Expression and prognostic analysis of PFKFB4 in oral squamous cell carcinoma

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Abstract

Fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase 4 (PFKFB4) is a crucial enzyme in the glycolysis pathway, possessing both kinase and phosphatase capabilities. Although it has emerged as an important oncogene in various cancer types, its function in oral squamous cell carcinoma (OSCC) is still not well understood. In our research, PFKFB4 expression was assessed via immunohistochemical (IHC) staining of tissue microarrays and OSCC patient specimens. The transcriptional expression of PFKFB4 in OSCC was analyzed by utilizing The Cancer Genome Atlas (TCGA) dataset. Correlation between PFKFB4 expression and clinicopathological features was examined using the \( \chi^2 \) test. Prognostic investigation of PFKFB4 was conducted via Kaplan-Meier and Cox analyses. PFKFB4 levels were notably elevated in OSCC samples in comparison to adjacent normal tissues (\( P < 0.001 \)). Elevated PFKFB4 expression was associated with higher histologic grade (\( P = 0.0438 \)), higher T stage (\( P = 0.031 \)), and more advanced clinical stage (\( P = 0.0063 \)). The ROC curve demonstrated the diagnostic potential of PFKFB4 (AUC = 0.827). Increased levels of PFKFB4 were linked to decreased overall survival (OS) (\( P = 0.04 \)), poorer disease-specific survival (DSS) (\( P = 0.04 \)), and shorter progression-free interval (PFI) (\( P < 0.001 \)). PFKFB4 expression was identified as an independent risk factor for OS based on Cox regression analysis [hazard ratio (HR) = 1.517, \( P = 0.044 \)]. An OS nomogram was constructed with a concordance index of 0.690. Our findings reveal that upregulated PFKFB4 expression in OSCC tissues could serve as a potential prognostic biomarker.

Keywords: PFKFB4, Oral squamous cell carcinoma, Prognosis, Metabolism.

1. Introduction

OSCC ranks among the most prevalent malignancies affecting the head and neck region. Typically, most OSCC patients are at an advanced stage when first diagnosed [1], contributing to a relatively low 5-year survival rate, which has remained around 60% [2]. Poor prognosis can mainly be attributed to late diagnosis, lack of personalized targeted therapies, and the development of drug resistance [3]. Therefore, there is a critical need to identify new markers that can facilitate the diagnosis and treatment of OSCC, ultimately improving patient outcomes.

Cancer is characterized by the alteration of glucose metabolism [4]. Cancer cells tend to use glycolysis over oxidative phosphorylation for energy production, even when oxygen is available. This is a phenomenon known as the Warburg effect or aerobic glycolysis [5]. The rewiring of glucose metabolism is also essential in OSCC tumorigenesis [6]. PFKFB4 is a critical metabolic enzyme that plays a key role in controlling glucose metabolism by dynamically mediating glycolysis [7]. The PFKFB4 gene encodes a PFKB isoenzyme which was first discovered in the testes [8]. Growing proof indicates that PFKFB4 is crucial in the glucose metabolism of tumor cells by shifting glucose to the pentose phosphate pathway. What’s more, PFKFB4 facilitates ROS detoxification as well as lipid and nucleotide synthesis in cancer cells [9,10].

PFKFB4 has been found to be upregulated in various cancers, including glioblastoma, gastric, breast, thyroid, bladder, lung, and colon cancer [10-12]. Moreover, its upregulation has been linked to tumorigenesis, cell proliferation, metastasis, and poorer survival outcomes [13-16], underscoring its pivotal role in cancer initiation and progression.

Previous research has shown the importance of PFKFB4 in various human cancers. However, clinical implications of PFKFB4 in OSCC remain elusive. Thus, our research examined PFKFB4 expression using immunohistochemistry in normal and malignant oral tissues. Additionally, we evaluated PFKFB4 mRNA expression in OSCC and its clinicopathology correlation using publicly available transcriptomic databases. Our results showed a notable upregulation of PFKFB4 in OSCC tissues compared to normal tissues. Upregulated expression of PFKFB4 was significantly associated with advanced tumor stage.

