Expression level of glutamine synthetase is increased in hepatocellular carcinoma and liver tissue with cirrhosis and chronic hepatitis B

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Abstract Studies have suggested that glutamine synthetase (GS) is a potential marker of hepatocellular carcinoma (HCC). We aimed to evaluate the expression of GS in non-malignant liver tissue and serum GS levels in HCC, liver cirrhosis (LC), chronic hepatitis B (CHB), five kinds of extrahepatic diseases patients and healthy subjects. Immunohistochemistry (IHC) was used to assess GS expression in 260 liver tissue samples (from 120 HCC, 90 CHB stage 4, and 50 CHB stage 1–3 patients). Enzyme-linked immunosorbent assays of 325 samples (from 100 healthy donors, 33 CHB stage 1–3, 43 CHB stage 4, 111

HCC, and 45 extrahepatic diseases patients) were used to further analyze GS levels in serum. IHC studies showed the expression of GS in 70% of HCC patients, 46.7% of CHB stage 4 patients and 38% of CHB stage 1–3 patients. The χ^2 tests showed significant difference between HCC samples and non-tumor tissues ($P = 0.001$ for HCC vs. CHB stage 4, $P = 0.000$ for HCC vs. CHB). Consistent with this, serum GS levels are increased in HCC and CHB stage 1–4 patients. There are significant differences among all samples ($P = 0.000$ for all), except CHB stage 1–3 versus CHB stage 4 ($P = 0.552$). Based on multiple linear regressions, HCC, CHB stage 1–4 and AFP were significantly associated with serum GS levels. In addition, in HCC group, TNM and Child-Pugh were significantly associated with GS levels. Expression of GS is increased in HCC, LC, and CHB. It may be a new serum marker for liver disease.

Keywords Glutamine synthetase · Hepatocellular carcinoma · Liver cirrhosis · Chronic hepatitis B · Regeneration · Receiver operating characteristic curve · Sensitivity and specificity

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Abbreviations

HCC	Hepatocellular carcinoma
GS	Glutamine synthetase
LC	Liver cirrhosis
CHB	Chronic hepatitis B
IHC	Immunohistochemistry
ELISA	Enzyme-linked immunosorbent assay
PBS	Phosphate-buffered saline
SI	Staining intensity
ROC	Receiver operating characteristic
AUC	Areas under the curve

Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths in Asia. In China, because of the high prevalence of chronic hepatitis B and hepatitis B cirrhosis infections, HCC became the second-most common cause of cancer-related death in the 1990s [1]. At present, only a few biomarkers are available for the diagnosis and monitoring of HCC. When considering sensitivity, specificity, and operability, α -fetoprotein (AFP) remains the best available marker for diagnosis of HCC clinically, although it does not fulfil the requirements of an ideal tumor marker [2]. Asymptomatic patients with early HCC who have negative serum markers (AFP and PIVKA-II) are usually not diagnosed until the advanced stage. Early diagnosis of HCC could greatly improve the outcome of surgical treatments for HCC, with better long-term survival and reduced recurrence risk [3]. Therefore, search for effective markers for HCC is a field of intense research.

Glutamine synthetase (GS) is a member of an enzyme family that catalyzes the synthesis of glutamine from glutamate and ammonia. It plays an important role in ammonia detoxification, nitrogen balance and pH regulation in the liver [4–6]. Christa et al. [7] demonstrated that levels of GS mRNA, GS protein, and GS activity are upregulated in human HCCs. Kuo [8] described earlier an equal presence of total GS mRNA in total liver mRNA late in fetal life and in adult life. Using immunohistochemistry (IHC) of HCC tissue, Di Tommaso et al. [9] further identified the overexpression of GS in HCC and proposed GS as a useful biomarker for early diagnosis and prognosis in HCC patients. However, it was not known whether GS expression is also increased in chronic hepatitis B (CHB) and liver cirrhosis (LC) patients, and whether serum GS levels could differentiate HCC from LC, CHB, colorectal carcinoma, lung carcinoma, breast carcinoma, cerebral vascular accident, and myositis. The aims of this study were to investigate GS expression in the tissue of HCC and CHB stage 1–4 patients, determine serum GS levels in HCC, CHB stage 1–4, colorectal carcinoma, lung carcinoma, breast carcinoma, cerebral vascular accident, myositis, and healthy subjects, and assess the possibility of GS as a serum marker for diagnosing HCC compared with α -fetoprotein (AFP).

