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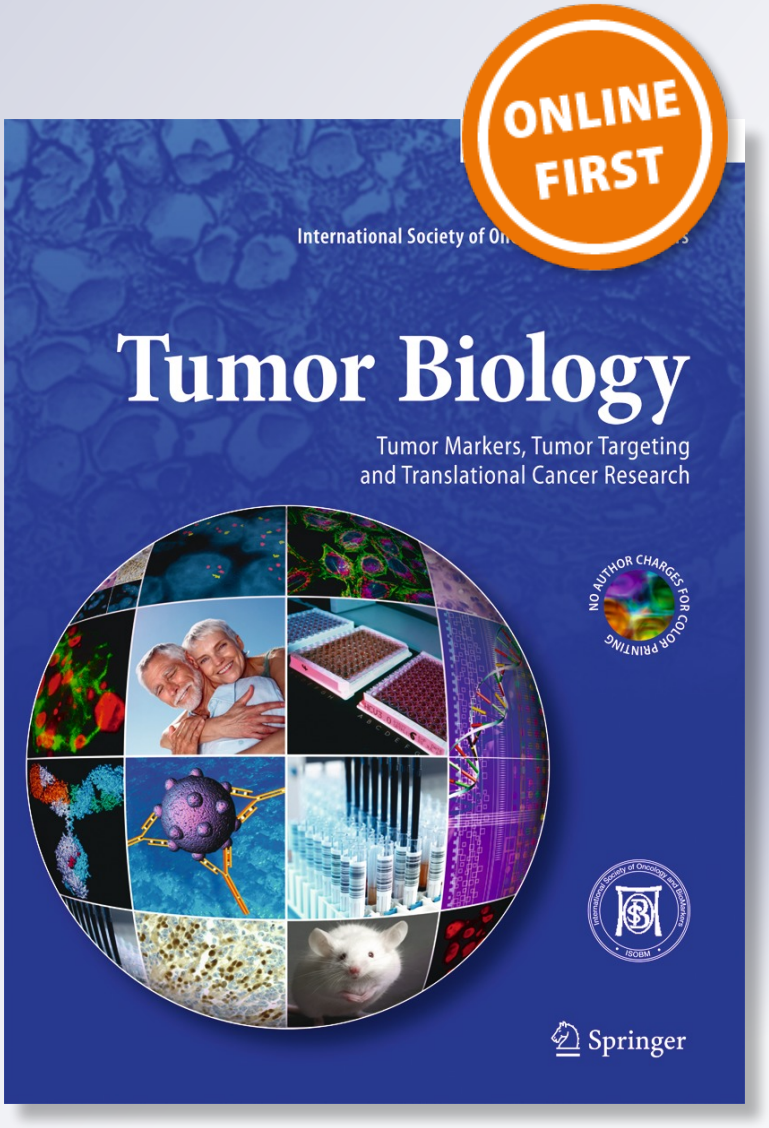

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RESEARCH ARTICLE

Expression of BTG1 in hepatocellular carcinoma and its correlation with cell cycles, cell apoptosis, and cell metastasis

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Abstract This study aimed to analyze the expression, clinical significance of B cell translocation gene 1 (BTG1) in hepatocellular carcinoma, and the biological effect in its cell line by BTG1 overexpression. Immunohistochemistry and Western blot were used to analyze BTG1 protein expression in 70 cases of hepatocellular cancer and 32 cases of normal tissues to study the relationship between BTG1 expression and clinical factors. Recombinant lentiviral vector was constructed to overexpress BTG1 and then infect hepatocellular cancer HepG2 cell line. The level of BTG1 protein expression was found to be significantly lower in hepatocellular cancer tissue than normal tissues ($P<0.05$). Decreased expression of BTG1 was significantly correlated with tumor invasion, lymph node metastasis, clinic stage, and histological grade of patients with hepatocellular cancer ($P<0.05$). Meanwhile, loss of BTG1 expression correlated significantly with poor overall survival time by Kaplan-Meier analysis ($P<0.05$). The result of biological function has shown that HepG2 cell-transfected BTG1 had a lower survival fraction; higher percentage of the G0/G1 phases; higher cell apoptosis; significant decrease in migration and invasion; and lower Cyclin D1 (CND1), B cell lymphoma 2 (Bcl-2), and matrix metalloproteinases (MMP)-9 protein expression compared with HepG2 cell-untransfected BTG1 ($P<0.05$). BTG1 expression decreased in hepatocellular cancer and correlated significantly with lymph node metastasis, clinic stage, histological grade, poor overall survival, proliferation, and metastasis in hepatocellular cancer cell by regulating CND1, Bcl-2, and MMP-9 protein expression, suggesting that BTG1 may play important roles as a negative regulator to hepatocellular cancer cell.

Keywords BTG1 · Hepatocellular carcinoma · CND1 · Bcl-2 · MMP-9 · Metastasis

Introduction

Tumor development and progression are closely related to tumor cell cycle regulation, as evidenced by the presence of impaired cell cycle regulation mechanisms in almost all tumors. Studies have shown that cell proliferation, differentiation, senescence, and apoptosis are cell cycle dependent. Many proto-oncogenes and tumor suppressor genes are directly involved in cell cycle regulation or function as major factors regulating cell cycle [1].

