Prognostic value of glucose transporter 3 expression in hepatocellular carcinoma

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Abstract. Determining an effective biomarker for predicting the prognosis of patients with hepatocellular carcinoma (HCC) may improve patient survival rates. The present study aimed to investigate the expression of glucose transporter 3 (GLUT-3) in HCC and to determine its predictive value for the survival of patients with HCC. Immunohistochemistry was used to detect GLUT-3 expression in HCC tissues of 275 and 140 patients with HCC from training and validation cohorts, respectively. The association between GLUT-3 expression and the clinicopathological characteristics of patients with HCC, and between GLUT-3 expression and patient survival rates were analyzed. The predictive value of GLUT-3 expression was confirmed using the validation cohort. The results demonstrated that the high GLUT-3 expression in HCC tissues was significantly associated with elevated α-fetoprotein level, large tumor size, poor histological differentiation and Tumor-Node-Metastasis stages III and IV ($P<0.05$). In addition, GLUT-3 high expression was also significantly associated with reduced overall survival of patients with HCC in the training and validation cohorts. In conclusion, the results from the present study suggested that GLUT-3 may be considered as a potential independent prognostic factor for predicting the survival of patients with HCC.

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Abbreviations: AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; DFS, disease-free survival; GLUT, glucose transporter; HCC, hepatocellular carcinoma; HIF-1, hypoxia-inducible factor-1; OS, overall survival; STAT3, signal transducer and activator of transcription3; TNM, Tumor-Node-Metastasis

Key words: GLUT3, hepatocellular carcinoma, prognosis, biomarker, mechanism

Introduction

Hepatocellular carcinoma (HCC) is the seventh most common malignant tumor and the second most frequent cause of cancer-associated mortality worldwide in 2016 (1). Although progress has been made in the diagnosis of HCC, the treatment and prevention of the disease and prognosis prediction remain poor (2). At present, the classification and prognosis evaluation of patients with HCC depend on clinical staging systems, including Tumor-Node-Metastasis (TNM) stage, Barcelona Clinic Liver Cancer (BCLC) stage and the Cancer of the Liver Italian Program stage (3). Although the clinical stage can predict the risk of tumor recurrence to a certain extent, it rarely directly reflects the prognosis of patients with HCC after hepatectomy. It is therefore crucial to identify an effective prognostic molecular marker to predict the clinical prognosis of patients with HCC.

A total of 14 subtypes of facilitative glucose transporters (GLUTs) have been described in humans, of which role is to transport glucose to different tissues in the body (4). Previous studies have reported that GLUT-3 is overexpressed in numerous solid tumors, including oral squamous cell carcinoma, laryngeal carcinoma, nonsmall cell lung carcinoma and bladder cancer, which may be due to the rapid proliferation of tumor cells in hypoxic condition (5-9). Since the rate of ATP produced by glycolysis under anaerobic conditions is significantly lower than during aerobic metabolism, high GLUTs expression is required by tumor cells to satisfy the increased need for glucose (4). GLUT-3 may therefore be a potential tumor cell marker. To the best of our knowledge, the expression of GLUT-3 in HCC and its association with the clinicopathological characteristics of patients have not yet been identified. In the present study, the association between GLUT-3 expression in HCC tissues and the clinicopathological characteristics and clinical prognosis of patients with HCC was evaluated.

Materials and methods

Patients and tissue specimens. Formalin-fixed paraffin-embedded tissues of 275 patients with HCC who underwent surgical resection between April 2003 and December 2008 at the Shandong Provincial Hospital Affiliated

to Shandong University (Shandong, China) were included in the training cohort. In parallel, in order to verify the prognostic efficacy of GLUT-3 as a predictive marker in HCC, 140 formalin-fixed paraffin-embedded tissues of patients with HCC who underwent surgery during the same period at the Sun Yat-Sen University Cancer Center (Guangdong, China) were randomly selected and included in the validation cohort. The inclusion criteria were as follows: i) Child-Pugh classification (10) was A or B; ii) patients did not receive antitumor therapy prior to surgery; iii) radical resection was performed; iv) HCC pathology was confirmed after surgery; v) no evidence of extrahepatic metastasis or primary cancer of other organs; and vi) complete follow-up information was available. The exclusion criteria were as follows: i) Patients received preoperative antitumor therapy, including radiotherapy or chemotherapy; ii) preoperative extrahepatic metastasis was observed; iii) malignant tumors associated with other organs were identified; and iv) follow-up information was missing.

