Medicine®

OPEN

The role of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 in esophageal squamous cell carcinoma

Junhui Bao, MD[a,b], Ya Wu, MD[c], Limei Wang, MD[b], Yaqun Zhu, MD[a,*]

Abstract

6-phosphofructo-kinase-2/fructose diphosphatase-2 isoenzyme 3 (PFKFB3) is closely related to the growth of many types of cancer cells. Glycolysis not only provides Adenosine triphosphate for the growth of tumor cells, but also protects them from acid products, which is beneficial to the invasion and metastasis of tumors. However, PFKFB3 expression in esophageal squamous cell carcinoma (ESCC) has been scarcely reported. In this study, the role of PFKFB3 was studied in 120 ESCC samples using immunohistochemistry technique (IHC), western blotting, and reverse transcriptase-polymerase chain reaction (RT-PCR). Both PFKFB3 protein and gene expression in ESCC tissues were significantly higher than in adjacent non-tumor tissues ($P < .05$). Single factor analysis showed that both PFKFB3 protein and gene expression are related to infiltration depth, stage, tumor metastasis, and the degree of tumor differentiation in ESCC. Multifactor Cox survival analysis revealed that PFKFB3 protein expression, tumor location, tumor metastasis, tumor differentiation degree, and tumor stage were independent factors affecting the overall survival of postoperative patients. Multivariate Cox survival analysis showed that PFKFB3 mRNA has a good performance for predicting 3-year survival of patients with ESCC 0.89 (0.79–0.99), with a sensitivity of 0.85 and specificity of 0.77. Encouragingly, the sensitivity and specificity of PFKFB3 in the diagnosis of early ESCC (stage I and stage II) can reach 87.8% and 91.5%. In conclusion, high PFKFB3 protein and gene expression may be associated with the occurrence, development, and prognosis of ESCC. PFKFB3 could be used to help develop new therapeutic and diagnostic strategies for ESCC patients.

Abbreviations: EAC = esophageal adenocarcinoma, ESCC = esophageal squamous cell carcinoma, IHC = immunohistochemistry technique, PFKFB3 = 6-phosphofructo kinase-2/fructose diphosphatase-2 isoenzyme 3, RT-PCR = reverse transcriptase-polymerase chain reaction.

Keywords: 6-phosphofructo kinase-2/fructose diphosphatase-2 isoenzyme 3, esophageal squamous cell carcinoma, glycolysis, prognosis

[a] Department of Radiotherapy & Oncology, The Second Affiliated Hospital of Soochow University, Suzhou, [b] Department of Radiotherapy & Oncology, Tong Liao City Hospital, Tong Liao, Inner Mongolia, [c] Department of Orthopedics, The First People's Hospital of Yancheng City, Yancheng, Jiangsu, China.

* Correspondence: Yaqun Zhu, Department of Radiotherapy & Oncology, the Second Affiliated Hospital of Soochow University, San Xiang Road No. 1055, Suzhou, Jiangsu 215004, China (e-mail: szzhuyaqun@sina.com).

1. Introduction

Esophageal cancer (EC) is a common malignant tumor of the digestive tract.[1,2] EC ranks 8th in incidence and 6th in fatality worldwide. China is a high incidence area of EC.[3] The incidence of EC in high incidence areas is about 121/10,000, which is >20 times higher than that in low incidence areas. Every year, there are over 270,000 new cases of EC, resulting in 200,000 deaths, with a mortality rate that ranks in fourth places.[4,5] There are 2 main tissue subtypes of EC, namely esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). In China, ESCC is the main subtype of EC.[6]

ESCC is a highly malignant subtype of tumor. At present, only about 1/3 of patients with ESCC qualify for surgical resection, with the majority requiring adjuvant chemotherapy postoperation.[7] The prognosis of ESCC is poor, with an average 5-year survival rate of only 35% to 45%. The treatment and prognosis associated with the different stages of ESCC vary greatly.[8] Patients with early ESCC have a good prognosis, particularly individuals who meet the requirements for endoscopic resection, which involves a minimally invasive endoscopic resection that avoid seriously damaging the patients' quality of life, unlike surgery and chemotherapy. Contrarily, patients with advanced ESCC have a poor prognosis that is often associated with a high medical burden.[9,10] Therefore, investigating the mechanism of the occurrence and development of ESCC may provide a scientific

basis for its diagnosis and treatment, especially due to the fact that an early diagnosis of ESCC is of high clinical significance.

6-phosphofructo kinase-2/fructose diphosphatase-2 isoenzyme 3 (PFKFB3) is a subtype of phosphofructokinase (PFK), which exists widely in various biological cells and plays an important role in the proliferation, migration, invasion, and metastasis of certain types of cancer cells.[11,12] PFKFB3 is a key rate-limiting enzyme in the process of glucose decomposition into adenosine triphosphate, which has dual activities of kinase and phosphatase.[13] The activity of PFKFB3 kinase is much higher than that of phosphatase, which can increase the rate of glycolysis. Several studies have shown that inhibiting the expression of PFKFB3 can significantly reduce the glycolysis rate and the growth of cancer cells,[14,15] which indicated that the PFKFB3 involved in glycolysis may be an important target for the treatment of malignant tumors. However, the expression of PFKFB3 in ESCC and their role in the development of ESCC have not been reported.

Defining the relationship between PFKFB3 and the occurrence and development of ESCC could provide an insight into the diagnosis, treatment, or prevention of ESCC. In this study, we evaluated the role of PFKFB3 expression in ESCC using immunohistochemistry (IHC), western blotting, and reversed transcriptase-polymerase chain reaction (RT-PCR) with the aim of proposing a potential prognostic diagnostic indicator for patients with ESCC.

