

B cell translocation gene 1 reduces the biological outcome of kidney cancer through induction of cell proliferation, cell cycle arrest, cell apoptosis and cell metastasis

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Abstract. The aim of the present study was to determine the expression and function of B cell translocation gene 1 (BTG1) in kidney carcinoma. Kidney samples were obtained from cancer lesions (n=85) and the adjacent normal tissue (n=40) in kidney cancer patients immediately following endoscopic biopsy. The effect of BTG1 overexpression was examined *in vitro* utilizing a human kidney cancer cell line, ACHN, stably transfected with a recombinant lentivirus (LeBTG1 cells) and compared to empty vector-transfected controls (LeEmpty). BTG1 protein expression was significantly lower in kidney cancer tissue biopsies compared to normal tissue, as measured by immunohistochemistry (34.1 vs. 77.8% of tissues; P<0.05) and western blotting (0.481±0.051 vs. 0.857±0.081; P<0.05). *In vitro* analyses revealed that LeBTG1 cells had a reduced survival fraction compared to control LeEmpty cells, with higher rates of apoptosis (16.6±2.5 vs. 6.1±0.7%; P<0.05). The proportion of LeBTG1 cells in G₀/G₁ stage and S phase was also significantly different from LeEmpty cells (66.8±5.3 and 22.2±1.5% vs. 44.4±3.1 and 34.5±2.3%, respectively; P<0.05), and the migration and invasion of LeBTG1 cells was significantly impaired with respect to LeEmpty cells (74.0±9.0 and 53.0±7.0 vs. 118.0±15.0 and 103.0±13.0, respectively; P<0.05). These effects were accompanied by decreased protein expression of cyclin D1, B-cell lymphoma 2 and matrix metalloproteinase 9 in LeBTG1 cells (0.118±0.018, 0.169±0.015 and 0.207±0.027, respectively) compared to control LeEmpty cells (0.632±0.061, 0.651±0.063 and 0.443±0.042, respectively; P<0.05). Reduced BTG1 expression is associated with increased disease severity, suggesting it is a negative regulator of kidney cancer and can serve as a

prognostic indicator. The results of the present study show that BTG1 protein levels were significantly reduced in kidney cancer biopsy specimens and were associated with disease progression and prognosis.

Introduction

Cellular proliferation, differentiation, senescence and apoptosis are cell cycle-dependent processes (1). Regulation of the cell cycle is closely linked to tumor development and progression, as it is impaired in almost all tumors (2,3). In addition, numerous proto-oncogenes and tumor suppressor genes function as major factors or are directly involved in the regulation of cell cycle (4). Carcinogenic factors can induce mutation, deletion, translocation or amplification of these genes, resulting in de-regulation of the cell cycle, abnormal cell proliferation and tumor development. A novel strategy for cancer therapy involves regulating cell cycle, proliferation and apoptosis of tumor cells and select inhibition of tumor tissue activity (5).

B cell translocation gene 1 (BTG1) is a member of the TOB/BTG family of proteins known to inhibit cell proliferation and negatively regulate the cell cycle that was first identified in B lymphoblastic leukemia (6-9). The TOB/BTG family members regulate these processes by acting on cell cycle genes in response to various internal and external stimuli. For example, high PC3 expression in NIH/3T3 cells arrests the cell cycle in the G₁ phase and inhibits cell growth (10). Enhanced BTG1 expression, which peaks in the G₀/G₁ phase, promotes the differentiation of neural stem and germ cells and plays an important role in angiogenesis. BTG1 also facilitates the formation of the CCR4/NOT transcriptional complex, which regulates the deadenylation and turnover of cytoplasmic mRNAs. BTG1 is structurally characterized by the presence of a specific BTG domain in the N-terminus along with a large anti-proliferative homologous region (11). Although BTG1 exhibits certain characteristics of tumor suppressor genes (12,13), it is not known if BTG1 is a kidney cancer suppressor gene. Therefore, the present study aimed to determine what roles BTG1 plays in the growth, proliferation, invasion, metastasis and apoptosis of kidney cancer cells.