In the training cohort, the median age of the patients was 55 years (age range, 24-74 years), 38 patients were women and 237 patients were men. In the validation cohort, the median age of the patients was 52 years (age range, 28-72 years), 15 patients were women and 125 patients were men. Clinical baseline and complete follow-up information were reviewed from the hospital databases. This study was approved by the Institutional Review Boards of Sun Yat-Sen University Cancer Center and Shandong Provincial Hospital Affiliated to Shandong University. Written informed consent was obtained from all patients included in this study.

Isolation of RNA and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the tissue samples using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The quality and quantity of RNA were assessed using the Agilent 2100 Bioanalyzer and NanoDrop ND-1000 Spectrophotometer (Agilent Technologies, Inc.). cDNA was synthesized from immunoprecipitated RNA using reverse transcriptase followed by second strand synthesis to generate double-stranded cDNA using SuperScript IV Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.) under the following conditions: 25˚C for 6 min, 55˚C for 20 min, and 80˚C for 10 min. The qPCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocols. The GAPDH was used as an endogenous control, and fold changes were calculated via relative quantification ($2^{-\Delta\Delta Cq}$). Transcripts were assessed using the following primers: GLUT-3 (forward, CAG CGAGACCCAGAGATGC; reverse, GACCCCAGTGTTGTA GCCAA) and GAPDH (forward, TGCACCACCAACTGCTTA GC; reverse, GGCATGGACTGTGGTCATGAG).

IHC staining. The formalin-fixed paraffin-embedded specimens were cut into 5-μm sections and placed on polylysine-coated slides (Sigma-Aldrich; Merck KGaA). Sections were deparaffinized in xylene and rehydrated using a gradient series of alcohol (100% for 5 min; 90% for 5 min; 80% for 5 min; and 70% for 5 min). Antigen retrieval was performed by heating sections in citrate buffer (pH 6.0; Dako; Agilent Technologies, Inc.) at 95˚C for 10 min. Samples were blocked with 10% goat serum (Beijing Solarbio Science & Technology Co., Ltd) at 37˚C for 2 h and with Peroxidase-Blocking Solution (Dako; Agilent Technologies, Inc.) at 37˚C for 30 min. Sections were incubated with the primary antibody against GLUT-3 (1:50; cat. no. ab95256; Abcam) and with an isotype-matched immunoglobulin G (1:100; cat. no. Ab83567; Abcam) used as a negative control at room temperature for 2 h. Immunohistochemical staining was performed using the Dako Envision Plus system [Dako; Agilent Technologies, Inc. Dako, EnVisio+System/HRP, Mo(DAB+), K400611-2] according to the manufacturer's instructions (magnification, x400). The number of tumor cells with a strong membrane signal for GLUT-3 was counted in ten low magnification fields with light microscope, and expressed as a percentage of the total number of cells. The mean percentage of immunoreactive tumor cells was calculated and scored according to the following 5-point scale: 0, 1, 2, 3 or 4 points for 0, 1-25, 26-50, 51-75 or 76-100% of positively stained cells, respectively. GLUT-3 was considered to be not expressed if the final score was 0. GLUT-3 expression was considered to be low if the final score was 1 or 2, and high if the final score was 3 or 4.

Statistical analysis. Statistical analyses were performed using SPSS 18.0 statistical software for Windows (SPSS, Inc.). GLUT-3 mRNA level in different types of tissue was analyzed using ANOVA followed by Scheffe post hoc test. χ^2 or Fisher's exact tests were used to determine the association between GLUT-3 expression levels and the clinicopathological characteristics of patients. Disease-free survival (DFS) time was calculated as the time between surgical resection and the appearance of recurrence evidence at any site or the last follow-up contact. Overall survival (OS) time was calculated as the time between surgical resection and the time of death or the last follow-up.

Receiver operating characteristic (ROC) curves were used in the training cohort and the validation cohort to validate the prognostic ability of GLUT-3 expression levels. Survival rate was calculated using Kaplan-Meier method, and log-rank test was used to compare differences in survival between groups. The Kaplan-Meier method was used for univariate analysis, whereas Cox proportional hazards regression model was used for multivariate analysis. Variables with $P<0.05$ in the univariate analysis were selected as variables for multivariate analysis. A two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics and expression of GLUT-3 in patients with HCC. The clinicopathological characteristics of all patients in the two cohorts included age, sex, etiology, liver cirrhosis, Child-Pugh classification, serum α-fetoprotein (AFP) level, tumor size, tumor number, vascular invasion, histological differentiation, BCLC stage and TNM stage, and are summarized in Tables I and II.

IHC was performed to investigate GLUT-3 expression. The results demonstrated that GLUT-3 was not expressed in normal liver (Fig. 1A) and paracancerous tissues of patients with HCC (Fig. 1B). However, GLUT-3 was expressed in variable ways in HCC tissues (Fig. 1C-E). Representative IHC images are presented in Fig. 1.

