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RESEARCH

Expression of SGTA Correlates with Prognosis and Tumor Cell Proliferation in Human Hepatocellular Carcinoma

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Abstract To investigate the potential role of small glutamine-rich TPR-containing protein A (SGTA) in hepatocarcinogenesis, immunohistochemistry and Western blot were performed to detect the expression of SGTA in clinical Hepatocellular carcinoma (HCC) samples, adjacent nontumorous liver tissues and HCC cell lines. In addition, expression of SGTA was correlated with clinicopathological variables and univariate and multivariate survival analyses were performed to determine the prognostic significance. Moreover, the biological significance of the aberrant expression of SGTA was investigated in vitro. Both immunohistochemistry evaluation and Western blot analyses demonstrated that SGTA was overexpressed in HCC tissues compared with adjacent nontumorous liver tissues. Expression of SGTA directly correlated with the histological grades of HCC and high expression of SGTA was associated with a poor prognosis. SGTA depletion by siRNA inhibited cell proliferation, blocked S-phase and mitotic entry in Huh7 cells. Western blot analyses showed that SGTA depletion decreased cyclin A and cyclin B levels. Taken together, owing to overexpression of SGTA in HCC and its important role in predicting poor prognosis and the development of HCC, SGTA could be a potential prognostic marker and therapeutic target of HCC.

Keywords Hepatocellular carcinoma · Small glutamine-rich TPR-containing protein A (SGTA) · Prognosis · Proliferation · Cell cycle

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Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death in China [1]. Most patients with HCC usually have a poor prognosis largely due to high incidence of tumor recurrence and intrahepatic metastasis after surgical resection and the 5-year postoperative survival rate is 30 % to 40 % [2]. It is commonly thought that chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections and chronic exposure to the aflatoxin B1 contribute to carcinogenesis of most HCC [3]. Although the various risk factors for HCC are well defined, the underlying molecular mechanism of HCC remains unclear. Moreover, hepatocarcinogenesis is a complex process associated with alterations in the structure or expression of several tumor suppressor genes and oncogenes and some cell cycle regulators during the process of initiation and progression of HCC, ultimately leading to the malignant transformation of normal liver cells [4, 5]. Identification of novel molecular mechanisms during the development and progression of HCC may provide new strategies for the diagnosis and medical treatments.

Small glutamine-rich TPR-containing protein A (SGTA, also known as SGT, hSGT or Vpu-binding protein), was originally discovered as a binding partner of the non-structural protein of autonomous parvovirus H-1 [6–8]. Human SGTA, as a member of TPR motif family, consists of three known TPR motifs. These TPR motifs are thought to mediate in protein–protein interaction and involved in a variety of processes, for instance, cell cycle regulation, protein folding, transcription, protein transport, ubiquitin-protesomes and several other

pathways [9]. Previous studies have investigated the role of SGTA in androgen signaling and raised the importance of SGTA in the development of prostate cancer [10]. More recently, studies have shown that phosphorylation of the protein chaperone SGTA at Ser305 is essential for PDGFRα stabilization and cell survival in PDGFRα-dependent cancer cells [11]. Given that cell cycle transitions depend on protein phosphorylation and dephosphorylation, SGTA protein phosphorylation may be a key regulatory event during mitosis [12]. To date, Winnefeld et al. have found that SGTA is required for progress through cell division (deficiency in SGTA led to partial cell cycle arrest in G2/M and unable to complete cell division due to mitotic arrest) and promotes cell proliferation. Moreover, SGTA localization also supports a role for this protein in cell division [13]. As a central process in cell division, mitosis together with cytokinesis can help cell coordinate the separation of sister chromatids and their distribution into the emerging two daughter cells, thus making sure that both daughter cells will contain a full set of genetic information [14]. However, disturbances in mitotic progression and aberrant mitosis may contribute to the development of cancer and that on the other hand molecules involved in mitosis constitute effective classical or promising new targets for cancer therapy [15, 16]. Therefore, it is of interest to see whether SGTA can also modulate cell cycle progress and cell proliferation of HCC, since uncontrolled cell division and increased cell proliferation activity are the hallmark of cancer.

Although studies have examined the important role of aberrant expression of SGTA in tumorigenesis such as human prostate cancer [10], research into the role of SGTA expression in human HCC is also lacking. In the present study, for the first time, we demonstrated that SGTA was significantly up-regulated in human HCC specimens and HCC cell lines. Then, we also investigated its associations with clinical and pathologic factors as well as the prognostic implications. Finally, using anti-SGTA small interfering RNA (siRNA), we further studied the roles of SGTA in HCC cell proliferation and cell cycle distribution.

Materials and Methods

Clinical Samples

All paired samples of HCC and their corresponding nontumorous liver tissues were obtained from HCC patients who had undergone curative partial hepatectomy at the Affiliated Hospital of Nantong University between the year of 2004 and 2005. The diagnosis was confirmed histologically in all cases, based mainly on examination of sections stained with hematoxylin and eosin (H&E). The 97 HCC cases comprised 78 males and 19 females. Their ages ranged from 31 to 69 years, with an average age of 48.19 years. According to the 2002 International Union against Cancer TNM classification system and the Edmondson grading system, histological grades were classified to well (grade I; n=12), moderately (grade II; n=41) and poorly differentiated (grade III; n=44). The main clinicopathological variables of the patients are shown in Table 1. All HCC tissues were collected using protocols approved by the Ethics Committee of AYliated Cancer Hospital of Nantong University and written informed consent was obtained from every patient. Tissue specimens were immediately processed after surgical removal. For histological examination, all tumorous and surrounding nontumorous tissue portions were processed into 10 % bufferedformalin-fixed, paraffin-embedded blocks.