2. Materials and methods

2.1. Patients and controls

We retrospectively analyzed a cross-sectional group of 120 eligible ESCC patients selected from the First People's Hospital of Yancheng between January 2015 and January 2018. All patients underwent surgical resection. The inclusion criteria for diagnosing ESCC was as follows: patients had primary esophageal cancer, excluding recurrence, metastasis, and other malignant tumors; cases that received radiotherapy, chemo-therapy, or other anti-cancer treatment before operation were excluded; patients were diagnosed with ESCC by pathologists in our hospital; complete clinical and pathological data were available.

Among the 120 ESCC patients, 85 were male and 35 were female (aged 32–87 years, mean age of 58 years). Tumor location: upper segment 40 cases, middle segment 49 cases, lower segment 31 cases. Infiltration depth: T1+T2 56 cases, T3+T4 64 cases. Poor differentiation: 58 cases, moderate differentiation: 27 cases, high differentiation: 35 cases. Forty-two cases were grade I and grade II, and 78 cases were grade III and grade IV. Paired adjacent tissues were collected from the 120 eligible ESCC patients as a control group.

The condition and survival of 120 ESCC patients were followed up by telephone. The total survival time (OS) is defined as the time from the date of operation to the last follow-up or death, and is calculated in terms of months. Postoperative follow-ups were performed every 2 months in the first year, every 3 months in the second year, every 6 months in the third year, and every 8 months until mortality. Informed consent was obtained from all patients prior to extraction of specimens. This study was approved by the Ethics Committee of the First People's Hospital of Yancheng (Identification No. HMU [Ethics] 2015004).

2.2. Detection of PFKFB3 expression in ESCC tissues by immunohistochemistry

IHC was performed to determine the distribution of PFKFB3. The Envision and DAB chromogenic reagent kits (antibody diagnostic inc, ADI) were used for immunohistochemical staining. Briefly, the specimens were cut into small pieces of tissue, fixed with 4% paraformaldehyde, and made into conventional sections (4 μm). The sections were dewaxed and gradient ethanol infused before performing high-pressure antigen repair by incubating in sheep serum 37°C for 20 minutes. This was followed by drop serum and the addition of PFKFB3-1 antibody (1: 200; Abcam Company, USA) before incubating the samples overnight. The sections were then incubated with second antibody labeled with corresponding horseradish peroxidase at 37°C for 20 minutes. After rinsing with phosphate buffer saline (PBS) buffer, streptavidin-peroxidase was added to the sections and incubated at 37°C for 20 minutes. The samples were then rinsed with PBS buffer and the color was developed under a light microscope. The samples were then re-dyed with hematoxylin, dehydrated, and sealed.

All staining results were determined by 2 independent pathologists blinded to the experiment. The cell staining reactions were evaluated based on the immunoreactive score (IRS) as follows[16]: IRS = staining intensity score × percentage of positive tumor cells. The score of the staining intensity was defined as follows: 0, no expression; 1, weak expression, 2, medium expression, and 3, strong expression. The percentage of positive stained cells was defined as follows: 0, no expression; 1, 1% to 10% positive tumor cells; 2, 11% to 50% positive tumor cells; 3, 51% to 80% tumor cells; 4, >80% positive tumor cells. An IRS value ≥4 was considered as high-PFKFB3-1 expression, whereas an IRS value <4 was considered low-PFKFB3-1 expression.

2.3. Detection of PFKFB3 protein expression by western blotting

The ESCC tissue samples were homogenized and lysed with cell lysate before centrifuging. The resulting supernatant was harvested as total protein, the concentration of which was determined using the bicinchoninic acid method. After calculating the volume of the sample and NaCl, 5 times buffer solution was measured according to the protein concentration, mixed with the sample, and placed in a 95°C water bath for 5 minutes. A polyacrylamide gel was prepared for protein electrophoresis. The protein sample was run at 80 V under constant pressure, then at 120 V at the interface between the separation gel and the concentrated glue. The resulting protein bands were transferred onto the polyvinylidene fluoride membrane under 200 mA for 2 hours with a constant current. After 2 hours of blocking, the gels were incubated with PFKFB3 (1:1000) and β-actin (1:1000) antibodies overnight at 4°C. Tris-Buffered Saline Tween (TBST) was washed 3 times, for 10 minutes each time. The second antibody was added to the samples and incubated for 2 hours, after which the TBST was washed 3 more times. The relative content of target protein in the sample was analyzed by visualization using a protein gel imaging system after coloring with chemiluminescence agent.

2.4. Detection of PFKFB3 mRNA by RT-PCR

Fresh frozen ESCC tissues were ground using glass grinder. The total RNA was extracted and reverse transcribed using a reverse transcription kit (Invitrogen Qiagen, Germany) to synthesize

Table 1

Primer sequences for RT-PCR analysis.

Primers	Primer sense	Primer sequences 5′-3′	Product size
PFKFB3	Forward	AGCCCGGATTACAAAGACTGC	158 bp
	Reverse	GGTAGCTGGCTTCATAGCAAC	158 bp
β-actin	Forward	TTCCAGCCTTCCTTCCTGGG	231 bp
	Reverse	TTGCGCTCAGGAGGAGGAAT	231 bp

RT-PCR = reverse transcriptase-polymerase chain reaction.

cDNA. β-actin was used as the internal reference. A PCR amplification kit was used for the amplification. The sequences of the primers used are provided in Table 1. The thermal cycling conditions were as follows: pre-denaturation at 95 °C for 3 minutes, 95 °C for 50 seconds, and 55 °C for 40 seconds, for a total of 40 cycles; extension at 72 °C for 60 seconds. The results demonstrated that the relative expression of PFKFB3 mRNA was expressed by $2^{-\Delta\Delta Ct}$, obtained by averaging the results of 3 independent experiments.

