Original Article

Elevated Sad1 and UNC84 Domain Containing 2 (SUN2) level inhibits cell growth and aerobic glycolysis in oral cancer through reducing the expressions of glucose transporter 1 (GLUT1) and lactate dehydrogenase A (LDHA)

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Abstract
Background/purpose: Oral cancer is a malignant tumor accompanied by high morbidity, mortality, and poor prognosis. Therefore, it is urgent to explore the precise regulation mechanisms underlying oral cancer. Sad1 and UNC84 Domain Containing 2 (SUN2) was considered as a tumor suppressor in some cancers. The purpose of the study was to define the role of SUN2 in oral cancer progression.

Materials and methods: Tumor tissues and paired paracancerous healthy tissues from 56 oral cancer patients were collected. Cell viability was measured using MTT assay. The colony formation assay was applied to determine cell proliferation ability. The mRNA and protein levels were assessed by qRT-PCR and Western blot, respectively.

Results: SUN2 expression was decreased in oral cancer tissues and cell models. SUN2 overexpression suppressed the growth of oral cancer cells, while the down-regulation of SUN2 promoted cell growth. SUN2 overexpression restrained the glucose uptake, lactate production, and ATP level of oral cancer cells, whereas down-regulation of SUN2 promoted glycolysis. Besides, elevated SUN2 inhibited the glucose transporter 1 (GLUT1) and lactate dehydrogenase A (LDHA) levels. However, SUN2 knockdown increased the levels of GLUT1 and LDHA.

Conclusion: SUN2 was decreased in oral cancer in vivo and in vitro. SUN2 overexpression suppressed cell growth and glycolysis via reducing the levels of GLUT1 and LDHA in oral cancer.
Introduction

Oral cancer is a type of common malignant neoplasia accompanied by high mortality and poor prognosis. The incidence of oral cancer ranked eighth among all cancer subtypes worldwide, and it is estimated that there are approximately 540,000 newly diagnosed cases each year. Despite considerable improvements have been achieved in the prognosis and treatment of oral cancer recently in the wake of the development of early screening and chemotherapy, the 5-year survival rate of oral cancer patients is still less than 63%. Therefore, it is urgent to investigate the regulation mechanism of oral cancer progression with the aim to develop novel therapeutic strategies.

Studies have proved that cancer cells can generate energy by glycolysis instead of oxidative phosphorylation, and such phenomenon was called Warburg effect. Glycolysis is considered as a major metabolic process for energy production and anabolic growth in cancer cells. Xian et al. reported 3-Bromopyruvate suppressed gastric cancer tumor growth through restraining glycolysis. Besides, Yu et al. demonstrated that glycolysis was related to shorter overall survival of oral cancer, and oral cancer progression was further mediated the carcinogenesis effects of increased glycolysis with the aim to develop novel therapeutic targets for oral cancer from bench to clinic.

Material and methods

Tissues collection and cell culture

Fifty-six patients diagnosed with oral cancer in the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology were recruited in the study. The cancer tissues and paracancerous healthy tissues of all cases were collected to detect the expressions of SUN2. All cases signed informed consent. The Ethics Committee of The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology approved the study.

Human oral cancer cell lines (HSC-6 and SCC-9) and human normal oral keratinocytes cell line hNOK were obtained from American Type Culture Collection (ATCC). Human oral cancer cell line HN13 was obtained from Shanghai Key Laboratory of Stomatology (Shanghai, China), and HSC-3 and TSCC1 cell lines were purchased from BeNa Culture Collection (Beijing, China). The HSC-6, SCC-9, HN13, HSC-3, TSCC1 and hNOK cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) at 37°C cell incubator with 5% CO₂.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA isolation from tissues and cells was conducted by TRizol (Yeasen, Shanghai, China). cDNA was obtained by First Strand cDNA Synthesis Kit (Yeasen, Shanghai, China). Then mRNA levels were determined using qPCR SYBR Green Master Mix system (Yeasen, Shanghai, China). The primers sequences used in the study were listed as below: SUN2: F, 5'-CTACTCCCGAGGTGACAG-3'; R, 5'-GGGTTCTCATGTTCTGGATT-3'; GLUT1: F, 5'-ACACTGGAGTCATCAATGCCC-3'; R, 5'-CAGGATGCTCTCCCATAGC-3'; LDHA: F, 5'-TACAGTTGGGTTTGGT-3'; R, 5'-CAGTTACGCTGACCAAT-3'; GAPDH: F, 5'-CTCCAAAATCAAGTGCGGCG-3'; R, 5'-ATGACGAACTGGGGCACT-3'. GAPDH was used as an internal control. 2⁻ΔΔCТ method was utilized to analyze mRNA expression.