GLUT-3 expression level in tumor tissues was significantly higher compared with normal liver tissues ($P<0.05$) and paracancerous tissues ($P<0.05$). However, there was no statistical

Table I. Expression of GLUT-3 and its relationship with clinicopathological characteristics of the training cohort.

Characteristic	GLUT-3 expression (n=275)		P-value
	No and low (n=193)	High (n=82)	
Age (≥55/<55 years)	60/133	29/53	0.489
Sex (F/M)	28/165	10/72	0.611
Etiology			0.473
Hepatitis B virus	176	74	
Hepatitis C virus	2	1	
Other	15	7	
Cirrhosis			0.487
Yes	143	64	
No	50	18	
Child-Pugh classification			0.381
A	191	80	
B	2	2	
AFP level			0.007
≤400 ng/ml	115	34	
>400 ng/ml	78	48	
Tumor size			0.166
≤5 cm	100	35	
>5 cm	93	47	
Tumor number			0.039
Single	160	59	
Multiple	33	23	
Vascular invasion			0.097
Yes	40	24	
No	153	58	
Histological differentiation			0.028
Well	33	6	
Moderate	125	54	
Poor	35	22	
TNM stage			0.018
I and II	153	54	
III and IV	40	28	
BCLC stage			0.227
0 and A	154	60	
B and C	39	22	

AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; F, female; GLUT-3, glucose transporter 3; M, male.

difference in GLUT-3 expression level between normal liver and paracancerous tissues (P>0.05; Fig. 1F).

High GLUT-3 expression tissue score in HCC was significantly and positively associated with elevated AFP level, large tumor size, poor histological differentiation and TNM stages III and IV (P<0.05).

Prognostic values of serum AFP level, GLUT-3 expression and tumor size for the OS and DFS of patients with HCC. The area under curves (AUCs) among serum AFP level, GLUT-3 expression and tumor size in predicting OS and DFS in patients with HCC were analyzed by ROC curves analysis in the training and validation cohorts. In the training cohort, the AUCs for GLUT-3 expression predicting the OS and DFS of patients with HCC were 0.59 [95% confidence interval (CI), 0.53-0.66] and 0.58 (95% CI, 0.51-0.65), respectively. In the validation cohort, the AUCs for GLUT-3 expression predicting the OS and DFS of patients with HCC were 0.61 (95% CI, 0.51-0.71) and 0.61 (95% CI, 0.51-0.70), respectively (Fig. 2).

Survival and expression of GLUT-3. In the training cohort, the prognostic ability of GLUT-3 expression was analyzed in

Table II. Expression of GLUT-3 and its relationship with clinicopathological characteristics of the validation cohort.

	GLUT-3 expression (n=140)		
Characteristic	No and low (n=99)	High (n=41)	P-value
Age (≥55/<55 years)	31/68	17/24	0.250
Sex (F/M)	9/90	6/35	0.335
Etiology			0.473
Hepatitis B virus	93	37	
Hepatitis C virus	1	1	
Others	5	3	
Cirrhosis			0.087
Yes	74	36	
No	25	5	
Child-Pugh classification			0.580
A	92	38	
B	7	3	
AFP level,			0.001
≤400 ng/ml	63	14	
>400 ng/ml	36	27	
Tumor size			0.031
≤5 cm	56	15	
>5 cm	43	26	
Tumor number			0.660
Single	80	31	
Multiple	19	10	
Vascular invasion			0.022
Yes	8	9	
No	91	32	
Histological differentiation			0.004
Well	21	0	
Moderate	60	26	
Poor	18	15	
TNM stage			0.144
I and II	79	28	
III and IV	20	13	
BCLC stage			0.403
0 and A	81	31	
B and C	18	10	

AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; F, female; GLUT-3, glucose transporter 3; M, male.

275 patients with HCC. High GLUT-3 tissue score was significantly associated with reduced DFS and OS (P<0.001; Fig. 3). To validate these findings, a validation cohort containing patients with HCC was tested. The results demonstrated that high GLUT-3 expression level in the validation cohort was also associated with poor DFS and OS (P<0.05; Fig. 4). The predictive value of GLUT-3 expression in the validation cohort was therefore validated for OS and DFS. The results from multivariate Cox regression analysis demonstrated that GLUT-3 expression level, BCLC, vascular invasion and tumor size were independent prognostic factors for the OS of patients with HCC (Table III).

Discussion

HCC is a common malignant tumor associated with high mortality rate (11). Surgical resection is the most effective treatment for patients with liver cancer; however, the postoperative long-term survival rate of patients is limited due to tumor recurrence (70% at 5 years) (12,13). Traditional stratification schemes that are based on clinical characteristics, including the American Joint Committee on Cancer (14), TNM and BCLC stages, provide limited prognostic guidance in the management of patients with HCC due to disease heterogeneity (3,15).