Materials and methods

The following points can be discussed under the above head:

Liver tissue specimens, serum specimens, and sample preparation

For IHC staining study, all liver tissue samples were collected from Beijing Youan Hospital and Chaoyang Hospital. Tissues of 36 patients with LC and 33 patients with CHB were biopsied, and the others were resected. Tissue sample was fixed in formalin and embedded in paraffin for routine histological examination.

Blood samples were obtained from 111 patients with HCC (Beijing Youan Hospital and Beijing Chaoyang Hospital), 36 patients with LC (Beijing Youan Hospital; 26 with Child-Pugh class A and 10 with Child-Pugh class B), 33 patients with CHB (Beijing Youan Hospital), 9 patients with colorectal carcinoma, 11 patients with lung carcinoma, 10 patients with breast carcinoma, 8 patients with cerebral vascular accident, 7 patients with myositis, and 100 healthy blood donors (the last six groups were from Beijing Friendship Hospital), with signed consent from the patients. The serum was immediately separated by centrifugation and frozen at -80°C . Sera from patients who were diagnosed with non-malignant liver disease (LC and CHB) at the time of serum collection were included in this study only if there was no evidence of malignant disease between enrollment and the start of this study.

Immunohistochemistry staining

Paraffin sections (5 μm) were deparaffinized with xylene and rehydrated in a graded alcohol series. Antigen retrieval was performed with a microwave oven in 1 mM EDTA solution, pH 8.0 (Zhongshan Golden Bridge Biotechnology, China). The activity of endogenous peroxidase was blocked by incubation in 3% H_2O_2 at room temperature for 10 min. After thoroughly washing with PBS, the slides were incubated with the primary antibody, polyclonal GS (1:400, Abcam) and PCNA (1:100, Zhongshan Golden Bridge Biotechnology), at 37°C for 2 h in a moist chamber. Following thorough washing with PBS, the slides were incubated with either the polymer Helper or poly-HRP anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology, China) for 20 min at 37°C . Brown signals were visualized with a 3,3'-diaminobenzidine tetrahydrochloride substrate kit (DAB kit, Zhongshan Golden Bridge Biotechnology, China); red signals were visualized with a 3-amino-9-ethylcarbazole substrate kit (AEC kit, Maixin Biotechnology, China). After counterstaining with hematoxylin, the slides were dehydrated and mounted for viewing with a microscope.

Enzyme-linked immunosorbent assay

GS levels in parallel serum samples were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Groundwork Biotechnology Diagnostic, Canada). AFP levels in parallel serum samples were determined using a commercially available ELISA kit (CanAg, Sweden).

Statistical analysis

Data were evaluated statistically using SPSS version 13.0 (SPSS Company). Quantitative data were summarized as means (SD). Categorical variables were reported as percentages. The χ^2 test was used to define significant differences among the pathological samples. The Kruskal–Wallis test was used to determine significant differences between the groups for GS in sera. Two diagnostic cutoff values for GS were set with two standard deviations above the mean of the healthy control and CHB. The cutoff value considered positive for AFP was 20 mg/ml, as described previously [10]. Multiple linear regression analysis was used to evaluate the relationship between serum GS levels and diagnosis of HCC, CHB stage 1–4, AFP levels, and various clinical variables, including sex, age, Child–Pugh stage, and TNM cancer stage. Logistic regression analysis was used to assess the efficacy of GS and AFP in differentiating HCC from CHB stage 4, HCC from healthy controls, and HCC from CHB stage 1–4 patients. $P \leq 0.05$ was set as a significant threshold.

Results

The result can be discussed under the following heads:

Clinical characteristics of HCC, CHB stage 1–4 patients and healthy controls

The demographic characteristics of the patients and controls are summarized in Tables 1 and 2. All cirrhosis is related to HBV infection. The group of patients with CHB were classified as four stages, and LC patients were classified into CHB stage 4. Patients with HCC were slightly older than CHB stage 1–4 patients and controls, and the proportion of male subjects was higher in HCC patients. The serum AFP level was significantly higher in HCC patients as compared with CHB stage 1–4 patients and healthy controls ($P = 0.000$ for HCC vs. CHB stage 1–4 and controls).

