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Original Article

Glutamine synthetase predicts adjuvant TACE response in hepatocellular carcinoma

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Abstract: Background: Adjuvant transcatheter arterial chemoembolization (TACE) is associated with better outcome and reduced tumor recurrence in hepatocellular carcinoma (HCC) patients. This study aimed to investigate the relationship between glutamine synthetase (GS) expression and survival of HCC patients after postoperative adjuvant TACE. Methods: We retrospectively analyzed 554 HCC patients in two independent cohorts who underwent curative resection. Immunohistochemistry assay was used to investigate the expression of GS protein and evaluate the association with survival and the response to adjuvant TACE. Results: In training cohort, patients with low GS expression who received postoperative adjuvant TACE showed a better overall survival (OS) (P<0.001) and less early phase recurrence (P=0.016). Adjuvant TACE was an independent prognostic factor for 5-year OS (HR=0.408, 95% CI 0.261-0.639, P<0.001) and early phase recurrence (HR=0.592, 95% CI 0.376-0.931, P=0.023). The same result was confirmed in validation cohort. Patients with high GS expression in both cohorts did not have a significant response to adjuvant TACE in OS and early phase recurrence. Conclusions: GS status in tumor might be a useful tool in the selection of HCC patients who would be likely to benefit from postoperative adjuvant TACE.

Keywords: Glutamine synthetase, hepatocellular carcinoma, transcatheter arterial chemoembolization, survival, prognosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and is the third leading cause of cancer-related death worldwide [1]. The main therapies for HCC are as follows: surgical interventions including transplantation, local ablative therapy, and transcatheter arterial chemoembolization (TACE). Among all treatments, hepatic resection is the first-line therapeutic option [2]. However, only about 10 to 20% of patients with HCC are currently eligible for surgical intervention [3]. Despite many types of available treatments, the prognosis of HCC is still unsatisfactory and it is reported that the 5-year overall survival rate is only 3-5% across the world [4]. The poor prognosis of patients with HCC is largely due to high rates of post-treatment recurrence and metastasis, in addition to resistance to systemic chemotherapy [5].

TACE, a kind of the regional therapies, which was initially studied for palliative treatment of unresectable HCC, has been reported to reduce the incidence of HCC recurrence and prolong survival as a kind of postoperative adjuvant therapies [6-9]. Meanwhile, many studies reported the controversial results [10-13]. The limited ways to identify patients who are most likely to benefit from such targeted adjuvant therapies have triggered a search for molecular markers that can be used to stratify patients with respect to prognosis and response to therapy.

Glutamine synthetase (GS, encoded by *GLUL*, glutamate-ammonia ligase) catalyses the ATP-dependent biosynthesis of glutamine from glutamate [14]. GS is present predominantly in the brain, kidneys, and liver [15]. In normal liver, GS is localized in the cells around the central vein [16]. Early studies demonstrated that absent

and low GS expression pattern in glioblastoma multiforme represented a valuable biomarker of both epilepsy and overall survival [17]. Additionally, GS may play a role in the pathogenesis of astrocytomas and can be used as a differential marker to distinguish astrocytic from oligodendroglial tumors [18]. GS has also been reported to have correlations with temporal lobe epilepsy [19]. Overexpressed GS was observed in solid pseudopapillary neoplasm of the pancreas [20]. And in human HCC samples, GS was found up-regulated in both mRNA and protein levels [16]. The immunoreactivity of GS protein was shown increased from precancerous lesions to early or advanced stages, indicating a role in the promotion of the metastatic potential of HCC [21]. Several reports have suggested that GS is an early marker of hepatocarcinogenesis [22]. Together with glypican 3 (GPC3) and heat shock protein 70 (HSP70) as a panel of markers, GS could contribute to the diagnosis of early HCC [23].

Previous studies discussed the significance of the GS expression level in the prognosis of HCC patients but the results were controversial. Osada found high GS expression group of HCC patients had a significantly shorter disease-free survival time than the low-GS group [24]. However, Dal Bello found that GS positive immunostaining was correlated with reduced specific and overall mortality [25]. Even more, GS was also reported not to be related with clinicopathological parameters and prognosis [26]. These conflicting results indicate that the roles of GS might vary in different patients or upon various treatments, and these differences need further investigation.

Given the therapeutic and prognostic potential of GS as biomarkers in HCC, we investigate the GS expression pattern and determine its contribution with clinical prognostic value in HCC patients. The results indicate that the expression of GS is able to predict HCC patients' outcomes after postoperative adjuvant TACE. This enzyme might be a useful marker to select HCC patients for adjuvant TACE therapy after hepatic surgery.