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Materials and methods

Reagents. The rabbit anti-human BTG1 monoclonal (14879-1-AP and 14102-1-AP) antibodies were purchased from Proteintech Group (Chicago, IL, USA). Rabbit anti-human cyclin D1 polyclonal (ab7958), mouse anti-human Bcl-2 monoclonal (ab117115), and rabbit anti-human MMP-9 polyclonal (ab7299) antibodies were purchased from Abcam (Cambridge, UK). Goat anti-rabbit fluorescent secondary antibody (IRDye800) was obtained from LI-COR Biosciences, Inc. (Lincoln, NE, USA). The β -actin primary antibody (A1978), polyoxymethylene and crystal violet were purchased from Sigma (St. Louis, MO, USA). The pLenti6/V5-DEST vector, Lentiviral Packaging Mix, Opti-MEM, Lipofectamine 2000 and TRIzol were obtained from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). An immunohistochemistry and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (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 (Thermo Fisher Scientific). Reverse transcriptase reagents and SYBR Premix ExTaq (Perfect real-time) were purchased from Takara (Shiga, Japan). Invasion chambers and Matrigel for invasion and migration assays were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Cell culture and gene transfection. The human kidney cancer cell line ACHN was maintained in RPMI-1640 medium supplemented with 10% FBS, which was changed every 2-3 days. Upon reaching confluence, cells were subcultured with 0.25% trypsin and 1% ethylenediaminetetraacetic acid. *BTG1* cDNA sequences were cloned into the *Bam*HI and *Asc*I sites of the pLenti6/V5-DEST vector, and cells were transfected with the pLenti6-BTG1 or pLenti6/V5-DEST vector using Lipofectamine 2000. Transfected cells were maintained in blastidicin (5 μ g/ml)-containing RPMI-1640 medium for selection of stable vector-containing sublines.

Immunohistochemistry. Immunohistochemistry was performed as previously described (14). Briefly, 4- μ m sections were prepared from paraffin-embedded biopsy samples and dehydrated. Sections were incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase, followed by 20 min in 0.05% trypsin. Sections were incubated for 20 min at room temperature in a blocking solution containing 10% goat serum followed by the BTG1 antibody (1:100) at 4°C overnight. For a negative control, the primary antibody was replaced with phosphate-buffered saline (PBS). Sections were subsequently incubated for 20 min each in secondary and tertiary antibodies at room temperature, visualized by 3,3'-diaminobenzidine staining and counterstained with a hematoxylin stain. Two pathologists blind to the patient condition examined and quantified the sections. Five randomly-selected fields from three slides for each specimen were examined under a microscope and counted. BTG1 expression was determined based on the percentage of positive cells (0 points, \leq 5%; 1 point, 5-25%; 2 points, 25-50%; and 3 points, >50% positive cells) and the staining intensity [0 points, no staining; 1 point, weak staining (light yellow); 2 points, moderate staining (yellowish-brown); and 3 points, strong staining (brown)]. The final score of BTG1 expression was the product of the BTG1 expression rate

(percentage score) and intensity: - for 0 points, + for 1-3 points, ++ for 4-6 points and +++ for 7-9 points.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from ACHN cells using the TRIzol reagent according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed using reverse transcriptase, and RT-qPCR was performed on an ABI Prism 7300 Real-Time PCR system (Applied Biosystems Inc., Life Technologies/Thermo Fisher Scientific) according to the standard manufacturer's instructions for SYBR Premix ExTaq. Gene specific primers used include: *BTG1*, sense, 5'-GGAATTCATGCATCCCTTCTACACCCGG; and anti-sense, 5'-CGACGCGTTTAACTGATACAGTCATCAT; and *β -actin* for normalization, sense, 5'-ATCGTCCACCGCAAATGCTTCTA and antisense, 5'-AGCCATGCCAATCTCATCTTGTT. Thermal cycling conditions were 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The expression level relative to *β -actin* was calculated using the $2^{-\Delta\Delta Ct}$ method in SDS 1.3 software (Applied Biosystems, Inc.).

Western blotting. Western blotting was performed as previously described (15). Briefly, 50 μ g of protein [determined using a bicinchoninic acid Protein Assay kit (Tiangen Biotech Co., Ltd., Beijing, China)] per samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were incubated for 2 h in 5% skimmed dry milk followed by an overnight incubation at 4°C in primary antibody (BTG1, 1:1,000; *β -actin*, 1:5,000). Subsequent to washing, the membranes were incubated with goat anti-rabbit fluorescent secondary antibody (IRDye800, 1:20,000 dilution) in the dark for 1 h at room temperature. The blots were scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Inc.). Western blot data were quantified by normalizing the BTG1 signal intensity of each sample to that of *β -actin*.