Immunohistochemistry Staining

Five micrometer-thick serial sections were mounted on glass slides coated with 10 % polylysine. These tissue sections were deparaffinized by incubation in xylene and rehydrated in a graded series of ethanol–water solutions. For the antigen retrieval, the sections were immersed in 0.01 M citrate buffer (pH 6.0) using a microwave vacuum histoprocessor (RHS-1, Milestone, Bergamo, Italy) at a controlled final temperature of 121 °C for 15 min. Endogenous peroxidase activity was blocked by immersion in 0.3 % methanolic peroxide for 30 min. Immunostaining was performed using the avidin biotin peroxidase complex method and antigen–antibody reactions were visualized with the chromogen diaminobenzadine. Briefly, the sections were incubated at room temperature for 2 h with anti-SGTA mouse monoclonal antibody (diluted 1:400; Santa Cruz Biotechnology, USA.), and anti-Ki-67 mouse monoclonal antibody (diluted 1:100; clone 7B11;Zymed Laboratories, San Francisco, Calif., USA). The sections were washed three times with PBS, incubated with horseradish peroxidase-conjugated secondary antibody (EnvisionTM Detection Kit, GK500705, Gene Tech) at 37 °C for 30 min, and then washed three more times with PBS. Finally, diaminobenzidine (DAB) was used for signal development, and the sections were counterstained with 20 % hematoxylin. The slides were dehydrated, cleared and evaluated. Each sample was incubated with an isotypic antibody dilution under the same experimental conditions as the negative control.

Immunohistochemical Evaluation

Two pathologists independently scored the results of the staining and similar results were obtained in these samples. For assessment of SGTA and Ki-67, at least five high-power fields were randomly chosen and cytoplasma (nuclear) staining was also examined under high power magnification. More than 500 cells were counted to determine the mean percent, which represented the percentage of immunostained cells relative to the total number of cells [17]. We defined SGTA expression levels according to the final score of each sample (low or high) by adding the scores for the intensity

Table 1 SGTA Ki-67 expression and clinicopathological features in 97 HCC specimens

Characteristics	Total	SGTA		*P*	Ki-67		*P*
		Low	High		Low	High	
Sex				0.305			0.608
Male	78	33	45		31	47	
Female	19	11	8		9	10	
Age				0.213			0.293
≤45	39	21	18		19	20	
>45	58	23	35		21	37	
Histological grade				0.004*			0.001*
Well	12	8	4		8	4	
Mod	41	24	17		23	18	
Poor	44	12	32		9	35	
Tumor size (cm)				0.310			0.538
≤5	49	25	24		22	27	
>5	48	19	29		18	30	
Metastasis				0.588			0.266
Absent	81	38	43		31	50	
Present	16	6	10		9	7	
HBsAG				0.814			0.812
Negative	24	10	14		9	15	
Positive	73	34	39		31	42	
Cirrhosis				1.000			1.000
Absent	21	9	12		9	12	
Present	76	35	41		31	45	
Serum AFP level(ng/mL)				0.296			0.833
≤50	37	14	23		16	21	
>50	60	30	30		24	36	
Ki-67				0.000*			
Low	40	31	9				
High	57	13	44				

Statistical analyses were performed by the Pearson χ^2 test

*$P<0.05$ was considered significant

and extent of staining. The intensity of staining was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). The extent of staining was scored based on the percentage of positive tumor cells: 0 (≤10 %), 1 (10–30 %), 2 (30–50 %), 3 (50–75 %), and 4 (75–100 %). Each case was finally considered low if the final score was 0 to 3 and high if the final score was 4 to 7. As for assessing the expression of Ki-67, the specific experimental methods can be found in the study by Ke et al. [18].

Cell Lines and Cell Culture

HCC cell lines (Huh7, HepG2, SMMC-7721 and BEL-7404) and normal hepatocyte cell lines (Chang and HL-7702) were obtained from the Shanghai Institute of Cell Biology, Academic Sinica and cultured in DMEM supplemented with 10 % fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin [19]. All the cells were incubated at 37 °C in a humidified chamber containing 5 % CO2.

Cell Cycle Analysis and Cell Synchronization

After cells were harvested at the proper time, they were fixed in 70 % ethanol for 1 h at 4 °C and then incubated with 1 mg/ml RNase A for 30 min at 37 °C. Subsequently, cells were collected by centrifugation at 2,000 rpm for 5 min and stained with propidium iodide (50ug/ml PI; Becton–Dickinson, San Jose, CA, USA) in PBS, 0.5 % Tween-20. At last, we analyzed the cells using a Becton–Dickinson BD FACScan flow cytometer and Cell Quest acquisition and analysis software. With regard to cell synchronization, serum deprivation or thymidine was used for cell cycle G1-S phase arrest according to previous two studies [18, 20].