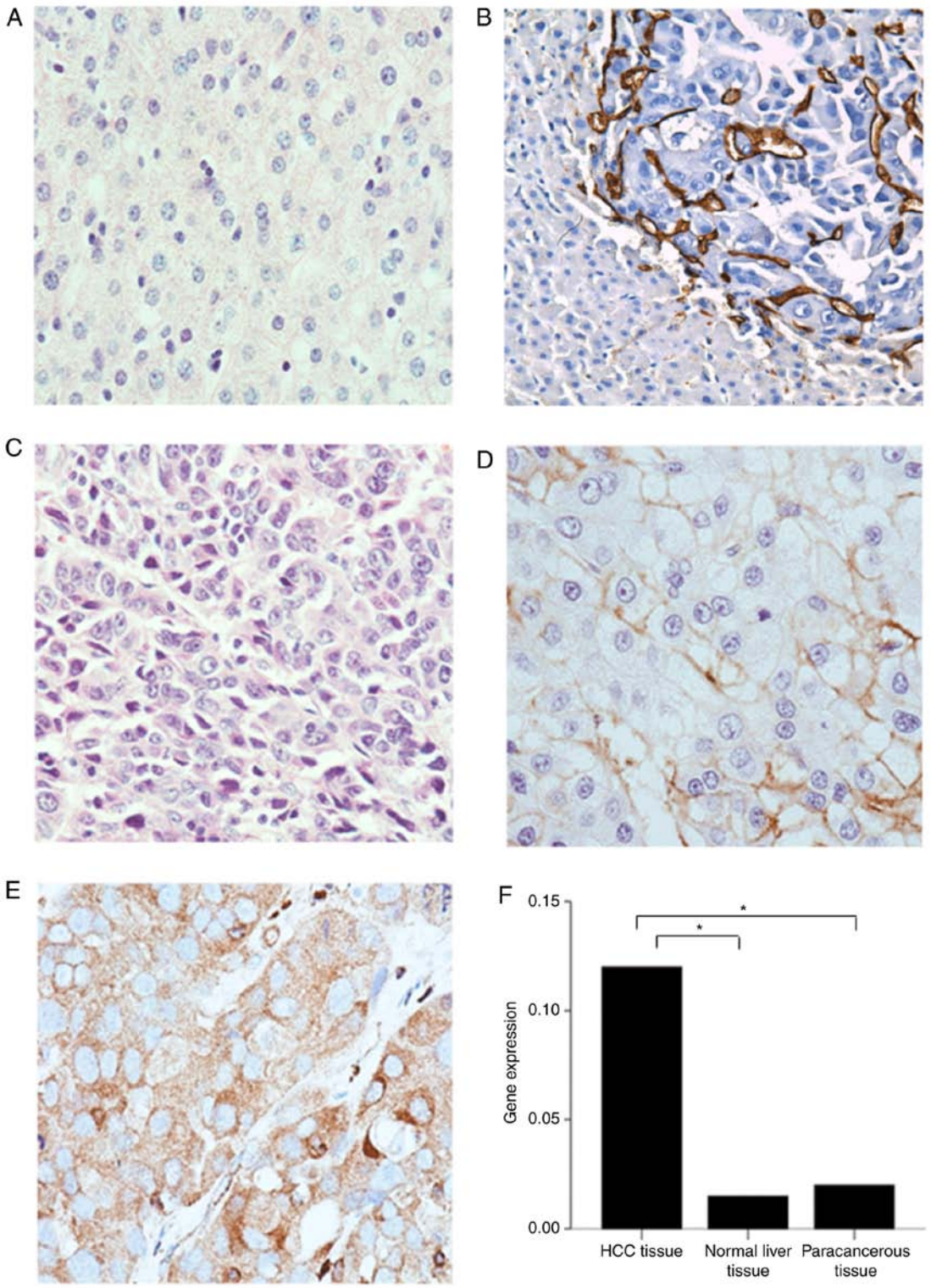

Figure 1. Representative immunohistochemistry images of GLUT-3 expression in normal liver and HCC tissues and GLUT-3 gene expression in different tissues. (A) Normal liver tissue with no GLUT-3 expression. (B) Paracancerous tissues with no GLUT-3 expression. (C) Liver cancer tissues with no GLUT-3 expression. (D) Liver cancer tissues with low GLUT-3 expression. (E) Liver cancer tissues with high GLUT-3 expression. Magnification, x40. (F) GLUT-3 mRNA level in different tissues. *P<0.05. GLUT-3, glucose transporter 3; HCC, hepatocellular carcinoma.

Specific biomarkers would therefore allow better stratification of the disease.