MTT assay. Cell viability was determined using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as previously described (16). Briefly, ACHN cells were plated into 96-well culture plates at an optimal density of 5×10^3 cells/ml with 200 μ l/well. After 24-96 h, 20 μ l of 5 mg/ml MTT was added to each well and incubated at 37°C for 4 h. Subsequently, the medium was gently aspirated and 150 μ l of dimethyl sulfoxide was added to each well to solubilize the formazan crystals. The optical density of each sample was immediately measured at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Flow cytometry. An Annexin V-FITC-flow cytometry assay was used as previously described (17) to detect the apoptosis rate. Cells were plated into 60-mm dishes for 48 h and grown to 70-75% confluency. Cells were subsequently collected, washed with ice-cold PBS and resuspended at a density of 1×10^6 cells/ml in a binding buffer and incubated for 15 min in the dark at 25°C with 5 μ l Annexin V-FITC and 10 μ l PI (20 μ g/ml). A total of 10,000 cells were analyzed with a FACScan flow cytometer with CellQuest software (BD Biosciences) for the apoptosis rate determination. For cell cycle distribution, 1×10^6 cells were fixed

in 70% ethanol and resuspended in 1 ml of a solution containing 3.8 mM sodium citrate, 50 $\mu\text{g/ml}$ PI, and 0.5 μg RNase A, and analyzed with the flow cytometer using the ModFit software program (Verity Software House, Topsham, ME, USA).

Invasion and migration assays. Invasion and migration assays were performed as previously described (18). Briefly, 8×10^5 cells were plated into Invasion Chambers with Costar Transwell 8- μm inserts coated with 50 μg reduced serum Matrigel according to the manufacturer's instructions. 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 and stained with 0.1% crystal violet for 30 min. 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 the statistical analyses were performed using the SPSS 16.0 software (IBM, Armonk, NY, USA), according to published guidelines (19). Survival distributions were estimated with the Kaplan-Meier method and compared with the log-rank test. Student's t-test, χ^2 and Fisher's exact tests were used to analyze the differences between groups. Data are presented as the mean \pm standard error, and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BTG1 protein expression in normal tissue and kidney cancer. Immunohistochemistry for BTG1 revealed light yellow to brown staining in 77.5% (31/40) of normal kidney tissues, and negative or weak staining in 34.1% (29/85) kidney cancer tissues ($P < 0.05$) (Table I, Fig. 1). Furthermore, BTG1 protein expression was significantly lower in cancer lesion samples compared to adjacent normal tissue, as determined by western blot analysis (0.481 ± 0.051 vs. 0.857 ± 0.081 ; $P < 0.05$) (Fig. 2). BTG1 expression levels correlated with T stage, lymph node metastasis, clinical stage and pathological differentiation ($P < 0.05$), regardless of age, gender, tumor size and pathological types ($P > 0.05$) (Table II).

BTG1 expression and prognosis. Follow-up examinations were performed on cancer patients for up to 60 months, with 37 patients remaining at the conclusion of the study. Overall survival (OS) rates were determined between patients positive for BTG1 expression and those negative for expression. Thirteen of the 56 individuals showing no BTG1 expression remained at the conclusion of the study, with an OS rate of 32.1%. Patients positive for BTG1 expression had a significantly higher OS rate of 65.5% (19/29) ($P < 0.05$) (Fig. 3).

Stable transfection of BTG1 in kidney cancer cells. BTG1 over-expressing ACHN cells (known as LeBTG1) were obtained by a stable transfection of BTG1 cDNA, and compared to ACHN cells overexpressing an empty vector (named LeEmpty) as a control. Analysis of RT-qPCR data showed that LeBTG1 cells had a significantly higher expression of BTG1 mRNA

Table I. BTG1 expression in normal and cancerous kidney tissue.

Groups	Case	Expression of BTG1 protein				χ^2	P-value
		-	+	++	+++		
Normal tissue	40	9	9	12	10	21.905	<0.0001
Cancer tissue	85	56	12	8	9		

BTG1, B cell translocation gene 1.

Table II. Association between BTG1 expression and kidney cancer characteristics.