Western Blot Analysis

Frozen liver tissues and harvested cells were carried out for immunoblot analysis. The tissues and cell proteins were

promptly homogenized in a homogenization buffer containing 1 M Tris–HCl pH7.5, 1 % TritonX-100, 1 % NP-40 (nonidet p-40), 10 % sodium dodecyl sulfate (SDS), 0.5 % sodium deoxycholate, 0.5 M EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM PMSF, then centrifuged at 10,000 g for 30 min to collect the supernatant. Before gel electrophoresis, total protein concentration determined with a Bio-Rad protein assay (BioRad, Hercules, CA, USA). The proteins were resolved by 10 % SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine diflouride filter (PVDF) membranes (Millipore, Bedford, MA). After blocked the non-specific binding sites for 2 h with 5 % dried skim milk in TBST (20 mM Tris, 150 mM NaCl, 0.05%Tween-20), the membranes were incubated overnight at 4°Cwith proper antibody against using the primary antibodies. Antibodies used were as follows: anti-SGTA (diluted1:500; Santa Cruz Biotechnology); anti-$P27^{Kip1}$ (1:1000; Santa Cruz Biotechnology); anti–proliferating cell nuclear antigen (PCNA; 1:1000; Santa Cruz Biotechnology); anti-cyclin A (1:1000; Abcam, Cambridge, UK); anti–cyclin B (1:500; Santa Cruz Biotechnology) and anti-GAPDH (1:10000; Sigma). Then the membranes were washed with TBST for three 5 min. Subsequently, the membranes were incubated with second antibody goat-anti-mouse or goat-anti-rabbit conjugated horseradish peroxidase (1:2,000; Southern-Biotech) for 2 h and visualized using an enhanced chemiluminescence system (ECL; PierceCompany, USA). At last, the membranes were subjected to three 15 min washes with TBST, signals were detected using enhanced chemiluminescence system (ECL, Cell Signaling Technologies).

Cell Proliferation Assay

Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay following the manufacturer's instructions. Briefly, cells were plated at a density of 2×10^4 cells/well in a 96 well plate in a volume of 100 μL and grown overnight. After adding Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) reagents to each well under different treatments and the cells were incubated for 2 h at 37 °C, we quantified the absorbance on an automated plate reader. Each experiment was performed in triplicate and repeated at least three times.

siRNA and Transfection

Small interference RNAs (siRNA) were chemically synthesized (GenePharma Co. Ltd.). The synthesized oligonucleotides for RNA interference (RNAi) SGTA targeted the sequence: 5′-CCGUGGCUUACUACAAGAA-3′ while a nonspecific scrambled siRNA with a sequence of 5′-UUCUCCGAACGUGUCACGU-3′ was used as a negative control. Huh7 cells were seeded the day before transfection using the Dulbeccomodified Eagle medium with 10 % FBS but without antibiotics. For transient transfection, the SGTA siRNA vector and the negative control vector were carried out by adding the mixture of siRNA and the siRNA transfection reagent (Santa Cruz Biotechnology) and plus siRNA dilution buffer as suggested by the manufacturer. Transfected cells were used for the subsequent experiments 48 h after transfection.

Statistical Analysis

All statistical analysis was performed using the Stat View 5.0 software package. The x^2 test was used to compare the association between SGTA and Ki-67expression and clinicopathological features. Survival curves were plotted using the Kaplan–Meier method and the log-rank test was used for univariate analysis. Multivariate analysis was performed using Cox's proportional hazards model and $P<0.05$ was considered to be statistically significant. The results are expressed as mean ± SE.

Results

The Expression of SGTA Protein in HCC, Adjacent Nontumorous Liver Tissues and HCC Cell Lines

In the present work, we first examined SGTA protein expression by immunohistochemistry in 97 samples from patients with HCC. Representative examples of reactivity for SGTA and Ki-67 are shown in Fig. 1. Among these adjacent nontumorous liver tissues, there was no or little SGTA and Ki-67 expression (Fig. 1b, d). In contrast, we found that SGTA and Ki-67 highly expressed in these HCC tissues (Fig. 1a, c). According to the statistical analysis, the positive expression rate of SGTA protein in the HCC tissues was significantly higher than that in the adjacent nontumorous liver tissues ($P<0.01$). Therefore, our data focused on the SGTA expression in HCC. In addition, SGTA was expressed mainly in the cytoplasm (Fig. 1a), whereas Ki-67 was expressed mainly in the nuclei (Fig. 1c). Its LI of SGTA ranged from 7.01 % to 84.11 %, whereas its LI of Ki-67 ranged from 0.91 % to 82.73 %. The mean percents of SGTA and Ki-67expression were 48 % and 35 %, respectively. For statistical analysis of the expression of SGTA and Ki-67, the HCC specimens were divided into two groups: low or high group mentioned in our "Materials and Methods". The results of immunohistochemistry staining are summarized in Table 1.

To confirm these observations, we explored the SGTA protein expression in 8 novel specimens by immunoblotting methods. In agreement with our immunohistochemistry data, Western blot analysis also showed significantly higher expression of SGTA in tumors as compared with that of corresponding noncancerous livers (Fig. 1e).

Furthermore, we investigated the expression of SGTA in available HCC cell lines to assess the biologic activities by Western blot analysis. Expression in Chang and HL-7702 cells was used as a reference for SGTA expression. The expression