2.5. Statistical methods

GraphPad (IBM, NY, USA) Prism 5 software and SPSS13.0 were used for the statistical analysis. The normal distribution data were expressed as the mean ± standard deviation (SD). The chi-square test was used to compare the association between the expression status of PFKFB3 and clinicopathological parameters in normal adjacent and cancer tissues. Kaplan–Meier survival analysis was used to analyze disease-specific and disease-free survival rates. The log-rank test was performed to analyze the difference in survival curves. Multivariable regression analysis was performed to detect prognostic factors using the Cox proportional hazards model. A value of $P < .05$ was considered statistically significant. The logistic regression model was used to draw receiver operator characteristic curve (ROC) and calculate the area under curve (AUC) to evaluate the efficiency of diagnosis.

3. Results

3.1. PFKFB3 protein expression in ESCC tissues and adjacent tissue

PFKFB3 protein is mainly expressed in the nucleus of ESCC tissue cells. The expression of PFKFB3 protein in ESCC tissues was significantly higher than in the normal adjacent tissues ($P < .05$; Fig. 1A–D). The positive expression rate of PFKFB3 protein in ESCC tissues was 90.83% (109/120) and 54.17% (65/120) in the adjacent tissues.

Further analysis showed that the immunostaining intensity ($t = 3.165$, $P < .05$) and the percentage of positive staining cells ($t = 6.776$, $P < .05$) of PFKFB3 in ESCC tissues were higher than those in adjacent tissues (Fig. 1E, F). The staining score of PFKFB3 in ESCC tissues was significantly higher than that in adjacent tissues ($t = 6.122$, $P < .05$; Fig. 1G).

Western blotting revealed that the expression of PFKFB3 protein in 120 ESCC tissues was significantly higher than that in the adjacent tissues ($P < .05$; Fig. 2A).

3.2. PFKFB3 mRNA expression in ESCC and adjacent tissues

Next, we evaluated the expression of PFKFB3 mRNA in 102 ESCC and adjacent tissues by RT-PCR. The results revealed a positive rate of PFKFB3 mRNA in ESCC tissues of 86.67% (104/
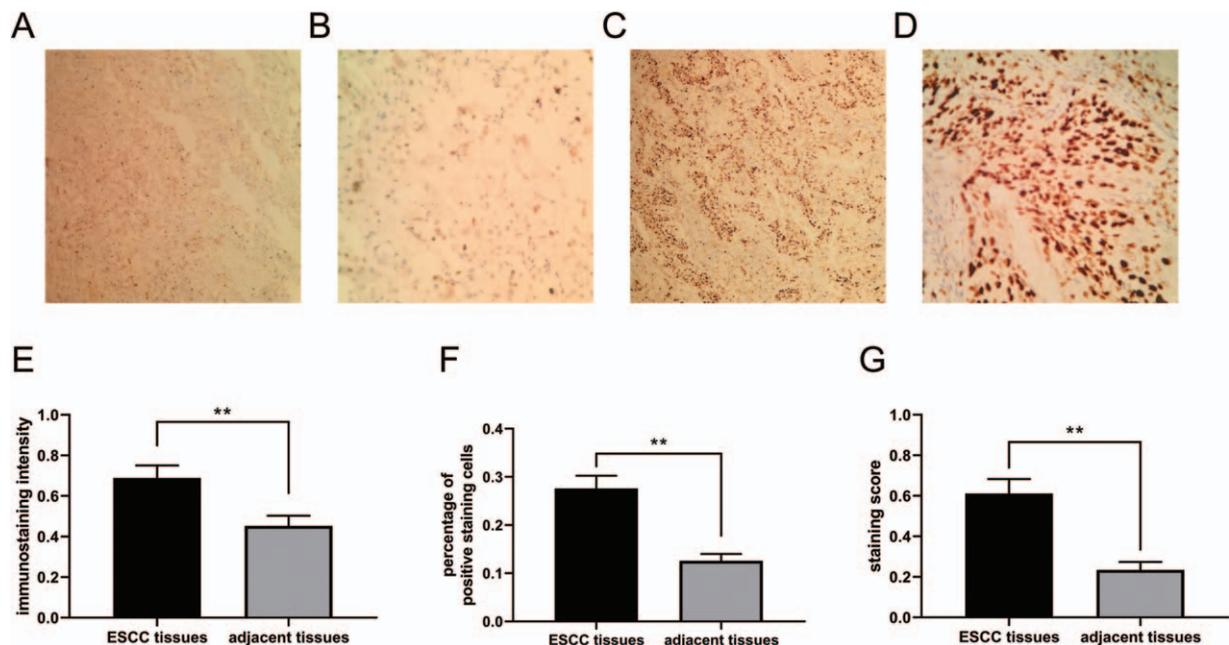