B cell translocation gene 1 (BTG1) is a member of human Transducer of ErbB-2 (TOB)/BTG family. Previous studies have shown that BTG1 gene has various biological functions. For instance, BTG1 has been found to mediate negative regulation of cell proliferation. Its expression varies with the progression of cell cycle, peaking in the G0/G1 phase before declining thereafter. In addition, enhanced BTG1 expression promotes the differentiation of neural stem cells and germ cells and plays an important role in angiogenesis. BTG1 also facilitates the formation of CCR4/NOT (CAF1), which is involved in nuclear transcription, deadenylation, and turnover of cytoplasmic messenger (m)RNAs. BTG1 is a member of antiproliferative BTG/TOB protein family. All members of the family can inhibit cell proliferation and exert negative regulation on cell cycle [2–6]. While these studies suggest

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that BTG1 displays some of the characteristics of tumor suppressor genes, no study to date has addressed whether BTG1 gene is a hepatocellular cancer suppressor gene and whether BTG1 plays a role in the growth and proliferation, invasion and metastasis, and apoptosis of hepatocellular cancer cells.

Materials and methods

Main reagents

Rabbit anti-human BTG1 polyclonal antibody, rabbit anti-human Cyclin D1 (CND1) monoclonal antibody, rabbit anti-human Bcl-2 polyclonal antibody, and rabbit anti-human matrix metalloproteinases (MMP)-9 polyclonal antibody were from Amcam Inc. (USA). β-Actin primary antibody was purchased from Sigma Chemical Company (St. Louis, MO, USA). pLenti6/V5-DEST vector, lentiviral packaging mix, Opti-MEM, and Lipofectamine 2000 were obtained from Invitrogen Corporation (Carlsbad, CA, USA). An immunohistochemistry kit and the Annexin V-FITC/PI apoptosis detection kit were purchased from 4A Biotech Co. Ltd. (Beijing, China). Fetal bovine serum (FBS), cell culture media, and supplementary materials were obtained from Gibco Co. (Grand Island, NY, USA).

Cell culture and gene transfection

Human hepatocellular cancer HepG2 cell was maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10 % fetal bovine serum (Gibco BRL, Grand Island, NY). Medium was changed every 2–3 days; when the cultures reached confluence, the cells were subcultured with 0.25 % trypsin and 1 % ethylenediaminetetraacetic acid. The pLenti6/V5-DEST vector (Invitrogen, CA, USA) was used to harbor BTG1 cDNA through cloning of BTG1 cDNA sequences into the *Bam*H I and *Asc* I sites of the pLenti6/V5-DEST vector. After amplification and DNA sequence confirmation, this vector was used to induce BTG1 expression in HepG2 cell. Briefly, HepG2 cell was grown and stably transfected with pLenti6-BTG1 or pLenti6/V5-DEST vector using Lipofectamine 2000 and grown in antibiotic Blasticidin (5 μg/ml)-containing RPMI-1640 medium for selection of stable sublines.

Immunohistochemistry

A 4-μm section was prepared from paraffin-embedded block and dehydrated, then incubated in 3 % hydrogen peroxide for 10 min to block endogenous peroxidase, followed by using trypsin for repair for 20 min; 10 % goat serum was introduced at room temperature for closure for 20 min, and BTG1 antibody (1: 100) was left in the wet box at 4 °C refrigerator for overnight. Then, the secondary and third antibodies were dropped into the wet box at room temperature for incubation for 20 min, respectively; DAB staining was again visualized by the hematoxylin stain and then came to normal dehydration with the coverslip sealed. BTG1 expression was determined based on the percentage of positive cells, combined with the staining intensity. The percentage of positive cells was divided into four levels: 0 point, ≤5 % of positive cells; 1 point, 5~25 %; 2 points, 25~50 %; and 3 points, >50 % of positive cells. The intensity of staining was classified as follows: 0 point: no staining, 1 point: weak staining (light yellow), 2 points: moderate staining (brown), and 3 points: strong staining (yellowish-brown). The final score of BTG1 expression was the product of the BTG1 expression rate and intensity, graded as 0 for negative and +~+++ for positive (+ for 1–3 points, ++ for 4–6 points, and +++ for 7–9 points). As for the negative control, the primary antibody was replaced with PBS.

Quantitative real-time RT-PCR

Total RNA was extracted from the hepatocellular cancer HepG2 cell using TRIzol reagent (Invitrogen; Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Five hundred nanograms of total RNA was reverse transcribed using TaKaRa Reverse Transcriptase Reagents (TaKaRa). Quantitative real-time RT-PCR (qRT-PCR) was performed on an ABI Prizm 7300 according to the standard protocol of SYBR Premix ExTaq perfect real-time system (TaKaRa). Primers for BTG1 and β-actin as a reference for normalization were as follows: BTG1 sense 5′- GGAATTCA TGCATCCCTTCTACACCCGG, antisense 5′- CGACGCGT TTAACCTGATACAGTCATCAT; β-actin sense 5′- ATCG TCCACCGCAAATGCTTCTA, antisense 5′- AGCCATGC CAAT CTCATC TTGTT. Thermal cycling conditions were 95 °C for 1 min, 95 °C for 15 s, and 40 cycles at 60 °C for 1 min. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method in SDS 1.3 software.