High serum AFP levels were associated with poor prognosis of patients with HCC (16); however, the optimal cut-off value of serum AFP level that could be used to predict a poor prognosis in patients with HCC has not yet been determined. To our knowledge, no molecular profiles have been established to date as the widely satisfactory prognostic biomarker in HCC, although some biomarkers have potentially predictive value (EpCAM signature, G3-proliferation subclass, and SUOX) (17-20). Prognostic molecular biomarkers should significantly predict the survival prognosis and be indicated for

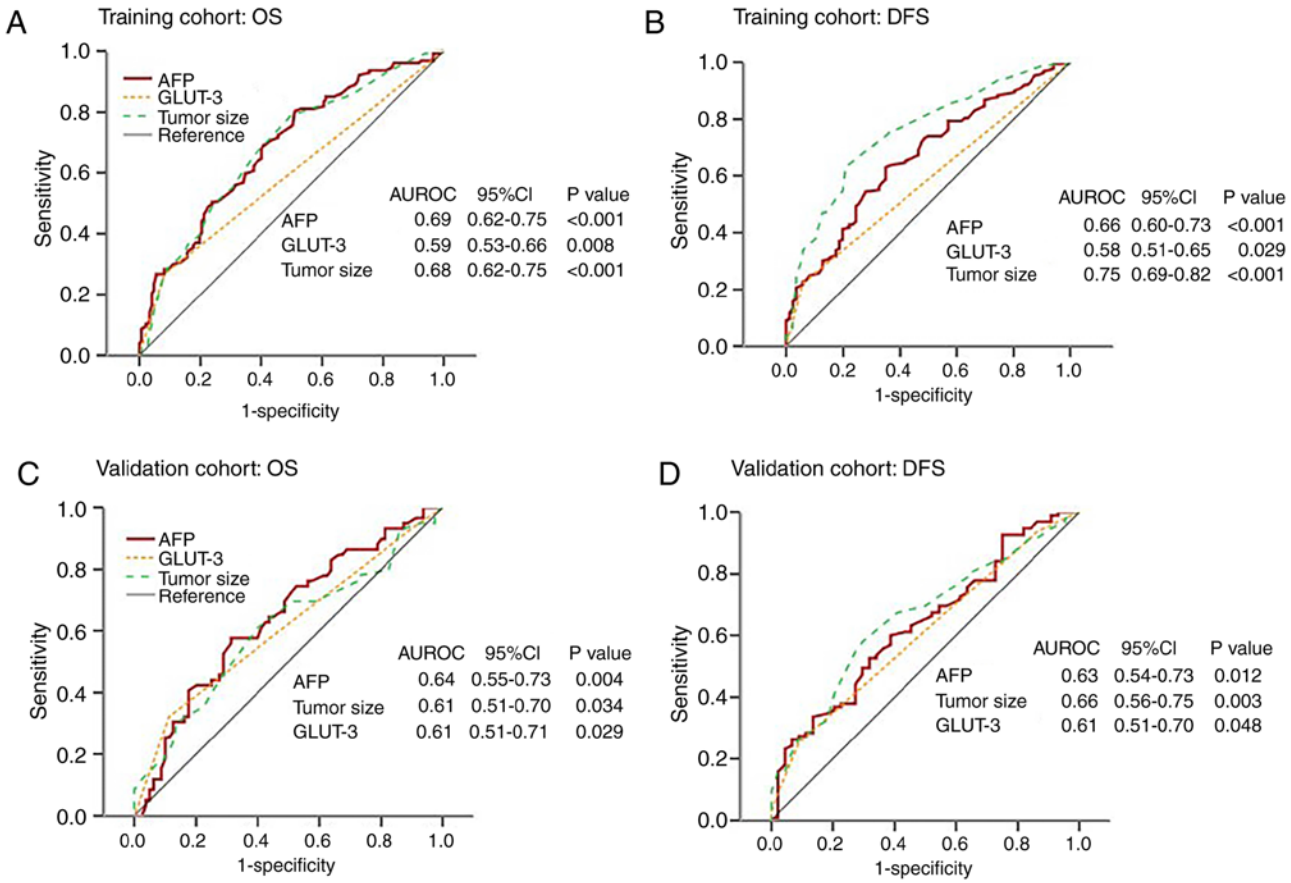

Figure 2. ROC curves of serum AFP level, GLUT-3 expression level and tumor size for predicting OS and DFS in the training and validation cohorts. (A) AUCs of AFP, GLUT-3 and tumor size were 0.69, 0.59 and 0.68, respectively. (B) AUCs of AFP, GLUT-3 and tumor size were 0.66, 0.58 and 0.75, respectively. (C) AUCs of AFP, GLUT-3 and tumor size were 0.64, 0.61 and 0.61, respectively. (D) AUCs of AFP, GLUT-3 and tumor size were 0.63, 0.61 and 0.66, respectively. AUC, area under curve; AFP, α-fetoprotein; CI, confidence interval; DFS, disease-free survival; GLUT-3, glucose transporter 3; HCC, hepatocellular carcinoma; OS, overall survival; ROC, receiver operating characteristic; AUROC, area under the receiver operating characteristic curve.