Figure 1. The staining result of Envision immunohistochemistry for PFKFB3 in ESCC and adjacent tissues.**$P < .05$. A, The staining weakly positive result for PFKFB3 in adjacent tissues (×100). B, The staining weakly positive result for PFKFB3 in adjacent tissues (×400). C, The staining strongly positive result for PFKFB3 in ESCC tissues (×100). D, The staining strongly positive result for PFKFB3 in ESCC tissues (×400). E, The immunostaining intensity of PFKFB3 in ESCC and adjacent tissues. F, The percentage of positive staining cells of PFKFB3 in ESCC and adjacent tissues. G, The staining score of PFKFB3 in ESCC and adjacent tissues. ESCC = esophageal squamous cell carcinoma, PFKFB3 = 6-phosphofructo kinase-2/fructose diphosphatase-2 isoenzyme 3.
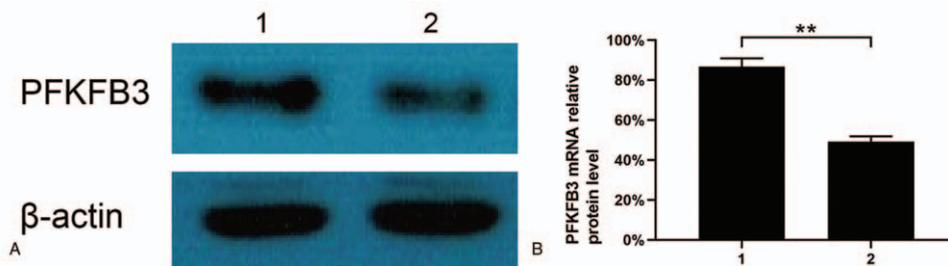
Figure 2. The expression of PFKFB3 protein and gene in ESCC and adjacent tissues. **$P < .05$. A, Expression of PFKFB3 protein in ESCC and adjacent tissues showed by western blot. B, Expression of PFKFB3 mRNA in ESCC and adjacent tissues showed by RT-PCR. 1: ESCC tissues group; 2: adjacent tissues group. ESCC = esophageal squamous cell carcinoma, PFKFB3 = 6-phosphofructo kinase-2/fructose diphosphatase-2 isoenzyme 3, RT-PCR = reverse transcriptase-polymerase chain reaction.

120), which was significantly higher than that in adjacent tissues, of 49.17% (59/120; $P < .05$; Fig. 2B).

3.3. Relationship between PFKFB3 mRNA and protein expression and clinicopathological features of ESCC

PFKFB3 mRNA and protein in ESCC tissues was consistently highly expressed. Single factor analysis showed that both PFKFB3 mRNA and protein are not associated with sex, age, or tumor location (all $P > .05$), but are related to infiltration depth, stage, tumor metastasis, and the degree of tumor differentiation (all $P < .05$; Table 2).

3.4. Prognostic value of PFKFB3 protein expression for the overall survival of postoperative patients with ESCC

Patients with higher levels of PFKFB3 protein expression in ESCC tissues had a significantly shorter disease-specific survival rate than other patients. Kaplan–Meier survival analysis showed that there was a significantly statistical difference between the 2 types of patients ($P < .05$) (Fig. 3). Among the 109 patients exhibiting higher levels of PFKFB3 protein expression in ESCC tissues, 91 patients died and 18 patients survived. Among the 11 patients with lower levels of PFKFB3 protein expression, 3 patients died and 8 patients survived. The median survival time of patients with higher and lower levels of PFKFB3 expression was 10.2 ± 1.1 months and 23.5 ± 6.4 months, respectively, which indicated that high levels of PFKFB3 expression affect the prognosis of ESCC patients.

Multivariate Cox survival analysis showed that PFKFB3 protein expression, tumor location, tumor metastasis, tumor differentiation degree, and tumor stage were independent factors affecting the overall survival of postoperative patients, while sex, age, and infiltration depth did not independently affect the overall survival of these patients (Fig. 4).

Table 2

Correlation of PFKFB3 protein and gene expression with clinicopathological features in ESCC (N [%]).

Characteristic	n	PFKFB3 protein positive rate	χ^2	P	PFKFB3 mRNA positive rate	χ^2	P
Gender							
Male	85	78 (91.76)	0.152	.593	74 (87.06)	0.021	.842
Female	35	31 (88.57)			30 (85.71)		
Age, y							
<60	44	40 (90.91)	0.190	.965	39 (88.64)	0.023	.804
≥60	76	69 (90.79)			65 (85.53)		
Infiltration depth							
T1+T2	56	47 (83.93)	0.149	.019	44 (78.57)	0.112	.021
T3+T4	64	62 (96.88)			60 (93.75)		
Tumor location							
Upper segment	40	36 (90.00)	0.217	.554	34 (85.00)	0.129	.497
Middle segment	49	46 (93.88)			44 (89.80)		
Lower segment	31	27 (87.10)			26 (83.87)		
Degree of tumor differentiation							
High differentiation	35	29 (82.86)	0.233	.027	27 (77.14)	0.239	.016
Moderate differentiation	27	23 (85.19)			21 (77.78)		
Poor differentiation	58	57 (98.28)			56 (96.55)		
Lymph node metastasis							
Yes	54	53 (98.15)	0.219	.018	51 (94.44)	0.201	.021
No	66	56 (84.85)			53 (80.30)		
Tumor stage							
I–II	42	33 (78.57)	7.129	.015	31 (73.81)	7.098	.022
III–IV	78	76 (97.44)			73 (93.59)		

ESCC = esophageal squamous cell carcinoma, PFKFB3 = 6-phosphofructo kinase-2/fructose diphosphatase-2 isoenzyme 3.
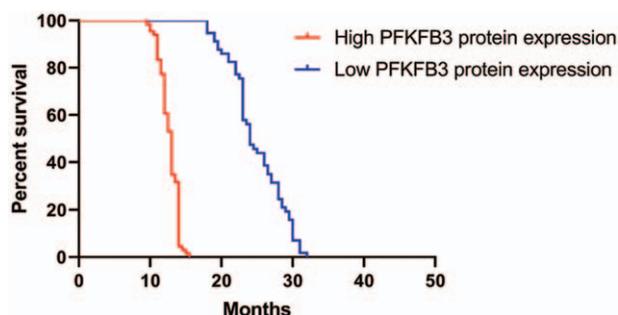
Figure 3. PFKFB3 expression and survival analysis of ESCC patients. ESCC = esophageal squamous cell carcinoma, PFKFB3 = 6-phosphofructo kinase-2/ fructose diphosphatase-2 isoenzyme 3.

