B-cell translocation gene 1 serves as a novel prognostic indicator of hepatocellular carcinoma

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Abstract. Although the B-cell translocation gene 1 (*BTG1*) plays an important role in apoptosis and negatively regulates cell proliferation, *BTG1* expression in hepatocellular carcinoma (HCC) has not been evaluated. In this study expression analysis of *BTG1* was conducted to clarify the role of *BTG1* in the initiation of HCC carcinogenesis and progression. *BTG1* mRNA expression levels were determined for HCC cell lines and 151 surgical specimen pairs using quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) assay. The mutational and methylation status of HCC cell lines were analyzed via high resolution melting (HRM) analysis and direct sequencing analysis to elucidate the regulatory mechanisms of *BTG1* expression. The expression and distribution of the *BTG1* protein in liver tissues were evaluated using immunohistochemistry (IHC). Decreased expression of *BTG1* mRNA was confirmed in the majority of HCC cell lines (89%) and clinical HCC tissues (85%) compared with non-cancerous liver tissues. Mutations or promoter hypermethylation were not identified in HCC cell lines. *BTG1* mRNA expression levels were not influenced by background liver status. The pattern of *BTG1* protein expression was consistent with that of *BTG1* mRNA. Downregulation of *BTG1* mRNA in HCC was significantly associated with shorter disease-specific and recurrence-free survival rates. Multivariate analysis of disease-specific survival rates identified *BTG1* mRNA downregulation as an independent prognostic factor for HCC (hazard ratio 2.12, 95% confidence interval 1.12-4.04, P=0.022). Our results indicate that altered *BTG1* expression might affect hepatocarcinogenesis and may represent a novel biomarker for HCC carcinogenesis and progression.

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Key words: B-cell translocation gene 1, hepatocellular carcinoma, tumor suppressor, expression, prognosis

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most common cause of cancer-related mortality worldwide. HCC is also the leading cause of death among cirrhotic patients (1,2). Most HCC cases occur in sub-Saharan Africa and Eastern Asia; however, the incidence rate has been increasing in some developed countries, including Japan, the United Kingdom, France, and the United States (3,4). All patients diagnosed with advanced stages of the disease exhibit very limited survival, and to date, only treatment with the multikinase inhibitor sorafenib has improved survival rates for these patients (5,6).

In contrast to most other solid tumors, the underlying cirrhotic liver disease found in HCC patients greatly complicates the tumor-related prognosis, which presents a unique situation in which accurate prognostic prediction is a relevant and unmet need (2,7,8). Chronic inflammation induced by chronic viral hepatitis, alcohol consumption or aflatoxin and subsequent hepatocyte regeneration are underlying causes of HCC (9). Continuous inflammation occasionally damages DNA in the hepatocytes of the regenerating liver and thereby increases the chance of developing a gene alteration that may lead to carcinogenesis. The molecular profiling of HCC has led to a better understanding of the physiopathology of this neoplasm and has allowed the development of novel therapeutic approaches (e.g., molecular targeted therapies) for tumors previously considered to be therapy-refractory (10,11). Integrative analyses of genetic and epigenetic information obtained for the tumor and the surrounding tissue should be used to identify novel biomarkers and therapeutic targets in HCC to improve existing treatment algorithms and eventually design a more personalized therapy for this devastating disease (12,13).

We have identified several HCC-related genes by expression and epigenetic analyses (14,15). From the exhaustive expression analysis obtained via our microarray data, the

B-cell translocation gene 1 (*BTG1*) was identified as a candidate tumor suppressor gene for HCC. Human *BTG1*, which is localized to chromosome 12q22, was originally identified as a translocation partner of the *c-Myc* gene in a case of B-cell chronic lymphocytic leukemia and belongs to a family of antiproliferative genes (16-18). *BTG1* is constitutively expressed in quiescent cells, and its expression is downregulated as cells enter the growth cycle (19,20). Experiments in which gene expression was induced showed that *BTG1* is a Bcl-2-regulated mediator of apoptosis and that it negatively regulates cell proliferation in breast and ovarian cancer (20,21). However, the role of *BTG1* in gastroenterological malignancies including HCC remains unclear.

