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Role of PFKFB3 and CD163 in Oral Squamous Cell Carcinoma Angiogenesis*

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Summary: 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3 (PFKFB3), an enzyme producing fructose 2, 6-bisphosphate (F-2, 6-BP), serves as a switch to activate phosphofructokinase-1, and is a critical enzyme for endothelial glycolysis, mediating circadian control of carcinogenesis. Also, tumor-associated macrophages (TAMs) play an important role in the progression and prognosis of numerous cancers. However, the role and clinical significance of PFKFB3 and TAMs in oral squamous cell carcinoma (OSCC) have not been elucidated. The present study was designed to investigate the correlation between PFKFB3 expression, CD163+ TAMs infiltration and tumor angiogenesis in OSCC by tissue microarray. Tissue microarrays containing 117 OSCC specimens and 56 matched paracarcinoma tissues were studied by immunohistochemistry. The expression levels of PFKFB3, CD163 and CD31 were significantly increased in OSCC specimens as compared with normal oral mucosa (P<0.05), and PFKFB was significantly correlated with tumor differentiation and tumor size (P<0.05), and CD163 was significantly correlated with areca nut chewing habit among OSCC tissues (P<0.05). Furthermore, Pearson's correlation analysis revealed that PFKFB3 was significantly correlated with both CD163 and CD31 (P<0.05), meanwhile CD163 was significantly correlated with CD31 (P<0.001), suggesting PFKFB3 may promote angiogenesis in tumor progression and metastases by regulating CD163+ TAMs infiltration in OSCC.
Key words: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; angiogenesis; CD163; CD31

Head and neck squamous cell carcinoma is the sixth most common cancer type and the eighth most frequent cause of cancer-related deaths worldwide[1]. Squamous cell carcinomas arising in oral cavity are termed as oral squamous cell carcinoma (OSCC), the most common cancer in the head and neck, which has the potential for rapid and unlimited growth, high metastasis and poor prognosis. Despite advances in the surgical, chemotherapy and radiotherapy treatment option, the five-year survival rate has not been improved significantly in the past years. Tumor progression is believed to be influenced by angiogenesis. Angiogenesis is a complex event mediated by angiogenic factors released from cancer cells and immune cells. It has been reported to be associated with progression, aggressiveness and metastases of various malignant tumors including OSCC. Endothelial cells are especially critical in the process of angiogenesis, and the migration and proliferation of endothelial cells initiate the formation of capillary networks, which provides a frame for further vascular maturation[2].

Increasing attention has been given to endothelial activities during angiogenesis, while endothelial cells have high glycolytic activity[3–5]. In tumor tissue, the level of glycolysis in endothelial cells is much higher than that in healthy tissue[5]. The 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase, isoform 3 (PFKFB3), is a critical enzyme for endothelial glycolysis. A recent study demonstrated that PFKFB3-driven glycolysis is important for the migration of endothelial cells. In response to angiogenic factors, PFKFB3-knockdown endothelial cells exhibit defects in the formation of filopodia and lamellipodia[5]. However, it remains unclear whether PFKFB3 is important for angiogenesis in OSCC.

Carcinogenesis is characteristically associated with macrophage-mediated smoldering inflammation, often caused by pathogens[6], or as a result of autoimmunity and inflammatory conditions of uncertain origin[7, 8].

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Macrophages are present in most human tumors and are often associated with bad prognosis, which come in many functional flavors ranging from what is known as classically activated macrophages (M1) associated with acute inflammation and T-cell immunity to immune suppressive macrophages (M2) associated with the promotion of tumor growth. Increasing evidence shows that CD163+ (M2) tumor-associated macrophages (TAMs) play an important role in the progression of OSCC[9, 10].

In this study, we examined the expression of PFKB3, CD163 and vascular endothelial cell marker CD31 in OSCC using tissue microarray and analyzed the association among these markers. In addition, we evaluated the association of the expression of TAM, pathological features and clinical outcomes to clarify their roles in OSCC prognosis.

1 MATERIALS AND METHODS

1.1 Ethics Statement

The present study was approved by the Medical Ethics Committee of Xiangya Hospital, Central South University (China) and was performed according to the Declaration of Helsinki guidelines on experimentation involving human subjects. Written informed consent was obtained from participants.