Groups	Case	Expression of BTG1 protein			χ^2	P-value
		-	+ to	+++		
Gender						
Male	57	37	20		0.072	0.788
Female	28	19	9			
Age, years						
≤ 50	34	24	10		0.558	0.455
> 50	51	32	19			
Tumor length, cm						
≤ 7	30	19	11		0.134	0.714
> 7	55	37	18			
Pathological types						
Clear cell type	51	31	20		1.971	0.373
Granule cell type	23	16	7			
Papillary cell type	11	9	2			
Lymph node metastasis						
N0	31	15	16		6.645	0.010
N+	54	41	13			
Clinical stages						
I-II	27	12	15		8.090	0.004
III-IV	58	44	14			
Histological grade						
I	33	17	16		4.954	0.026
II-III	52	39	13			

BTG1, B cell translocation gene 1.

compared to LeEmpty cells (0.911 ± 0.095 vs. 0.508 ± 0.055 ; $P < 0.05$) (Fig. 4A). Furthermore, western blot analysis showed that LeBTG1 cells had a significantly higher expression of BTG1 protein compared to LeEmpty cells (0.871 ± 0.089 vs. 0.429 ± 0.045 ; $P < 0.05$) (Fig. 4B).

Cellular effects of BTG1 overexpression. LeBTG1 cells had a significantly lower viability at 24, 48, 72 and 96 h compared to LeEmpty cells as assessed by an MTT assay ($P < 0.05$) (Fig. 5). Cell cycle analysis using flow cytometry showed that the proportion of LeBTG1 cells in the G_0/G_1 and S phases

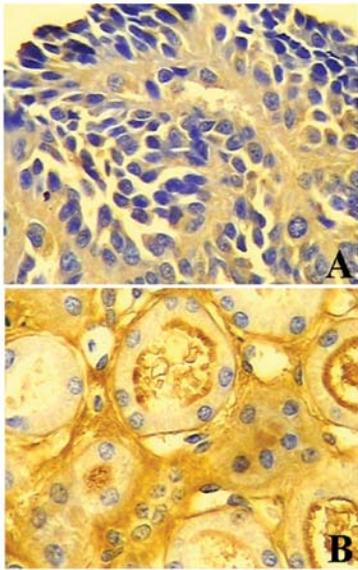


Figure 1. BTG1 immunohistochemistry in kidney cancer tissues compared to normal tissue. (A) BTG1 staining in kidney carcinoma (magnification, x100); (B) BTG1 staining in normal tissue (magnification, x400). BTG1, B cell translocation gene 1.

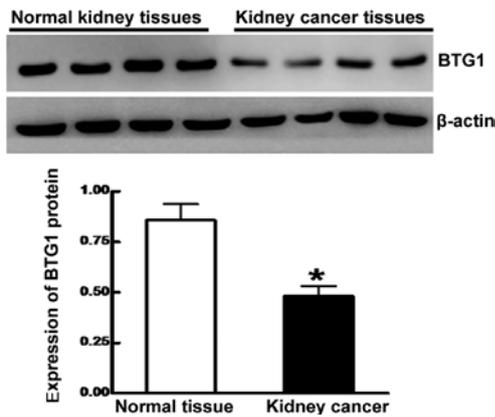


Figure 2. BTG1 protein levels in kidney cancer tissues compared to normal tissue. BTG1 protein levels were assessed by western blot analysis (upper panel). Quantification (lower panel) revealed that protein levels were significantly reduced relative to β-actin in cancer tissues compared to normal tissues; *P<0.05. BTG1, B cell translocation gene 1.

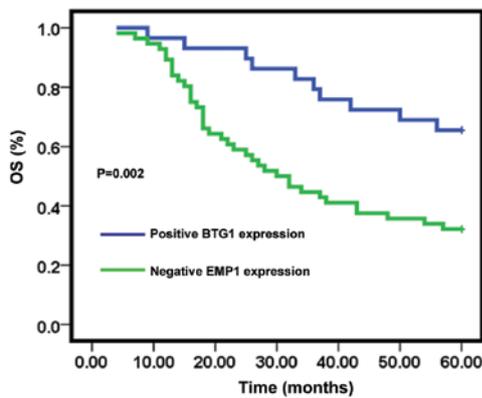


Figure 3. Overall survival time in kidney cancer patients. Kidney cancer patients positive for BTG1 expression had a significantly higher survival rate compared to those negative for expression; P<0.05. BTG1, B cell translocation gene 1.