Patients and methods

Patients and tissue samples

A total of 285 adult HCC patients who underwent hepatectomy in Eastern Hepatobiliary Surgery Hospital during July 1, 2003 to June 30, 2005, were recruited to a training cohort. Another independent cohort of 269 patients from January, 2006, to November, 2008 was collected as validation cohort of this study. The histological grade of tumor differentiation was defined according to the Edmondson grading system. Tumor staging was determined according to the 7th edition of TNM classification of the International Union Against Cancer and the Barcelona Clinic Liver Cancer (BCLC) staging systems. The patients included in the series had available paraffin embedded tumor tissues following the inclusion: preoperative World Health Organization performance status of 0-1; curative resection; no encephalopathy, visualizable ascites, or distant metastases; Child-Pugh class A; no chemotherapy or radiotherapy before surgery; and resected lesions identified as HCC on pathological examination. Curative resection of HCC was performed as described before [27]. All the tissue samples were obtained following written informed consent according to an established protocol approved by the Ethic Committee of Second Military Medical University.

Adjuvant TACE

In this article, adjuvant TACE was preventive TACE and was performed 4-6 weeks after hepatic resection following the techniques previously described [8]. Hepatic arterial angiography was firstly performed and then preventive chemoembolization was done among the patients without tumor stain in the remnant liver. The regimen for preventive adjuvant TACE consisted of 5-fluorouracil (5-FU) 0.75 g, cisplatin (DDP) 60 mg, and the emulsion mixed with mitomycin C (MMC) 16 mg and lipiodol 5 ml. Therapeutic TACE was performed in the patients with tumor stain according to the size and number of the tumor, and in this article, this is not mentioned. One month later, contrast-enhanced CT or magnetic resonance imaging (MRI) was performed and the regimen was finished.

Follow-up

The patients were followed up every 2 months during the first 2 years after surgery and every 3-6 months afterwards. Each follow-up visit included a complete physical examination, serum AFP level test, liver function tests, and an abdominal ultrasound. Contrast-enhanced

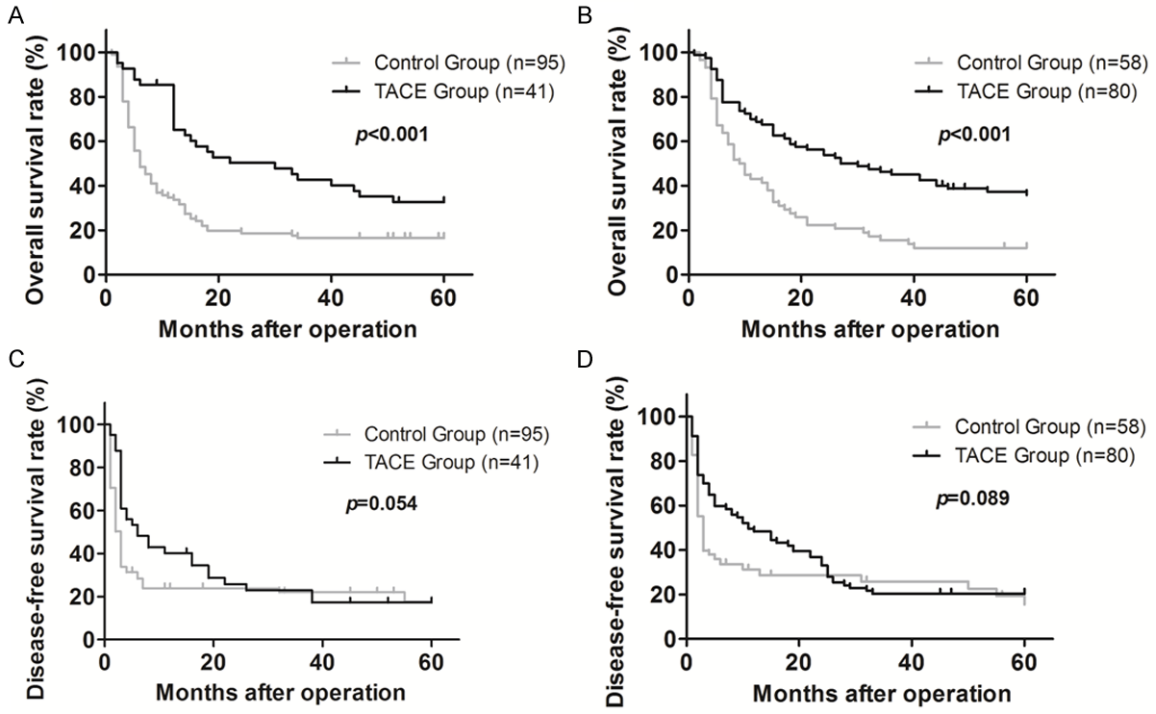

Figure 1. Response to adjuvant TACE in HCC patients with low GS expression. The 5-year overall survival rate between adjuvant TACE and control group in patients with low GS expression were analyzed by Kaplan-Meier in training cohort (A) and validation cohort (B). The 5-year disease-free survival rate between adjuvant TACE and control group in patients with low GS expression were analyzed by Kaplan-Meier in training cohort (C) and validation cohort (D).