1.2 Patient Samples and Tissue Microarray

A panel of OSCC and matched paracarcinoma tissues at the Department of Pathology, Center of Stomatology, Xiangya Hospital, Central South University were identified by two independent pathologists according to the 2006 World Health Organization classification system[11]. Tumor tissue microarrays were constructed in collaboration with Shanghai Biochip Co., Ltd. (China) and included 117 OSCC and 56 matched paracarcinoma tissues.

1.3 Immunohistochemistry and Scoring System

Immunohistochemistry was performed as protocols, using an appropriate biotin-conjugated secondary antibody and a Vectastain ABC Elite kit (Vector Laboratories, USA). Briefly, all slides were rehydrated and antigen retrieval was performed using sodium citrate (pH=6.0) in a pressure cooker with the exception of EGFR (EDTA buffer, pH=8.4). All slides were blocked with endogenous peroxidase with 3% hydrogen peroxide and non-specific protein was blocked with 2.5% bovine serum albumin in phosphate-buffered saline. The following primary antibodies were used: CD31 (1:200; BD Pharmingen, USA), PFKFB3 (1:200; Cell Signaling Technology, USA), CD163 (1:200; Cell Signaling Technology, USA), and slides were incubated at 4°C overnight with the diluted primary antibody. Slides were incubated with biotin-labeled secondary antibody [UltraSensitive™ S-P kit (mouse/rabbit); Fuzhou Maixin Biotechnology Co., Ltd., China] and streptavidin peroxidase, visualized by 3,3'-diaminobenzidine and counterstained with hematoxylin. All slides were scanned using the Aperio ScanScope CS whole slice scanner (Aperio Technologies, USA) with background substrate. The settings of the Aperio MVD algorithm were modified to allow identification of all CD31 stained blood vessels based on brown thresholds. The positive result was quantified using Aperio Quantification software (version 9.1; Aperio Technologies) for membrane, nuclear or pixel quantification and the membrane v9 algorithm was used to quantify the membranous expression of EGFR. Histoscore was calculated as a percentage of different positive cells using the formula (3+)×3+(2+)×2+(1+)×1. Histoscore of pixel quantification was calculated as total intensity/total cell number. The threshold for scanning of different positive cells was set according to the standard controls provided by Aperio.

1.4 Statistical Analysis

Data analysis was performed using GraphPad Prism 5.00 for Windows (GraphPad Software, Inc., USA). One-way analysis of variance followed by Tukey's post-hoc test or Bonferroni multiple comparison was used to analyze the differences in immunohistochemical staining. The relative expression of these markers was calculated using two-tailed Pearson's correlation following confirmation of the sample with Gaussian distribution. All values are expressed as the mean ± standard error of the mean (SEM). $P<0.05$ was considered to indicate a statistically significant difference.

2 RESULTS

2.1 Association between Expression of PFKFB3, CD163 and CD31 in OSCC and Matched Paracarcinoma Tissues

The expression of PFKFB3, CD163 and CD31 was initially evaluated by immunohistochemical staining. Representative immunostained PFKFB3, CD163 and CD31 in OSCC and matched paracarcinoma tissues are shown in fig. 1. The expression levels of PFKFB3, CD163 and CD31 were all increased in OSCC tissues as compared with matched paracarcinoma tissues (fig. 1A), and quantification of PFKFB3, CD163 and CD31 expression levels in 117 OSCC tissues and 56 matched paracarcinoma tissues was performed (fig. 1B).

