doi:10.4149/neo_2014_012

Decreased expression of Small glutamine-rich tetratricopeptide repeat-containing protein (SGT) correlated with prognosis of Hepatocellular carcinoma

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Received January 6, 2013 / Accepted May 27, 2013

Small glutamine-rich tetratricopeptide repeat-containing protein (SGT) is an ubiquitously expressed cochaperone of heat shock cognate protein of 70 kDa (Hsc70). SGT binds to the C terminus of Hsc70 to recruit Hsc70 into complexes of diverse function. SGTB was identified as an isoform of SGT with 60% amino acid sequence homology. To investigate the expression of SGTB in hepatocellular carcinoma (HCC) and determine its correlation with tumor progression and prognosis, we evaluated the expression levels of SGTB in HCCs and corresponding adjacent non-tumor liver tissues. We also assessed the association between their expression and clinicopathologic parameters. The expression of SGTB was absent or low in HCCs while it was notable in paracancerous tissues from 108 patients by western blotting and immunochemistry ($P < 0.05$). Among the 108 HCCs, low expression of SGTB was associated with gender, histological grade ($P<0.001$) and HBsAg expression ($P=0.002$). Univariate analysis showed that the low SGTB expression was associated with poor prognosis ($P<0.001$). Thus, decreased expression of SGTB may be a favorable independent poor prognostic parameter for hepatocellular carcinoma.

Key words: SGTB, Heat stress cognate 70 (Hsc70), HBV, Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, and the third most common cause of cancer mortality [1, 2]. This tumor, which arises from hepatocytes, is often associated with liver cirrhosis resulting from chronic liver diseases. When intermediate-to-advanced cancer is found at diagnosis, the majority of HCC patients are eligible only for nonsurgical treatment which is less efficacious. Thus detecting HCC at an earlier stage is important so that the use of curative treatments is possible [3].

Heat shock cognate protein of 70 kDa (Hsc70) is a constitutive member of the heat shock-induced Hsp70 protein family. Despite of their high degree of sequence homology, Hsp70 is induced by stress (e.g., heat shock) whereas Hsc70 is constitutively expressed in cells. Hsc70 interacts with many other molecules as well and regulates various cellular functions. It is also involved in various diseases such as cancer, cardiovascular, neurological, hepatic and many other diseases [5]. In particular, Hsc70 has been implicated in the pathogenesis and the pathophysiology of hepatic diseases such as hepatitis B and C, nonalcoholic steatohepatitis autoimmune hepatitis, and primary biliary cirrhosis. It plays an important role in the replication of hepatitis B virus (HBV) and hepatitis C virus (HCV) [5]. Some investigations supported that down regulation of this protein in the host would inhibit HBV replication in either wild-type or drug-resistant strains [6]. Nevertheless knock down of Hsc70 may be not appropriate as the depletion of the major housekeeping chaperone Hsc70 killed tumorigenic as well as nontumorigenic cells [7]. Therefore, we need to find another more specific target in HCC.

Abbreviations: HCC: hepatocellular carcinoma; SGT: small glutamine-rich tetratricopeptide repeat-containing protein; Hsc70: heat shock cognate protein of 70 kDa; Hsp70: heat stress protein 70; CTD: C-terminal domain; ABD: ATP- binding domain; PBD: peptide-binding domain; CHB: chronic hepatitis B; CC: coiled-coil region

Small glutamine-rich tetratricopeptide repeat-containing protein (SGT) was first identified as a protein putatively interacting with envelope proteins of two viruses, HIV type 1 and parvovirus H-1 [8]. Rat SGT consists of 314 amino acid residues that are arranged in three domains of equal size: an N-terminal region, a central TPR domain, and a C-terminal region. The central TPR domain consists of three repeats, which are sufficient for binding to the C terminus of Hsc70 [9]. In an in- vitro study, SGT negatively influenced the ability of Hsc70 to refold a denatured model protein substrate [10]. Our data suggested the expression of SGTB, which is an isoform of SGT [11], and a heat-shock protein cognate 70 (Hsc70) co-chaperone [12], showed significant expression difference between normal liver tissue and HCC.