CT or MRI was performed once every 6 months or when tumor recurrence or metastasis was suspected. Patients with tumor recurrences were treated with different therapeutic treatment including hepatic resection, local ablative therapy, and TACE et al, according to the tumor stage, liver functional reserve and the general condition of the patient. The follow-up period was defined as the interval from the date of operation to the date of death or the last follow-up. Deaths from other causes were treated as censored cases. All patients were observed until March 2014. Overall survival (OS) was defined as the interval between the dates of surgery and death. Disease-free survival (DFS) was defined as the interval between the dates of surgery and recurrence; if recurrence was not diagnosed, patients were censored on the date of death or the last follow-up.

Tissue microarray and immunohistochemistry analysis

After screening hematoxylin and eosin-stained slides for optimal tumor content, we constructed tissue microarray (TMA) slides (Shanghai Biochip Company, Ltd., Shanghai, China). Immunohistochemistry was performed as described before [28]. The sections were incubated in a primary polyclonal antibodies against GS (Epitomics, USA, Catalog: T1821 Lot: SA-091106AM) applied at 1:100 dilution. Finally, the visualization signal was developed with diaminobenzidine and the slides were counterstained in hematoxylin. Stained sections were evaluated in a blinded manner without prior knowledge of the clinical information using the German immunoreactive score (IRS) as described before [28]. Cases with discrepancies in IRS score were discussed together with other pathologists until consensus was reached.

Statistical analysis

All data are presented as the percentage of patients or means with standard deviation. Continuous variables were compared by the Student t-test, and categorical variables were compared by the Pearson's χ^2 test or Fisher's exact test. Survival curves were calculated using the Kaplan-Meier method and compared by the log-rank test. The Cox proportional-haz-

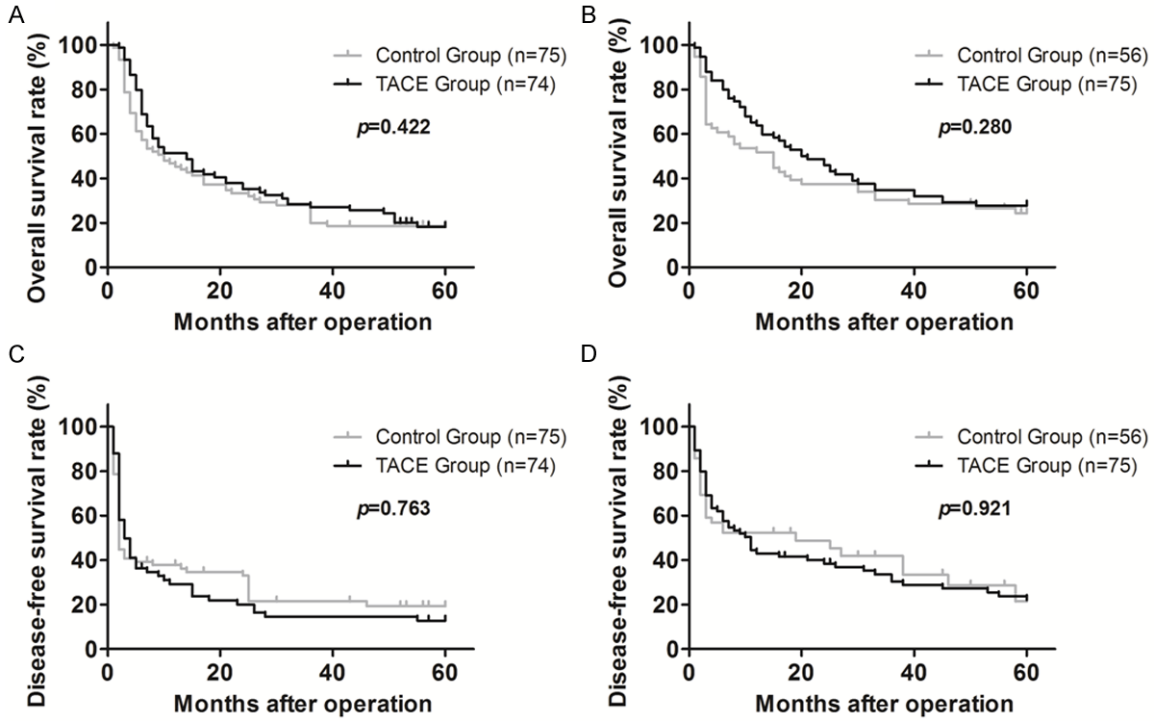

Figure 2. Response to adjuvant TACE in HCC patients with high GS expression. The 5-year overall survival rate between adjuvant TACE and control group in patients with high GS expression were analyzed by Kaplan-Meier in training cohort (A) and validation cohort (B). The 5-year disease-free survival rate between adjuvant TACE and control group in patients with high GS expression were analyzed by Kaplan-Meier in training cohort (C) and validation cohort (D).

ard regression model was used for analyses to explore the independent factors on survival based on the variables selected on univariate analysis. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses and a *P* value <0.05 was considered to be statistically significant.