Western blot

All operations were completed on the ice. At 4 °C, 12,000 r/min centrifugation lasted for 20 min, and then, the supernatant was taken for backup at −20 °C. After the detection of protein concentration with BCA Protein Assay Kit, each hole was given a sample amount of 50 μg for SDS-PAGE electrophoresis. Regulator power for ice bath was transferred to nitrocellulose membrane, followed by closure for 2 h with 5 % skim milk, subsequent to anti-1 overnight incubation at 4 °C (BTG1 1:1,000, CND1 1:500, Bcl-2 1:500, MMP-9 1:500. β-actin 1:5,000). The latter was from Sigma Chemical Company (St. Louis, MO, USA) in 5 % non-fat dry milk for 1 h at room temperature. After washing, the membrane was incubated

with goat anti-rabbit fluorescent secondary antibody (IRDye800, 1:20,000 dilution; the DyLight Fluor conjugated to goat anti-rabbit IgG was obtained from LI-COR Biosciences, Inc., Lincoln, Nebraska, USA) in the dark for 1 h at room temperature.

MTT assay

Cell viability was determined by using the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HepG2 cells were plated into 96-well culture plates at an optimal density of 5×10^3 cells/ml in 200 μl of culture medium per well. After 24–96 h of culture, 20 μl of 5 mg/ml MTT was added to each well and incubated at 37ºC for 4 h. The medium was then gently aspirated, and 150 μl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The optical density of each sample was immediately measured using a microplate reader (BioRad) at 570 nm.

Flow cytometry assay

An Annexin V-FITC-flow cytometry assay was used to detect the apoptosis rate in the cells after BTG1 transfection. Cells were seeded into 60-mm dishes for 48 h and grown to approximately 70–75 % confluence. After quick detachment from the plate, cells were collected, washed with ice-cold PBS, and resuspended at a cell density of 1×10^6/ml in a binding buffer from the Annexin V-FITC apoptosis detection kit (4A Biotech Co. Ltd., Beijing, China) and then stained with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI, 20 μg/ml). The cells were then incubated in the dark at 25ºC for 15 min before 10,000 cells were analyzed by a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and Cellquest software (Becton-Dickinson) for apoptosis rate determination.

Invasion and migration assays

The Costar Transwell 8-μm inserts were coated with 50 μg reduced serum Matrigel (BD Biosciences Franklin Lakes, NJ) for invasion assay according to the manufacturer's instruction, Invasion Chambers (BD China, Shanghai, China) at 8×10^5 cells per chamber. The membrane in the chamber was coated with Matrigel (BD China). Medium supplemented with 10 % FBS was used in the lower chamber. Migration assays were performed in the same manner excluding the Matrigel. After 12 h, non-invading cells and media were removed with a cotton swab. Cells on the lower surface of the membrane were fixed with polyoxymethylene (Sigma) and stained with 0.1 % crystal violet (Sigma) for 0.5 h. Stained cells were counted under a microscope in four randomly selected fields, and the average was used to indicate cell migration and invasion.

Statistical analyses

All statistical analyses were performed using SPSS16.0 software. For the clinicopathologic features, P values were calculated using the χ^2 test. Student t test was used to analyze the difference between groups. A 5 % or lower P value was considered to be statistically significant.

Results

BTG1 protein expression in hepatocellular cancer and normal tissue

In hepatocellular cancer tissues, BTG1 staining was negative or weak. In normal hepatocellular tissues, BTG1 staining ranged from light yellow to brown. Statistically, BTG1 was expressed in 32.9 % (23/70) of hepatocellular cancer tissues, which was lower than the 87.5 % (28/32) in normal tissues. The difference was statistically significant ($P<0.05$, Fig. 1). While Western blot showed that the relative expression of BTG1 protein presented volume between cancer lesion and adjacent normal tissue were 0.255±0.027 and 0.776 ±0.082, showing the difference with statistical significance ($P<0.05$, Fig. 2). The expression of BTG1 was correlated with lymph node metastasis, clinical stages, and pathological differentiation ($P<0.05$), regardless of age, gender, tumor invasion, and pathological types ($P>0.05$, Table 1).

BTG1 expression and prognosis

Survival analysis was performed in all the patients and follow-up data were collected. All patient follow-ups ended in 2012 after a revisit time of 60 months. Among all cases, 13 were still alive at this time and 57 were dead. Patients were divided into two groups according to BTG1 expression level. There were 23 individuals with positive levels of BTG1 expression; among whom, eight were still alive and 15 were dead. The survival rate was 34.8 %. There were 47 individuals with negative levels of BTG1 expression; among whom, five were still alive and 42 were dead. The survival rate was 10.6 %. Patients with high levels of BTG1 expression had significantly higher 5-year survival rates than those patients with low levels of BTG1 expression ($P<0.05$, Fig. 3).