3.5. The accuracy of PFKFB3 mRNA for predicting 3-year survival of patients with ESCC

To further clarify the role of PFKFB3 in the overall survival of postoperative patients, area under the receiver operating characteristic (AUROCs) were calculated to determine the value of PFKFB3 mRNA for predicting the 3-year survival of patients with ESCC. The AUROC of PFKFB3 mRNA for predicting the 3-year survival of patients with ESCC was 0.89 (0.79–0.99), with a sensitivity of 0.85 and a specificity of 0.77 (Fig. 5).

3.6. Diagnostic value of PFKFB3 in early ESCC

We further studied the diagnostic ability of PFKFB3 in early ESCC (stage I and stage II). ROC curve analysis showed that the percentage of positive stained cells of PFKFB3 was 0.940 (95% CI: 0.87–1.00) for early ESCC, with a sensitivity and specificity of

87.8% and 91.5%, respectively (Fig. 6A). The AUC of the staining score of PFKFB3 for early ESCC was 0.89 (95% CI: 0.80–0.98), with a sensitivity and specificity of 83.2% and 91.3% respectively (Fig. 6B).

4. Discussion

The proliferation, invasion, and distant metastasis of malignant tumors require adequate nutrition and energy. Tumor cells are metabolically active.[17] In order to meet the needs of rapid proliferation, invasion, and metastasis, the energy metabolism must be carefully regulated, which was denoted as metabolic reprogramming.[18] Among them, aerobic glycolysis is the most important metabolic mode. Tumor cells are able to maintain a high level of anaerobic glycolysis under aerobic conditions. Glucose can be decomposed into lactic acid via the so-called "Warburg effect."[19] Therefore, inhibiting the glycolysis of tumor cells can inhibit their proliferation, differentiation, and distant metastasis. There are 4 subtypes of PFKFB family, namely PFKFB1, PFKFB2, PFKFB3, and PFKFB4, which have different expression levels and functions in tissues.[20]

PFKFB3 was first isolated from brain tissue in 1995. It has the dual activity of a kinase and phosphatase,[21] with a kinase activity that is stronger than its phosphatase activity. It can be divided into spectrum type and induction type due to differences in the terminal sequence of PFKFB3C.[22] The expression level of PFKFB3 in normal tissue is very low, but is highly expressed in many cancer cells, such as gastric,[23] lung,[24] and prostate cancer.[25] The mechanism of PFKFB3 is as follows[26]: an increase in PFKFB3, a phosphofructokinase-1 (PFK-1) activator, enhances the activity of PFK-1 to promote the rapid hydrolysis of glucose uptake by cancer cells, providing sufficient energy for cell proliferation; PFKFB3 is necessary for the growth of tumors
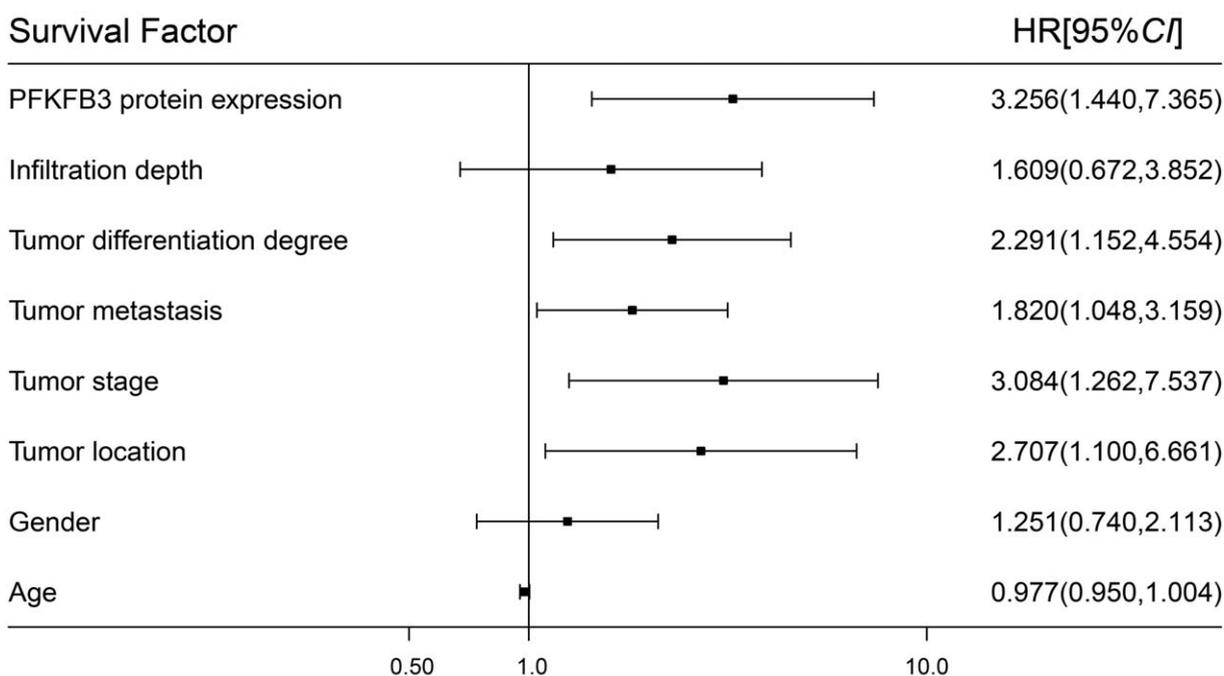