Results

Characteristics of the study population

Patient characteristics of both cohorts are shown in Supplementary Table 1. All the patients were underwent curative liver resection and diagnosed by radiologic imaging plus pathology. In the training cohort (n=285), most of the patients were men (88.4%, n=252), were long-term carriers of hepatitis B virus (HBV) (87%, n=248), and had an elevated serum level of AFP (alpha-fetoprotein) (69.1%, n=197); 91.2% of the patients (n=260) had a single tumor nodule and 46% of the patients (n=131) had intrahepatic metastasis; 57.5% of the patients (n=164) had portal venous invasion at the time of resection; 52.3% of patients (n=149) were GS-high staining; 40.4% of patients (n=115) received adjuvant TACE after the surgery within 1-2 months, while 59.6% patients (n=170) followed up only at the first two months after resection. In the validation cohort (n=269), 131 patients (48.7%) were GS-high staining and 155 patients (57.6%) were received TACE after liver resection (Supplementary Table 1).

Expression of GS and response to adjuvant TACE in 5-year OS and DFS

We analyzed associations between GS expression and the response to postoperative adjuvant TACE through the Kaplan-Meier method and the log-rank test. Baseline of patient characteristics was shown in Supplementary Tables 2 and 3. In the training cohort, patients with low GS expression in tumors had a significant improvement in 5-year OS after receiving postoperative adjuvant TACE, as compared with those without adjuvant TACE therapy (median OS for adjuvant TACE versus control group: 32.1 versus 16.3 months; 95% confidence

Table 1. Univariate and multivariate Cox regression analyses GS for 5-year OS of patients with low GS expression in the training and validation cohort

Variables	Training cohort		Validation cohort	
	Hazard ration (95% CI)*	*P* Value	Hazard ration (95% CI)*	*P* Value
Univariate analysis				
Age (>50 years vs ≤50 years)	0.906 (0.614-1.337)	0.619	0.661 (0.448-0.977)	0.038
Gender (male vs female)	0.622 (0.341-1.136)	0.123	1.633 (0.823-3.239)	0.161
HBs Ag (positive vs negative)	1.860 (0.937-3.691)	0.076	1.061 (0.552-2.038)	0.860
Serum AFP (>400 ng/ml vs ≤400 ng/ml)	1.140 (0.740-1.757)	0.551	1.445 (0.943-2.215)	0.091
Largest tumor size (>5 cm vs ≤5 cm)	2.143 (1.218-3.769)	0.008	2.333 (1.462-3.723)	<0.001
Tumor number(multiple vs single)	1.546 (0.715-3.344)	0.268	1.029 (0.585-1.810)	0.920
Intrahepatic metastasis (positive vs negative)	1.628 (1.109-2.389)	0.013	1.612 (1.091-2.383)	0.017
Portal venous invasion (positive vs negative)	2.636 (1.741-3.991)	<0.001	2.156 (1.456-3.193)	<0.001
Microscopic vascular invasion (positive vs negative)	2.581 (1.254-5.315)	0.010	4.110 (2.394-7.055)	<0.001
Cirrhosis (positive vs negative)	1.909 (1.235-2.949)	0.004	1.745 (1.138-2.676)	0.011
Tumour encapsulation (yes vs no)	0.404 (0.249-0.654)	<0.001	0.461 (0.304-0.697)	<0.001
BCLC stage (C vs B vs A)	2.942 (1.947-4.444)	<0.001	1.827 (1.403-2.379)	<0.001
TNM (III+IV vs I+II)	2.719 (1.754-4.214)	<0.001	2.654 (1.763-3.995)	<0.001
Adjuvant TACE (yes vs no)	0.473 (0.304-0.735)	0.001	0.450 (0.303-0.668)	<0.001
Multivariate analysis				
Microscopic vascular invasion (positive vs negative)	NA		4.001 (2.276-7.034)	<0.001
Cirrhosis (positive vs negative)	1.694 (1.091-2.630)	0.019	NA	
Tumour encapsulation (yes vs no)	NA		NA	
BCLC stage (C vs B vs A)	2.885 (1.912-4.354)	<0.001	NA	
TNM (III+IV vs I+II)	NA		1.903 (1.249-2.899)	0.003
Adjuvant TACE (yes vs no)	0.408 (0.261-0.639)	<0.001	0.404 (0.268-0.608)	<0.001

Variables' *P* value less than 0.30 were brought into further multivariate analysis. **P* value less than 0.05 was considered statistically significant.

interval, CI: 25.2-39.1 versus 12.2-20.4, P< 0.001, **Figure 1A**). This finding was confirmed in the validation cohort (median OS for adjuvant TACE versus control group: 33.2 versus 17.4 months, 95% CI: 30.0-38.3 versus 12.8-22.1, P<0.001, **Figure 1B**). Additionally, Patients with low GS expression in both cohorts had longer 5-year disease-free survival (DFS) time in TACE groups than in control groups, but the differences were not significant (**Figure 1C** and **1D**). In contrast, patients with high GS expression in both cohorts did not have a significant response to adjuvant TACE in 5-year OS and DFS (**Figure 2A-D**).