Western blot

Proteins were extracted utilizing RIPA lysis buffer (Thermo Scientific, Waltham, MA, USA). To separate proteins, the proteins sample was separated by SDS-PAGE gels and transferred onto the PVDF membrane, following by probing to anti-SUN2 (ab65447, 1:500), GLUT1 (ab652, 1:500), and LDHA (ab125683, 1:500) and β-actin (ab8227, 1:500) antibodies (Abcam, Cambridge, UK) at 4°C for 12h after blocking by 5% skim milk. Subsequently, the membrane was
incubated with appropriate secondary antibody (Abcam, Cambridge, UK). After the membrane incubated with ECL Detection Reagent (Yeasen, Shanghai, China), the blots were visualized. The density of bands was quantified using Image J software.

Cell transfection

The short hairpin RNA (shRNA), negative control (shNC), shRNA of SUN2 (shSUN2), pcDNA3.1 control vector, and pcDNA3.1-SUN2 were generated in RiboBio (RiboBio, Guangzhou, China). Before transfection, cells were inoculated into 6-wells plates with 5×10^5 cells per well and maintained in 37°C cell incubator with 5% CO₂ for 24hr. HSC-6 cells were transfected with pcDNA3.1 control vector or pcDNA3.1-SUN2, and SCC-9 cells were transfected with shNC or shSUN2 using Lipofectamine 3000 (Thermo Scientific, Waltham, MA, USA) following instruction of manufacturer. Cells were collected for subsequent experiments 48 h post-transfection.

(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were inoculated into 6-wells plates with 1×10^4 cells each well and cultured in 37°C cell incubator with 5% CO₂ for 24hr. Cells were maintained with MTT for 4h, following by adding 100 μl DMSO to solubilize formazan.

The absorbance at 490 nm was measured by the microplate reader.

Colony formation assay

Cells were seeded into 6-wells plates (300 cells/well) and cultured in 37°C cell incubator with 5% CO₂ for two weeks. Colonies were fixed in 4% paraformaldehyde, and dyed with 0.1% crystal violet. The number of colonies (>50 cells) was calculated.

Detection of glucose uptake, lactate production, and ATP level

The glucose uptake, lactate secretion and ATP level of HSC-6 and SCC-9 cells upon SUN2 overexpression or knockdown, were detected using Glucose Uptake Fluorometric Assay Kit, Lactate Assay Kit and ATP Assay Kit (Sigma, St. Louis, MO, USA) respectively based on the procedure of manufacturers.

Statistical analysis

SPSS Statistics (Chicago, IL, USA) was used to conduct data statistics analysis. All data were presented as mean ± standard deviation (SD). Student’s t-test or one-way ANOVA with LSD post hoc test were selected to elevate group differences. A P value < 0.05 was considered statistically significant in this study.
Results

SUN2 was down-regulated in oral cancer tissues and cells

To elucidate the influence of SUN2 on oral cancer, SUN2 expression was firstly determined in patients. The mRNA expressions of SUN2 in tumor tissues of 56 oral cancer patients were decreased versus paracancerous normal tissues (P < 0.001, Fig. 1A). In addition, the protein levels of SUN2 were decreased in tumor tissues of oral cancer patients as well (Fig. 1B). Furthermore, SUN2 levels also reduced in human oral cancer cell lines, named HSC-6, SCC-9, HN13, HSC-3 and TSCC1 compared to the human normal oral keratinocytes hNOK cells (Fig. 1C, all P < 0.001). Therefore, SUN2 level was decreased in patients’ tissues and cell models of oral cancer.

SUN2 overexpression suppressed oral cancer cell growth

Subsequently, the effects of SUN2 on oral cancer progression were determined. As shown in Fig. 1C, SUN2 expression in HSC-6 cells was lower than that in SCC-9, HN13, HSC-3 and TSCC1 cells, and SUN2 expression in SCC-9 cells was higher than that in HSC-6, HN13, HSC-3 and TSCC1 cells. Therefore, HSC-6 cells and SCC-9 cells were selected for following experiments.