Figure 4. Association of PFKFB3 expression and prognosis of ESCC patients: multifactor Cox survival. ESCC = esophageal squamous cell carcinoma, PFKFB3 = 6-phosphofructo kinase-2/fructose diphosphatase-2 isoenzyme 3.
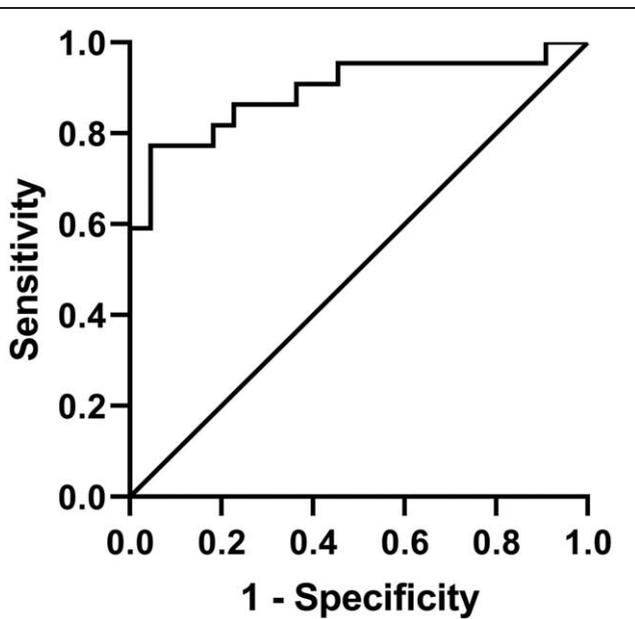
Figure 5. AUROC of PFKFB3 mRNA for predicting 3-year survival of patients with ESCC. ESCC = esophageal squamous cell carcinoma, PFKFB3 = 6-phosphofructo kinase-2/fructose diphosphatase-2 isoenzyme 3.

containing Ras-transformed cells, promoting normal angiogenesis and the growth of tumor cells, as well as accelerating the occurrence and development of tumors; the activation of the HIF-1α and p38/MK2 signaling pathways and the phosphorylation of serine at 461 site of PFKFB3 upregulates the expression of PFKFB3, thus promoting the proliferation of cancer cells; PFKFB3 also plays a role in cell cycle and cell apoptosis. Therefore, PFKFB3 plays an important role in maintaining the

development and progress of cancer and is a potential target for cancer treatment.

Li et al[27] found that the expression of PFKFB3 in head and neck squamous cell carcinoma (HNSCC) tissues was significantly higher than in adjacent mucosal tissues. The pharmacological inhibition of PFKFB3 via PFK15 was found to suppress tumor growth and alleviate metastasis in HNSCC. Ko et al[28] found that fibroblasts cocultured with TIGAR-overexpressing breast carcinoma cells induce hypoxia-inducible factor (HIF) activation via increased glucose uptake, increased PFKFB3 expression, and increased lactate dehydrogenase-A expression. Zhang et al[23] found that protein kinase D3 (PRKD3) upregulated PFKFB3 and activated glycolysis, as shown by an increased glucose consumption and lactate production. The knockdown of PFKFB3 suppressed glycolysis in gastric cancer (GC) cells with highly expressed PRKD3 but not in PRKD3 silenced cells. Furthermore, PRKD and PFKFB3 inhibitor suppressed the viability of GC cells. PFKFB3 is also considered an important prognostic indicator of cancer. It was found[24] that both PFKFB3 mRNA and protein expression were significantly high in lung adenocarcinoma cells. A high expression of PFKFB3 protein was an independent prognostic marker in lung adenocarcinoma. Chen et al[29] found that the levels of PFKFB3+ CD68+ cell infiltration in peritumoral tissues were negatively correlated with overall survival and could serve as an independent prognostic factor for survival in patients with hepatocellular carcinoma. Therefore, PFKFB3 not only reflects the occurrence and development of cancers, but can also be used as an alternative endpoint marker for cancer chemoprevention.

In our study, we investigated the role of PFKFB3 expression in ESCC tissues. Our study showed that PFKFB3 protein was localized in the nucleus of ESCC tissue cells. The expression of PFKFB3 in ESCC was higher than that in the adjacent tissue. The immunostaining intensity, the percentage of positive staining cells, and the staining score of PFKFB3 in ESCC tissues were all higher than those in adjacent tissues. The expression of PFKFB3
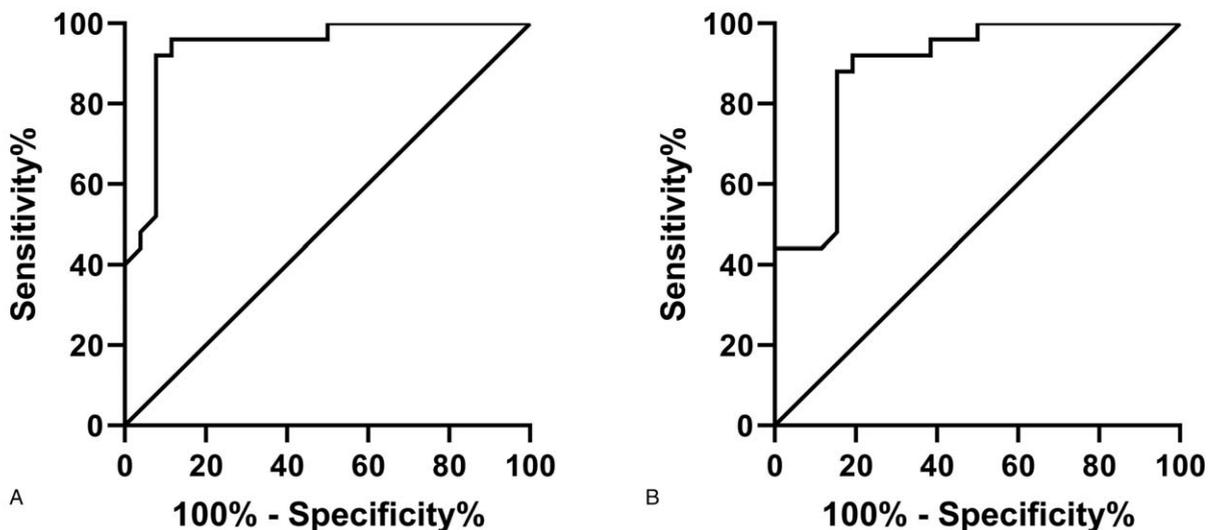