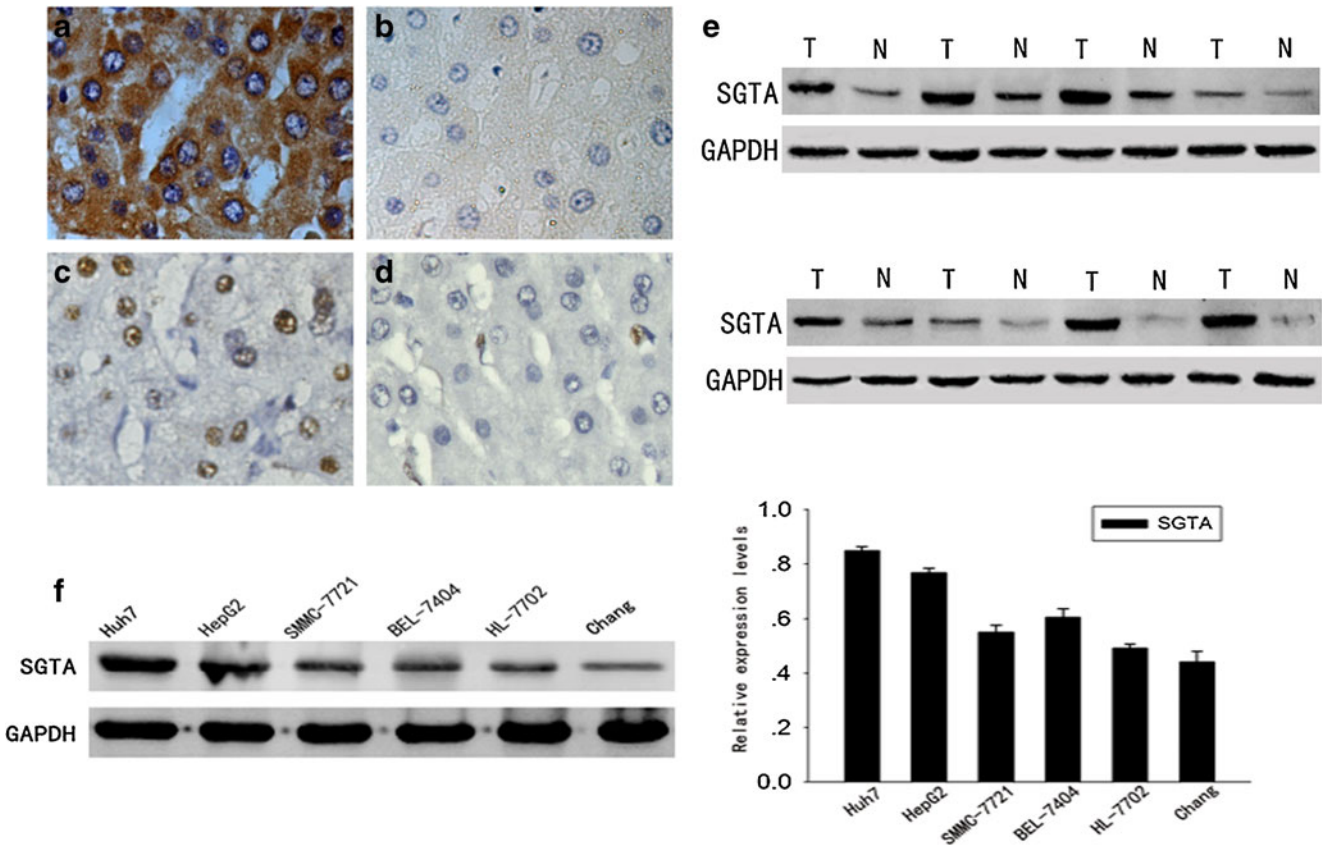

Fig. 1 Expression of SGTA in human hepatocellular carcinoma tissues and cell lines. **a-d** Immunohistochemical analysis of SGTA and Ki-67 expression in HCC and adjacent noncancerous liver tissues. Paraffin-embedded tissue sections were stained with antibodies for SGTA and Ki-67 and counterstained with hematoxylin. High expression of SGTA (**a**) and Ki-67(**c**) is showed in tumor cells. Low concentrations of SGTA (**b**) and Ki-67(**d**) in adjacent normal tissues cells (SP×400). **e** Expression of SGTA in eight representative matched samples of hepatocellular carcinoma tissue (T) and adjacent noncancerous liver tissues (N). **f** Western blot analysis shows that SGTA protein expression is increased in HCC cells compared with the normal hepatocyte cell lines (Chang and HL-7702). The bar chart demonstrates the ratio of SGTA protein to GAPDH by densitometry. The data are means ± SEM. GAPDH was used as a loading control. The same experiment was repeated at least 3 times

of SGTA in HCC cells increased dramatically, especially in Huh7 cells, as compared with these two normal hepatocyte cell lines (Fig. 1f). Tumorigenesis is associated with multiple factors and SGTA overexpression may be an important factor in HCC. Therefore, we can hypothesize that overexpression of SGTA may contribute to cell proliferation of HCC.

Correlation of SGTA Expression with the Clinicopathological Features of HCC

To understand the clinicopathologic significance of SGTA expression in HCC, we evaluated the association of SGTA and Ki-67 expression with clinicopathologic variables. The clinicopathologic data of the patients are summarized in Table 1. The result of statistical analysis showed that expression of SGTA was significantly associated with histological grade (P=0.004), but there was no relation between SGTA expression and other prognostic factors (Table 1). Moreover, in most specimens, the high expression of SGTA was similar to the high expression of Ki-67 (Table 1). And we found that

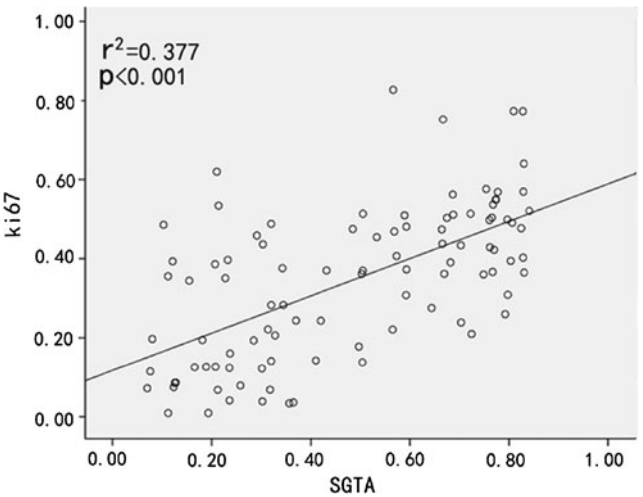

Fig. 2 Relationship between SGTA and Ki-67 proliferation index expression in HCC. Scatterplot of SGTA versus Ki-67 with regression line showing a correlation using the Spearman's correlation coefficient

there was a positive correlation between SGTA expression and Ki-67–based proliferative activity ($P<0.001$; Fig. 2).