Patients and methods

Patients and tissue samples. HCC and adjacent non-tumor liver tissues specimens were obtained from 108 patients. All underwent hepatic surgical resection without postoperative systemic chemotherapy at the Surgery Department, the Affiliated Hospital of Nantong University between April 2004 and May 2007. The diagnosis was confirmed histologically in all cases, based mainly on examination of sections stained with H&E. The main clinical and pathologic variables of the patients are shown in Table 1. Eighty-six patients were men and twenty-two were women; their ages ranged from 21 to 69 (mean = 54.18 years old). Seventy-six patients were positive for HBV surface antigen, eighty-nine were positive for cirrhosis. Histological grades were classified to well differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III).Of the 108 patients examined, 11 were classified as grade I, 51 were classified as grade II, and 46 were classified as grade III. The main clinicopathological variables of the patients are shown in Table 1. Tissue samples were immediately processed after surgical removal. For histological examination, all tumorous and surrounding nontumorous tissue portions were fixed in formalin and embedded in paraffin. Informed consent was obtained from all patients.

Table 1. SGTB expression and clinicopathological parameters in 108 HCC specimens

Parameters	Total	SGTB		P[a]
		Low	High	
Age(years)				
≤50	55	44	11	0.102
>50	53	35	18	
Gender				
Male	86	69	17	0.001
Female	22	10	12	
Histological grade				
Well	11	4	7	<0.001
Mod	51	31	20	
Poor	46	44	2	
Metastasis				
Positive	16	14	2	0.160
Negative	92	65	27	
Tumor size(cm)				
≤5	63	46	17	0.971
>5	45	33	12	
HBsAg				
(-)	32	17	15	0.002
(+)	76	62	14	
Cirrosis				
Positive	89	66	23	0.609
Negative	19	13	6	
AFP(ng/ml)				
≤50	42	28	14	0.225
>50	66	51	15	

a Statistical analyses were performed by the Pearson $\chi 2$ test. $P<0.05$ was considered significant.

Immunoblot analysis. Tissue and cell protein were promptly homogenized in a buffer containing 1 M Tris–HCl pH 7.5, 1% Triton X-100, 1% NP-40 (nonidet p-40), 10% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.5 MEDTA, 10 lg/ml leupeptin, 10 lg/ml aprotinin, and 1 mMPMSF, then centrifuged at 10,000 g for 30 min to collect the supernatant. Protein concentrations were determined with a Bio-Rad protein assay (BioRad, Hercules, CA, USA). The supernatant diluted in 2xSDS loading buffer and boiled. Proteins were separated with SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride filter (PVDF) membranes (Millipore, Bedford, MA). The membranes were blocked with 5% dried skim milk in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20). After 2 h at room temperature, the filters were washed by TBST for three times and then incubated overnight with polyclonal antibody against using the primary antibodies described later and horseradish peroxidase-linked IgG as the secondary antibodies. Immunoreactive bands were visualized by chemiluminescence (NEN Life Science Products, Boston,MA) Antibodies used were as follows: anti-SGTB (AP5076a; 1:500; Abgent); anti- β-actin (1:4000; Sigma).

Immunohistochemistry(IHC). The sections were deparaffinized using a graded ethanol series, and endogenous peroxidase activity was blocked by soaking in 0.3% hydrogen peroxide. Thereafter, the sections were processed in 10mmol/L citrate buffer (pH 6.0) and three minutes of high temperature and pressure to retrieve the antigen. After rinsing in PBS (pH 7.2), 10% goat serum was applied for 1 hour at room temperature to block any nonspecific reactions. After that the sections were incubated overnight at 4 °C with anti-SGTB rabbit polyclonal antibody (diluted 1:100; Novus Biologicals), Negative control slides were also processed in parallel using a nonspecific immunoglobulin IgG (Sigma Chemical Co., St. Louis, MO) at the same concentration as the primary antibody. All slides were processed using the peroxidase antiperoxidase method (Dako, Hamburg, Germany). After rinsing in PBS, the peroxidase reaction was visualized by incubating the sections

with diaminobenzidine tetrahydrochloride in 0.05 mol/L Tris buffer (pH7.6) containing 0.03% H2O2. After rinsing in water, the sections were counterstained with hematoxylin, dehydrated, and coverslipped.

Immunohistochemical evaluation. Two observers (Q.K and M.D.L.) independently evaluated the immunostaining results, similar results were obtained in these samples. For assessment of SGTB, five highpower fields in each specimen were selected randomly, and cytoplasma (nuclear) staining was examined under high power magnification. All sections were scored by means of the immunoreactivity score (IRS) according to Remmele & Stegner [13]. The intensity of immunostaining in each tumor section was assessed as strong (3), moderate (2), weak (1), or negative (0); semi-quantitatively using the following scale: < 10% of cells (0), 10–30% (1), 30–50% (2), 50–70% (3), and> 70% (4) of cells, and then combined these values. This resulted in an overall SGTB immunohistochemical score ranging from 0 to 12. SGTB expression was considered high when scores were > 3, and low when scores were ≤ 3.