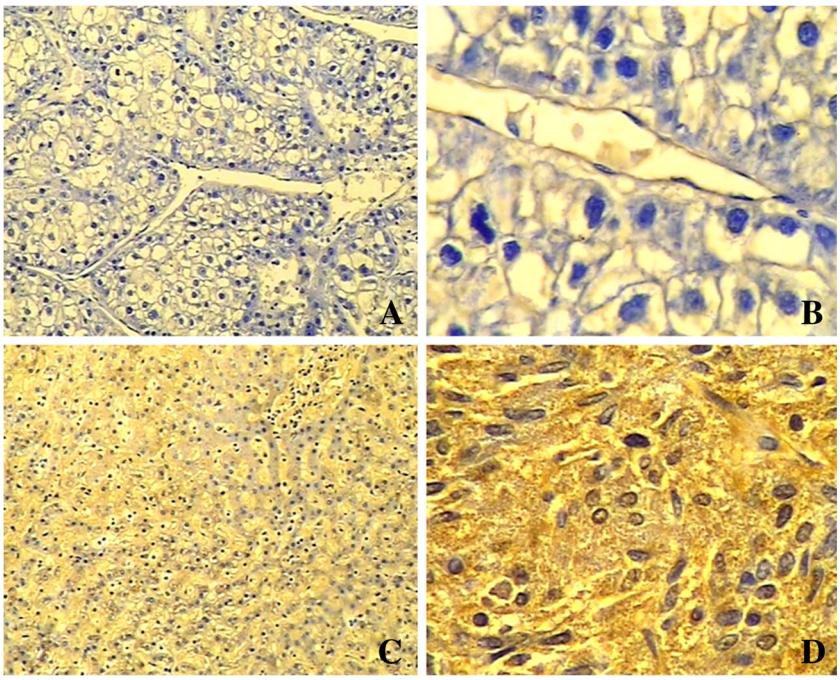

Fig. 1 Expressions of BTG1 protein in hepatocellular carcinoma and normal tissue. **a**, **b** Hepatocellular carcinoma (**a** SP×100, **b** SP×400). **c**, **d** Normal tissue (**c** SP×100, **d** SP×400)

Stable transfection of BTG1 cDNA in hepatocellular cancer cells

In this study, we first stably transfected BTG1 cDNA into HepG2 cells and obtained overexpressed BTG1 HepG2 sublines (named as LeBTG1 cell) and empty vector-transfected HepG2 cell (named as LeEmpty cell) as the control. qRT-PCR data showed that the BTG1 mRNA expression level was 0.419±0.041 in empty vector-transfected cells. In contrast, the amount of BTG1 mRNA in the BTG1-transfected cell lines was 0.837±0.085. The difference was statistically significant ($P<0.05$, Fig. 4a). Furthermore, Western blot analysis showed that the control cells had approximately equal amounts of immunoreactive protein (0.422±0.051). In contrast, the amount of BTG1 protein in the BTG1-transfected cell lines was 0.818±0.084. The difference was statistically significant ($P<0.05$, Fig. 4b).

Effects of BTG1 overexpression on effect of hepatocellular cancer cell

Next, we assessed the effect of BTG1 expression on the regulation of hepatocellular cancer cell viability. MTT assay

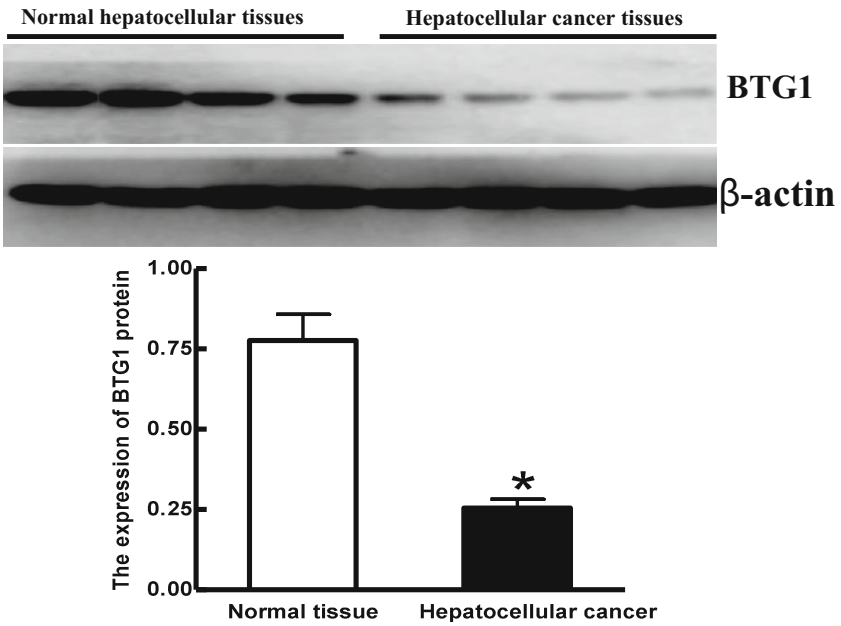

Fig. 2 Expressions of BTG1 protein in hepatocellular carcinoma and normal tissue. *$P<0.05$ compared to the normal tissue

Table 1 Relation between BTG1 expression and clinical characteristics in hepatocellular cancer tissue