Figure 6. AUROC of PFKFB3 for predicting early ESCC patients (stage I and stage II). A, The percentage of positive staining cells; B, the staining score. ESCC = esophageal squamous cell carcinoma, PFKFB3 = 6-phosphofructo kinase-2/fructose diphosphatase-2 isoenzyme 3.

mRNA in ESCC tissues was also significantly higher than that in adjacent tissues. The relationship between PFKFB3 protein and gene expression and the clinical pathological features of ESCC patients are consistent. Our study also found that the PFKFB3 protein and gene expression in ESCC were not related to sex, age, or tumor location, but were related to infiltration depth, stage, tumor metastasis, and the degree of tumor differentiation. The expression of PFKFB3 protein and gene in the poor differentiation group and the lymph node metastasis group was significantly higher than in the high and moderate differentiation group and the group without lymph node metastasis. The expression of PFKFB3 protein and gene in the stage III–IV group and infiltration depth T3+T4 group was significantly higher than that in the stage I–II group and infiltration depth T1+T2 group. These results suggest a relationship between the formation, invasion, and metastasis of ESCC and high levels of PFKFB3 expression.

Moreover, we also studied the relationship between PFKFB3 expression and the overall survival of ESCC patients. The median survival time for patients with high levels of PFKFB3 expression was significantly shorter than patients with lower levels of PFKFB3 expression in ESCC. Multivariate Cox survival analysis revealed that PFKFB3 protein expression, tumor location, tumor metastasis, tumor differentiation degree, and tumor stage were independent factors affecting the overall survival of ESCC patients. These results indicate that an increased PFKFB3 expression was related to the prognosis of ESCC patients. AUROC analysis revealed that PFKFB3 mRNA had a good performance for predicting 3-year survival of patients with ESCC 0.89 (0.79–0.99), with a sensitivity of 0.85 and a specificity of 0.77, which suggests that PFKFB3 mRNA can be used to effectively predict the prognosis of ESCC patients. Encouragingly, PFKFB3 showed a good AUROC 0.940 (95% CI: 0.87–1.00) in the diagnosis of early ESCC (stage I and stage II), with a sensitivity of 87.8% and a specificity of 91.5%.

The present study also had a number of limitations that warrant consideration. Firstly, this work includes the largest cohort for the study of PFKFB3 protein and gene expression in ESCC. However, more clinical patient validation will be necessary. Secondly, both the comprehensive ability of PFKFB3 mRNA as a clinical predictor for 3-year survival of ESCC patients and PFKFB3 in diagnosis of early ESCC (stage I and stage II) will need to be further confirmed and validated. Lastly, further cell and animal experiments will need to be used to further elucidate the mechanism of action of PFKFB3.

In conclusion, our study demonstrated that high levels of PFKFB3 protein and gene expression are closely related to the occurrence, development, and prognosis of ESCC. The findings presented in this study provide a basis for the development of new therapeutic and diagnostic strategies for the treatment of patients with ESCC.

Author contributions

Conceptualization: Junhui Bao, Yaqun Zhu.
Data curation: Junhui Bao, Li Mei Wang.
Funding acquisition: Yaqun Zhu.
Investigation: Junhui Bao, Ya Wu, Yaqun Zhu.
Methodology: Junhui Bao, Ya Wu, Yaqun Zhu.
Software: Junhui Bao, Li Mei Wang.
Supervision: Yaqun Zhu.

Validation: Ya Wu, Li Mei Wang.
Writing – original draft: Junhui Bao.
Writing – review & editing: Yaqun Zhu.

References

[1] Sakamoto T, Fujiogi M, Matsui H, et al. Comparing perioperative mortality and morbidity of minimally invasive esophagectomy versus open esophagectomy for esophageal cancer: a nationwide retrospective analysis. Ann Surg 2019; doi: 10.1097/SLA.0000000000003500.

[2] Du J, Li G, He R, et al. Association between potentially functional polymorphisms of chemokine family members and the survival of esophageal cancer patients in a Chinese population. Onco Targets Ther 2019;12:4631–41.

[3] Yang C, Shen S, Zheng X, et al. Long noncoding RNA HAGLR acts as a microRNA-143-5p sponge to regulate epithelial-mesenchymal transition and metastatic potential in esophageal cancer by regulating LAMP3. FASEB J 2019;33:10490–504.

[4] Harada G, Bonadio RRdCC, de Araújo FCC, et al. Induction chemotherapy for locally advanced esophageal cancer. J Gastrointest Cancer 2019; doi: 10.1007/s12029-019-00266-1.

[5] Li W, Huang K, Wen F, et al. LINC00184 silencing inhibits glycolysis and restores mitochondrial oxidative phosphorylation in esophageal cancer through demethylation of PTEN. EBioMedicine 2019;44:298–310.

[6] Yamamura K, Izumi D, Kandimalla R, et al. Intratumoral Fusobacterium nucleatum levels predict therapeutic response to neoadjuvant chemotherapy in esophageal squamous cell carcinoma. Clin Cancer Res 2019;25:6170–9.