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and histologic grade. Moreover, PFKFB4 overexpression predicted poor prognosis. Our research indicates that PFKFB4 could be a useful diagnostic and prognostic biomarker for OSCC patients.

2. Materials and methods

2.1. Clinical specimens and tissue microarray

45 patients with primary OSCC tumors were included in the research conducted at the Stomatological Hospital, Southern Medical University. 45 OSCC tissues and 11 adjacent normal tissues were collected postoperatively. Informed consent was given by all participants. Additionally, the human OSCC tissue microarray (HN1920e01, Biotech Company, Xi’an, China) was utilized, comprising 175 tumor tissues and 12 adjacent normal tissues. The Ethics Committee of Stomatological Hospital, Southern Medical University approved the study protocol (AFSQ-0105).

2.2. Immunohistochemical analysis

OSCC samples embedded by paraffin were deparaffinized using xylene. Then, they were rehydrated with gradient concentrations of alcohol. The antigen retrieval process involved heating in EDTA buffer. Then endogenous peroxidase activity was inhibited and the slides were blocked, followed by overnight incubation with anti-PFKFB4 antibody (Proteintech, Rosemont, IL, USA). Afterward, the slides were treated with HRP-conjugated secondary antibody. The reactions for PFKFB4 were visualized by 3,3-diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA) and the nuclei were visualized by counterstaining using hematoxylin. Stained slides were scanned and digital images were generated by the Aperio ePathology eH IC IVD System (Leica, Leica Biosystems Imaging, Inc., Wetzlar, Germany). PFKFB4 expression was measured utilizing the Imagescope software according to Aperio ePathology eH IC Cytoplasm Image Analysis. The image analysis was conducted based on the following algorithm: quantify the pixel intensity of the selected area, identify the nuclei and cytoplasm, detect the intensity of nuclei and cytoplasm and set threshold. The algorithm provides an H-Score, a final co-localized result and a histogram for the IHC staining of the cytoplasm and nuclei. The H-scores ranged from 0 to 105. Five representative fields were evaluated (400× magnification) for each slide. Then, the final score was assigned to each sample. The median score was employed as the threshold to categorize PFKFB4 expression into high and low levels in the samples.

2.3. Cell culture

The HSC6, SCC4, SCC9, CAL33, CAL27, and CAL27 cell lines were maintained in our laboratory. The CAL27, SCC4, and SCC9 cell lines were obtained from the American Type Culture Collection. HSC6 was generously provided by Prof. J Silvio Gutkind at the National Institutes of Health in the USA. CAL33 cells were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures). HOKs were acquired from ScienCell Research Laboratories through commercial purchase. CAL27, CAL33, and HSC6 cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). SCC4 and SCC9 cells were cultivated in DMEM/F12 1:1 (Gibco, Rockville, MD, USA) supplemented with 10% FBS and 400 ng/ml hydrocortisone (Solarbio, Beijing, China). HOKs were maintained in Keratinocyte-SFM (Gibco, Rockville, MD, USA) medium supplemented with penicillin-streptomycin (100 U/mL).

2.4. Western blot assay

Cellular proteins were fully lysed using RIPA buffer with protease inhibitor (Roche, Basel, Switzerland) and qualified with BCA protein assay kit (Sigma-Aldrich, St. Louis, MO, USA). After performing SDS-PAGE, the extracted proteins were transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked using nonfat milk and then subjected to incubation with primary antibodies targeting PFKFB4 (Proteintech, Rosemont, IL, USA) and GAPDH (Proteintech, Rosemont, IL, USA). Then, secondary antibodies were used to incubate the membranes and they were detected using enhanced chemiluminescence (ECL) assay (Thermo, Waltham, MA, USA).