Uni- and multivariate analysis of prognostic factors of 5-year OS in patients with low GS expression

We used Cox proportional-hazards regression to evaluate the effect of treatment on survival in patients of both cohorts who had low GS expression. In training cohort, as is shown in **Table 1**, univariate analysis revealed that largest tumor size, intrahepatic metastasis, portal venous invasion, microscopic vascular invasion, cirrhosis, encapsulation, BCLC stage, TNM stage and adjuvant TACE were statistically correlated with overall survival. The individual parameters were further subjected to multivariate Cox proportional hazards model, which demonstrated that adjuvant TACE, together with cirrhosis and BCLC stage was strongly associated with 5-year OS. Adjuvant TACE was an independent prognostic indicator for the 5-year OS of HCC patients with low GS expression (hazard ratio [HR] 0.408, 95% CI: 0.261-0.639, P<0.001). In the validation set, similar result was also observed. Ages, largest tumor size, intrahepatic metastasis, portal venous invasion, microscopic vascular invasion, cirrhosis, encapsulation, BCLC stage, TNM stage and adjuvant TACE were statistically correlated with overall survival by univariate analysis. Multivariate analysis showed that together with TNM stage and microscopic vascular invasion, adjuvant TACE was an independent predictors for 5-year OS in low GS expression patients (HR

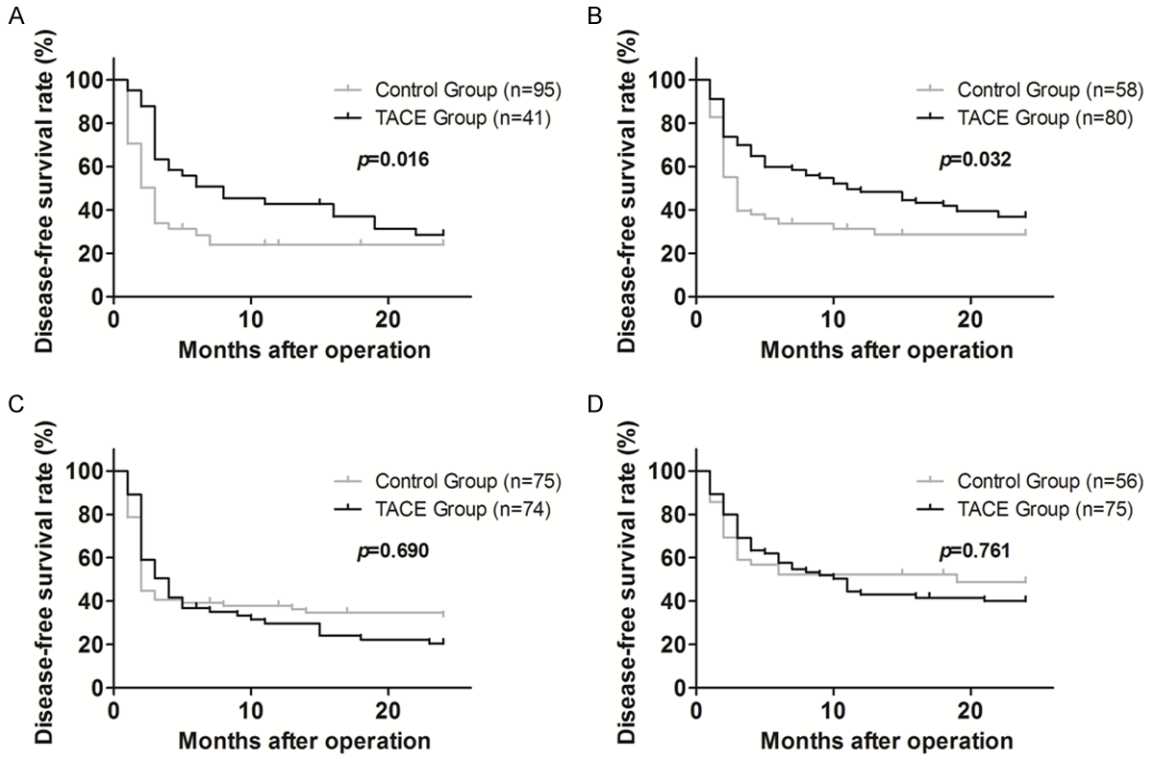

Figure 3. Adjuvant TACE decreased early phase recurrence in patients with low GS expression. The 2-year disease-free survival rate between adjuvant TACE and control group in patients with low GS expression were analyzed by Kaplan-Meier in training cohort (A) and validation cohort (B). The 2-year disease-free survival rate between adjuvant TACE and control group in patients with high GS expression were analyzed by Kaplan-Meier in training cohort (C) and validation cohort (D).