2.2 PFKFB3 and CD163 Expression and Clinicopathological Characteristics

Considering the significant role of PFKFB3 in tumorigenesis, we examined the expression of PFKFB3 grouped by tumor differentiation and tumor size among OSCC tissues. PFKFB3 statistically increased with the tumor differentiation grade and also the tumor size (fig. 2A and 2B). The OSCC patients were divided into

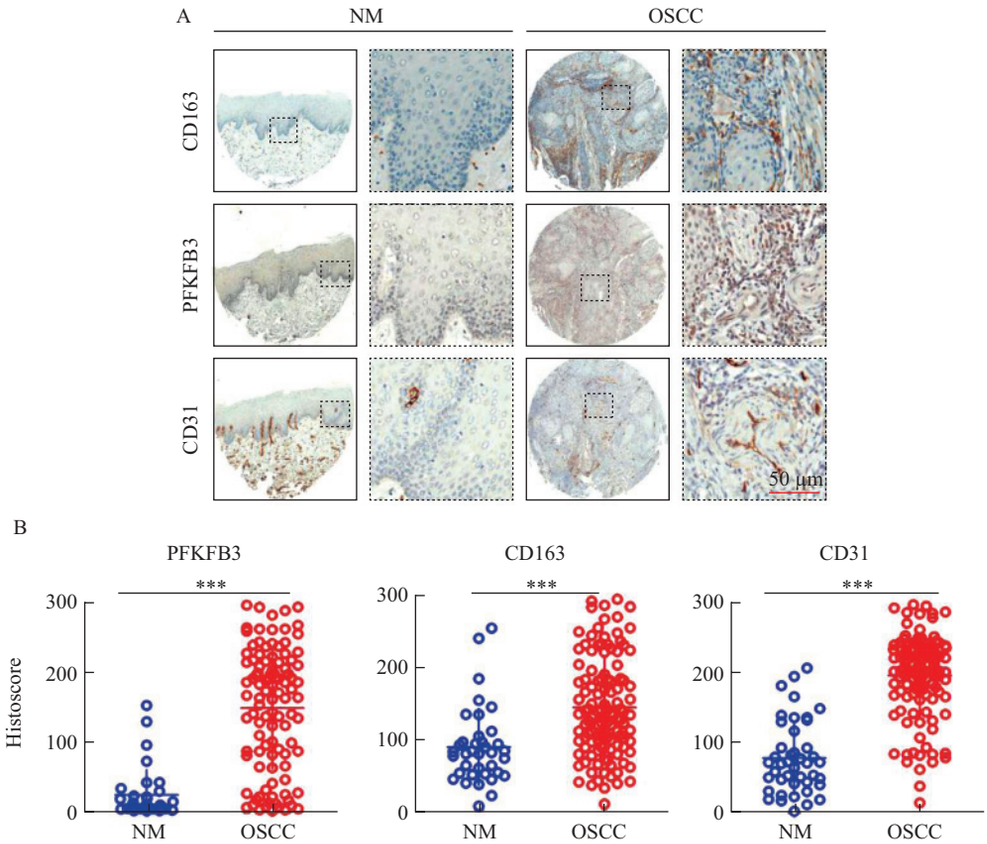

Fig. 1 Association between the expression of PFKFB3, CD163 and CD31 in OSCC and matched paracarcinoma tissues
A: representative immunohistochemical staining of PFKFB3, CD163 and CD31 expression in OSCC and matched paracarcinoma tissues (scale bar=50 μm). B: quantification of PFKFB3, CD163 and CD31 expression levels using an AperioScanscope scanner and software. Data were analyzed by Graph Pad Prism 5 software. Data are presented as the mean±standard error of the mean. $^{***}P<0.01$