Statistical analysis

Statistical analysis was performed using the SPSS Statistics17.0 software package. SGTB expression in HCCs was studied using the Spearman rank correlation test because the data were not normally distributed. Survival curves were calculated using the Kaplan–Meier method, and the log-rank test was used for analysis. The results of the HCC cells are expressed as the mean±SE. P<0.05 was considered statistically significant.

Results

The expression of SGTB protein in HCC and the adjacent non-tumor liver tissue. Expression of SGTB was first studied by immune blotting method in 8 novel specimens, we showed that SGTB expression was different in paired non-tumor liver and HCC biopsy samples, with a dramatically decreased expression in eight tumors compared with the adjacent non-tumor liver tissues, (Fig1). In order to identify the expression of SGTB, we further investigated 108 paired tissue samples by immunohistochemistry (Fig2). SGTB immunoreactivity was identified in 99 cases (91.7%) of paracancerous tissues, while it was recognized in 29 cases (26.9%) of HCCs. SGTB was low expressed or absent in most specimens and mainly in cytoplasm (Fig2a-d) compared with corresponding adjacent

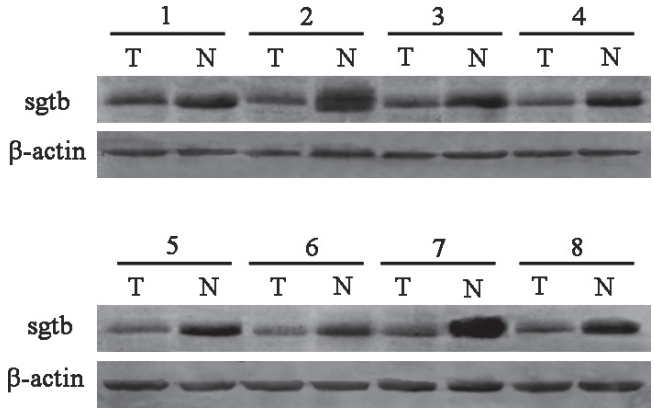

Figure 1. SGTB is low expressed in hepatocellular carcinoma but high in paired adjacent non-tumor liver tissues. Western blot of eight representative paired samples of hepatocellular carcinoma tissue (T) and adjacent non-tumor liver tissues (N) immunoblotted against SGTB. Whole-cell lysates were prepared from tissue specimens obtained from hepatocellular carcinoma and adjacent non-tumor liver tissues. In all samples tested, SGTB expression was obviously lower in hepatocellular carcinoma than in paired adjacent non-tumor liver tissues. β-actin was used as a control for protein load and integrity.

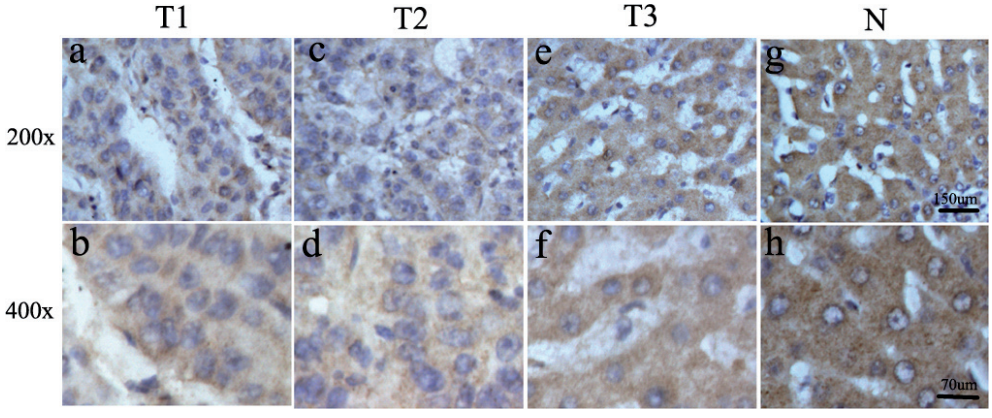

Figure 2. Immunohistochemical staining of SGTB in HCC tissues (a-f) and non-tumor liver tissues (g, h). Paraffin-embedded tissue sections were stained with antibodies for SGTB and counterstained with hematoxylin. (a and c) SGTB weak staining was shown in HCC tissues (200×), (b and d) with staining predominant in the cytoplasm (400×). (e) SGTB strong staining was shown in HCC tissues (200×), (f) with staining predominant in the cytoplasm (400×). (g) SGTB immunoreactivity was identified in non-tumor liver tissues (200×), (h) with staining predominant in the cytoplasm (400×).

liver samples (Fig2g-h), and it was high expressed in only a few specimens (Fig2e-f). This difference indicated that low expression of SGTB may be associated with HCC occurrence.