2.5. Gene expression analysis of PFKFB4 in OSCC

The TCGA database was used to obtain the transcriptional expression levels of PFKFB4 in patients with OSCC and HNSC. The OSCC data were extracted from the HNSC dataset. The analysis included 504 TCGA-HNSC samples and 330 TCGA-OSCC samples. The predictive sensitivity and specificity of PFKFB4 gene were evaluated by ROC analysis using pROC package in R. The AUC value was calculated to assess the effectiveness of the ROC analysis.

2.6. Prognostic value of PFKFB4 gene in OSCC

Prognostic analysis was conducted using Kaplan-Meier analysis. R packages “survival” and “survminer” were utilized to generate Kaplan-Meier curves. Patients were categorized into high and low-expression groups using the optimal threshold of PFKFB4 expression [17]. The effects of clinical characteristics on patient outcomes were assessed through Cox regression analyses. Then, a nomogram was established using independent prognostic factors, with the aim of predicting overall survival probability. Additionally, calibration plots were utilized to assess the nomogram’s performance.

2.7. Statistical analysis

The correlation between PFKFB4 gene expression and various clinicopathological characteristics was assessed using the χ2 test. PFKFB4 gene expression was also compared between OSCC and normal tissues utilizing t-test. Statistic Package for Social Science (SPSS) 22.0 (IBM, Armonk, NY, USA) was used to analyze the data and P < 0.05 was considered statistically significant.

3. Results

3.1. Upregulation of PFKFB4 protein expression in OSCC tissues

The study included a total of 220 OSCC tissues and 23 normal tissues. Among these specimens, 190 cases were male, and 30 cases were female, with ages ranging from 27 to 78 years old and an average age of 53.4 ± 9.913 years old (Table 1).

To investigate the clinical significance of PFKFB4 in OSCC, we conducted IHC staining to assess its expression in tumor samples and normal tissues. Positive staining for PFKFB4 protein was predominantly observed in the...
cytoplasm of OSCC cells, whereas the majority of stromal cells displayed negative staining for PFKFB4 (Figure 1A, B, C).

Moreover, PFKFB4 was overexpressed in OSCC samples relative to adjacent normal tissues (Figure 1D, P < 0.0001). PFKFB4 levels were correlated with the T stage (Table 1, P = 0.031) and showed a potential link with TNM stage (Table 1, P = 0.074). Besides, PFKFB4 protein levels in OSCC cell lines were also determined by Western blot. OSCC cell lines (CAL27, CAL33, and SCC9) exhibited higher levels of PFKFB4 protein than HOKs (Figure 1E).

3.2. Expression pattern of PFKFB4 transcript in TCGA OSCC patients

To further validate our observations on PFKFB4 in OSCC, UALCAN transcriptome analysis was conducted using TCGA database. A significant upregulation of PFKFB4 transcripts was observed in HNSCC samples in comparison with normal tissues (P < 0.001, Figure 2A). Transcriptional expression analysis revealed elevated levels of PFKFB4 in OSCC specimens compared to adjacent normal tissues, as indicated by both unpaired and paired sample analyses (P < 0.001, Figure 2B, 2C).

To assess the diagnostic performance of PFKFB4, a ROC curve was used, demonstrating high predictive accuracy in distinguishing OSCC tissues from adjacent normal tissues (AUC = 0.827, 95% CI = 0.751-0.902, Figure 2D).

3.3. Correlation between PFKFB4 expression and clinical characteristics of patients with OSCC

The χ2 test was employed to investigate the correlation between PFKFB4 levels and clinical features (Table 2). Elevated levels of PFKFB4 were linked to clinical stage (P = 0.0063, Figure 3A) and histologic grade (P = 0.0438, Figure 3B). However, no significant correlations were observed between PFKFB4 expression and pathologic stage, pathologic T stage, age, and alcohol history (Figure 3C-F).