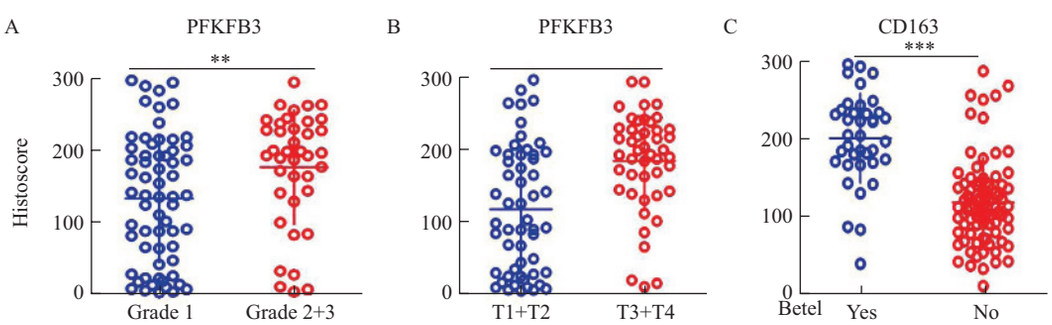

Fig. 2 Quantification of PFKFB3 expression levels in 117 OSCC tissues
A: grouped by tumor differentiation (G1: n=73; G2+3: n=44); B: grouped by tumor size (T1+2: n=65; T3+4: n=52)]. C: quantification of CD163 expression levels in 117 OSCC tissues (grouped by areca nut chewing habit, yes: n=34; no: n=83). The quantification study was done using an AperioScanscope scanner and software, and data were analyzed by Graph Pad Prism 5 software. Data are presented as the mean ± standard error of the mean. $^{**}P<0.05$, $^{***}P<0.01$

two groups according to the areca nut chewing habit (areca nut chewing more than 10 pieces per day and more than 1 year), and it was found that the CD163 expression was significantly correlated with the areca nut chewing habit (fig. 2C).

2.3 Involvement of PFKFB3 and CD163 in OSCC Angiogenesis

To explore whether or not PFKFB3 and CD31 are implicated in OSCC angiogenesis, we analyzed the immunohistochemial staining scores of PFKFB3, CD163, and angiogenic factor by conducting Spearman rank correlation coefficient test and linear tendency test. Spearman rank correlation coefficient test of cases with interpretable scores of CD31 and PFKFB3 showed positive correlation (fig. 3A; $P<0.05$, $r=0.2318$, $n=117$). Similar results were also observed in CD31 and CD163

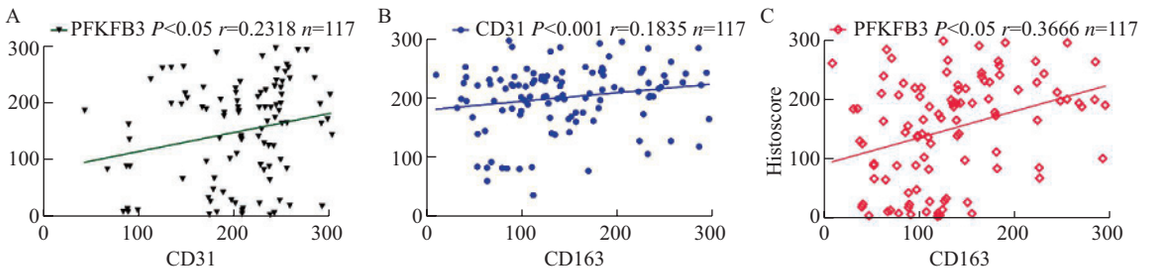

Fig. 3 Correlation and regression of PFKFB3, CD163 and CD31 in OSCC tissues using two-tailed Pearson's test
A: correlation between PFKFB3 and CD31expression levels in OSCC tissues (P<0.05, r=0.2318, n=117); B: correlation between CD163 and CD31 expression levels in human OSCC tissues (P<0.001, r=0.1835, n=117). C: correlation between PFKFB3 and CD163 expression levels in human OSCC tissues (P<0.05, r=0.3666, n=117)

(fig. 3B, P<0.001, r=0.1835, n=117). These results suggested that PFKFB3 and CD163 may be involved in OSCC angiogenesis. To verify the relationship between PFKFB3 with CD163 in OSCC, we then performed a correlation analysis between PFKFB3 with CD163. The levels of PFKFB3 with CD163 were positively correlated (fig. 3C; P<0.05, r=0.3666, n=117). This indicated CD163 may attend the process of PFKFB3 regulating OSCC angiogenesis.

3 DISCUSSION

Increasing evidence showed that many tumors are characterized by a metabolic rewiring with increased glucose uptake and lactate production, termed as aerobic glycolysis[12, 13]. Targeting aerobic glycolysis presents a promising strategy for cancer therapy. PFKFB3 bears an oncogene-like regulatory element and benefits the synthesis of F26BP to promote glycolytic flux with its high kinase activity[14]. Given that this gene is commonly overexpressed in human cancers, including breast, colon, ovarian and thyroid carcinomas, but is insufficiently expressed in normal tissues, targeting PFKFB3 presents a promising strategy for cancer treatment[14]. However, whether PFKFB3 is overexpressed in OSCC tissues remains unclear. In the present study, the positive immunoreactivity staining of PFKFB3 was highly expressed in OSCC as compared with that in the matched paracarcinoma tissues, also the expression of PFKFB3 was statistically related with the tumor differentiation and tumor size.