Correlation between SGTB expression and clinicopathological parameters of HCC. The clinicopathological data of the 108 patients are summarized in Table 1. As shown in Table 1, we evaluated the association of SGTB expression with clinicopathologic parameters. SGTB expression did not correlate with age, metastasis, tumor size, cirrhosis, the level of AFP, but notably correlated with gender, histological grade (P<0.001) and HBsAg (P=0.002) (Table 1). Although the sgtb gene is considered as weakly female specific in mouse liver in some research [14], we haven't find the discrepancy between the male and female human paracancerous liver tissues.

Survival analysis. At the end of clinical follow-up, survival information was available in 90 cases of 108 patients (83.3%). For statistical analysis of the expression of SGTB, the carcinoma specimens were divided into two groups: high expressors and low expressors, according to the immunohistochemical score using the semi-quantitative immunoreactive score method of Remmele and Stegner. SGTB expression was considered high when scores were > 3, and low when scores were ≤ 3. Of these 90 patients, the survival rate of the SGTB low-expresser group was far below the SGTB high-expresser group (Table 2). When all variables were compared separately to survival status, SGTB (P<0.001) and histological grade (P<0.001) were clearly influenced survival status (Table 2A). Interestingly, gender factor also shows the correlation with patients' survival probably be attributed to high incidence of liver cancer in male. As the low expression of SGTB was associate with gender, statistical analysis on the male patients was further performed. Results proved SGTB (P=0.001) and histological grade (P=0.001) influenced survival significantly which was consistent with above research (Table 2B). In univariate analysis either on the whole 90 patients or just on the male, the Kaplan–Meier survival curves showed that low SGTB expression related to a poor survival with statistical significance (Fig3). The Cox's proportional hazards regression model proved that SGTB and histological grade were independent prognostic factors in the 90 patients with HCC (Table 3).

Table 2. A. Survival status and clinicopathological parameters in 90 HCC specimens

Parameters	Total	Survival status		p[a]
		Alive	Dead	
Age(years)				
≤50	48	25	23	0.978
>50	42	22	20	
Gender				
Male	74	34	40	0.010
Female	16	13	3	
Histological grade				
Well	10	10	0	<0.001
Mod	37	23	14	
Poor	43	14	29	
Metastasis				
Positive	14	4	10	0.054
Negative	76	43	33	
Tumor size(cm)				
≤5	50	34	16	0.001
>5	40	13	27	
HBsAg				
(-)	29	18	11	0.197
(+)	61	29	32	
Cirrosis				
Negative	16	10	6	0.364
Positive	74	37	37	
AFP(ng/ml)				
≤50	37	19	18	0.890
>50	53	28	25	
Sgtb				
Low expression	69	29	40	<0.001
High expression	21	18	3	

B. Survival status and clinicopathological parameters in 74 male HCC specimens

Parameters	Total	Survival status		p[a]
		Alive	Dead	
Age(years)				
≤50	41	20	21	0.585
>50	33	14	19	
Histological grade				
Well	8	8	0	0.001
Mod	28	15	13	
Poor	38	11	27	
Metastasis				
Positive	13	3	10	0.068
Negative	61	31	30	
Tumor size				
≤5	42	27	15	<0.001
>5	32	7	25	
HBsAg				
(-)	25	14	11	0.215
(+)	49	20	29	
Cirrosis				
Negative	11	6	5	0.535
Positive	63	28	35	
AFP(ng/ml)				
≤50	31	15	16	0.721
>50	43	19	24	
SGTB				
Low expression	60	22	38	0.001
High expression	14	12	2	

a Statistical analyses were performed by the Pearson $\chi 2$ test. P<0.05 was considered significant