Group	Case	Expression of BTG1 protein			
		–	+~+++	χ^2	*P*
Sex					
Male	52	37	15	1.475	0.225
Female	18	10	8		
Age (year)					
≤45	47	32	15	0.058	0.810
>45	23	15	8		
Pathological types					
Hepatocellular type	48	32	16	2.985	0.225
Cholangiocarcinoma type	16	10	6		
Mixed cell type	6	5	1		
Tumor invasion					
T1+T2	24	13	11	2.390	0.122
T3+T4	46	34	12		
Lymph node metastasis					
N0	26	12	14	8.260	0.004
N+	44	35	9		
Clinic stages					
I~II	21	10	11	5.183	0.023
III~IV	49	37	12		
Histological grade					
I	19	9	10	4.622	0.032
I~III	51	38	13		

showed that relative proliferative capacity of the LeBTG1 cell relatively grew slower at 24, 48, 72, and 96 h compared with the parental LeEmpty cell. The difference was statistically significant ($P<0.05$, Fig. 5). Moreover, cell cycle analysis showed that the G0/G1 and S phases of the cell cycle were significantly different in Le BTG1 cell compared to the control cell lines (84.5±5.7 and 7.9±0.7 % vs. 64.2±3.1 and 23.0 ±2.0 %), showing the difference with statistical significance ($P<0.05$, Fig. 6). Meanwhile, there was a relative large increase in the early apoptosis rate, 10.4±1.1 % in LeBTG1 cell compared to control cells (2.8±0.4 %), indicating a difference with statistical significance ($P<0.05$, Fig. 7). Furthermore, the HepG2 cells transfected with BTG1 or empty vector were transferred to transwell chambers or Matrigel-coated transwell chambers to evaluate the effect of BTG1 on cell invasion potential. Introduction of BTG1 clearly led to a significant decrease in HepG2 cell migration and invasion (102.0±18.0 and 63.0±11.0, respectively) compared with the cells transfected with empty vector (162.0 ±22.0 and 103.0±14.0, respectively). The difference was statistically significant ($P<0.05$, Fig. 8). To further identify the mechanisms by which BTG1 inhibited hepatocellular cancer cell proliferation, changed cell cycles, promoted cell apoptosis, and decreased migration and invasion, we analyzed the expression level of CylinD1, Bcl-2, and MMP-9 protein due to their critical roles in cell proliferation, cell cycles, cell apoptosis, and migration and invasion. Western blot analysis revealed that BTG1 significantly downregulated CND1, Bcl-2, and MMP-9 protein expression in HepG2 cell transfected with BTG1 (0.123±0.015, 0.213±0.024, 0.112±0.011, respectively) compared to control cell (0.665±0.061, 0.805±0.081, 0.763±0.077, respectively). The difference was respectively statistically significant ($P<0.05$, Fig. 9).

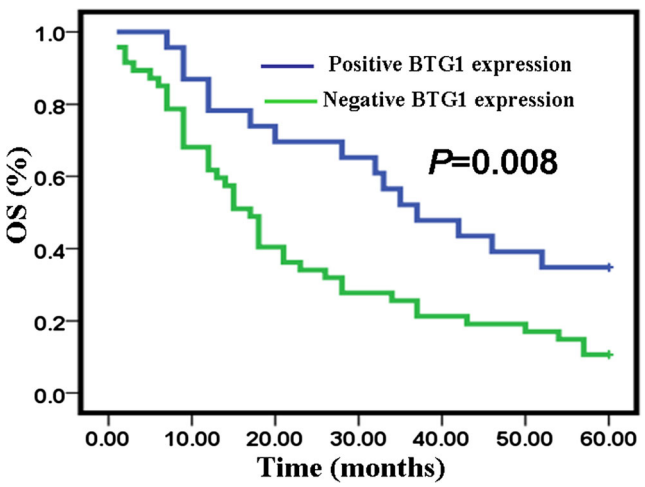

Fig. 3 Relation between BTG1 expression and overall survival in hepatocellular carcinoma by Kaplan-Meier analysis

Discussion

Tumor development and progression are associated with uncontrolled proliferation and reduced apoptosis of tumor cells. BTG1 can inhibit proliferation, regulate cell cycle, and induce apoptosis by acting as a tumor suppressor gene, whose role has been confirmed in breast cancer (Fig. 7) [7]. This study located and quantified for the first time BTG1 protein expression in hepatocellular cancer tissue and normal hepatocellular tissue using immunohistochemistry and Western blot techniques. Our results showed that BTG1 protein levels were significantly lower in hepatocellular cancer than in normal tissue. Our univariate analysis also showed that BTG1 protein levels were correlated with lymph node metastasis, clinical stage, and differentiation of hepatocellular cancer. This result suggests that BTG1 deletion is a major contributor to the development and progression of hepatocellular cancer and that dedifferentiation is one of the hallmarks of tumor cells. This study showed that BTG1 in hepatocellular cancer was negatively correlated with histological differentiation, with lower differentiation associated with lower expression. The determination of the prognosis of patients with hepatocellular cancer is an extremely important part of clinical work. Current studies have shown tumor stage as the preferred prognostic indicator [8]. However, for cancer patients in the same stage