The aims of this study were to evaluate the clinical significance of *BTG1* expression, examine the regulatory factors involved in *BTG1* transcription, clarify the roles of *BTG1* in hepatocarcinogenesis and its subsequent progression, and propose a potential diagnostic and therapeutic molecular target for HCC.

Materials and methods

Ethics. This study conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects and has been approved by the Institutional Review Board of Nagoya University, Aichi, Japan (no. 2013-0295). Written informed consent for usage of clinical samples and data, as required by the Institutional Review Board, was obtained from all patients.

Sample collection. Nine HCC cell lines (Hep3B, HepG2, HLE, HLF, HuH1, HuH2, HuH7, PLC/PRF/5 and SK-Hep1) were obtained from the American Type Culture Collection (Manassas, VA, USA). Primary HCC tissues and corresponding non-cancerous tissues were collected from 151 consecutive patients undergoing liver resection for HCC at Nagoya University Hospital between January, 1998 and January, 2012. Treatment after recurrence generally included the following options: surgery, radiofrequency ablation, transcatheter arterial chemoembolization, and chemotherapy according to tumor status and liver function.

Tissue samples were collected, immediately flash frozen in liquid nitrogen and stored at -80˚C until RNA extraction (28 days on average) was performed. Tumor samples ~5 mm^2 in size that did not contain a necrotic component and were confirmed to contain >80% tumor cells by definition were used for RNA extraction. Corresponding non-cancerous liver tissue samples were collected >2 cm away from the edge of the tumor, were obtained from the same patient and did not contain any regenerative or dysplastic nodules.

Microarray procedure. Sample collection, RNA extraction, and Affymetrix HG-U133A and HG-U133B GeneChip (Affymetrix, Inc., Santa Clara, CA, USA) gene expression arrays were performed as previously described (22-24).

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR). The *BTG1* mRNA expression levels were analyzed by RT-qPCR. Total RNA (10 μg per sample) was isolated and used to generate complementary DNA. The primer sequences used in this study are listed in Table I. RT-qPCR was performed on nine HCC cell lines and 151 pairs of clinical samples with a SYBR-Green PCR Core Reagents kit (Perkin-Elmer/Applied Biosystems, Inc., Foster City, CA, USA) and included no-template samples as a negative control. Real-time detection of the SYBR-Green emission intensity was conducted with an ABI StepOnePlus Real-Time PCR system (Perkin-Elmer/Applied Biosystems, Inc.). The expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was quantified in each sample for standardization. For cell lines, biological replicates were tested in triplicate. Technical replicates were performed in triplicate for both cell lines and HCC tissues. The expression levels for each sample are shown as the *BTG1* value divided by the *GAPDH* value. *BTG1* mRNA expression was considered to be downregulated in tumor tissues when its level was <40% of the level in the corresponding non-cancerous tissues.

Mutational analysis. The *BTG1* gene consists of two exons. Mutational surveillance of HCC cell lines was performed in exon 1 and 2 of the *BTG1* gene by high resolution melting (HRM) analysis. HRM is known to be a reliable and concise technique for the detection of genetic alterations (25-27). Genomic DNA obtained from HCC cell lines was amplified with specific primer pairs according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). All samples were tested in triplicate. Eight wells of each PCR plate were allocated to wild-type control DNA, and one well contained a non-template control to validate the PCR. HRM was conducted using a StepOnePlus instrument (Life Technologies) with a melting temperature range set between 60 and 98˚C. Scanning data were analyzed by HRM software v3.0.1 (Life Technologies). The primers used for mutational analysis are listed in Table I.

Bisulfite sequencing analysis. The *BTG1* gene contains a CpG island near the promoter region; thus, we hypothesized that aberrant methylation is responsible for regulating *BTG1* transcription in HCC. Genomic bisulfite-treated DNA from HCC cell lines was sequenced to ascertain the levels of DNA methylation. The bisulfite treatment and sequencing procedures were performed as previously reported (28,29).