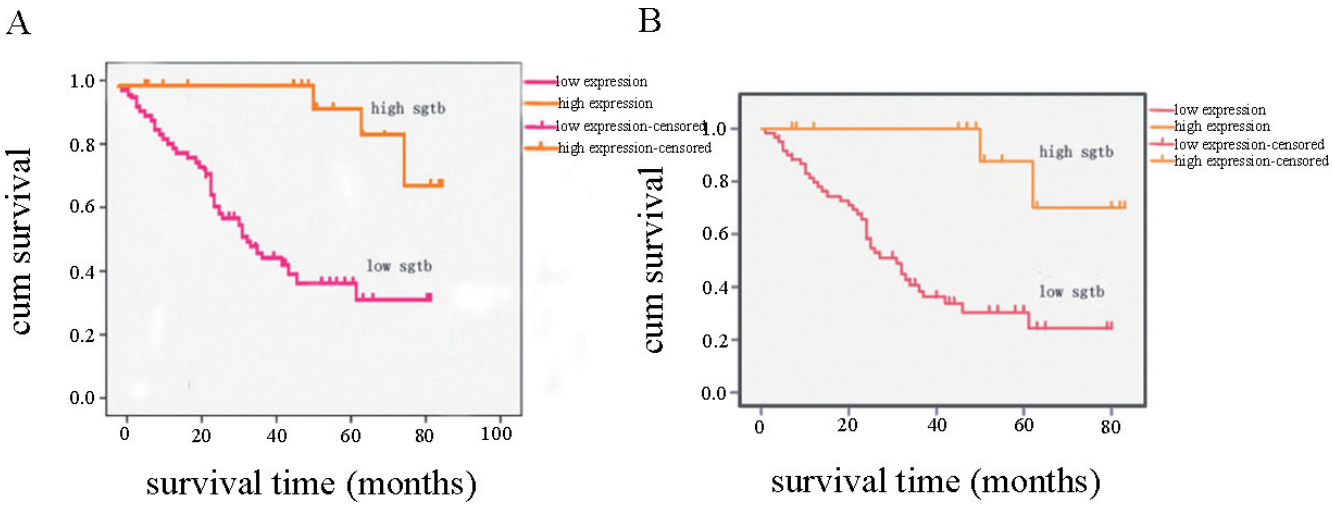

Figure 3. A Kaplan–Meier survival curves for low SGTB expression versus high SGTB expression in 90 patients of HCC showed a highly significant separation (P<0.001, logrank test). B Kaplan–Meier survival curves for low SGTB expression versus high SGTB expression in 74 male patients of HCC showed a highly significant separation (P<0.001, logrank test).

Discussion

HCC is a potentially fatal consequence of HBV infection. Approximately 25% of chronically HBV-infected individuals will develop HCC [15]. Chronic carriers of HBV have up to a 30-fold increased risk of HCC [16]. Clinical, pathological, epidemiological, molecular, and animal studies clearly show that HBV infection is the most important risk factor for HCC and that it is the etiology underlying approximately 50% of all cases [17, 18]. Viral factors associated with a greater risk of HCC include HBeAg positivity, serum HBV DNA levels, and HBV genotypes. Increasing evidence suggests that persistent HBV replication is a predictor of HCC [3]. Thus, efforts to prevent HCC in chronic hepatitis B (CHB) patients should begin by controlling HBV infection and resistance to HBV replication maybe more effective.

In present study, we find SGTB down regulated obviously in HCC. Although the mechanism of low SGTB expression increases the risk of HCC occurrence remains unclear, it influence the infections of HBV according to our statistical correlation analysis of the clinical factors. Consequently, SGTB down regulation leading to occurrence of HCC could be achieved by increasing the risk of HBV infection.

Previous studies have reported that SGTB is a heat-shock protein cognate 70 (Hsc70) co-chaperone [12]. Hsc70 is an ATP-binding protein of the heat stress protein 70 (Hsp70) family [19]. Human Hsc70 protein contains an ATP-binding domain (ABD) (nucleotides [nt] 1 to 1146; amino acids [aa] 1 to 382), a peptide-binding domain (PBD) (nt 1155 to 1629; aa 385 to 543), and a C-terminal domain (CTD) (nt 1620 to 1938; aa 540 to 646) [22, 23]. Within the PBD, there are a nuclear localization signal (NLS) region (nt 1419 to 1476; aa 473 to 492) and a coiled-coil (CC) region (nt 1533 to 1608; aa 511 to 536) [23, 24]. It is constitutively expressed and only mildly induced during stress situation while Hsp70 is highly inducible during stress [20]. Hsc70 was found to be required for the reverse transcription process in experiments using duck HBV DNA polymerase [21, 22].