Survival Analysis

Based on the results of the immunohistochemical assay, survival information was available in 81 cases of 97 patients (84 %). Of these 81 HCC patients, only 15 of 44 (34 %) patients in the SGTA high-expresser group were alive versus 26 of 37 (70 %) in the SGTA low-expresser group (Table 2). Further univariate and multivariate analyses were conducted to examine the correlation between SGTA expression and other clinical pathological parameters in HCC patients. When all variables were compared separately to survival status, only SGTA ($P=0.002$), Ki-67($P=0.004$), cirrhosis ($P=0.027$) and histological grade ($P=0.000$) significantly influenced survival (Table 2). Univariate analysis showed that high SGTA expression related to a poor survival with statistical significance ($P=0.002$; Fig. 3). The Cox's proportional hazards regression model proved that SGTA, Ki-67 expression, cirrhosis, metastasis and histological grade were independent prognostic factors in patients with HCC (Table 3).

Expression of SGTA in Proliferating HCC Cells

Previous studies demonstrated the presence of SGTA protein throughout the cell cycle [13]. Here, we further detected the expression of SGTA during cell cycle progression in HCC cells. Huh7 cells were arrested in G1 phase by serum deprivation for 72 h and the G1 phase increased from 48.02 % to 69.85 %. Upon serum addition, the cells were released from G1 phase and reentered S phase (Fig. 4a). Western blots showed

Table 2 Survival status and clinicopathologic parameters in 81 HCC specimens

Parameters	Total	Survival status		*P*
		Alive	Dead	
Sex				0.295
Male	62	29	33	
Female	19	12	7	
Age (y)				0.071
≤45	33	21	12	
>45	48	20	28	
Histological grade				0.000*
Well	9	9	0	
Mod	33	20	13	
Poor	39	12	27	
Tumor size (cm)				0.184
≤5	39	23	16	
>5	42	18	24	
Metastasis				0.162
Absent	66	36	30	
Present	15	5	10	
HBsAG				0.295
Negative	19	12	7	
Positive	62	29	33	
Cirrhosis				0.027*
Absent	17	13	4	
Present	64	28	36	
Serum AFP level (ng/mL)				1.000
≤50	33	17	16	
>50	48	24	24	
SGTA				0.002*
Low expression	37	26	11	
High expression	44	15	29	
Ki-67				0.004*
Low expression	33	23	10	
High expression	48	18	30	

Statistical analyses were performed by the Pearson χ^2 test

*$P<0.05$ was considered significant

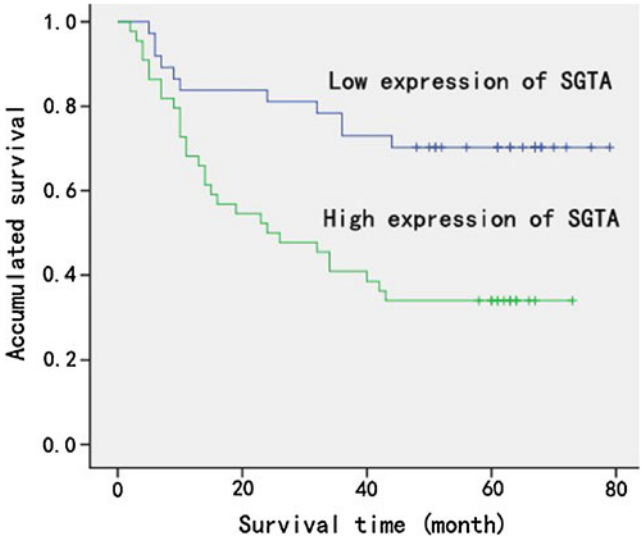

Fig. 3 Kaplan-Meier survival curves for low versus high SGTA expression in 81 patients with HCC show a highly significant separation between curves (P=0.002, log-rank test). On the basis of final score of each sample, the patients were divided into 2 groups: low (scores 0–3) and high (scores 4–7)

that the expression of SGTA was increased after serum stimulation in Huh7 cells. The expression of SGTA was higher in S phase compared with the expression of G1 phase. Meanwhile, the expression of PCNA was up-regulated, whereas the expression of CDK inhibitor $P27^{Kip1}$, a major regulator of G1–S transition in the cell cycle [21, 22], was inversely diminished (Fig. 4b). These results highlight the importance of SGTA in regulating HCC cell cycle progress and cell proliferation.

SGTA Knockdown Inhibits Cellular Proliferation and Promotes Cell Cycle Arrest

To further investigate the effect of SGTA on cellular proliferation, we used chemically synthesized siRNA to knock down endogenous SGTA in Huh7 cells that displayed highest abundance of SGTA in these available cells (Fig. 1f). The efficiency of the SGTA-targeted siRNA-mediated down-regulation was assessed by Western blot analysis. As predicted, siRNA knocked down the protein expression of SGTA as compared with negative control siRNA and mock treatment (Fig. 5a). To determine the effect of SGTA knockout on cell proliferation, SGTA-siRNA, negative control-siRNA and mock treatment Huh7 cell proteins were tested 48 h post-transfection by Western blot. It showed that the PCNA protein correlated positively with SGTA expression (Fig. 5a). Additionally, using CCK-8 assay, we also found that cell proliferation rate of Huh7 cells treated with siRNA exhibited a significant decrease compared with the negative control siRNA or mock-treated cells (Fig. 5b).