Immunohistochemistry (IHC). IHC was performed to investigate *BTG1* protein localization in 48 representative sections of well-preserved HCC tissue as described previously (30). Sections were incubated for 1 h at room temperature with a rabbit antibody directed against *BTG1* (PA5-25035; Thermo Fisher Scientific, Inc., Rockford, IL, USA) diluted 1:100 in Antibody Diluent (Dako, Carpinteria, CA, USA) and then developed for 2 min using liquid 3,3'-diaminobenzidine as the substrate (Nichirei Corp., Tokyo, Japan). The staining patterns were compared between HCC tissue and the corresponding non-cancerous tissue. The intensity of *BTG1* protein expression was graded depending on the percentage of stained cells as follows: no staining, minimal (<30%); focal (30-70%); and diffuse (>70%) (31). To avoid subjectivity, specimens were randomized and coded before analysis by two independent observers blinded to the status of the samples. Each observer

Table I. Primers and annealing temperature.

Gene	Experiment	Type	Sequence (5'→3')	Product size (bp)	Annealing temperature (˚C)
BTG1	RT-qPCR	Forward	CTGCAGACCTTCAGCCAGA	104	60
		Reverse	CGAATACAACGGTAACCCGA		
	Exon 1; HRM sequencing	Forwarvd	CATCGCTCGTCTCTTCCTCT	419	54
		Reverse	GACTCTGACCCAGGGATGTG		
	Exon 2; HRM sequencing	Forward	CGATCCTAAGCGTTGTTTCTC	497	56
		Reverse	TCCATAATCCATCCCCAAGA		
	Bisulfite sequencing	Forward	GTGGTATTATAAAGGGTGTTG	118	62
		Reverse	AACCTCCCCAACTACCTCC		
GAPDH	RT-qPCR	Forward	GAAGGTGAAGGTCGGAGTC	226	60
		Probe	CAAGCTTCCCGTTCTCAGCC		
		Reverse	GAAGATGGTGATGGGATTTC		

BTG1, B-cell translocation gene 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, quantitative real-time reverse transcription polymerase chain reaction; HRM, high resolution melting.

Table II. Microarray results for *BTG1* expression.

Gene	Log_2 ratio	Normal signal	Detection	Tumor signal	Detection	Probe ID	Chromosomal location
BTG1	-1.6	2544.9	Positive	757.1	Positive	HU133p2 _10370	Chr 12q22
	-1.5	1389	Positive	418.8	Positive	HU133p2 _10369	Chr 12q22

BTG1, B-cell translocation gene 1.

evaluated all the specimens at least twice within a given time interval to minimize intra-observer variation.

Statistical analysis. The values between the two groups were analyzed using the Mann-Whitney U test. The χ^2 test was used to analyze the association between the expression status of *BTG1* and clinicopathological parameters. The strength of the correlation between two variables was assessed by Spearman's rank correlation coefficient. Disease-specific and -free survival rates were calculated using the Kaplan-Meier method, and the difference in survival curves was analyzed using the log-rank test. We performed multivariable regression analysis to detect prognostic factors using the Cox proportional hazards model, and variables with $P<0.05$ were entered into the final model. All statistical analysis was performed using JMP 10 software (SAS Institute, Inc., Cary, NC, USA). $P<0.05$ was considered statistically significant.

Results

Patient characteristics. The age of the 151 patients ranged from 34-84 years (median 64 years), and the male-to-female ratio was 126:25. Thirty-seven patients presented with hepatitis B infections, and 84 patients presented with hepatitis C infections. In terms of the non-cancerous liver samples, the number of patients with normal liver, chronic hepatitis, and cirrhosis were 10, 87, and 54, respectively. When classified according to the 7th edition of the UICC classification, 84, 39 and 18 patients were in stages I, II and III, respectively.

Expression array. Gene expression that was reduced further in tumor tissues than in the corresponding non-cancerous tissues was used to identify new candidate tumor suppressors in HCC. *BTG1* expression was reduced in HCC compared with normal tissue, with a log_2 ratio of -1.6 and -1.5 (Table II).

BTG1 mRNA expression and regulatory mechanisms in HCC cell lines. Decreases in *BTG1* mRNA were confirmed in eight (89%) of the nine HCC cell lines compared with the median expression level in non-cancerous liver tissues; these results demonstrate the heterogeneity of *BTG1* expression in HCC cell lines (Fig. 1A). No mutations were detected by the HRM analysis of *BTG1* exons 1 and 2 (Fig. 1B). Direct nucleotide sequence analysis of bisulfite-treated GC cell lines showed absence of hypermethylation of *BTG1* promoter region in all GC cell lines (Fig. 1C).