SGT contains three domains including an amino-terminal domain, a central tetratricopeptide repeat (TPR) domain, and a carboxyl-terminal domain [25]. The TPR motifs might give an indication to its function. Since there is extensive evidence demonstrating that TPR motifs are important in the function of chaperones, cell cycle, transcription and protein transport complexes [26]. SGTB is actually an isoform of SGT with 60% amino acid sequence homology harboring similar binding properties. Some data indicate that SGTs couple Hsc70 to client proteins and downstream-acting proteins [12]. It binds to Hsc70 via a two-carboxylate clamp mechanism involving the

Table 3. Contribution of various potential prognostic factors to survival by Cox regression analysis in 90 HCC specimens.

	Hazard ratio	95%Confidence interval	p[a]
Gender	0.273	0.074-1.005	0.051
Age(years)	1.344	0.640-2.824	0.435
Histological grade	2.011	1.026-3.943	0.042
Metastasis	0.969	0.269-3.488	0.962
Tumor size	0.244	0.101-0.593	0.002
HBsAg	1.377	0.510-3.722	0.528
Cirrhosis	0.965	0.295-3.162	0.953
AFP	1.262	0.601-2.651	0.539
SGTB	0.191	0.047-0.782	0.021

a Statistical analyses were performed by the log-rank test. P<0.05 was considered significant.

TPR domain [12]. Since SGTB was down regulated in HCC, we would like to know whether SGT expression changes. The paired HCC and normal liver tissues' SGT expression was evaluated with western blotting method. Actually, the results did show the differential expression. The role of its abnormal expression in HCC is followed up. Most important results show that the ability of SGT to interact with Hsc70 and to negatively influence the capacity of Hsc70 together with DnaJ to refold an unfolded substrate indicates a role as a negative regulator of Hsc70 function [26]. The C terminus of Hsc70 was the binding domain interacting with SGT [25]. In addition, recent researches suggested the Hsc70 mutant with deletion of the CTD or NLS caused partial loss of support for HBV replication compared to wild-type Hsc70 [6]. Since our data show the correlation between SGTB expression and HBV infection, we speculate that CTD of Hsc70 occupied by SGTB may partially prevent it from interacting with HBV, then repress the replication of HBV in host cells, and reduce the occurrence of HCC.

Although some investigations support that direct down regulation of Hsc70 in the host would inhibit HBV replication in either wild-type or drug-resistant strains [6]. However, unlike majority other families of Hsp70 expressed only at low or undetectable levels in most unstressed normal cells and tissues, which is rapidly induced by variety of physical and chemical stresses [7], Hsc70 is the only cytosolic Hsp70 protein that is abundantly and ubiquitously expressed in all cells [27]. Therefore, utilizing Hsc70 as the target of drug may disturb the functions of normal host cells. Comparatively directing SGTB probably has more practical value.

Besides that, the interaction between SGTB and Hsc70 probably modulate the function of p53 which is often expressed aberrantly in HCC. Previous evident a good correlation exists between high levels of p53 and the presence of p53-Hsc70 complexes [28]. Over expression of p53 or structural alterations of p53 likely mediate the interaction of Hsc70 and p53, which is a functionally normal response to an altered oncogene product. Hsc70-p53 complexes could result in the activation of a pathway that leads to the transformed phenotype [28]. If this pathway is activated in the development of HCC, overexpression of SGTB may disturb the formation of p53-Hsc70 complexes to some extent by binding to Hsc70 competitively, and have negative effects on HCC.

In line with the correlation analysis of clinical factors, SGTB expression was also obviously correlated with histological grade ($P<0.001$) and gender. Although there is no present survey reveals the function of SGTB in cell differentiation directly, an integrated approach of differential mass spectrometry and gene oncology analysis identified SGT as one of novel proteins regulating neuronal differentiation and survival, it was up regulated during NGF-induced PC12 cell neuronal differentiation process [29]. SGTB may play a similar role in the differentiation of liver cells. However, knowledge of the precise dynamic molecular events remains far from complete. Interestingly, the reason of SGTB expression correlated with gender remains unclear. Our reports on the male patients are consistent with the investigations on the whole samples. Further study on female should be followed up.

Taken together, our findings demonstrated that compared with paracancerous tissue, SGTB was significantly down-regulated in HCC, its expression in HCC shows remarkable relations with histological grade ($P<0.001$) and gender. Low SGTB expression related to a poor survival with statistical significance.

Acknowledgments: This work was supported by the National Basic Research Program of China (973 Program, No.2011CB910604, and No.2012CB822104); the National Natural Science Foundation of China (No. 81172879); Natural Science Foundation of Jiangsu province (No.BK2009156); A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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