Previous studies showed that hSGT depletion specifically caused reduced proliferation and resulted in mitotic arrest of cultured cells [13]. Thus, we considered the possibility that SGTA knockdown might exert its inhibition of cell proliferation by affecting the cell cycle distribution. Huh7 cells were synchronized through thymidine (2 mmol/L) treatment [20] and flow cytometry was performed 48 h after transient transfection of the hSGTA siRNA. Cell cycle analysis showed a significantly decreased population in the S phase whereas a remarkable increase in G2 phase after transfection of SGTA siRNA into the Huh7 cell line, as compared with negative control and mock cells (Fig. 5c). To investigate the mechanism by which down-regulated SGTA is related to cell cycle arrest, we examined the expression of mitotic cyclins (cyclin A, cyclin B) and $P27^{Kip1}$ by Western blot. Knocking down SGTA by siRNA caused failure to accumulate cyclin A and cyclin B while the levels of $P27^{Kip1}$ almost had no variation compared with negative control and mock cells (Fig. 5d). Taken together, these data suggested that the siRNA targeting SGTA could exhibit a specific inhibitory effect on cell proliferation associated with cell cycle arrest in HCC cells.

Table 3 Contribution of various potential prognostic factors to survival by Cox regression analysis in 81 HCC specimens

	Hazard ratio	95 % confidence interval	P
Sex	1.538	0.680–3.478	0.301
Age	1.812	0.920–3.567	0.085
Histological grade	3.200	1.765–5.799	0.000*
Tumor size	1.865	0.988–3.520	0.054
Metastasis	2.225	1.083–4.572	0.030*
HBsAG	1.580	0.698–3.575	0.272
Cirrhosis	0.318	0.113–0.895	0.030*
Serum AFP level	1.114	0.592–2.098	0.738
SGTA	2.978	1.482–5.984	0.002*
Ki-67	2.567	1.253–5.260	0.010*

Statistical analyses were performed by the Cox regression analysis
*P<0.05 was considered significant

Discussion

As known, carcinogenesis and development of tumor are a complicated biological process, including HCC that is characterized by mainly uncontrolled proliferation. It is becoming increasingly apparent that the proliferation of cancer and carcinogenesis could never be documented without referring to altered regulation of the cell cycle which may lead to uncontrolled growth and contribute to tumor formation [23]. During multistep carcinogenesis, the dysregulation of multiple cell cycle regulators is often manifested as an accumulation of genetic alterations highly associated with the development and progression of tumors [24, 25]. Although numerous studies have documented that inappropriate expression of cell cycle regulatory proteins can contribute to human hepatocarcinogenesis, research into the connection between cell cycle regulator SGTA and HCC is also