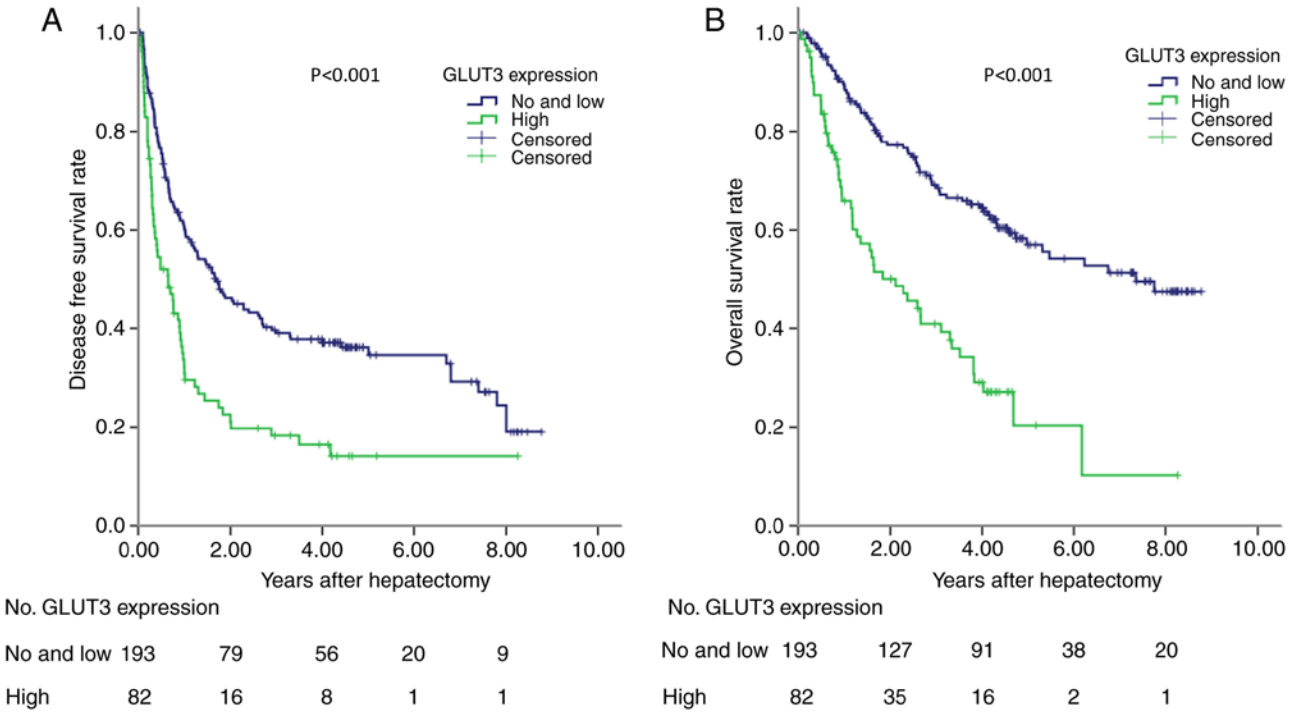

Figure 3. DFS and OS in the training cohort. (A) DFS of 275 patients with HCC according to GLUT-3 expression. (B) OS of 275 patients with HCC according to GLUT-3 expression. GLUT-3 expression was predictive for DFS and OS in patients with HCC. P<0.001. DFS, disease-free survival; GLUT-3, glucose transporter 3; HCC, hepatocellular carcinoma; OS, overall survival.

Table III. Cox regression model analysis in training cohort.

Characteristic	B	SE	Wald	P-value	Exp (B)	95.0% CI for Exp (B)	
						Down	Upper
BCLC stage	0.671	0.232	8.368	0.004	1.957	1.242	3.084
GLUT-3 expression	0.891	0.208	18.388	<0.001	2.436	1.622	3.660
Vascular invasion	0.636	0.253	6.341	0.012	1.889	1.151	3.099
Tumor size	0.687	0.208	10.957	0.001	1.988	1.323	2.985

BCLC, Barcelona Clinic Liver Cancer; CI, confidence interval; GLUT-3, glucose transporter 3.

