (Zhong et al. 2020), and SMAD5-AS1 (Zheng

et al. 2019), which are related to such patients from the perspectives of stage, grade, radiosensitivity together with poor prognosis. Down-regulating these lncRNAs *via* their expressions can inhibit NPC cells in terms of the migration, invasion and multiplication (Wang et al. 2019; Jiang et al. 2021).

LncRNA DLEU2 is an lncRNAs family member and miR-15a/16-1 cluster host gene (Liu et al. 2019). Located on the chromosome 13q14, it can mediate and make contributions to biological processes like invasion and propagation of cells (Xu et al. 2021). DLEU2 not only presented a reduced expression in laryngeal carcinoma, but also exerted tumor suppressor's functions by regulating miR-16-1, as reported by Xie et al. (2018). Besides, the tissues and cell lines of pancreatic cancer are found to have an up-regulated DLEU2 expression, while DLEU2 acts as an endogenous "sponge" of miR-455 to regulate SMAD2 expression and promote pancreatic cancer by enhancing cell proliferation plus invasion (Xu et al. 2019a). DLEU2 has dysregulated expressions in many tumors, displaying intimate correlations with the metastasis besides growth of tumors (Wu et al. 2020a). According to the findings of Zhang et al. (2016), compared with that in adjacent tissues and normal cervical cell lines, DLEU2 was lowly expressed in cervical cancer cells and tissues, whereas DLEU2 at an overexpressed level inhibited the ability of cervical cancer cells to proliferate, migrate and invade, so DLEU2 functioned as a tumor suppressor gene. In hepatocellular carcinoma tissues, especially in tumors >5 cm and advanced tumor tissues, nevertheless, DLEU2 expression has been verified to be up-regulated, and DLEU2 promotes hepatocellular carcinoma cells to propagate, migrate and invade through binding EZH2, thus facilitating carcinogenesis (Guo et al. 2019). Hence, DLEU2 may play various roles in different tumors. Pitifully, it is still unknown whether DLEU2 takes part in NPC onset and progression. CNE2 cells in this study manifested a prominently raised DLEU2 expression in comparison to HNEpCs. The multiplication, invasion as well as angiogenesis of CNE2 cells were dramatically repressed, while their apoptosis was strengthened by down-regulating DLEU2 expression.

Moreover, suppressing tumor angiogenesis is beneficial to the development of human tumor therapies (Hu et al. 2018). The mechanism of tumor neovascularization is complex, which involves VEGF (Viallard et al. 2017). As a common regulator of neovascularization, VEGF can promote vascular endothelial cell division, increase vascular permeability, and exert important effects on malignant tumors from the aspects of propagation, metastasis and differentiation (Kong et al. 2020; Murahashi et al. 2021). Uncontrollable angiogenesis during tumor formation can form new vessels, as a driving force for the nutritional supply to tumor cells, thus leading to rapid tumor progression, invasion and metastasis (Ho et al. 2018). Inhibiting VEGF expression or blocking its function is helpful for the regulation of anti-angiogenesis in NPC, thus ameliorating the internal balance between angiogenic factors and inhibitors (Miles et al. 2014). In this study, VEGF expression decreased significantly after the down-regulation of DLEU2 expression. The PI3K/Akt signaling pathway activation and inactivation substantially affect tumor onset and progression, and many lncRNAs regulate malignant tumors via this pathway (Wu et al. 2020b). Herein, the phosphorylation levels of regulatory subunits p85 and Akt decreased after the down-regulation of DLEU2 expression, implying attenuated PI3K/Akt signaling pathway activity.

CONCLUSION

In conclusion, DLEU2 expression increases in NPC cells, and DLEU2 works as an oncogene, which may enhance the cell proliferation and invasion, promote the angiogenesis, and inhibit the apoptosis by activating the PI3K/Akt signaling pathway. Hence, DLEU2 serves as a latent target in curing NPC.

RECOMMENDATIONS

These study findings are conducive to the exploration of the molecular mechanisms of lncRNAs in NPC incidence and development, which offer a theoretical basis for NPC diagnosis and treatment using DLEU2 as the potential target.

ABBREVIATIONS

Akt: Protein kinase B;
DLEU2: deleted in lymphocytic leukemia 2;
HNEpC: human nasal epithelial cell;
MTT: methyl thiazolyl tetrazolium;
NC: negative control; ncRNA: non-coding ribonucleic acid;
NPC: nasopharyngeal carcinoma;

PI3K: phosphatidylinositol 3-kinase;
qPCR: quantitative polymerase chain reaction;
siRNA: small interference RNA;
VEGF: vascular endothelial growth factor.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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