SUN2 overexpression plasmid were transfected into HSC-6 cells increase the level of SUN2, which was verified by Western blot (Fig. 2A, P < 0.001). Two shRNAs for SUN2 were transfected into SCC-9 cells to knockdown SUN2 expression. The two shSUN2 both decreased the SUN2 expressions (Fig. 2A, two P < 0.01). The shSUN2 with higher knockdown efficiency was used for further experiments.
Figure 3  SUN2 overexpression reduced glycolysis of oral cancer cells. A. The glucose uptake was detected in HSC-6 and SCC-9 cells expressing pcDNA-SUN2 and shSUN2, respectively. B. The lactate production was determined in HSC-6 and SCC-9 cells expressing pcDNA-SUN2 and shSUN2, respectively. C. The intracellular ATP concentration was determined in HSC-6 and SCC-9 cells expressing pcDNA-SUN2 and shSUN2, respectively (*: \( P < 0.05 \). **: \( P < 0.01 \). ***: \( P < 0.001 \)).

Figure 4  SUN2 overexpression inhibited GLUT1 and LDHA levels in oral cancer cells. A. The mRNA levels of GLUT1 in HSC-6 and SCC-9 cells expressing pcDNA-SUN2 or shSUN2 were determined by qRT-PCR. B. The mRNA levels of LDHA in HSC-6 and SCC-9 cells expressing pcDNA-SUN2 or shSUN2 were determined by qRT-PCR. C. The protein levels of LDHA in HSC-6 and SCC-9 cells expressing pcDNA-SUN2 or shSUN2 were determined by Western blot (**: \( P < 0.01 \). ***: \( P < 0.001 \)).
Afterward, results proved that elevated SUN2 inhibited the cell viability of HSC-6 cells \( (P < 0.001) \), while SUN2 knockdown significantly increased cell viability of SCC-9 cells \( (P < 0.001) \) (Fig. 2B). In addition, SUN2 overexpression suppressed the colony formation ability of HSC-6 cells \( (P < 0.01) \), and SUN2 knockdown promoted the colony formation of SCC-9 cells \( (P < 0.01) \) (Fig. 2C). Thus, SUN2 overexpression suppressed the growth of oral cancer cell models.

**SUN2 overexpression reduced glycolysis of oral cancer cells**

To clarify the roles of SUN2 in oral cancer, the influence of SUN2 on glycolysis was investigated. Results showed that SUN2 overexpression suppressed glucose uptake in HSC-6 cells \( (P < 0.05) \), and SUN2 silence promoted the glucose uptake in SCC-9 cells \( (P < 0.001) \) (Fig. 3A). In addition, the lactate production could also be inhibited by SUN2 overexpression in HSC-6 cells \( (P < 0.001, \text{Fig. 3B}) \). Similarly, SUN2 knockdown promoted the lactate production in SCC-9 cells \( (P < 0.001, \text{Fig. 3B}) \). Furthermore, SUN2 overexpression decreased the ATP concentration of HSC-6 cells \( (P < 0.01) \), and SUN2 knockdown increased the ATP concentration of SCC-9 cells \( (P < 0.01, \text{Fig. 3C}) \). Collectively, SUN2 overexpression reduced glycolysis of oral cancer cell models.

**SUN2 overexpression inhibited the expression of GLUT1 and LDHA in oral cancer cells**

To interpret the influence of SUN2 on glycolysis, the expression levels of GLUT1 and LDHA was detected after SUN2 expression was changed. Results revealed that SUN2 overexpression reduced the GLUT1 mRNA level \( (P < 0.01) \), while SUN2 knockdown elevated the mRNA level of GLUT1 \( (P < 0.001) \) (Fig. 4A). The mRNA level of LDHA also was inhibited by overexpression of SUN2 and increased by down-regulation of SUN2 (all \( P < 0.01, \text{Fig. 4B} \)). Moreover, Western blot results found that the GLUT1 and LDHA also decreased by SUN2 overexpression and increased by SUN2 knockdown at protein level (all \( P < 0.001, \text{Fig. 4C} \)). Hence, SUN2 overexpression inhibited GLUT1 and LDHA levels in oral cancer.