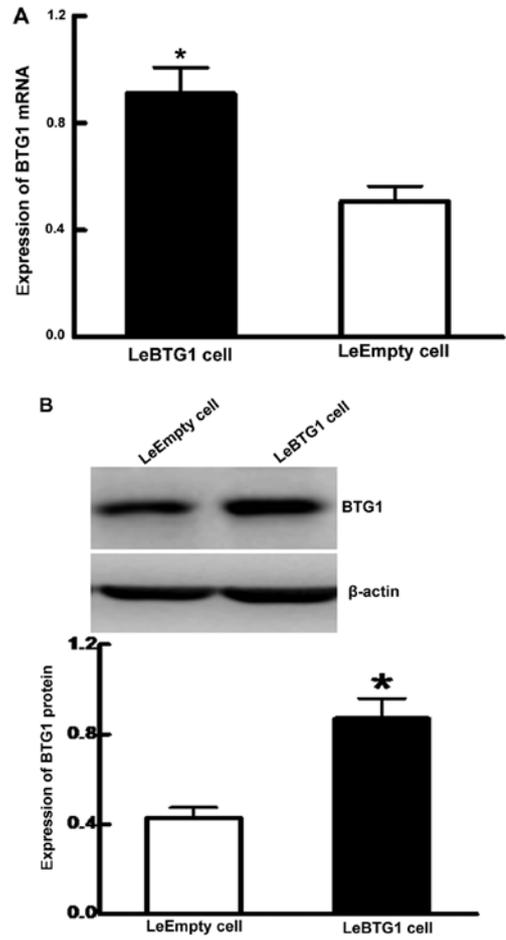


Figure 4. *BTG1* expression in a stable transfected cell line. (A) LeBTG1 cells expressed significantly more *BTG1* mRNA as determined by qRT-PCR. (B) Western blot analysis revealed that LeBTG1 cells expressed significantly more BTG1 protein; levels relative to β-actin, *P<0.05 compared to LeEmpty cells. BTG1, B cell translocation gene 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

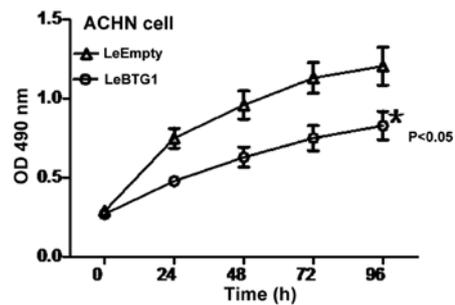


Figure 5. Reduced cell viability with *BTG1* overexpression. An MTT assay demonstrated that LeBTG1 cells had significantly reduced activity levels at 24, 48, 72 and 96 h, indicating reduced viability and slower growth (P<0.05). BTG1, B cell translocation gene 1.

of the cell cycle were significantly different compared to the control LeEmpty cells (66.8±5.3 and 22.2±1.5 vs. 44.4±3.1 and 34.5±2.3%, respectively; P<0.05) (Fig. 6). In addition, there was a large increase in the early apoptosis rate in LeBTG1 cells compared to control LeEmpty cells (16.6±2.5 vs. 6.1±0.7%; P<0.05) (Fig. 7). Furthermore, LeBTG1 cells had a reduced capability for invasion and migration through Transwell