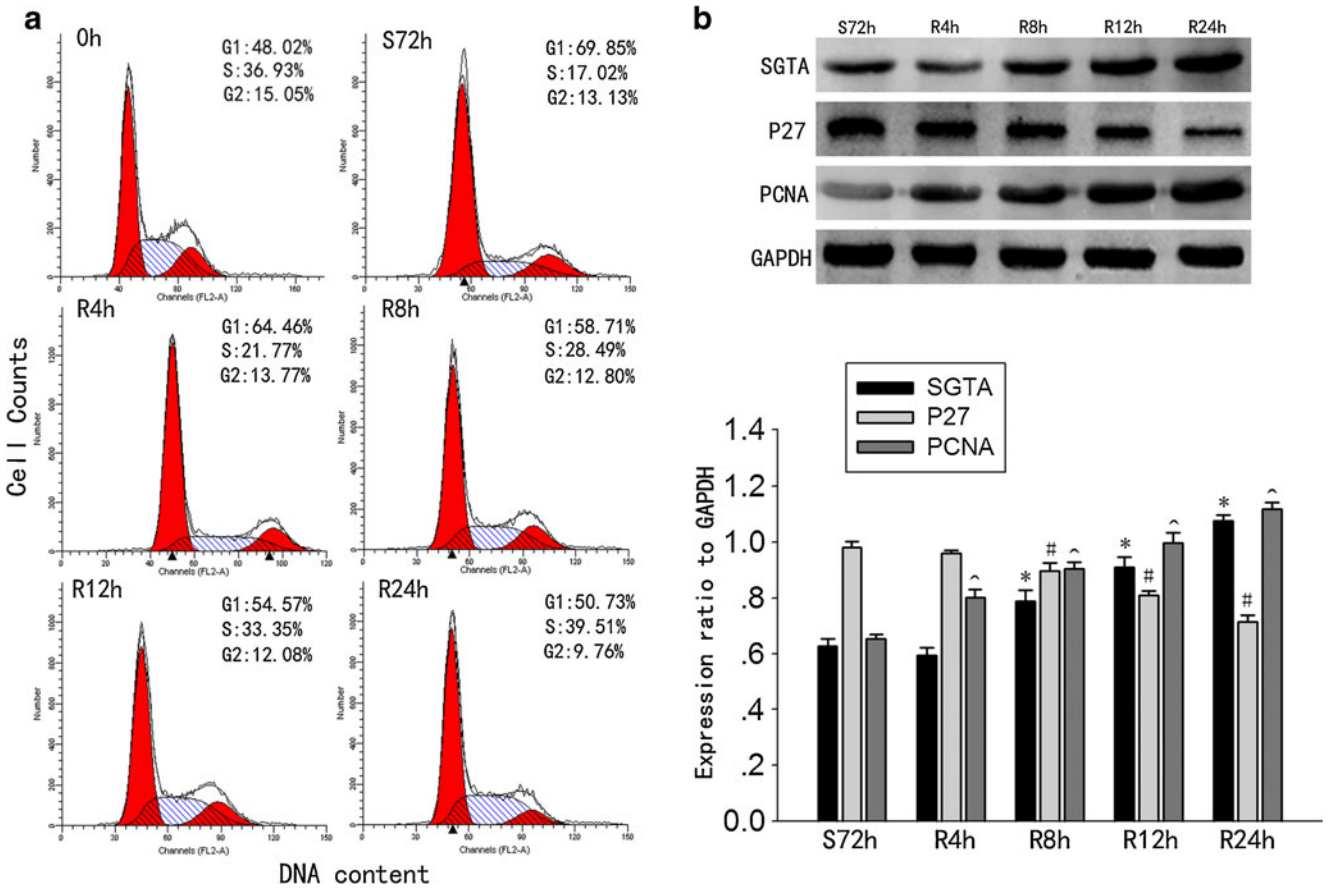

Fig. 4 Expression of SGTA and cell cycle related molecules in proliferating HCC cells. **a** Flow cytometry quantitation of cell cycle progress in Huh7 cells. Cells were synchronized at G1 after serum starvation for 72 h, then progressed into cell cycle by adding medium containing 10 % FBS for the indicated times. **b** Huh7 cells were synchronized by serum starvation for 72 h and upon serum releasing, cell lysates were prepared and analyzed by Western blot using antibodies directed against SGTA, $P27^{Kip1}$ and PCNA. GAPDH was used as a control for protein load and integrity. The bar chart below demonstrates the ratio of SGTA, $P27^{Kip1}$ and PCNA protein to GAPDH for each time point by densitometry. The data are means ± SEM (n=3, *, #, ^P<0.05, compared with control: S72h). S: serum starvation; R: serum release

lacking and extensive efforts should be necessary to know the connection between SGTA and HCC in depth.

SGTA, referred to as a trigger of mitotic entry [13], was recently identified to be involved in cancer-relevant process [9–11], however its expression, clinical significances and biological function in HCC progression are still unknown. Thus, our study is the first time to analyze SGTA expression in HCC with respect to possible associations with clinicopathologic data as well as prognosis. We observed that SGTA overexpression occurred in HCC compared with the corresponding nontumorous tissues, which showed no or low SGTA expression. In accordance with the results of immunohistochemistry, Western blot analysis also confirmed that SGTA was up-regulated in tumors and HCC cell lines, compared with the corresponding nontumorous tissues and normal hepatocyte cell lines. Malignant conversion is a complex process. Overexpression of SGTA may represent an important mechanism in the development of HCC. With regard to the correlation between the SGTA expression and clinicopathological features as well as prognosis in HCC after surgical resection, we found that SGTA expression was significantly associated with histological grade, and high expression of SGTA was significantly associated with a poor prognosis. These findings suggested that SGTA contributes to the malignant progression of HCC and become a novel prognostic marker for HCC patients. In addition, we also found a positive correlation between SGTA expression and ki-67 immunoreactivity, a useful marker of tumor proliferative activity.

To date, the functions of SGTA on tumorigenesis and cell cycle progression of HCC are still unclear. In the present work, we first detected the expression of SGTA during cell cycle progression in HCC cells and found that the expression of SGTA was up-regulated during G1 to S phase. Our data also revealed that PCNA, an indicator of cell proliferation, was up-regulated whereas $P27^{Kip1}$, a major regulator of G1–S transition in the cell cycle [21, 22], was declined during the G1 to S phase. This result indicated that SGTA might contribute to hepatocarcinogenesis