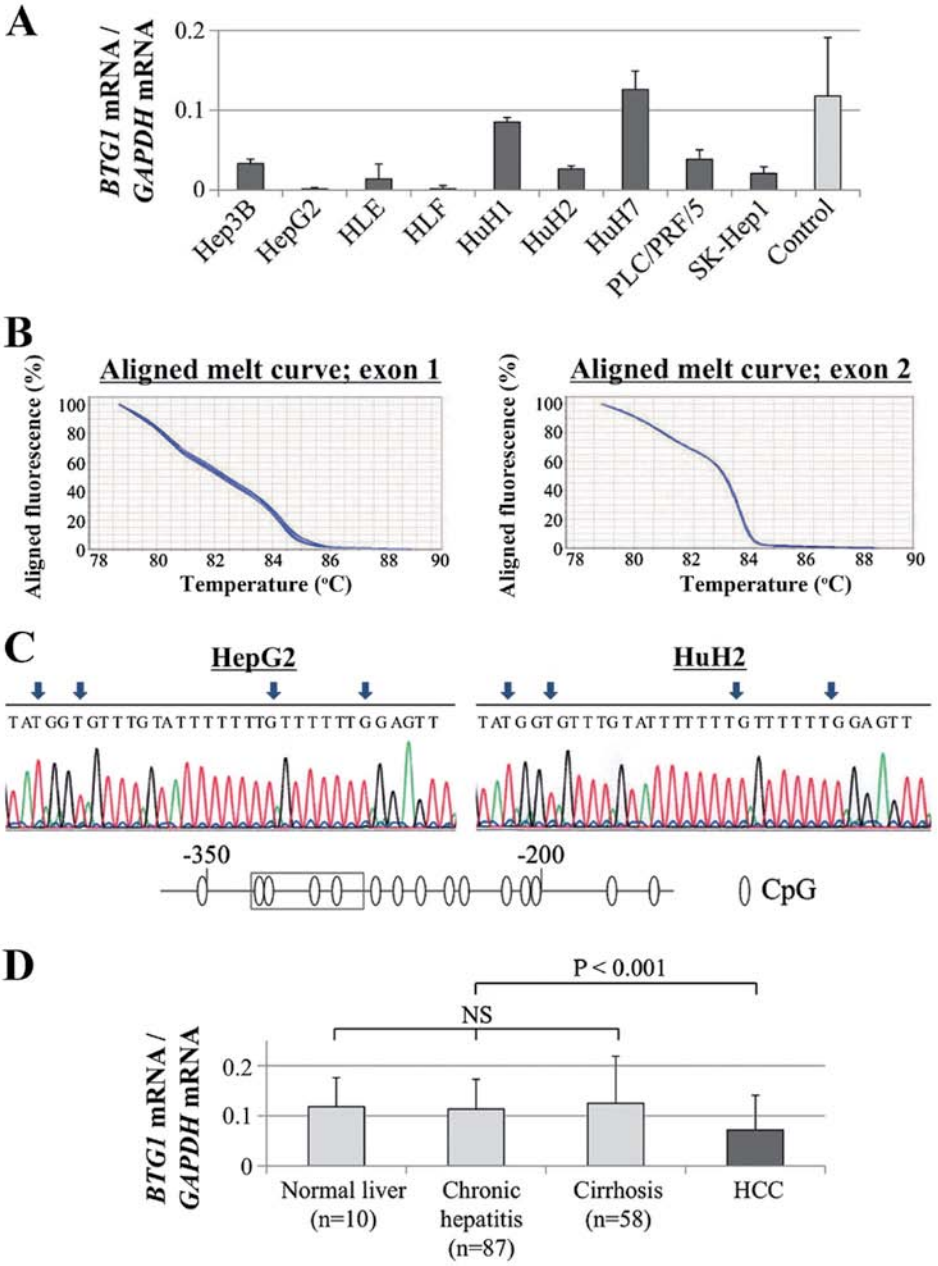

Figure 1. (A) Expression status of B-cell translocation gene 1 (*BTG1*) in hepatocellular carcinoma (HCC) cell lines. *BTG1* mRNA expression in HCC cell lines and control samples (median value of 151 non-cancerous tissues) by quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR). Compared with the control cell lines, reduced expression was confirmed in all the HCC cell lines except for HuH1 and HuH7. (B) Results of the high-resolution melting (HRM) analysis for *BTG1* exon 1 and 2. No mutations were detected. (C) Representative results of the bisulfite sequencing analysis. All CpGs were converted to TG. (D) *BTG1* expression in clinical specimens. There were no significant differences in the *BTG1* mRNA expression levels observed in non-cancerous tissues among the three patient groups categorized by background liver status. HCC tissues showed a lower mean expression of *BTG1* mRNA than the corresponding normal tissues. NS, not significant.

Expression status of BTG1 in 151 clinical HCC samples. The *BTG1* mRNA expression levels of non-cancerous tissue samples were categorized pathologically into normal liver (n=10), chronic hepatitis (n=87), and cirrhosis (n=58). Upon evaluation of these samples, no significant differences were found, suggesting that the expression of *BTG1* mRNA in non-cancerous liver was not affected by background liver fibrosis (Fig. 1D). In 129 (85%) of 151 patients, the expression level of *BTG1* mRNA was lower in the HCC tissues than in the corresponding normal tissues. The HCC tissues exhibited significantly lower expression levels of *BTG1* mRNA than the corresponding normal tissues (P<0.001, Fig. 1D).

Expression patterns of the *BTG1* protein were evaluated by IHC. Representative cases with downregulated *BTG1* mRNA expression in HCC tissues exhibited reduced expression of the *BTG1* protein in the cytoplasm of the cancerous tissues compared with the adjacent non-cancerous tissues (Fig. 2A). Overall, the staining intensity (shown in Fig. 2B) of the *BTG1* protein in 48 patients was consistent with the RT-qPCR data (Fig. 2C).

Prognostic values of the expression status of BTG1. Fifty-four of 151 HCC patients showed substantial downregulation (<40%) of *BTG1* mRNA in HCC tissues compared with non-cancerous