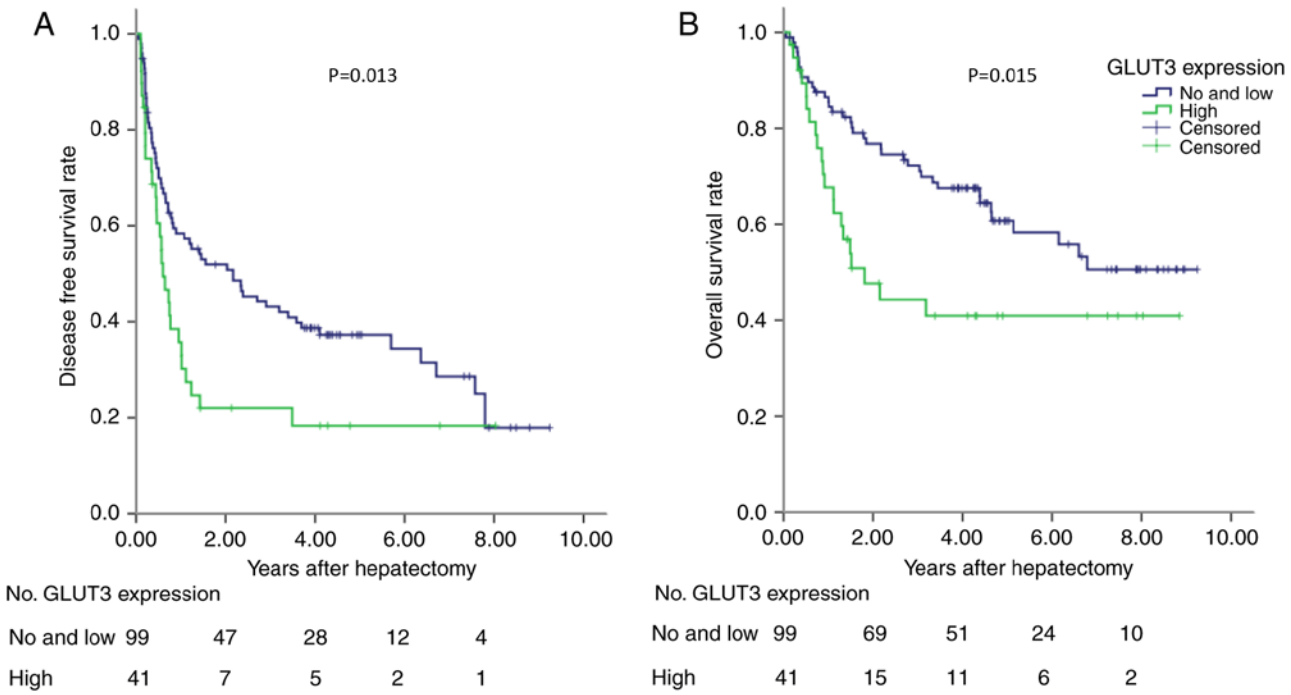

Figure 4. DFS and OS in the validation cohort. (A) DFS of 140 patients with HCC according to GLUT-3 expression. (B) OS of 140 patients with HCC according to GLUT-3 expression. GLUT-3 expression was predictive for DFS (P=0.013) and OS (P=0.015) in patients with HCC. DFS, disease-free survival; GLUT-3, glucose transporter 3; HCC, hepatocellular carcinoma; OS, overall survival.

most patients in clinical practice. Therefore, more acceptable markers should be explored according to standard criteria. In the present study, GLUT-3 expression and its prognostic value in patients with HCC were analyzed. The results demonstrated that increased GLUT-3 expression level was associated with decreased OS in patients with HCC following tumor resection. In addition, GLUT-3 expression level was also associated with elevated serum AFP level, large tumor size, poor histological differentiation and TNM stages III and IV. Taken together, these results demonstrated that GLUT-3 overexpression may be considered as a biomarker for predicting the survival of patients with HCC.

Increased energy metabolism has been accepted as a hallmark of cancer, and is widely observed in cancer cells (21). Increased glucose use by glycolysis is an exclusive property of invasive cancer cells (22). In tumor cells, glucose uptake across the plasma membrane, which is mediated by facilitative GLUTs, is thought to be the rate-limiting step of glucose metabolism (23). Enhanced glucose uptake in tumors can be therefore mediated by overexpression of GLUTs overexpression (4). Of the 14 subtypes of human GLUTs, the most closely associated with glucose metabolism are GLUT-1-5, which have different body distributions under physiological conditions. For instance, GLUT-l and 3 are the two most widely studied GLUTs, and the ones that are most strongly associated with malignant tumors. It has been reported that the upregulation of specific glucose transporters may represent a key mechanism by which malignant cells may achieve increased glucose uptake to support the high rate of glycolysis (24,25). In addition, GLUT-3 is overexpressed in human brain tumors, oral tongue carcinoma, endometrial and breast cancers, non-small lung carcinoma, oral squamous cell carcinoma and laryngeal carcinoma (5,6,8,26,27). To the best of our knowledge, the present study was the first to analyze GLUT-3 expression and its association with the prognosis of patients with HCC.