0.404, 95% CI: 0.268-0.608, P<0.001) (**Table 1**).

Adjuvant TACE decreased early phase recurrence in patients with low GS expression

Kaplan-Meier survival curves with comparisons of early (<2 years) phase recurrence in adjuvant TACE group versus control group in low GS expression of HCC patients in both cohorts are shown in **Figure 3**. In training cohort, patients with low GS expression in tumors had a significant improvement in 2-year DFS time after receiving postoperative adjuvant TACE, as compared with those without adjuvant TACE therapy (median DFS for adjuvant TACE versus control group: 11.7 versus 7.5 months, 95% CI: 8.8-14.6 versus 5.5-9.5, P=0.016, **Figure 3A**). We confirm the results in the validation cohort that TACE group has longer 2-year DFS time than control group in HCC patients with low GS expression (median DFS for adjuvant TACE versus control group: 13.0 versus 9.0 months, 95% CI: 10.9-15.1 versus 6.4-11.5, P=0.032, **Figure 3B**). Additionally, patients with high GS expression in both cohorts did not have a favorable response to adjuvant TACE in 2-year DFS (**Figure 3C** and **3D**).

We then used Cox proportional-hazards regression to evaluate the effect of TACE on 2-year DFS in the patients with low GS expression of both cohorts. In training cohort, univariate analysis revealed that HBsAg, largest tumor size, portal venous invasion, microscopic vascular invasion, cirrhosis, encapsulation, BCLC stage, TNM stage and adjuvant TACE were statistically correlated with 2-year DFS. Multivariate analysis showed that adjuvant TACE, together with HBsAg, largest tumor size, cirrhosis and BCLC stage was strongly associated with 2-year DFS. Adjuvant TACE was an independent prognostic indicator for the 2-year DFS of HCC patients with low GS expression (HR 0.592, 95% CI: 0.376-0.931, P=0.023) (**Table 2**). Similar result was conformed in the validation cohort. Multivariate analysis showed adjuvant TACE was an independent predictors for 2-year DFS in low

Table 2. Univariate and multivariate Cox regression analyses GS for 2-year DFS of patients with low GS expression in the training and validation cohort

Variables	Training cohort		Validation cohort	
	Hazard ration (95% CI)*	*P* Value	Hazard ration (95% CI)*	*P* Value
Univariate analysis				
Age (>50 years vs ≤50 years)	0.969 (0.645-1.454)	0.879	0.857 (0.563-1.303)	0.469
Gender (male vs female)	0.606 (0.315-1.168)	0.135	1.228 (0.566-2.664)	0.603
HBs Ag (positive vs negative)	2.630 (1.147-6.031)	0.022	1.152 (0.579-2.294)	0.687
Serum AFP (>400 ng/ml vs ≤400 ng/ml)	1.173 (0.743-1.852)	0.494	1.772 (1.102-2.851)	0.018
Largest tumor size (>5 cm vs ≤5 cm)	2.031 (1.128-3.656)	0.018	2.043 (1.249-3.342)	0.004
Tumor number (multiple vs single)	1.415 (0.616-3.254)	0.413	0.916 (0.487-1.722)	0.784
Intrahepatic metastasis (positive vs negative)	1.349 (0.901-2.018)	0.146	1.365 (0.900-2.070)	0.143
Portal venous invasion (positive vs negative)	1.614 (1.064-2.451)	0.024	2.079 (1.365-3.167)	0.001
Microscopic vascular invasion (positive vs negative)	2.657 (1.228-5.751)	0.013	3.673 (2.060-6.550)	<0.001
Cirrhosis (positive vs negative)	1.666 (1.068-2.601)	0.025	1.770 (1.112-2.818)	0.016
Tumour encapsulation (yes vs no)	0.510 (0.313-0.833)	0.007	0.491 (0.316-0.766)	0.002
BCLC stage (C vs B vs A)	1.684 (1.145-2.479)	0.008	1.871 (1.407-2.488)	<0.001
TNM (III+IV vs I+II)	1.742 (1.127-2.695)	0.013	2.714 (1.746-4.220)	<0.001
Adjuvant TACE (yes vs no)	0.615 (0.394-0.960)	0.032	0.654 (0.429-0.996)	0.048
Multivariate analysis				
HBs Ag (positive vs negative)	3.570 (1.535-8.305)	0.003	NA	
Largest tumor size (>5 cm vs ≤5 cm)	1.941 (1.066-3.536)	0.030	NA	
Microscopic vascular invasion (positive vs negative)	2.323 (1.036-5.205)	0.041	3.166 (1.742-5.754)	<0.001
Tumour encapsulation (yes vs no)	0.591 (0.353-0.990)	0.046	NA	
TNM (III+IV vs I+II)	NA		2.095 (1.332-3.296)	0.001
Adjuvant TACE (yes vs no)	0.592 (0.376-0.931)	0.023	0.644 (0.419-0.990)	0.045

Variables' *P* value less than 0.30 were brought into further multivariate analysis. **P* value less than 0.05 was considered statistically significant.