**Discussion**

Oral cancer is a common malignant neoplasia accompanied by high morbidity, mortality, and poor prognosis.\(^ {1-3} \) It is urgent to investigate regulation mechanisms of oral cancer development and screen novel therapeutic strategies. SUN2 is reported as a tumor suppressor in several types of cancer, such as atypical teratoid/rhabdoid tumors, prostate cancer, and lung cancer.\(^ {10-12} \) Loss of SUN2 was found in breast cancer tissues and cells.\(^ {14} \) Low level of SUN2 was presented in atypical teratoid/rhabdoid tumors, prostate cancer, lung cancer tissue, etc.\(^ {10-12} \) Taken together, these evidences suggested SUN2 expression might be specifically decreased in cancer tissues. Here, we hypothesize SUN2 level was inhibited in oral cancer, and overexpression of SUN2 might be beneficial for oral cancer. Therefore, we explored the influence of SUN2 on oral cancer progression in this study.

In current research, we confirmed SUN2 was down-regulated in patients with oral cancer. The aberrant expression of SUN2 implied that SUN2 might participate in the modulation of oral cancer development. Here, we found that SUN2 overexpression suppressed cell growth. Similarly, SUN2 knockdown promoted cell growth, which were in accordance with previous studies.\(^ {11,12} \) Yajun et al. revealed that elevated SUN2 restrained cell proliferation and tumor growth of prostate cancer.\(^ {11} \) Besides, SUN2 overexpression inhibited lung cancer proliferation, migration, and invasion ability.\(^ {12} \) Thus, we concluded that SUN2 overexpression suppressed the oral cancer cell growth \textit{in vitro}.

Studies demonstrated that glucose metabolism pattern was commonly changed to aerobic glycolysis instead of oxidative phosphorylation pathway in cancer cells (Warburg effect).\(^ {15-18} \) Glycolysis is considered as a major metabolic process for energy production and anabolic growth in cancer cells.\(^ {15} \) Usually, glycolysis was enhanced in cancer cells, such as in oral cancer cells.\(^ {19,20} \) Furthermore, SUN2 was proved to modulate lung cancer glycolysis.\(^ {12} \) Combined, we conjectured that SUN2 might participate in the modulation of glycolysis in oral cancer. In this study, we found that SUN2 suppressed glucose uptake, lactate production and intracellular ATP levels, and SUN2 silence promoted glucose uptake, lactate production, and increased ATP levels. In other words, SUN2 overexpression reduced glycolysis of oral cancer cells, which was consistent with the findings revealed by Lv et al.\(^ {12} \) Lv and colleagues illustrated SUN2 overexpression inhibited glycolytic rate, lactate level and ATP level in lung cancer cells.\(^ {12} \) To interpret the regulation mechanism of SUN2 on glycolysis, expressions of two vital glycolysis related genes named GLUT1 and LDHA were measured, which were essential for glucose absorption and lactate conversion process.\(^ {18-22} \) In the study, SUN2 overexpression inhibited the GLUT1 and LDHA levels in oral cancer. In other words, SUN2 reduced the aerobic glycolysis of oral cancer cells via inhibiting GLUT1 and LDHA levels. The results were similar to the results in lung cancer.\(^ {12} \) However, the influence of SUN2 on glycolysis in oral cancer was contrary to the findings in prostate cancer.\(^ {11} \) SUN2 exhibited no influences on glycolytic rate and glucose uptake in prostate cancer cells.\(^ {11} \) The difference of SUN2-mediated effects on glycolysis between oral cancer and prostate cancer might be caused by the specificity of tumor tissue types, which requires further investigations.

In view of the reduced SUN2 levels in various cancer tissues, including oral cancer,\(^ {10-12} \) we speculated that SUN2 may be decreased in cancer tissues specifically. Besides, SUN2 overexpression was demonstrated to suppress cancer progression in several types of cancers.\(^ {10-12} \) Therefore, the specific expression of SUN2 in cancers as well as the consistence of its regulatory effect on cancer progression make SUN2 a potential therapeutic target for cancers. In conclusion, this study demonstrated SUN2 was down-regulated in oral cancer. SUN2 overexpression suppressed cell growth and aerobic glycolysis via reducing GLUT1 and LDHA levels, shedding lights on SUN2 as a potential therapeutic target for oral cancer treatment from bench to clinic.
Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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