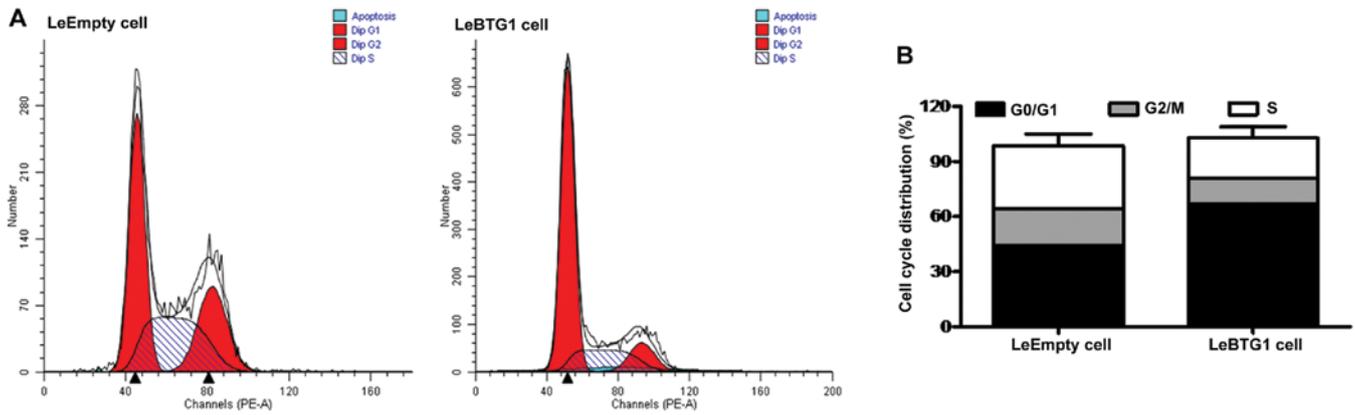


Figure 6. Cell cycle distribution with *BTG1* overexpression. (A) Results of flow cytometry cell sorting of the LeBTG1 and LeEmpty cells; (B) the cell cycle phase distribution was significantly altered in LeBTG1 cells; * $P < 0.05$ compared to the LeEmpty cells. *BTG1*, B cell translocation gene 1.

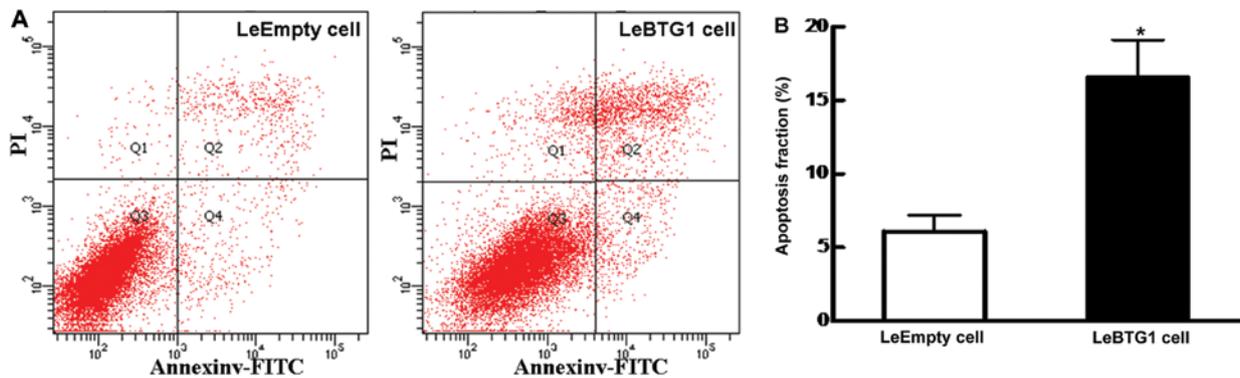


Figure 7. Increased apoptosis with *BTG1* overexpression. (A) Results of flow cytometry cell sorting of the LeBTG1 and LeEmpty cells. (B) A higher percentage of LeBTG1 cells were apoptotic compared to LeEmpty cells; * $P < 0.05$. *BTG1*, B cell translocation gene 1; FITC, fluorescein isothiocyanate.