Expression of GS in HCC and non-malignant liver tissues by immunohistochemistry

IHC staining of GS was performed on paraffin sections that contained tumor tissue from 120 HCC patients, and liver tissue from 90 CHB stage 4 patients, and 50 CHB stage 1–3 patients. GS has been shown by IHC and in situ hybridization to sublocate in a small population of hepatocytes (<8% of total hepatocytes) surrounding the terminal hepatic venules in human liver [11–13]. Cases were semi-quantitatively evaluated by two of us and semi-quantitatively scored according to the number of immunoreactive (IR) cell. Individual cases were considered IR when more than 5% of cells were IR. Positive signal for GS was in the cytoplasm and for PCNA was in the nucleus. Immunoreactive tissues were further subclassified as follows: + = 5–10% IR cells (low expresser, LE); ++ = 11–50% IR cells (intermediate expresser, IE); +++ $\geq$ 50% IR cells (high expresser, HE). We also evaluated the staining intensity (SI) of GS as faint, moderate, and strong, with the latter being detectable in control pericentral and periseptal hepatocytes [4]. Three sections from each tissue sample were evaluated for GS and PCNA expression.

The positive staining rate of GS was 38% (19 of 50) in CHB (stage 1–3) samples, 46.7% (42 of 90) in CHB (stage 4) (Fig. 1a) and 70% (84 of 120) in tumor tissues (Fig. 1b). HCC tissue showed diffuse and strong nuclear immunoreactivity for PCNA (Fig. 1c). Furthermore, it is noteworthy that most non-tumor tissues (56 of 61) showed positive cytoplasmic immunoreactivity for GS accompanied by nuclear immunoreactivity for PCNA (Fig. 1d). In non-malignant liver diseases patients, GS overexpression was restricted to 11–50% hepatocytes, whereas the majority of HCC showed diffuse immunostaining (>50% IR cells). GS immunoreactivity did not correlate with tumor dedifferentiation (Table 3). The χ^2 tests showed significant expression differences between HCC and non-tumor tissues ($P = 0.001$ for HCC vs. CHB stage 4; $P = 0.000$ for HCC vs. CHB stage 1–4), but no significant difference between CHB stage 1–3 and stage 4 ($P = 0.322$).

Serum levels of GS in HCC, non-malignant liver patients, colorectal carcinoma, lung carcinoma, breast carcinoma, cerebral vascular accident, myositis, and control subjects

The serum GS levels in 100 healthy individuals, 9 colorectal carcinoma patients, 11 lung carcinoma patients, 10 breast carcinoma patients, 8 cerebral vascular accident

Table 1 Clinical characteristics of HCC, LC, and CHB patients, and healthy controls

Characteristics	HCC (<i>n</i> = 111)	CHB (<i>n</i> = 69)		Control (<i>n</i> = 100)
		Stage 1–3 (<i>n</i> = 26)	Stage 4 (<i>n</i> = 43)	
Sex, male (%)	82 (73.9%) ^c	15 (57.7%)	28 (65.1%)	63 (63%)
Mean age (SD)	54.8 (10.5) ^{b,d}	40.8 (11.7)	50 (15.1)	32.3 (10.9)
AFP (mg/ml) ^a	33.7 (6.6–368.8) ^d	4.9 (2.8–7.3) ^{e,f}	6.7 (3.7–29.6)	2.9 (2.1–4.6)

LC were classified as CHB stage 4

^a AFP serum level is expressed as the median (interquartile range)

^b $P = 0.024$ for HCC versus CHB stage 4 (age)

^c $P = 0.548$ for HCC versus all other groups

^d $P = 0.000$ for HCC versus CHB stage 1–3 and HCC versus control (age)

^d $P = 0.000$ for HCC versus CHB stage 1–3, CHB stage 4 and Control, and CHB stage 4 versus control (AFP)

^e $P = 0.114$ for CHB stage 1–3 versus CHB stage 4 (AFP)

^f $P = 0.001$ for CHB stage 1–3 versus control (AFP)

Table 2 Clinical characteristics of colorectal carcinoma, lung carcinoma, breast carcinoma, cerebral vascular accident, and myositis

Characteristics	Colorectal carcinoma (<i>n</i> = 9)	Lung carcinoma (<i>n</i> = 11)	Breast carcinoma (<i>n</i> = 10)	Cerebral vascular accident (<i>n</i> = 8)	Myositis (<i>n</i> = 7)
Sex, male (%)	7 (77.8%)	8 (72.7%)	10 (100%)	5 (62.5%)	4 (57.1%)
Mean age (SD)	61.4 (11.1)	64.2 (8.9)	56.7 (14.2)	60.4 (23.0)	29.4 (14.0)