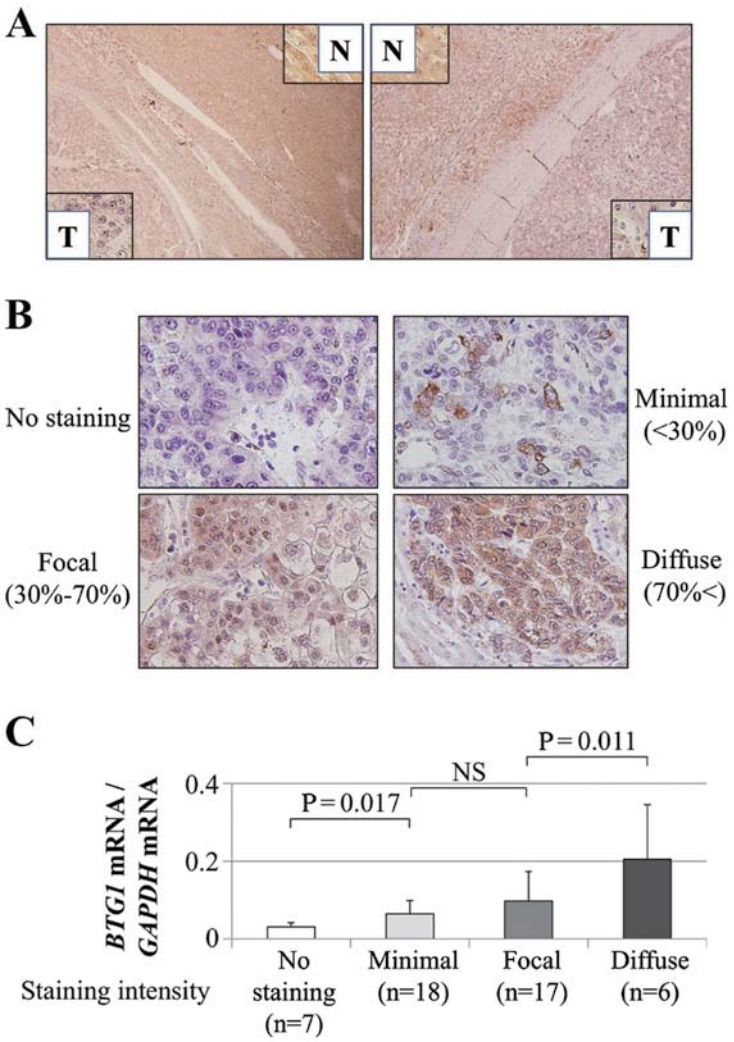

Figure 2. (A) Detection of B-cell translocation gene 1 (*BTG1*) protein by immunohistochemical staining in two representative hepatocellular carcinoma (HCC) patients. In both samples, the cancerous tissues exhibited reduced *BTG1* protein expression compared with the adjacent noncancerous tissues (x100 and x400 magnification). N, non-cancerous tissue component; T, tumor tissue component. (B) Representative cases of each *BTG1* staining intensity; no staining, minimal, focal and diffuse (x400 magnification). (C) Staining intensity of *BTG1* was associated directly with the expression level of *BTG1* mRNA in HCC tissues. NS, not significant.

tissues. The downregulation of *BTG1* mRNA in the HCC samples was significantly associated with male gender, protein induced by vitamin K antagonists (PIVKA) II >40 mAU/ml, tumor size ≥3 cm, tumor differentiation (poorly to moderately differentiated), serosal infiltration, vascular invasion, advanced UICC stage, and extra-hepatic recurrence (Table III). The *BTG1* mRNA expression levels in HCC tissues were inversely correlated with preoperative PIVKA II levels (Fig. 3A). Patients exhibiting a downregulation of *BTG1* mRNA expression in the HCC samples had a significantly shorter disease-specific survival rate than the other patients (2-year survival rates, 67 and 82%, respectively, Fig. 3B). Multivariate analysis identified the downregulation of *BTG1* mRNA as an independent prognostic factor for HCC (hazard ratio 2.12, 95% confidence interval 1.12-4.04, P=0.022, Table IV). In terms of recurrence-free survival rates, patients with a substantial downregulation of *BTG1* mRNA in the HCC samples had significantly earlier recurrence rates after surgery than the other patients (2-year recurrence-free survival rates: 39 and 65%, respectively, P=0.032, Fig. 3C).

Discussion