Table 1. Analysis of PFKFB4 protein expression and clinical features (n=220).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases</th>
<th>Low expression of PFKFB4</th>
<th>High expression of PFKFB4</th>
<th>P value</th>
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<tr>
<td>Age, n (%)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>168</td>
<td>84 (50.0%)</td>
<td>84 (50.0%)</td>
<td>0.874</td>
</tr>
<tr>
<td>≥60</td>
<td>52</td>
<td>27 (51.9%)</td>
<td>25 (48.1%)</td>
<td></td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Male</td>
<td>190</td>
<td>96 (50.5%)</td>
<td>94 (49.5%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>15 (50.0%)</td>
<td>15 (50.0%)</td>
<td></td>
</tr>
<tr>
<td>T stage, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.031*</td>
</tr>
<tr>
<td>T1 + T2</td>
<td>112</td>
<td>65 (58.0%)</td>
<td>47 (42.0%)</td>
<td></td>
</tr>
<tr>
<td>T3 + T4</td>
<td>108</td>
<td>46 (42.6%)</td>
<td>62 (57.4%)</td>
<td></td>
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<tr>
<td>N stage, n (%)</td>
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<td></td>
<td></td>
<td>0.686</td>
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<tr>
<td>N0</td>
<td>115</td>
<td>60 (52.2%)</td>
<td>55 (47.8%)</td>
<td></td>
</tr>
<tr>
<td>N+</td>
<td>105</td>
<td>51 (48.6%)</td>
<td>54 (51.4%)</td>
<td></td>
</tr>
<tr>
<td>TNM stage, n (%)</td>
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<td></td>
<td></td>
<td>0.074</td>
</tr>
<tr>
<td>I + II</td>
<td>63</td>
<td>38 (60.3%)</td>
<td>25 (39.7%)</td>
<td></td>
</tr>
<tr>
<td>III + IV</td>
<td>157</td>
<td>73 (46.5%)</td>
<td>84 (53.5%)</td>
<td></td>
</tr>
<tr>
<td>Histologic grade, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>0.547</td>
</tr>
<tr>
<td>Well</td>
<td>159</td>
<td>77 (48.4%)</td>
<td>82 (51.6%)</td>
<td></td>
</tr>
<tr>
<td>Moderate + poor</td>
<td>61</td>
<td>33 (54.1%)</td>
<td>28 (45.9%)</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05; T, tumor; N, node.

Fig. 1. PFKFB4 levels are increased in both OSCC tissues and cell lines. (A-C) Representative immunohistochemistry images of PFKFB4 staining in normal and OSCC tissues. Staining intensity: (A) Negative, (B) Weak, (C) Strong. (D) Statistical analysis of PFKFB4 expression in normal and OSCC tissues based on staining scores. **** indicates P < 0.0001.

Fig. 2. The transcriptional expression of PFKFB4 was compared between OSCC and normal tissues in TCGA cohort. (A) Transcriptional expression analysis revealed up-regulation of PFKFB4 in HNSCC tissues. (B) Transcriptional expression of PFKFB4 was higher in OSCC tissues than in non-matched normal tissues. (C) The expression of PFKFB4 was elevated in OSCC tissues compared to normal tissues. (D) The ROC curve was utilized to assess PFKFB4’s diagnostic performance in OSCC patients. *** indicates P < 0.001.
3.4. Prognostic value of PFKFB4 in patients with OSCC

The Kaplan-Meier analysis showed that increased levels of PFKFB4 were associated with lower overall survival (OS) rates (HR = 1.44, 95% CI = 1.02-2.03, \( P = 0.04 \)), worse disease-specific survival (DSS) (HR = 1.57, 95% CI = 1.02-2.41, \( P = 0.04 \)), and shorter progression-free interval (PFI) (HR = 1.83, 95% CI = 1.02-2.95, \( P < 0.001 \)) (Figure 4A-C), suggesting a connection between elevated PFKFB4 expression and poor OSCC prognosis.