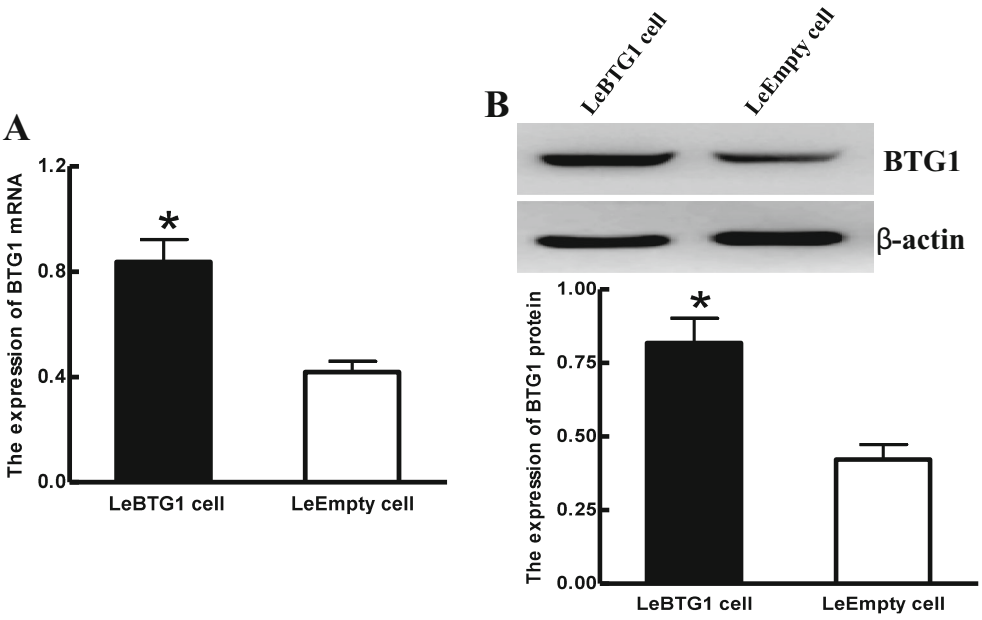

Fig. 4 Expression and identification of the BTG1 gene. **a** qRT-PCT. **b** Western blot. *$P<0.05$ compared to the LeEmpty cell

and with a similar state, their prognosis can still vary considerably. Therefore, it is of particular significance to find an ideal molecular marker in the clinical practice. This study showed in survival analysis that BTG1 expression-positive patients had a significantly higher 5-year overall survival rate than patients without BTG1 expression. Therefore, the combination of TNM classification system and BTG1 expression scores may provide some valuable information for clinicians in the choice of treatment options, prognosis judgment, and prediction of disease severity.

Abnormal proliferation of tumor cells often plays a key role in the development of hepatocellular cancer, as cells that are supposed to undergo apoptosis under normal circumstances continue to survive and show malignant growth behavior [9]. This study found for the first time in in vitro tests that hepatocellular cancer cells with high BTG1 expression had significantly weakened proliferation potential. CND1 is considered a proto-oncogene product and is highly expressed or mutated in a variety of human tumors [10, 11]. In our study, increased

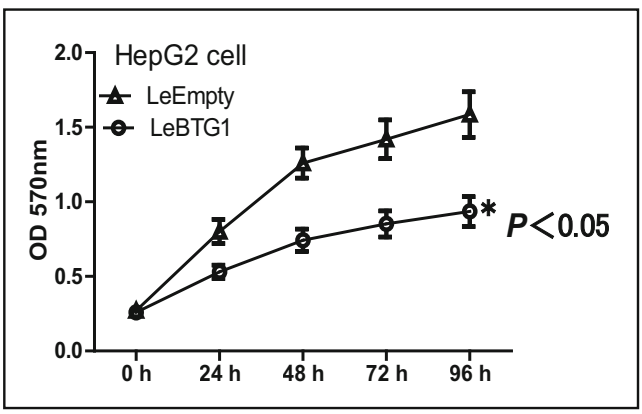

Fig. 5 The effects of BTG1 overexpression on the cell proliferation. *$P<0.05$ compared to the LeEmpty cell

BTG1 expression in hepatocellular cancer HepG2 cells resulted in a higher proportion of cells in G0/G1 phase, suggesting the occurrence of notable G0/G1 arrest. These results demonstrate that high BTG1 expression inhibits the growth of hepatocellular cancer HepG2 cells. A possible mechanism may be the induction of cell G0/G1 arrest. We also found that CND1 protein expression decreased in transfected cells, suggesting that cell growth inhibition as a result of high BTG1 expression may be related to the fact that BTG1 involves in cell cycle regulation by inhibiting G1/S phase transition and downregulating cyclin expression.

Apoptosis is a spontaneous, programmed death process that occurs in cells under certain physiological or pathological conditions. Its occurrence is modulated by numerous internal and external factors and involves a series of changes in relevant genes [12]. The apoptotic process is under tight regulation by a number of genes, which are highly conserved across species, such as Bcl-2 family, caspase family, oncogenes like C-myc, and tumor suppressor gene P53 [13]. In this study, the increase in BTG1 expression in hepatocellular cancer cells induced apoptosis and the expression of antiapoptotic protein Bcl-2 decreased significantly. Corjay et al. reported that high expression of BTG1 was present in apoptotic cells within macrophage-rich tissues in patients with hereditary hyperlipidemia [14]. Lee et al. showed that BTG1 could induce apoptosis in glioma cells [15]. Nahta et al. confirmed that BTG1 showed high expression in Bcl-2 antisense oligodeoxynucleotide-mediated apoptosis in breast cancer MCF7 cells [16]. Therefore, when put in the context of these findings reported in previously published studies, this study showed that high expression of BTG1 inhibited the growth of hepatocellular cancer cells possibly by reducing Bcl-2 expression [17, 18].