BTG is a nuclear protein that is imported into the nucleus through a nuclear localization signal; its nucleocytoplasmic translocation depends on the stage of cell growth and is mediated by a nuclear export signal (32-34). Accordingly, the BTG family, which is thought to play an intimate role in the proliferation of cancer cells, has attracted attention in recent years (34,35). *BTG1* has been shown to enhance homeobox B9-mediated transcription in transfected cells and mediate its antiproliferative function. As shown by DNA fragmentation and nuclear condensation, *BTG1* localizes to specific macrophage-rich regions in human lesions and apoptotic cells (36). *BTG1* mRNA is abundantly expressed in quiescent endothelial cells and is decreased upon the addition of angiogenic growth factors (19).

In this study, the expression status and regulatory mechanisms of *BTG1* were investigated in HCC. Following the confirmation that *BTG1* mRNA expression is remarkably suppressed in most HCC cell lines, the somatic mutation and

Table III. Association between expression status of *BTG1* mRNA and clinicopathological parameters in 151 HCC patients.

Clinicopathological parameters	Downregulation of *BTG1* mRNA (n)	Others (n)	P-value
Age			
<65 year	19	48	0.088
≥65 year	35	49	
Gender			
Male	51	75	0.004[a]
Female	3	22	
Background liver			
Normal liver	4	6	0.497
Chronic hepatitis	34	53	
Cirrhosis	16	38	
Pugh-Childs classification			
A	48	92	0.187
B	6	5	
Hepatitis virus			
Absent	14	16	0.387
HBV	12	25	
HCV	28	56	
AFP (ng/ml)			
≤20	24	57	0.091
>20	30	40	
PIVKA II (mAU/ml)			
≤40	9	49	<0.001[a]
>40	45	48	
Tumor multiplicity			
Solitary	39	78	0.253
Multiple	15	19	
Tumor size			
<3.0 cm	10	37	0.010[a]
≥3.0 cm	44	60	
Differentiation			
Well	7	28	0.022[a]
Moderate to poor	47	69	
Growth type			
Expansive growth	45	82	0.847
Invasive growth	9	15	
Serosal infiltration			
Absent	34	80	0.009[a]
Present	20	17	
Formation of capsule			
Absent	14	33	0.299
Present	40	64	
Infiltration to capsule			
Absent	22	46	0.428
Present	32	51	
Septum formation			
Absent	14	39	0.074
Present	40	58	
Vascular invasion			
Absent	35	79	0.025[a]
Present	19	18	
Margin status			
Negative	43	80	0.668
Positive	11	17	
UICC pathological stage			
I	26	68	0.014[a]
II	17	22	
III	11	7	
Extra-hepatic recurrence			
Absent	33	79	0.007[a]
Present	21	18	