Fig. 1 IHC analysis of GS and PCNA: **a** periportal cytoplasmic immunoreactivity of CHB stage 4 tissue in a diffuse pattern with inflammation and necrosis for GS (magnification $\times 200$), **b** HCC tissue showing diffuse and strong cytoplasmic immunoreactivity for GS (magnification $\times 200$), **c** HCC tissue showing diffuse and strong nuclear immunoreactivity for PCNA (magnification $\times 200$), **d** liver tissue with CHB showing positive cytoplasmic immunoreactivity for GS accompanied by nuclear immunoreactivity for PCNA (magnification $\times 200$) (reviewer 2, question 3)

patients, 7 myositis patients, 43 CHB (stage 4) patients, 26 CHB (stage 1–3) patients, and 111 HCC patients (organized by cancer stage) are summarized in Table 4 and Fig. 2. Statistical analysis showed that the distribution of serological levels of GS in HCC, CHB (stage 1–3), and

CHB (stage 4) patients is significantly higher than in controls ($P = 0.000$ for all). There are significant differences between all of them ($P < 0.05$ for all), except for HCC (stage I) versus CHB (stage 4) ($P = 0.131$); for CHB stage 1–3 versus CHB stage 4 ($P = 0.552$), and for HCC

Table 3 Immunohistochemical results in HCC and CHB patients

Staining intensity	CHB (<i>n</i> = 140)						HCC (<i>n</i> = 120)								
	S _{1–3} (<i>n</i> = 50)			S ₄ (<i>n</i> = 90)			G ₁ (<i>n</i> = 46)			G _{2–3} (<i>n</i> = 59)			G ₄ (<i>n</i> = 15)		
	Number of IR			Number of IR			Number of IR			Number of IR			Number of IR		
	+	++*	+++*	+	++*	+++*	+	++*	+++*	+	++*	+++*	+	++*	+++*
1+	13	10	6	12	21	10	3	6	4	1	7	4	0	1	2
2+*	2	12*	4*	3	22*	4*	1	9*	7*	0	6*	8*	0	4*	2*
3+*	0	3*	0*	2	15*	1*	0	1*	15*	6	7*	20*	1	3*	2*

LC liver cirrhosis, CHB chronic hepatitis B, IR immunoreactive cases

* indicate intermediate expresser or high expresser for immunoreactive tissues, and moderate or strong for evaluation the staining intensity
Staining intensity: 1+, faint; 2+, moderate; 3+, strong; +, number of cases with 5–10% immunoreactive cells; ++, number of cases with 11–50%; +++, number of cases with >50% immunoreactive cells. LC patients were classified as CHB, stage 4

versus CHB (stage 1) ($P = 0.073$); $P = 0.011$ for colorectal carcinoma versus control; $P > 0.05$ for control versus lung carcinoma, breast carcinoma, cerebral vascular accident, and myositis, respectively.

The results of multiple linear regression analysis with serum GS level as the response variable are summarized in Table 5. HCC, CHB, and AFP were significantly associated with serum GS levels. In HCC patients, TNM and

Child-Pugh (indicators of cancer progression) were significantly associated with GS levels.

Evaluation of GS and AFP as markers of HCC

We also measured serum AFP levels in the sample set. AFP values were normal in healthy donors but elevated in 7 out

Table 4 Serum GS levels of patients and controls (mg/ml)

Characteristic	Mean	SD	Median	Interquartile range
HCC (<i>n</i> = 111)	29.2	29.1	18.0	4.7–49.6
Stage I (<i>n</i> = 9)	11.9	15.1	6.8	2.8–15.5
Stage II (<i>n</i> = 59)	18.4	22.1	12.4	0.8–31.0
Stage III (<i>n</i> = 30)	37.3	22.9	35.9	16.0–54.0
Stage IV (<i>n</i> = 13)	72.6	30.4	69.8	56.9–101.3
CHB (<i>n</i> = 69)	7.6	13.8	2.6	1.2–5.6
Stage 1 (<i>n</i> = 6)	8.7	13.7	4.0	1.3–13.4
Stage 2 (<i>n</i> = 9)	3.4	4.2	2.6	0.6–4.7
Stage 3 (<i>n</i> = 11)	6.1	8.3	2.6	1.8–6.9
Stage 4 (<i>n</i> = 43)	8.8	16.1	2.8	1.3–7.7
Colorectal carcinoma (<i>n</i> = 9)	0.8	0.7	0.8	0–1.4
Lung carcinoma (<i>n</i> = 11)	0.5	0.8	0.2	0–0.8
Breast carcinoma (<i>n</i> = 10)	0.6	1.0	0	0–1.4
Cerebral vascular accident (<i>n</i> = 8)	0.1	0.2	0	0–0
Myositis (<i>n</i> = 7)	0	0	0	0–0
Control (<i>n</i> = 100)	0.3	0.8	0	0–0.3