Tumor invasion and metastasis share common molecular mechanisms and involve a number of changes in tumor cells

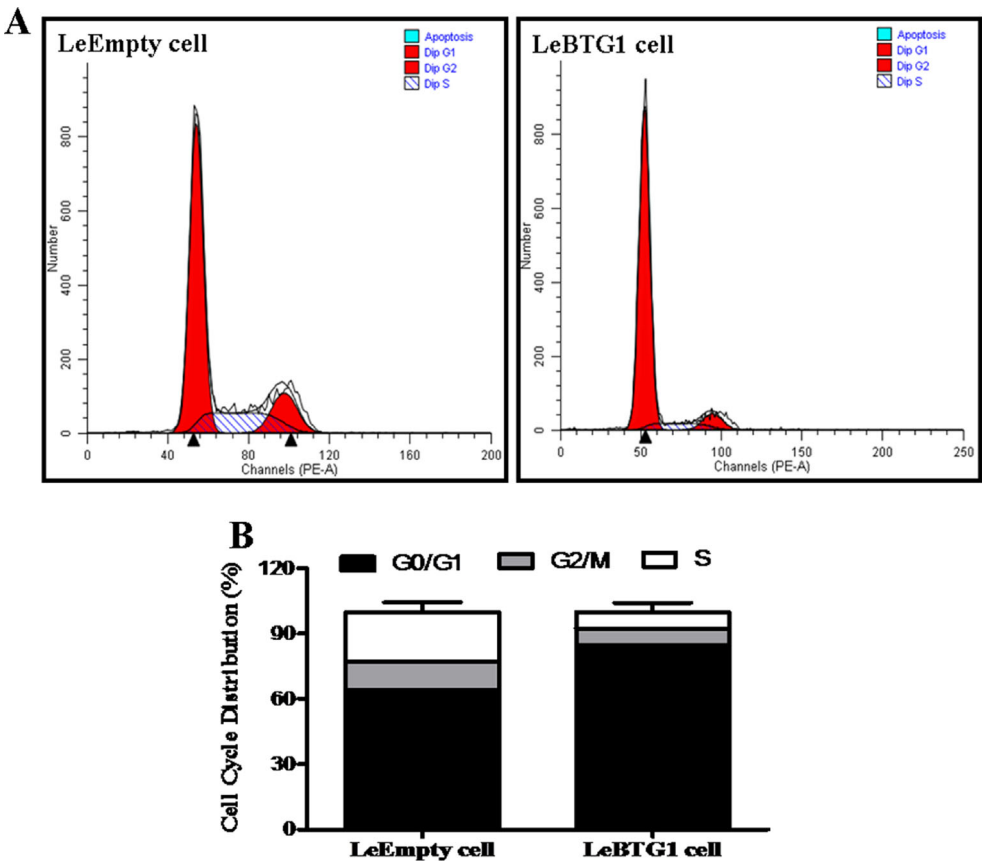

Fig. 6 The effects of BTG1 overexpression on the cell cycle. *$P<0.05$ compared to the LeEmpty cell

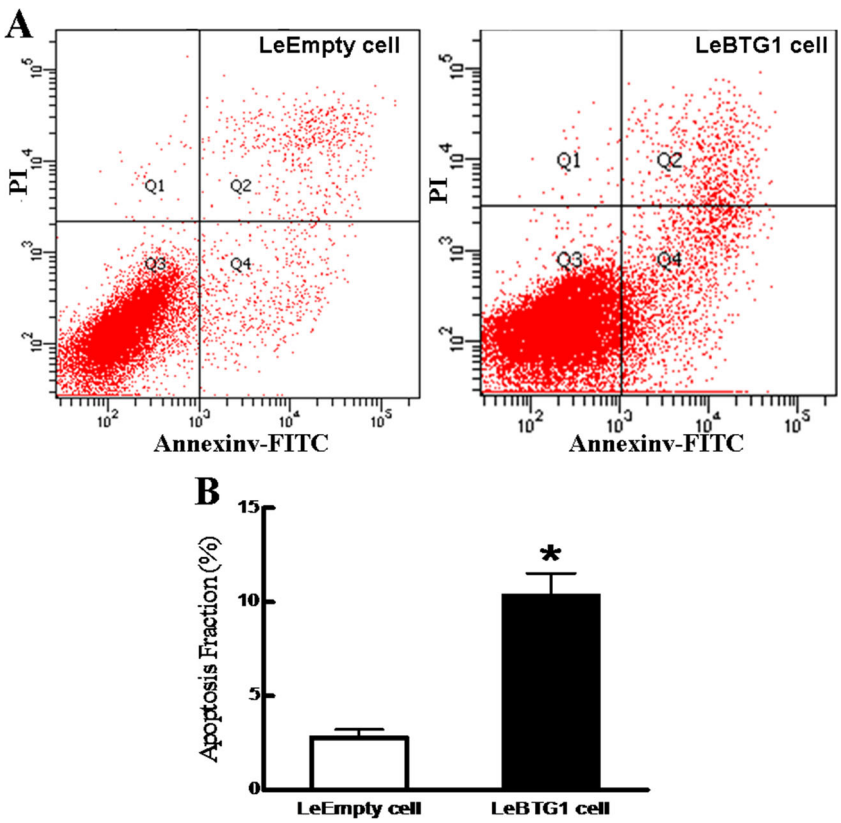

Fig. 7 The effects of BTG1 overexpression on the cell apoptosis. *$P<0.05$ compared to the LeEmpty cell