[a]Statistically significant difference (P<0.05). *BTG1*, B-cell translocation gene 1; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; PIVKA, protein induced by vitamin K antagonists; UICC, Union for International Cancer Control.

DNA methylation statuses were evaluated as possible mechanisms of suppression. No mutations were detected in any of the HCC cell lines examined by the HRM. In addition, bisulfite sequencing showed absence of hypermethylation in the *BTG1* promoter in all GC cell lines. These findings were consistent with those in acute lymphoblastic leukemia (37), and further study will be needed to elucidate the alterative underlying molecular pathway suppressing *BTG1* transcription.

Interestingly, the expression analysis of clinical samples demonstrated important clinical implications for the expression of *BTG1*. *BTG1* was downregulated in most HCC tissues, and the strong suppression of *BTG1* was an independent prognostic factor associated with early recurrence. These results indicate that *BTG1* is a putative tumor suppressor gene that affects both carcinogenesis and the subsequent progression of HCC. The potential use of *BTG1* expression as a prognostic biomarker is supported by the finding that *BTG1* expression in HCC tissues was inversely correlated with the serum levels of PIVKA II, an important HCC tumor marker.

In clinical practice, aggressive pre- and post-operative systemic therapy could be considered for patients exhibiting strong downregulation of *BTG1* identified in biopsies or surgical specimens in anticipation of early recurrence and an adverse prognosis. *BTG1* interacts with and regulates the activity of protein arginine methyl transferase (PRMT)1 (38,39). Members of this enzyme family, including PRMT1, are considered global regulators of gene expression that act as transcriptional coregulators of the arginine methylation of histone tails and are critical regulators of transcription (40). Based on our results, the forced expression or artificial modification of interacting molecules (including PRMT1) of

Table IV. Prognostic factors in 151 patients with hepatocellular carcinoma.

Variable	n	Univariate			Multivariable		
		HR	95% CI	P-value	HR	95% CI	P-value
Age (≥65)	84	1.92	1.07-3.57	0.030[a]	1.77	0.96-3.38	0.069
Gender (male)	126	1.27	0.60-3.13	0.553			
Background liver (cirrhosis)	54	1.58	0.88-2.81	0.123			
Pugh-Childs classification (B)	11	0.93	0.28-2.32	0.889			
AFP (>20 ng/ml)	70	1.90	1.07-3.42	0.029[a]	1.55	0.81-2.96	0.181
PIVKA II (>40 mAU/ml)	93	2.10	1.14-4.07	0.016[a]	1.16	0.56-2.51	0.695
Tumor multiplicity (multiple)	34	2.09	1.11-3.76	0.023[a]	1.77	0.91-3.32	0.092
Tumor size (≥3.0 cm)	104	2.20	1.13-4.71	0.020[a]	1.11	0.50-2.62	0.810
Tumor differentiation (well)	35	0.55	0.25-1.10	0.095			
Growth type (invasive growth)	24	1.44	0.69-2.76	0.318			
Serosal infiltration	37	2.51	1.32-4.61	0.006[a]	1.12	0.55-2.24	0.742
Formation of capsule	104	1.05	0.57-2.02	0.884			
Infiltration to capsule	83	1.20	0.67-2.18	0.537			
Septum formation	98	0.87	0.49-1.60	0.651			
Vascular invasion	37	3.40	1.87-6.07	<0.001[a]	2.26	1.13-4.55	0.022[a]
Margin status (positive)	28	2.64	1.42-4.73	0.003[a]	2.30	1.21-4.23	0.012[a]
Downregulation of *BTG1* mRNA	54	2.55	1.43-4.57	0.002[a]	2.12	1.12-4.04	0.022[a]

Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. [a]Statistically significant (P<0.05). HR, hazard ratio; CI, confidence interval; AFP, α-fetoprotein; PIVKA, protein induced by vitamin K antagonists; *BTG1*, B-cell translocation gene 1.