Angiogenesis has long been known to aid in progression and metastasis of malignant tumors. An adequate vascular response is essential for initial development as well as continued growth of solid tumors[15]. Tumor angiogenesis is a complex event mediated by angiogenic factors released from cancer cells and/or by host immune cells[16]. CD31 is a common endothelial cell marker that is widely used to monitor blood vessels. In the present study, CD31 was used as an angiogenic marker in order to analyze the blood vessel distribution and status in this carcinoma. The expression of CD31 was higher in the OSCC tissues than in the matched paracarcinoma tissues. Therefore, the high expression of CD31 in OSCC may explain the invasive tendency of OSCC and early hematogenous metastasis. In the present immunohistochemical analysis, the correlation between PFKFB3 and CD31 showed the important role of PFKFB3 in the angiogenesis of human OSCC. PFKFB3 regulated the formation of filopodia and lamellipodia and directional migration by compartmentalizing with F-actin in motile protrusions, which affects endothelial migration[5]. Increasing evidence showed that PFKFB3 is also important for endothelial proliferation, silencing PFKFB3 dramatically reduced T-lymphocyte proliferation[17], PFKFB3 appears in mid-to-late G1 and is vital for cell division in both HeLa cells and lymphocytes[18], the knockdown of endothelial PFKFB3 prevented endothelial cells from proceeding to the S phase, resulting in a much lower proliferative rate than control endothelial cells under both normoxic and hypoxic conditions[19]. In this study the correlation analysis suggested a significant association between PFKFB3 and CD31, which indicated that PFKFB3 leads to pathological angiogenesis, thus PFKFB3 is a promising target for the treatment of pathological angiogenesis and its associated diseases.

TAMs, especially the CD163+ alternatively activated phenotype (M2), are associated with poor outcome in multiple solid cancers and play important roles in cancer progression and prognosis. Our previous studies have clarified CD163+ tumor-associated macrophages are correlated with poor prognosis and CSCs in OSCC[20], suggesting their potential prognostic value in OSCC. In the present study, we found that the expression levels of CD163 among the areca nut consumers (chewing more than 10 pieces per day, chewing habit more than 1 year) were significantly higher than those in other OSCC tissues. Habit of chewing areca nut can induce oral submucous fibrosis (OSMF), which is a premalignant condition of oral mucosa. The cellular immune responses by macrophages do play an important role in pathogenesis

of OSMF, resulting from elevated production of fibrogenic cytokines and reduced production of antifibrotic cytokines. Pereira *et al*[21] investigated the possible correlation of macrophages using CD68 in various histopathological grades of OSMF, and found CD68 plays a vital role in the pathogenesis of OSMF and can be regarded as a useful marker for assessing the progress of the disease. These foundings were in accordance with our present study.

The significant relevance between the expression of CD163 and CD31 indicated that CD163+ TAMs might promote tumor angiogenesis, and the progression of OSCC. Recently, it was found that CD163 as a marker of M2 macrophage was correlated with high expression of MMP9 and high micro vessel density[22], which may contribute to the tumor aggressiveness and angiogenesis, and these findings were in accordance with our results.

In our present study, we examined the relevance between the expression of PFKFB3 and CD163 to check the relationship between PFKFB3 and CD163+ TAMs infiltration. The significant relevance between the expression of PFKFB3 and CD163 indicated that PFKFB3 may promote angiogenesis in tumor progression and metastases by regulating CD163+ TAMs infiltration.

In conclusion, PFKFB3 levels were positively correlated with the expression of CD163 and CD31 in human OSCC. These results revealed that PFKFB3 is possibly involved in the angiogenesis by affecting the expression of CD163 in human OSCC. Thus, targeting PFKFB3 or blocking CD163+ TAMs infiltration may provide a possible novel therapeutic strategy for the treatment of human OSCC.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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