LC patients were classified as CHB stage 4

$P = 0.758$ for stage I HCC versus stage II; $P = 0.001$ for stage III versus stage IV, and stage I versus stage III; $P = 0.000$ for stage II versus stage III, stage II versus stage IV, and stage I versus stage IV

$P = 0.748$ for different stage among CHB; $P = 0.552$ for CHB stage 1–3 versus CHB stage 4; $P = 0.000$ for control versus CHB (stage 1–3) and CHB (stage 4); $P = 0.131$ for HCC (stage 1) versus CHB (stage 4)

$P = 0.000$ for HCC versus control, HCC versus CHB (stage 4) and HCC versus CHB; $P = 0.073$ for HCC versus CHB (stage 1); $P = 0.003$ for HCC versus CHB (stage 2); $P = 0.006$ for HCC versus CHB (stage 3)

$P = 0.011$ for colorectal carcinoma versus control, $P > 0.05$ for control versus lung carcinoma, breast carcinoma, cerebral vascular accident, and myositis

Fig. 2 Box plot of serum GS levels: CHB chronic hepatitis B; LC patients were classified as CHB stage 4; HCC hepatocellular carcinoma; CVA cerebral vascular accident

Table 5 Multiple linear regression analysis of predictors of serum GS level

Factor	Regression coefficient	P
Overall (n = 280)		
Age (years)	−0.036	0.705
Sex (male vs. female)	−0.664	0.779
AFP	0.001	0.000
HCC (n = 211) ^a	8.334	0.000
HCC (n = 111)		
TNM stage 1–2 versus stage 3–4	20.354	0.000
Child-Pugh 1–2 versus 3–4	31.438	0.000
CHB (n = 169) ^a	8.613	0.000
Stage 1–3 (n = 126) ^a	10.919	0.000
Stage 4 (n = 143) ^a	6.985	0.000

^a Healthy control as the reference group

LC patients were classified as CHB stage 4

of 43 CHB (stage 4) patients (24.5–278.9 mg/ml) and 2 out of 26 CHB (stage 1–3) patients (28.2–51.1 mg/ml). Logistic regression analysis showed that both GS and AFP are predictors of differentiation between HCC versus non-HCC ($P = 0.01$ for GS and $P = 0.029$ for AFP). GS, but not AFP, was a predictor of differentiation between HCC versus healthy controls ($P = 0.000$ for GS and $P = 0.653$ for AFP). Neither GS nor AFP was a predictor of differentiation between HCC versus CHB stage 4 ($P = 0.127$ for GS and $P = 0.110$ for AFP). The results of a receiver operating

characteristic (ROC) analysis of the diagnostic performance of GS and AFP, and a combination of both markers are summarized in Table 6 and Fig. 3. AFP was more effective than GS in differentiating between HCC versus CHB stage 1–4 patients, HCC versus CHB stage 4, and HCC versus non-HCC. In contrast, GS was more effective than AFP in differentiating between HCC versus healthy controls. Although the simultaneous use of the two markers can increase the sensitivity between HCC versus CHB (stage 4) and HCC versus CHB stage 1–4, it cannot increase the efficiency of diagnosis. In the HCC group, elevated GS values correlate with AFP values (Pearson correlation coefficient for GS and AFP values = 0.404, $P = 0.000$). Using a cutoff value of 1.9 mg/ml, GS is elevated in 1 out of 11 lung carcinoma patients (2.2 mg/ml, 9.1%) and 1 out of 10 breast carcinoma (2.96 mg/ml, 10%). In addition, using a cutoff value of 35.2 mg/ml, GS is elevated in 4 out of 69 CHB patients (5.8%), 6 out of 26 HCC patients ($20 < \text{AFP} \leq 100$ mg/ml) (23.1%) and 5 out of 7 HCC patients ($100 < \text{AFP} \leq 200$ mg/ml) (71.4%), respectively.