[7] Natsuizaka M, Whelan K, Kagawa S, et al. Interplay between Notch1 and Notch3 promotes EMT and tumor initiation in squamous cell carcinoma. Nat Commun 2017;8:1758. doi: 10.1038/s41467-017-01500-9.

[8] Fu X, Li T, Dai Y, et al. Preoperative systemic inflammation score (SIS) is superior to neutrophil to lymphocyte ratio (NLR) as a predicting indicator in patients with esophageal squamous cell carcinoma. BMC Cancer 2019;19:721. doi: 10.1186/s12885-019-5940-6.

[9] Lu Z, Lu C, Li C, et al. Dracorhodin perchlorate induces apoptosis and G2/M cell cycle arrest in human esophageal squamous cell carcinoma through inhibition of the JAK2/STAT3 and AKT/FOXO3a pathways. Mol Med Rep 2019;20:2091–100.

[10] Liu X, Gao Z, Zhang Q, et al. Serum pepsinogen assay is not recommended for the diagnosis of esophageal squamous cell carcinoma: a systematic review and meta-analysis. Cancer Manag Res 2019;11:5643–54.

[11] Gu M, Li L, Zhang Z, et al. PFKFB3 promotes proliferation, migration and angiogenesis in nasopharyngeal carcinoma. J Cancer 2017;8:3887–96.

[12] Montemurro C, Nomoto H, Pei L, et al. Publisher correction: IAPP toxicity activates HIF1α/PFKFB3 signaling delaying β-cell loss at the expense of β-cell function. Nat Commun 2019;10:3507. doi: 10.1038/s41467-019-11516-y.

[13] Li J, Mao X, Tian T, et al. Role of PFKFB3 and CD163 in oral squamous cell carcinoma angiogenesis. Curr Med Sci 2019;39:410–4.

[14] Finucane O, Sugrue J, Rubio-Araiz A, et al. The NLRP3 inflammasome modulates glycolysis by increasing PFKFB3 in an IL-1β-dependent manner in macrophages. Sci Rep 2019;9:4034. doi: 10.1038/s41598-019-40619-1.

[15] Gustafsson N, Färnegårdh K, Bonagas N, et al. Targeting PFKFB3 radiosensitizes cancer cells and suppresses homologous recombination. Nat Commun 2018;9:3872. doi: 10.1038/s41467-018-06287-x.

[16] Yasuhiro H, Yuichi S, Masaaki I, et al. Prognostic significance of IMMT expression in surgically-resected lung adenocarcinoma. Thorac Cancer 2019;10:2142–51.

[17] Mondal S, Roy D, Sarkar B, et al. Therapeutic targeting of PFKFB3 with a novel glycolytic inhibitor PFK158 promotes lipophagy and chemosensitivity in gynecologic cancers. Int J Cancer 2019;144:178–89.

[18] Zhang R, Li R, Liu Y, et al. The glycolytic enzyme PFKFB3 controls TNF-α-induced endothelial proinflammatory responses. Inflammation 2019;42:146–55.

[19] Wang C, Qu J, Yan S, et al. PFK15, a PFKFB3 antagonist, inhibits autophagy and proliferation in rhabdomyosarcoma cells. Int J Mol Med 2018;42:359–67.

[20] Zhu Y, Lu L, Qiao C, et al. Targeting PFKFB3 sensitizes chronic myelogenous leukemia cells to tyrosine kinase inhibitor. Oncogene 2018;37:2837–49.

[21] Jiang H, Shi H, Sun M, et al. PFKFB3-driven macrophage glycolytic metabolism is a crucial component of innate antiviral defense. J Immunol 2016;197:2880–90.

[22] Hu K, Wang D, Liu P, et al. Targeting of MCT1 and PFKFB3 influences cell proliferation and apoptosis in bladder cancer by altering the tumor microenvironment. Oncol Rep 2016;36:945–51.

[23] Zhang J, Zhang Y, Wang J, et al. Protein kinase D3 promotes gastric cancer development through p65/6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 activation of glycolysis. Exp Cell Res 2019;380:188–97.

[24] Li X, Liu J, Qian L, et al. Expression of PFKFB3 and Ki67 in lung adenocarcinomas and targeting PFKFB3 as a therapeutic strategy. Mol Cell Biochem 2018;445:123–34.

[25] Ragnum H, Røe K, Holm R, et al. Hypoxia-independent downregulation of hypoxia-inducible factor 1 targets by androgen deprivation therapy in prostate cancer. Int J Radiat Oncol Biol Phys 2013;87:753–60.

[26] Trenti A, Tedesco S, Boscaro C, et al. The glycolytic enzyme PFKFB3 is involved in estrogen-mediated angiogenesis via GPER1. J Pharmacol Exp Ther 2017;361:398–407.

[27] Li Hui-Min, Yang Jie-Gang, Liu Zhuo-Jue, et al. Blockage of glycolysis by targeting PFKFB3 suppresses tumor growth and metastasis in head and neck squamous cell carcinoma. J Exp Clin Cancer Res 2017;36:7. doi: 10.1186/s13046-016-0481-1.

[28] Ko Y, Domingo-Vidal M, Roche M, et al. TP53-inducible glycolysis and apoptosis regulator (TIGAR) metabolically reprograms carcinoma and stromal cells in breast cancer. J Biol Chem 2016;291:26291–303.

[29] Chen D, Ning W, Jiang Z, et al. Glycolytic activation of peritumoral monocytes fosters immune privilege via the PFKFB3-PD-L1 axis in human hepatocellular carcinoma. J Hepatol 2019;71:333–43.