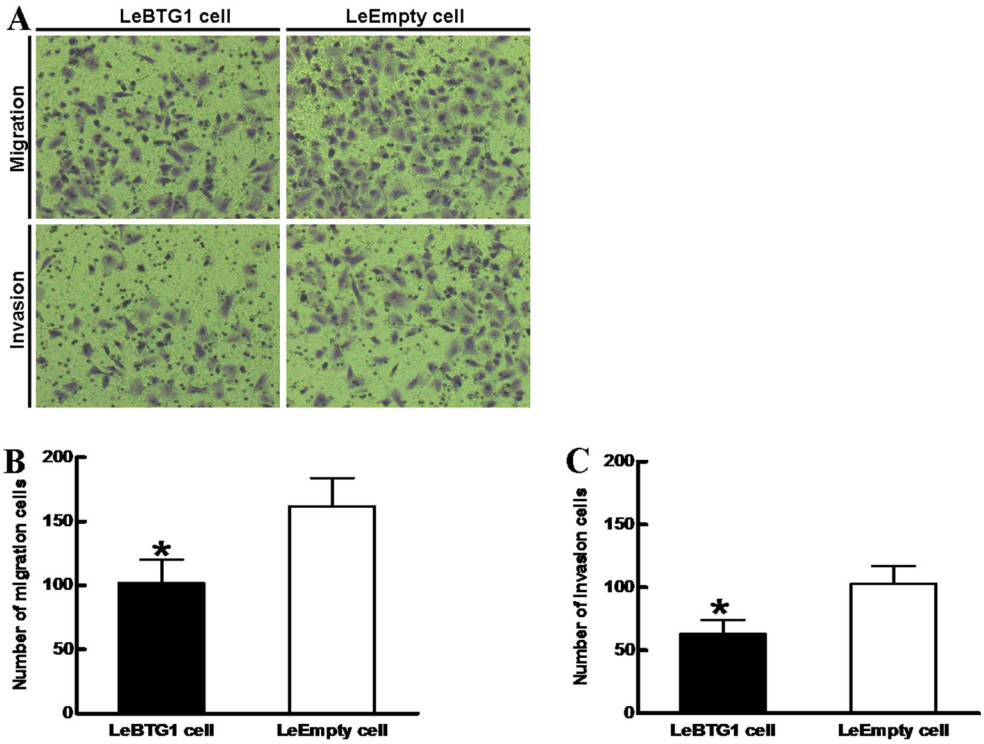

Fig. 8 The effects of BTG1 overexpression on the cell migration and invasion. *$P<0.05$ compared to the LeEmpty cell

and microenvironment, such as altered adhesion properties of tumor cells, hydrolysis of matrix proteins around tumor cells, enhanced tumor cell proliferation and survival potential, tumor cell migration, lymphangiogenesis, evasion of immune attack, and chemotaxis and growth of target organs of metastasis (Fig. 8) [17]. MMP remodels extracellular matrix and basement membrane through proteolytic degradation, which is a key step in tumor invasion and clonal growth at distant sites. Most of these enzymes are produced by stromal cells instead of tumor cells. Tumor cells with malignant phenotype or invasion and metastasis phenotype have highly expressed MMPs, and the degree of malignancy of breast cancer is associated with excessive expression of MMP-2 and MMP-9 [18]. This study showed that compared with cells transfected with empty vector, the number of BTG1 high-expressing cells that invaded and migrated through the basement membrane of transwell chamber decreased significantly, suggesting that high expression of BTG1 reduces cell invasion and metastasis. BTG1 high-expressing cells showed decreased MMP-9 protein levels. This suggests that BTG1 overexpression-conferred inhibition of cell invasion and metastasis is associated with its role in downregulating MMP-9 expression (Fig. 9).

The results of this study showed that BTG1 protein levels were significantly reduced in hepatocellular cancer and were associated with lymph node metastasis, clinical stage, cell differentiation, and prognosis. BTG1 may involve in the proliferation, apoptosis, invasion, and metastasis of hepatocellular cancer cells by regulating the protein expression of CND1, Bcl-2, and MMP-9.

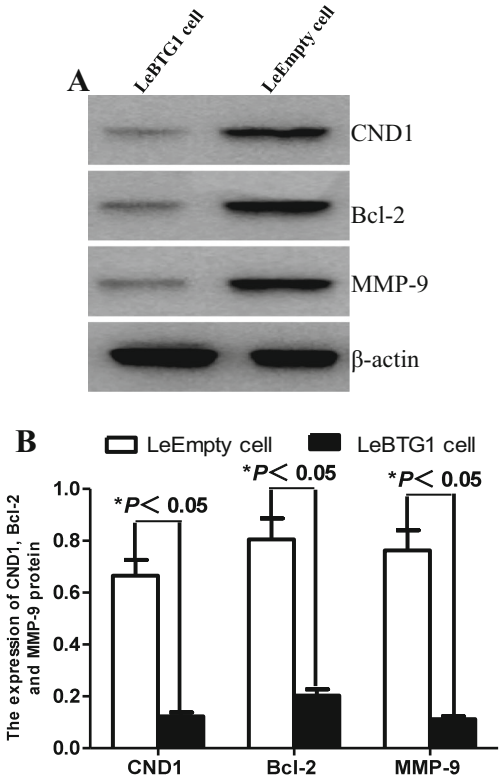

Fig. 9 The effects of BTG1 overexpression on CND1, Bcl-2, and MMP-9. *$P<0.05$ compared to the LeEmpty cell

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