Correlation of serum GS level with IHC staining

Owing to the fact that the serum samples (Fig. 2) are from the same subsets of tissue samples (Fig. 1), we compared the correlation of serum GS level with IHC staining. In HCC and CHB stage 1–4 groups, serum GS values correlate with immunohistochemical IR (Pearson correlation coefficient for GS and immunohistochemical IR = 0.550, $P = 0.000$).

Table 6 Properties of ROC curves and sensitivity/specificity of GS and AFP

Comparison	AUC (95% CI)			
	GS	AFP	Combined	
HCC versus healthy control	0.918 (0.876–0.959)	0.901 (0.861–0.942)	0.914 (0.871–0.957)	
HCC versus CHB stage 4	0.725 (0.642–0.809)	0.758 (0.682–0.833)	0.725 (0.639–0.811)	
HCC versus CHB stage 1–4	0.739 (0.665–0.813)	0.780 (0.713–0.846)	0.741 (0.668–0.814)	
HCC versus non-HCC	0.845 (0.793–0.896)	0.852 (0.804–0.900)	0.783 (0.727–0.839)	
Sensitivity/specificity (%)				
	GS	AFP	In parallel	In series
HCC versus healthy control	82.9/98.0	62.2/99	83.8/99	61.3/100
HCC versus CHB stage 4	72.1/74.4	66.7/79.1	83.8/37.2	61.3/83.7
HCC versus CHB stage 1–4	72.1/76.8	68.5/81.2	82.9/37.7	61.3/87
HCC versus non-HCC	82.9/73.4	62.2/94.1	83.8/72.8	61.3/94.7

Cutoffs for GS and AFP set at 1.9 mg/ml and 20 mg/ml, respectively. LC patients were classified as CHB stage 4

AUC Areas under the curve, CI confidence interval

Fig. 3 ROC curves for GS and AFP: **a** HCC versus healthy controls; **b** HCC versus non-malignant controls (healthy and CHB stage 1–4); **c** HCC versus CHB stage 1–4; **d** HCC versus CHB (stage 4). LC patients were classified as CHB stage 4

Discussion

To our knowledge, this is the first study to systematically analyze GS expression in CHB, LC, and HCC patients using IHC, and investigate serum GS levels as a marker of

differentiation among HCC, LC, CHB, colorectal carcinoma, lung carcinoma, breast carcinoma, cerebral vascular accident, myositis patients, and healthy subjects. Because all cirrhosis is due to HBV infection, LC patients were classified as CHB, stage 4. The finding that GS

overexpression is associated with necroinflammation and regeneration in the liver tissue of CHB stage 1–4 patients, and that serum GS levels are also increased in HCC and CHB stage 1–4 patients is novel and has not been reported to date.

The finding that GS expression is increased to different degrees in HCC and CHB patients has important implications. GS is highly correlated with HCC carcinogenesis and its progression. In addition, GS is closely associated with necroinflammation and regeneration in CHB stage 1–4 patients. This has further implications for the direction of future research. CHB is risk factor for the development of HCC; HBV incites chronic injury to hepatocytes, with continuous necroinflammation and regeneration activity, and increased hepatocyte turnover. The ultimate outcome of this is the accumulation of potential critical mutations in the hepatocyte genome, with subsequent malignant transformation and clonal expansion, leading to HCC [14]. Further study of the role of GS in CHB should focus on the mechanisms sustaining chronic inflammation and the promotion of subsequent malignant transformation.

In this study, the level of GS in stage I and stage II HCC is significantly lower than in stage III and stage IV HCC. This observation indicates that GS correlates with HCC progression and thus could be a reliable prognostic marker for HCC; this is consistent with the opinion of Di Tommaso et al. [9].

Medina MA et al. [15] noticed that the serum concentration of glutamine is decreased in advanced cancers, especially in the parenchyma of cirrhotic liver in HCC patients. Glutamine is the end product of GS activity, which is the main energy source of tumor cells [16–18]. It is reasonable to hypothesize that increased GS might be the result of glutamine deficiency. As a chief component of the canonical Wnt signalling pathway, β -catenin is implicated in a variety of cancers, including HCC. The GS gene, the target gene of β -catenin, plays a crucial role in protein metabolism in the liver or in other tissues [19]. Cieply et al. [20] noticed that the overexpression of GS is highly correlated with β -catenin mutation and proposed GS as an overall reliable marker of β -catenin activation secondary to its mutation. Behari et al. [21] proposed that targeting β -catenin might be an attractive approach to HCC treatment. Luca DT et al. suggested that, in keeping with this early role in hepatic tumorigenesis, GS overexpression did not associate with tumor dedifferentiation [22], which is confirmed by the present findings.