Univariate Cox analysis demonstrated that high PFKFB4 expression (HR = 1.633, 95% CI = 1.143-2.334, \( P = 0.007 \)), along with pathologic N1-3 stage (HR = 1.785, 95% CI = 1.223-2.605, \( P = 0.003 \)), pathologic T2-4 stage (HR = 3.057, 95% CI = 1.347-6.936, \( P = 0.008 \)), and pathologic stage III/IV (HR = 2.174, 95% CI = 1.388-3.405, \( P < 0.001 \)) were associated with shorter OS time (Table 3).

Multivariate analysis further identified PFKFB4 expression (HR = 1.517, 95% CI = 1.011-2.275, \( P = 0.044 \)) and
pathologic stage (HR = 2.107, 95% CI = 1.051-4.223, \( P = 0.036 \)) as independent prognostic factors (Table 3).

We establish a nomogram to account for the predictive capacity of PFKFB4 expression and pathologic stage. Total score of each individual was calculated and patients with a higher score exhibited poorer survival (Figure 4D). The calibration curves demonstrated the powerful predictive ability of PFKFB4 gene for overall survival at 1, 3, and 5 years (Figure 4E-G). The nomogram was constructed with a concordance index 0.690 (95% CI = 0.659-0.721), indicating that the Cox proportional hazard model had moderate accuracy in predicting OS.

### 4. Discussion

OSCC poses a significant global healthcare challenge due to late diagnosis and poor survival rates. Early detection and diagnosis of early-stage OSCC are crucial for improving patient outcomes. Therefore, it is essential to delve into the oncology and pathogenesis of OSCC and discover novel molecular biomarkers, in the hope of facilitating early detection and enhancing personalized therapies. PFKFB4, a critical regulator of glycolysis, has been reported to function as an important oncogene in various malignancies [15,18-22]. Hence, we explored the diagnostic and prognostic role of PFKFB4 in OSCC patients. Our findings showed that PFKFB4 expression was elevated in OSCC. Besides, up-regulation of PFKFB4 was correlated with unfavorable clinicopathological variables, such as higher histologic grade and advanced tumor stage. Moreover, relevance between upregulated PFKFB4 expression and poor survival was proven. According to Cox analysis, PFKFB4 may act as an independent predictor of prognosis in OSCC.

### Table 3. Cox analysis of clinicopathological features and PFKFB4 expression for overall survival.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;=60</td>
<td>158</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>172</td>
<td>1.261 (0.912 - 1.744)</td>
<td>0.161</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>225</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>105</td>
<td>1.101 (0.786 – 1.544)</td>
<td>0.576</td>
</tr>
<tr>
<td>Pathologic stage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Stage I &amp; Stage II</td>
<td>74</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Stage III &amp; Stage IV</td>
<td>225</td>
<td>2.174 (1.388 - 3.405)</td>
<td>&lt; 0.001*</td>
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<tr>
<td>Pathologic stage N stage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>120</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>N1&amp;N2&amp;N3</td>
<td>156</td>
<td>1.785 (1.223 - 2.605)</td>
<td>0.003*</td>
</tr>
<tr>
<td>Pathologic T stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>31</td>
<td>Reference</td>
<td></td>
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<tr>
<td>T2&amp;T3&amp;T4</td>
<td>273</td>
<td>3.057 (1.347 - 6.936)</td>
<td>0.008*</td>
</tr>
<tr>
<td>PFKFB4</td>
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<tr>
<td>low</td>
<td>247</td>
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<tr>
<td>High</td>
<td>83</td>
<td>1.633 (1.143 – 2.334)</td>
<td>0.007*</td>
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<td>Histologic grade</td>
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<td></td>
</tr>
<tr>
<td>G1</td>
<td>52</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>G2&amp;G3&amp;G4</td>
<td>269</td>
<td>1.517 (0.946 - 2.432)</td>
<td>0.084</td>
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</tbody>
</table>

* \( P < 0.05; T, \) tumor; \( N, \) node.