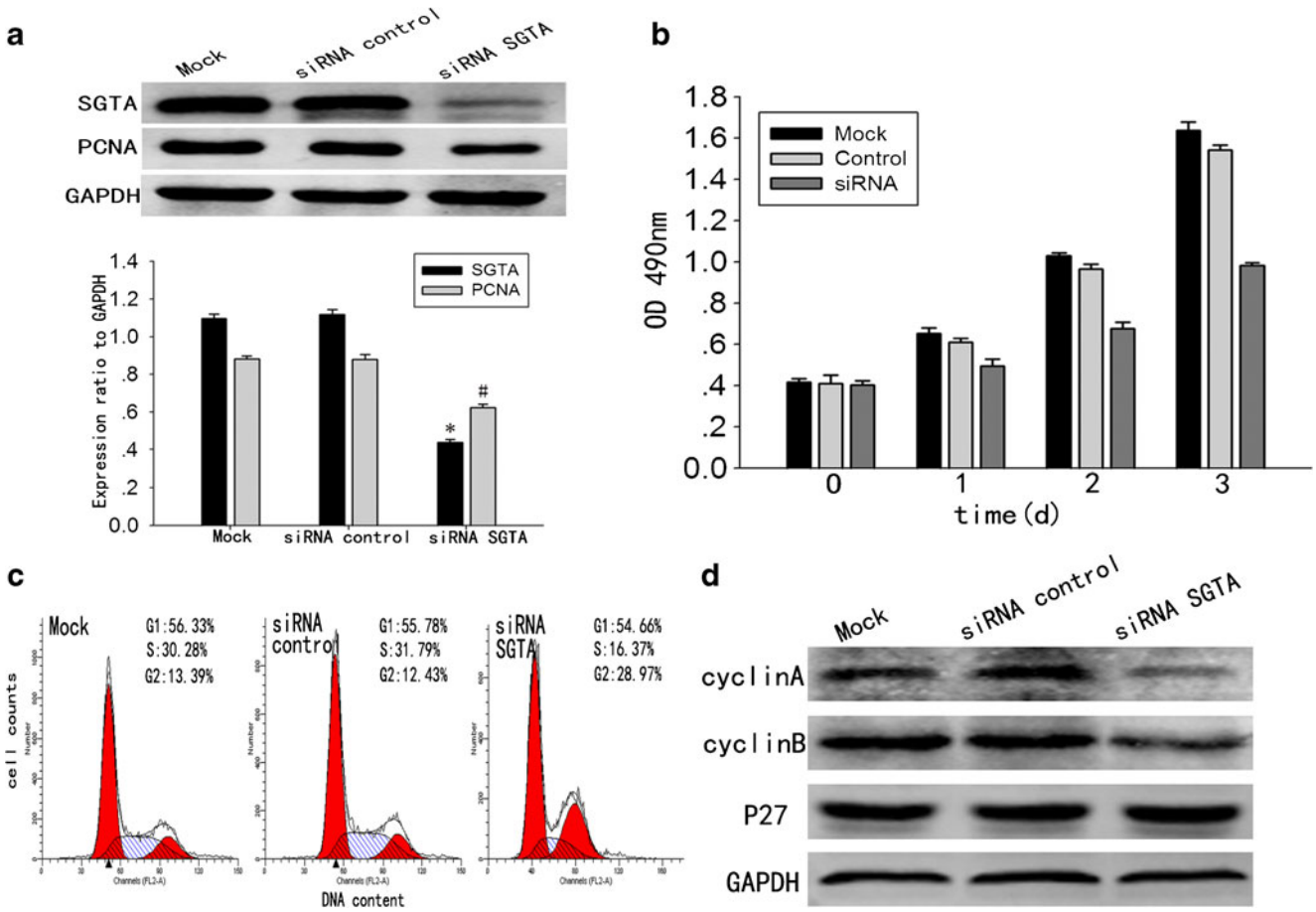

Fig. 5 SGTA knockdown inhibited cell proliferation and effects of altered SGTA expression on cell cycle in HCC cells. **a** Western blot analysis showed that siRNA treatment of SGTA markedly decreased SGTA and PCNA levels 48 h after siRNA transfection in Huh7 cells. The *bar chart* below demonstrates the ratio of SGTA and PCNA protein to GAPDH by densitometry. The data are means ± SEM (*, #P <0 .01 compared with the negative control [mock-treated cells]). **b** CCK-8 assay showed that SGTA knockdown inhibited cell proliferation. Cell Counting Kit-8 reagents were added to the medium and incubated for additional 2 h. Absorbance was measured at each indicated time (0, 1, 2, and 3 day). Each time point was derived from three independent experiments. **c** SGTA knockdown resulted in the delay of G1-S transition and significant arrest in G2 phase of Huh7 cells, after these HCC cells were released from the synchronous status. The data are showed as mean ± standard error for three experiments. **d** Western blot analysis of a series of cell cycle related molecules in SGTA depletion Huh7 cells

by regulating cell proliferation of HCC. Therefore, to further determine whether SGTA plays a role in the proliferation of HCC, we knocked down the expression of SGTA in Huh7 cells using siRNA. Western blot showed an obviously decrease of PCNA protein expression, which indirectly suggested the role of SGTA in the proliferation of HCC cells. Consistent with the Western blot results, CCK-8 assay of Huh7 cells treated with siRNA exhibited a significant decrease of the proliferation rate compared with the negative control siRNA- or mock-treated cells. Herein, we may raise the possibility that SGTA knockdown inhibited the cellular proliferation of HCC. Interestingly, in contrast to the effect of overexpression SGTA in SMMC-7721 cells [9], our data proposed a possibility that the up-regulation of SGTA could contribute to cellular proliferation of Huh7 cells and oncogenesis and progression of HCC. A rational explanation for above controversial observations is that SGTA exerts its function through different pathways, respectively, in different cells. Moreover, it is unlikely that SGTA plays a direct role in apoptosis according to our known knowledge that the known TPR motif proteins are not the effectors [26–29]. On the contrary, the regulator role of SGTA in apoptosis depends on its ability to interact with other proteins such as Hsp70 and Hsc70 [9] and further work need to be performed to elucidate the underlying molecular mechanism. Our present study is similar with the observation reported by Winnefeld et al., which shows that hSGT knockdown reduces cell proliferation in all human cell lines tested and this is mainly due to hSGT depletion-specific arrests in mitosis [13]. To explore the possible mechanism relevant to up-regulation of SGTA in regulating cell proliferation of HCC, cell cycle analysis demonstrated that down-regulation of SGTA decreased cell population in the S phase as well as augmented G2 cycle arrest, leading to unable to complete cell division in Huh7 cells. Subsequent Western blot analysis revealed that siRNA-SGTA down-regulated the protein levels of cyclin A and cyclin B but with scarcely any change in the expression level of $P27^{Kip1}$. Absence of cyclin A and cyclin B after siRNA-SGTA

might contribute to a decrease in S phase and G2 phase arrest as a late event. Taken together, these findings indicated that SGTA might contribute to tumor cell proliferation through the regulation of cell cycle distribution. However, the detailed underlying molecular mechanisms that SGTA stimulates cell cycle progression and proliferation in HCC cells need to be further studied.

In conclusion, the present study showed that SGTA expression was high in HCC specimens and high expression of SGTA was associated with histological grade as well as poor prognosis. Additionally, we found that the expression of SGTA increased during cell cycle progression in Huh7 cells. Furthermore, SGTA depletion by siRNA inhibited cell proliferation and resulted in cell cycle arrest. Given these results, SGTA might serve as a molecular target closely associated with cell proliferation and cell cycle for future development of HCC therapeutics.

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Conflict of interest statement None

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