This study revealed the important phenomenon that strong and diffuse cytoplasmic immunoreactivity frequently occurred not only in tumor tissue, but also in non-malignant tissues with necroinflammation and regeneration. This indicates that GS is associated with necroinflammation and regeneration. Recently, Niva et al. [23] found that

E. japonensis GS may play role in regeneration, which is consistent with our result. By performing a dose response study, where CD1-mice injected with non-lethal or lethal doses of acetaminophen, Apte et al. [24] identified that β -catenin activation is related to increased liver regeneration and that a positive role of β -catenin in impelling regeneration following toxicant-induced liver failure by mediating proliferation through regulation of cyclin-D1 levels. They noticed that β -catenin activation is an early event and is vital for regeneration. Sekine et al. also demonstrated that β -catenin activation is crucial for liver regeneration and plays a key role in regulating expression of various cyclins (A, D, and E), which are critical for G1 to S phase transition of cell cycle [25, 26]. This is of special interest since there is currently no sensitive marker for liver regeneration except AFP [27]. In the light of GS overexpression highly correlated with β -catenin mutation, it is reasonable to believe that GS and β -catenin may play potential role in regeneration and they may be sensitive markers for liver regeneration besides AFP. We hypothesized that GS overexpression in CHB may be attributable to its association with regeneration.

In summary, GS expression is increased in HCC and CHB stage 1–4 patients. Results suggest that GS plays a potential role in the pathogenesis of chronic inflammation and subsequent malignant transformation, probably through its role in protein metabolism secondary to β -catenin mutation in the liver. Further studies to elucidate the function of GS and its underlying mechanism might help to understand the progression from CHB to HCC, and provide a potential therapeutic target. More importantly, this study confirmed the findings that serum GS level is increased in HCC and CHB (stage 1–4) patients, serum GS level may be recommended as a diagnostic marker for liver diseases, and it is a serum marker for diagnosis of HCC, especially for those patients with borderline AFP value ($100 < \text{AFP} \leq 200$ mg/ml).

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References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;55:74–108 (PubMed: 15761078)
2. Seow TK, Liang RC, Leow CK, Chung MC. Hepatocellular carcinoma: from bedside to proteomics. *Proteomics* 2001;1: 1249–1263 (PubMed: 11721636)
3. Zhou XD, Tang ZY, Yang BH, Lin ZY, Ma ZC, Ye SL, Wu ZQ, Fan J, Qin LX, Zheng BH. Experience of 1000 patients who