GS expression patients (HR 0.644, 95% CI: 0.419-0.990, P=0.045) (**Table 2**).

Discussion

We analyzed in HCC patients the association of GS expression levels and response to adjuvant TACE therapy in two independent cohorts. A significant interaction was observed between GS expression and adjuvant TACE therapy with respect to the effect on survival. Patients whose tumors had low GS expression were more likely to have a response to TACE, as compared with patients of high GS expression. In both univariate and multivariate analyzes, adjuvant TACE was associated with significant improvement in overall survival and early phase recurrence in patients with low GS expression. Thus, GS expression might be a predictor of the response to postoperative adjuvant TACE.

The incidence of HCC recurrence after curative resection is to 70% at 5 years because of the dissemination of microscopically viable tumor remnants or de novo tumors [29, 30]. In general, tumor remnants and intrahepatic metastasis account for early (<2 years) phase recurrence, whereas, de novo tumors from multicentric carcinogenesis are contributing to late (>2 years) phase recurrence [31, 32]. Due to the poor prognosis, attempts have been made to develop adjuvant therapies to reduce recurrence rates. As an adjuvant therapy, TACE may have some effects in preventing postoperative recurrence, however, the effectiveness still remains uncertain. Some studies suggest that TACE may suppress recurrence and prolong survival while others don't support the benefits of adjuvant TACE to patients with resectable HCC [6, 7, 9-12]. Additionally, due to the toxicity and expense, it is meaningful to find the appropriate patients who would be likely to benefit from postoperative TACE therapy.

In the present study, we discussed the roles of GS in HCC patients receiving TACE after hepatic resection. We found that, the patients with low GS expression in their tumor samples have significantly improved overall survival when treated with postoperative adjuvant TACE, indicating

that GS might be an effective biomarker to select HCC patients for adjuvant TACE therapy. Compared with those without TACE therapy, these patients with postoperative adjuvant TACE have less early phase recurrence and prolonged survival. The better overall survival might result from the lower early phase recurrence rates. While, in the patients with high GS expression, it did not show significant difference among the treatments with or without TACE in both survival and recurrence, suggesting that high expression of GS identified by postoperative immunostaining might be an early warning sign that the patients should receive other appropriate adjuvant therapies due to the invalid effect of postoperative adjuvant TACE for these patients. It has become increasingly common in cancer management that using molecular diagnostics to detect variations of specific genes or signatures in order to guide targeted therapies, such as HER2 gene amplification test in breast cancer, KRAS and NRAS mutation test in colorectal cancer and BRAF mutation test in melanoma and colorectal cancer [33-35]. However, biomarker-guided therapy for HCC patients is still not available in current clinical treatment. To our best knowledge, it is the first time to propose that GS might be a useful marker to stratify patients with respect to prognosis and response to postoperative adjuvant TACE therapy.

GS catalyzes the synthesis of glutamine, which is the crucial energy source for the growth of both neoplastic and normal cells, and the upregulation of GS is related to higher tumor growth and proliferation [36]. It has been proved that the metabolic pathway, including glutamine, glutamine synthetase, and glutamate, has played an increasing role in the growth and metabolism of tumor [37]. So, we believe that there is a plausible biochemical explanation of the favorable prognostic value of GS deficiency in HCC patients receiving postoperative adjuvant TACE.

The mechanisms behind the sensitivity of tumors with low-GS expression to postoperative TACE are unclear. We surmised that, in tumor tissues with low GS expression, for the decreased ability to synthesize glutamine, the cancer cells should obtain essential glutamine from blood supply. Once TACE performed, acquisition of glutamine dropped because of the arterial embolism and the cancer cells could not maintain the metabolism for the lack of glutamine. While in the tumor tissue with high GS expression, glutamine could be synthesized by the cancer cells themselves and did not entirely rely on blood supply. This might be an explanation of the different response to TACE therapy. Furthermore, there is still no direct evidence about the relationship between GS and drug resistance. The hypotheses require evaluation and the mechanism needs further investigations.