Compared with other GLUTs, GLUT-1 and 3 have a higher affinity for glucose under physiological conditions (4). GLUT-3 is mainly expressed in the brain and testicles (28). In addition, GLUT-3 is present in the intracellular vesicles of various types of leukocyte and can be transferred to plasma membrane under the activation of proliferative stimuli. For instance, in T-lymphocytes, activation is characterized by the emergence of insulin receptors on the plasma membrane; however, their physiological significance is unclear (29). As aforementioned, numerous studies demonstrated that GLUT-3 is also expressed in various types of tumor tissue. Malignant cells grow faster and require more oxygen and glucose than normal cells. Although mitochondrial oxidative phosphorylation is considered to be a more efficient metabolic process for ATP synthesis compared with glycolysis (30), tumor cells use glycolysis as the main metabolic mode, even when sufficient oxygen is present. This phenomenon is known as the Warburg effect (31). Although glycolysis produces less ATP, a large number of intermediate metabolites can be used to construct macromolecular structures, including RNA, proteins, lipids and NADP (30). As tumors grow, cells may encounter hypoxic conditions that lead to the induction of the hypoxia inducible factor 1 (HIF-1) transcription factor, which increases the transcription of glucose transporters (32). The decrease of ATP production efficiency and the high energy requirement of tumor cells can stimulate the increase of glucose uptake by malignant tumor cells as aforementioned (30,32). Furthermore, GLUT-3 overexpression can participate in the transport of more glucose into tumor cells in order to satisfy their high metabolism and rapid growth. However, the mechanism of GLUT-3 overexpression in tumor cells is unknown, particularly in HCC, which was investigated, to the best of our knowledge, in only one study to date (33). At present, there are several hypotheses about the role of GLUT-3 overexpression in tumor cells, including IL-6/signal transducer and activator of transcription 3 (STAT3), PI3K-Akt and hypoxia-induciblefactor-1 (HIF-1) signaling pathways. A previous study demonstrated that activation of IL-6/STAT3 pathway can stimulate expression of GLUT isoforms, and therefore increase glucose uptake capacity in HCCs (33). STAT3 is a membrane receptor-mediated nuclear transcription factor (34). Cytokines, including IL-6, and growth factors (such as epidermal growth factor and platelet-derived growth factor) activate STAT3 through phosphorylation. Phosphorylated STAT3 enters then the nucleus, binds to the DNA regulatory regions of target genes and induces their expression (35). High expression of GLUT-3 may therefore be facilitated by the activation of the IL-6/STAT3 pathway. The involvement of PI3K-Akt in GLUTs regulation suggests that uncontrolled Akt activation, caused by disturbances in PI3K α subunit or phosphate and tension homolog, may mediate the increased glucose uptake and overexpression of GLUTs observed in tumors. A previous study reported that in hypoxic BeWo choriocarcinoma cells, HIF-1 mediates transcriptional regulation of glycolytic genes with hypoxia-response elements in their promoter regions, including GLUT-1 and GLUT-3 (36). GLUT-3 is overexpressed following HIF-1α complex stabilization in response to hypoxia in BeWo choriocarcinoma cells (36). However, the underlying mechanisms of GLUT-3 overexpression in HCC remain unclear and require further investigation.

The current study presented some limitations. Firstly, there were inherent biases due to the retrospective nature of the study. Secondly, the number of patients involved in this study was relatively small, and results should be confirmed in a larger patient cohort. Thirdly, the molecular mechanism of GLUT-3 overexpression in liver cancer tissues remains unclear and requires further investigations.

In conclusion, the present study demonstrated the association between GLUT-3 expression level and the clinical prognosis of patients with HCC. Furthermore, the results demonstrated that increased GLUT-3 expression level was associated with poor prognosis of patients with HCC, suggesting that GLUT-3 may be considered as a potential prognostic in HCC. This finding provided a basis for investigating GLUT-3 as a potential target in the treatment of HCC, which may lead to the development of novel treatment strategies.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HG, JL and YH participated in the conception and design of the study. All authors collected and interpreted the data. HG, XZ, MC, HL, FL, YH, XS, HZ, ZN and QN performed the statistical analysis. YH drafted the manuscript, and HG and JL edited it critically. All authors gave final approval of the version to be published.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Sun Yat-Sen University Cancer Center and Shandong Provincial Hospital Affiliated to Shandong University. Written informed consent was obtained from all the patients who participated in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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