![Fig. 4. Prognostic value of PFKFB4 expression in OSCC patients measured by Kaplan-Meier analysis. OS in (A), DSS in (B), PFI in (C) for OSCC patients with high versus low PFKFB4. (D) Nomogram was generated to predict OS probability. (E-G) Calibration curves demonstrated good predictive ability for overall survival at 1, 3, and 5 years.](image-url)
and higher histologic grade (grade 2-4) in OSCC patients. Similar correlation between PFKFB4 expression and tumor stage has been reported in lung cancer [24]. Additionally, upregulation of PFKFB4 expression was observed in gastric cancer tissues and PFKFB4 upregulation was correlated with age, pathologic tumor stage, and tumor-node-metastasis stage [25]. Collectively, these studies indicated that PFKFB4 overexpression was significantly relevant to clinicopathological characteristics in various solid cancers.

Multiple studies have shown that increased expression of PFKFB4 promotes cancer cell proliferation, migration and invasion, as well as plays a role in developing resistance to chemotherapy [11,14,26]. Silencing or inhibiting PFKFB4 gene was shown to suppress glycolysis and proliferation in various types of human cancer cells [14,21,23]. In a preclinical animal model, depletion of PFKFB4 results in tumor growth arrest [27]. Besides, PFKFB4 has been demonstrated to promote tumor progression by regulating glycolytic flux or glycolysis-independent mechanism. Alterations in PFKFB4 expression affect glucose metabolism, release of lactate, and ROS production in breast cancer cells [28]. PFKFB4 plays a role in promoting cancer tumorigenesis and progression by phosphorylating and activating steroid receptor coactivators (SRCs) in breast cancer and lung adenocarcinoma [18,21]. Furthermore, PFKFB4 affects chemoresistance in lung cancer through regulation of autophagy [26]. PFKFB4 also stimulates synthesis of hyaluronan through activation of p38 pathway, thereby promoting breast cancer metastasis [14]. Further research is needed to clarify the functional profiles and precise mechanisms of PFKFB4 upregulation in OSCC.

We analyzed the diagnostic capacity of PFKFB4 in OSCC through ROC analysis, revealing its high accuracy in distinguishing OSCC tissue from normal tissue. Subsequently, the prognostic significance of PFKFB4 was assessed using Kaplan-Meier analysis. The results revealed that PFKFB4 overexpression was found to be associated with reduced OS, DSS, and PFI. Similar correlations between PFKFB4 overexpression and poor clinical outcomes have been observed in several other cancers. Besides, patients with decreased PFKFB4 expression exhibited notably improved overall survival in gastric cancer [25]. Moreover, our multivariate Cox analysis identified PFKFB4 expression and pathologic stage as independent predictors in OSCC. Calibration curves indicated that PFKFB4 gene had high predictive accuracy in OSCC. Taken together, we unravel the role of PFKFB4 in OSCC progression and indicates that it can be used as an independent prognostic indicator.

5. Conclusions
In our study, PFKFB4 was markedly overexpressed in OSCC and significantly correlated with poor prognosis, serving as an independent adverse prognostic factor. Moreover, our research adds to a more comprehensive interpretation of the function role of PFKFB4 in OSCC. Additional comprehensive investigations are needed to fully elucidate the precise mechanisms by which PFKFB4 contributes to the tumorigenesis and progression of OSCC.

Data availability
The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author upon reasonable request.

Conflicts of interest
The authors declare no conflicts of interest.

Ethics approval
The study was conducted in accordance with the Declaration of Helsinki, and approved by the institutional Ethics Committee of Stomatological Hospital, School of Stomatology, Southern Medical University (No. AFSQ-0105).

Consent to participate
Informed consent was obtained from all subjects involved in the study.

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Author contributions
S.C. and J.H. contributed to designing the study plan, performing the experiments, analyzing the data and drafting the manuscript. W.W. and Z.W. assisted in performing the experiments and data interpretation. B.C. and J.W. contributed to the project conception, drafting the manuscript and supervision. All authors read and approved the final manuscript.

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