In conclusion, we observed the expression of GS in HCC patients and the association of GS expression levels with survival and response to therapy with TACE. Tumors with low GS had a favorable response to adjuvant TACE therapy. GS might be a useful marker to stratify patients with respect to response to postoperative adjuvant TACE therapy.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Clinical characteristics of the enrolled patients in both cohorts

Classification	Training cohort (n=285)	Validation cohort (n=269)
Age (years)*	49.6±10.6	52.1±9.5
Sex: male/female (n/n)	252/33	242/27
HBsAg positive [n (%)]	248 (87.0)	240 (89.2)
Serum AFP [n (%)]		
≤400 (ng/ml)	88 (30.9)	89 (33.1)
>400 (ng/ml)	197 (69.1)	180 (66.9)
Tumor size (cm)*	9.0±4.2	7.5±3.9
Tumor number [n (%)]		
Single	260 (91.2)	230 (85.5)
Multiple	25 (8.8)	39 (14.5)
Intrahepatic metastasis [n (%)]	131 (46.0)	142 (52.8)
Portal venous invasion [n (%)]	164 (57.5)	100 (37.2)
Microscopic vascular invasion [n (%)]	252 (88.4)	187 (69.5)
Cirrhosis [n (%)]	202 (70.9)	175 (65.1)
Tumor encapsulation [n (%)] yes/no (n/n)	105 (36.8)	130 (48.3)
BCLC stage [n (%)]		
A	11 (3.9)	83 (30.9)
B	100 (35.1)	88 (32.7)
C	174 (61.0)	98 (36.4)
TNM stage [n (%)]		
I/II	104 (36.5)	136 (50.6)
III/IV	181 (63.5)	133 (49.4)
GS staining: high/low (n/n)	149/136	131/138
Adjuvant TACE: yes/no (n/n)	115/170	155/114

*The data are presented as means and standard deviations.

Supplementary Table 2. Baseline of clinicopathological characteristic in training cohort

Characteristics	Low GS expression		*P* value	High GS expression		*P* value
	Control Group (n=95)	TACE Group (n=41)		Control Group (n=75)	TACE Group (n=74)	
Age (years)*	48.3±9.7	50.6±9.8	0.201	50.9±12.2	49.2±10.2	0.362
Gender			0.695			0.593
Male	81	36		67	68	
Female	14	5		8	6	
HBsAg			0.378			0.374
Negative	9	6		13	9	
Positive	86	35		62	65	
Serum AFP			0.627			0.455
≤400 (ng/ml)	24	12		24	28	
>400 (ng/ml)	71	29		51	46	
Largest tumor size (cm)*	9.4±3.6	8.5±4.5	0.202	9.1±4.2	8.8±4.7	0.735
Tumor number			0.697			0.457
Single	90	38		65	67	
Multiple	5	3		10	7	
Intrahepatic metastasis			0.998			0.800
Negative	51	22		40	41	
Positive	44	19		35	33	
Portal venous invasion			0.671			0.219
Negative	38	18		29	36	
Positive	57	23		46	38	
Microscopic vascular invasion			1.000			0.819
Negative	11	5		9	8	
Positive	84	36		66	66	
Cirrhosis			0.235			0.743
Negative	15	10		20	18	
Positive	80	31		55	56	
Tumour encapsulation			0.608			0.797
No	69	28		41	42	
Yes	26	13		34	32	
BCLC stage			0.765			0.187
A	3	1		5	2	
B	31	16		22	31	
C	61	24		48	41	
TNM stage			0.550			0.281
I/II	32	16		25	31	
III/IV	63	25		50	43	

*The data are presented as means and standard deviations. *P* values are derived from two-tailed tests.

Supplementary Table 3. Baseline of clinicopathological characteristics in validation cohort

Characteristics	Low GS expression		*P* value	High GS expression		*P* value
	Control Group (n=58)	TACE Group (n=80)		Control Group (n=56)	TACE Group (n=75)	
Age (years)*	51.0±10.1	51.6±9.4	0.708	51.5±10.3	53.8±8.6	0.172
Gender			0.761			0.531
Male	54	73		48	67	
Female	4	7		8	8	
HBsAg			0.700			0.993
Negative	7	8		6	8	
Positive	51	72		50	67	
Serum AFP			0.974			0.294
≤400 (ng/ml)	19	26		16	28	
>400 (ng/ml)	39	54		40	47	
Largest tumour size (cm)*	8.4±4.0	7.4±3.9	0.121	7.6±4.3	6.8±3.7	0.247
Tumour number			0.772			0.638
Single	51	69		48	62	
Multiple	7	11		8	13	
Intrahepatic metastasis			0.435			0.108
Negative	28	44		28	27	
Positive	30	36		28	48	
Portal venous invasion			0.895			0.126
Negative	34	46		34	55	
Positive	24	34		22	20	
Microscopic vascular invasion			0.491			0.986
Negative	15	25		18	24	
Positive	43	55		38	51	
Cirrhosis			0.671			0.175
Negative	19	29		16	30	
Positive	39	51		40	45	
Tumour encapsulation			0.828			0.324
No	33	47		28	31	
Yes	25	33		28	44	
BCLC stage			0.311			0.157
A	12	24		18	19	
B	23	23		15	27	
C	23	33		23	19	
TNM stage			0.344			0.165
I/II	25	41		26	44	
III/IV	33	39		30	31	

*The data are presented as means and standard deviations. *P* values are derived from two-tailed tests.