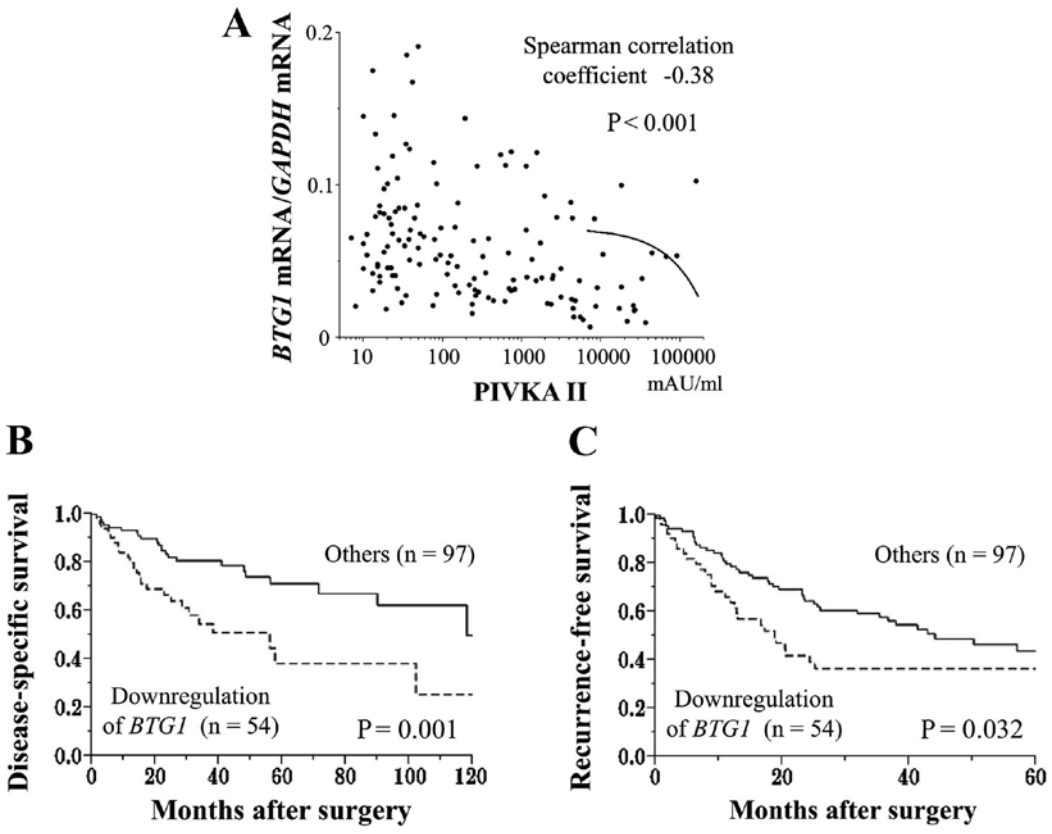

Figure 3. (A) Correlative evaluation between the B-cell translocation gene 1 (*BTG1*) mRNA expression levels in hepatocellular carcinoma (HCC) tissues and preoperative protein induced by vitamin K antagonist (PIVKA) II levels. (B and C) Prognostic impact of *BTG1* mRNA expression in HCC patients. HCC patients with downregulated *BTG1* mRNA had significantly shorter survival rates than patients with normal *BTG1* mRNA levels. (B) Disease-specific and (C) recurrence-free survival rates. P-values were calculated using the log-rank test.

BTG1 may be used as novel therapeutic approaches for the treatment of HCC. For future consideration, external validation is necessary, and functional analysis of the *BTG1* gene could help to further clarify the role that *BTG1* plays in the progression of HCC.

In summary, the reduced expression of *BTG1* mRNA was associated with early recurrence rates and subsequent poor prognoses in patients with HCC. Our results indicate that altered *BTG1* expression might affect hepatocarcinogenesis and may represent a novel biomarker for the initiation of carcinogenesis and the progression of HCC.

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