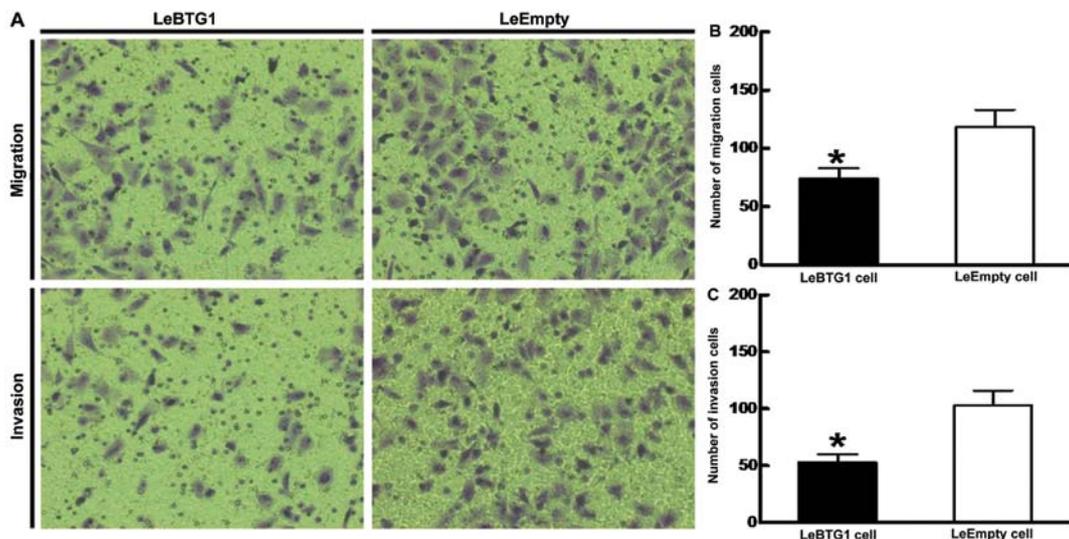


Figure 8. Reduced cell migration and invasion with *BTG1* overexpression. (A) Photomicrographs showing cellular invasion and migration of LeBTG1 and LeEmpty cells through Transwell 8- μ m inserts (magnification, x400). (B) Quantification of migrating cells and (C) invading cells; * $P < 0.05$ compared to control LeEmpty cells. *BTG1*, B cell translocation gene 1.

inserts (74.0 ± 9.0 and 53.0 ± 7.0 , respectively) compared to control LeEmpty cells (118.0 ± 15.0 and 103.0 ± 13.0 , respectively; $P < 0.05$) (Fig. 8).

To further identify the mechanisms by which *BTG1* overexpression regulated these cellular changes in cancer cells, expression levels of proteins critical for the regulation of cell

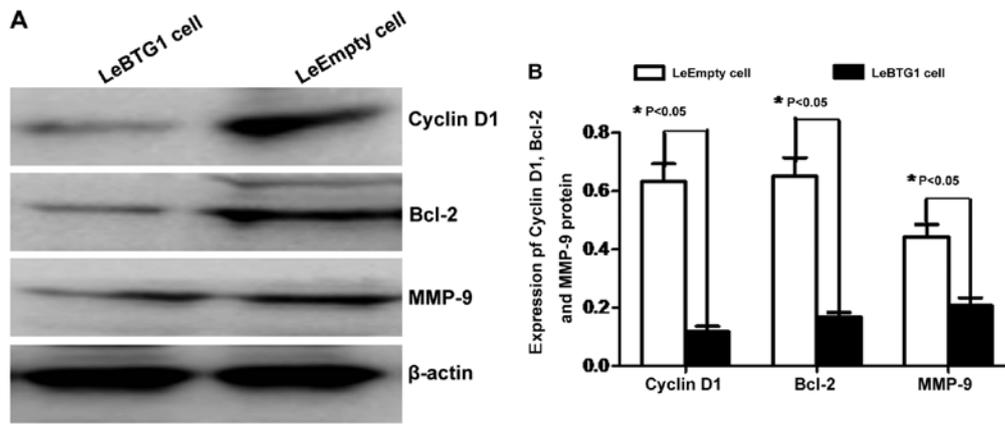


Figure 9. Reduced protein levels of cyclin D1, Bcl-2 and MMP-9 with *BTG1* overexpression. (A) Representative western blots showing protein expression in LeBTG1 and LeEmpty cells. (B) Quantification of cyclin D1, Bcl-2 and MMP-9 expression relative to β -actin; * $P < 0.05$ compared to LeEmpty cells. *BTG1*, B cell translocation gene 1; Bcl-2, B-cell lymphoma 2; MMP-9, matrix metalloproteinase 9.

cycle, apoptosis and migration were examined. Western blot analysis revealed that LeBTG1 cells had significantly reduced levels of cyclin D1, Bcl-2 and MMP-9 (0.118 ± 0.018 , 0.169 ± 0.015 and 0.207 ± 0.027 , respectively) compared to control LeEmpty cells (0.632 ± 0.061 , 0.651 ± 0.063 and 0.443 ± 0.042 , respectively; $P < 0.05$) (Fig. 9).