- underwent hepatectomy for BlnaU hepatocellular carcinoma. *Cancer* 2001;91:1479–1486 (PubMed: 11301395)
4. Bioulac-Sage P, Laumonier H, Rullier A, Cubel G, Laurent C, Zucman-Rossi J, Balabaud C. Over-expression of glutamine synthetase in focal nodular regeneration: a novel easy diagnostic tool in surgical pathology. *Liver Int* 2009;29:459–465 (PubMed: 18803590)
 5. Brosnan ME, Brosnan JT. Hepatic glutamate metabolism: a tale of 2 hepatocytes. *Am J Clin Nutr* 2009;90:857–861 (PubMed: 19625684)
 6. Gebhardt R, Alicja BF, Krügel V, Ueberham E, Gaunitz F. Hepatocellular expression of glutamine synthetase: an indicator of morphogen actions as master regulators of zonation in adult liver. *Prog Histochem Cytochem* 2007;41:201–266 (PubMed: 17368308)
 7. Christa L, Simon MT, Flinois JP, Gebhardt R, Brechot C, Lasserre C. Overexpression of glutamine synthetase in human primary liver cancer. *Gastroenterology* 1994;106:1312–1320 (PubMed: 7909780)
 8. Kuo CF, Paulson KE, Darnell JE Jr. Positional and developmental regulation of glutamine synthetase expression in mouse liver. *Mol Cell Biol* 1988;8:4966–4971 (PubMed: 2905422)
 9. Di Tommaso L, Destro A, Seok JY, Balladore E, Terracciano L, Sangiovanni A, Iavarone M, Colombo M, Jang JJ, Yu E, Jin SY, Morenghi E, Park YN, Roncalli M. The application of markers (HSP70 GPC3 and GS) in liver biopsies is useful for detection of hepatocellular carcinoma. *J Hepatol* 2009;50:746–754 (PubMed: 19231003)
 10. Oka H, Tamori A, Kuroki T, Kobayashi K, Yamamoto S. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. *Hepatology* 1994;19:61–66 (PubMed: 7506227)
 11. Häussinger D, Schliess F. Glutamine metabolism and signaling in the liver. *Front Biosci* 2007;12:371–391 (PubMed: 17127305)
 12. Poyck PP, Hoekstra R, Vermeulen JL, van Wijk AC, Chamuleau RA, Hakvoort TB, van Gulik TM, Lamers WH. Expression of glutamine synthetase and carbamoylphosphate synthetase in a bioartificial liver: markers for the development of zonation in vitro. *Cells Tissues Organs* 2008;188:259–269 (PubMed: 18354250)
 13. Malarkey DE, Johnson K, Ryan L, Boorman G, Maronpot RR. New insights into functional aspects of liver morphology. *Toxicol Pathol* 2005;33:27–34 (PubMed: 15805053)
 14. Fung J, Lai CL, Yuen MF. Hepatitis B and C virus-related carcinogenesis. *Clin Microbiol Infect* 2009;15:964–970 (PubMed: 19874379)
 15. Medina MA. Glutamine and cancer. *J Nutr* 2001;131:2539–2542 (PubMed: 11533309)
 16. Yang C, Sudderth J, Dang T, Bachoo RG, McDonald JG, DeBerardinis RJ. Glioblastoma cells require glutamate dehydrogenase to survive impairments of glucose metabolism or Akt signaling. *Cancer Res* 2009;69:7986–7993 (PubMed: 19826036)
 17. Dang CV. MYC, microRNAs and glutamine addiction in cancers. *Cell Cycle* 2009;8:3243–3245 (PubMed: 19806017)
 18. DeBerardinis RJ, Cheng T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene* 2010;29:313–324 (PubMed: 19881548)
 19. Cadoret A, Ovejero C, Terris B, Souil E, Lévy L, Lamers WH, Kitajewski J, Kahn A, Perret C. New targets of beta-catenin signaling in the liver are involved in the glutamine metabolism. *Oncogene* 2002;21:8293–8301 (PubMed: 12447692)
 20. Cieply B, Zeng G, Proverbs-Singh T, Geller DA, Monga SP. Unique phenotype of hepatocellular cancers with exon-3 mutations in beta-catenin gene. *Hepatology* 2009;49:821–831 (PubMed: 19101982)
 21. Behari J, Zeng G, Otruba W, Thompson MD, Muller P, Micsenyi A, Sekhon SS, Leoni L, Monga SP. R-Etodolac decreases beta-catenin levels along with survival and proliferation of hepatoma cells. *J Hepatol* 2007;46:849–857 (PubMed: 17275129)
 22. Di Tommaso L, Franchi G, Park YN, Fiamengo B, Destro A, Morenghi E, Montorsi M, Torzilli G, Tommasini M, Terracciano L, Tornillo L, Vecchione R, Roncalli M. Diagnostic value of HSP70, Glypican 3, and Glutamine synthetase in hepatocellular nodules in cirrhosis. *Hepatology* 2007;45:725–734 (PubMed: 17326147)
 23. Niva CC, Lee JM, Myohara M. Glutamine synthetase gene expression during the regeneration of the annelid *Enchytraeus japonensis*. *Dev Genes Evol* 2008;218:39–46. (PubMed: 18183418)
 24. Apte U, Singh S, Zeng G, Cieply B, Virji MA, Wu T. Beta-catenin activation promotes liver regeneration after acetaminophen-induced injury. *Am J Pathol* 2009;175:1056–1065 (PubMed: 19679878)
 25. Sekine S, Gutierrez PJ, Lan BY, Feng S, Hebrok M. Liver-specific loss of beta-catenin results in delayed hepatocyte proliferation after partial hepatectomy. *Hepatology* 2007;45:361–368 (PubMed: 17256747)
 26. Tan X, Behari J, Cieply B, Michalopoulos GK, Monga SP. Conditional deletion of beta-catenin reveals its role in liver growth and regeneration. *Gastroenterology* 2006;131:1561–1572 (PubMed: 17101329)
 27. Schmidt LE, Dalhoff K. Alpha-fetoprotein is a predictor of outcome in acetaminophen-induced liver injury. *Hepatology* 2005;41:26–31 (PubMed: 15690478)