Discussion

Tumor development and progression are associated with uncontrolled proliferation and reduced apoptosis of tumor cells. *BTG1* has been shown to act as a tumor suppressor gene in breast cancer, by inhibiting proliferation, regulating cell cycle and inducing apoptosis (20). The present study examined *BTG1* protein expression in kidney cancer tissue and showed that levels were significantly lower and correlated with tumor invasion, lymph node metastasis, clinical stage and cancer differentiation. A recent study suggests that tumor stage is the preferred prognostic indicator (21), although prognoses can vary considerably among patients in the same stage. Therefore, it is of particular importance to identify reliable molecular markers for use in clinical practice. The results of the present study indicate that *BTG1* deletion is a major contributor to the development and progression of kidney cancer. As expression-positive patients had a significantly higher 5-year overall survival rate, *BTG1* may be a useful prognostic indicator for patients with kidney cancer. The combination of the tumor-node-metastasis classification system and *BTG1* expression scores may provide certain valuable information for clinicians in choosing treatment options, and for predicting disease severity and prognosis.

The development of kidney cancer is driven by the abnormal proliferation of cells that normally undergo apoptosis (22). A recent study has indicated that overexpression of *BTG1* can affect the cell cycle and suppress tumor growth (20). The present study utilized *in vitro* tests to confirm that kidney cancer cells with a high *BTG1* expression had significantly weakened viability and proliferation potential. In addition, *BTG1* overexpression resulted in decreased protein levels of cyclin D1, which is considered to be a proto-oncogene product that is highly expressed or mutated in a variety of human tumors (23). The increased *BTG1* expression in kidney cancer

ACHN cells resulted in a higher proportion of cells in the G_0/G_1 phase, indicating the occurrence of G_0/G_1 arrest and inhibition of growth. Taken together, the data implicate *BTG1* in cell cycle regulation and cyclin D1 expression.

In the present study, increased *BTG1* expression in kidney cancer cells reduced the amount of the anti-apoptotic protein Bcl-2 and induced apoptosis. Apoptosis is a programmed death process that involves a series of changes in relevant genes, including Bcl-2 and caspase family genes, oncogenes such as C-myc, and the tumor suppressor gene p53 (24), and is regulated by numerous internal and external factors (25). These results are in agreement with previous works linking *BTG1* expression with apoptosis. Corjay *et al* (26) demonstrated a high level of *BTG1* expression in apoptotic cells within macrophage-rich tissues in patients with hereditary hyperlipidemia, and Lee *et al* (27) showed that *BTG1* could induce apoptosis in glioma cells. Additionally, a study by Nahta *et al* (28) found that apoptosis in kidney cancer MCF7 cells induced by Bcl-2 knockdown was regulated by *BTG1* expression. Therefore, the evidence indicates that *BTG1* can inhibit the growth of kidney cancer cells by reducing Bcl-2 expression.

Tumor invasion and metastasis are major causes for treatment failures, thus, the main research aims are to identify the molecular mechanisms underlying metastasis and target key pathways that inhibit this process. Tumor invasion and metastasis share common molecular mechanisms and involve a number of changes in tumor cells and the microenvironment, including altered tumor cell adhesion properties, enhanced proliferation, survival, chemotaxis and migration of tumor cells, lymphangiogenesis, evasion of immune attack and hydrolysis of surrounding matrix proteins (29). A key step in tumor invasion and clonal growth is the remodeling of the extracellular matrix and basement membranes through proteolytic degradation by MMPs, which are highly expressed by tumor cells with malignant, invasive and metastatic phenotypes. Additionally, the degree of malignancy and patient prognosis are associated with excessive expression of MMP-2 and MMP-9 (30,31). Tumor cells can also regulate the expression of MMPs produced by stromal cells by secreting chemokines, cytokines and extracellular MMP inducer, a cell surface glycoprotein. The present study showed that *BTG1*-overexpressing kidney cancer cells

had decreased MMP-9 protein levels and reduced invasion and migration *in vitro*, indicating that *BTG1* modulated tumor cell metastasis by downregulating MMP-9 expression.

In conclusion, the present study provides clinical and *in vitro* evidence implicating *BTG1* in the development and progression of kidney cancer. The results of this study show that *BTG1* protein levels were significantly reduced in kidney cancer biopsy specimens and were associated with disease progression and prognosis. Furthermore, the effects on the regulation of cancer cell proliferation, apoptosis, invasion and metastasis indicate that *BTG1* expression may serve as a prognostic marker for kidney cancer patients.

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