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Frequent promoter hypermethylation and transcriptional downregulation of BTG4 gene in gastric cancer

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ABSTRACT

The BTG4 gene belongs to the BTG family of genes endowed with antiproliferative properties. In this study, we have found that BTG4 undergoes promoter CpG island hypermethylation-associated inactivation in gastric cancer and 5′-aza-2′-deoxycytidine (DAC) treatment restores BTG4 expression. We also found BTG4 levels were significantly reduced in primary gastric cancer but not in normal gastric tissues. BTG4 reexpression in gastric cancer causes growth inhibition of colony assays and nude mice. Taken together, our data support BTG4 as a candidate tumor suppressor gene that is epigenetically silenced in the majority of gastric cancers.

The BTG4 gene maps to 11q23 and encodes a 26 kDa protein of the BTG family of growth inhibitor genes. BTG2 (PC3) was the founding member of the BTG family of genes endowed with antiproliferative properties, namely, BTG1, ANA/BTG3, PC3B, TOB, and TOB2 [1]. PC3 was originally isolated as a gene induced by nerve growth factor during neuronal differentiation of rat PC12 cells, or by TPA in NIH3T3 cells (named TIS21), and is a marker for neuronal birth in vivo. BTG2, the human homolog of PC3, regulated by p53, was likely relevant to cell cycle control and cellular response to genotoxic damage [2]. BTG2 was also identified as an important downstream effector of p53-dependent arrest of proliferation in mouse and human fibroblasts transduced by oncogenic Ras [3]. Similarly, BTG3, another target of p53, was reported to negatively regulate cell proliferation by binding to and inhibiting E2F1 [4].

Although much remains unknown about the function of BTG4, the BTG4 gene has been found to be completely absent or barely detectable in colorectal cancer cell lines, and BTG4 overexpression suppresses colony formation in colorectal cancer cells [5]. To characterize the role of BTG4 in gastric cancer, we examined a series of primary gastric cancers and control tissues for BTG4 expression and promoter methylation. Our results confirm frequent downregulation of BTG4 expression in primary gastric cancers and identify hypermethylation of the BTG4 gene as a common epigenetic aberration in these tumors.

Materials and methods

Cell lines and tissue specimens. The SV40-transformed immortal human gastric mucosal epithelial cell line (GES-1) and five gastric cancer cell lines (AGS, SGC-7901, MKN-28, MKN-45, BCG-823) were all preserved in our laboratory and maintained in RPMI 1640 with 10% FBS. BCG-823 is an adherent, poorly differentiated, human gastric cancer cell line with mutant p53, and MKN-45 cell line expresses wild-type p53 [6]. Cells were treated with 5 μM/L 5′-aza-2′-deoxycytidine (DAC; Sigma) for 48 h. The Adriamycin (ADR) was added at a concentration of 0.5 μg/ml for 24 h. 38 gastric cancer samples were obtained from the Department of Pathology, Rui-jin Hospital. Samples of adjacent normal gastric mucosa from 15 patients, at least 2 cm distant from the tumor, were also collected. The adjacent non-tumor area subsequently was verified by histology to be free of tumor infiltration. The clinicopathological characteristics were analyzed according to tumor size, histological grading and presence of nodal metastasis.

DNA methylation analysis of the BTG4 gene. Genomic DNA (2 μg) was modified with sodium bisulfite using EpiTect Bisulfite kit (Qiagen). Methylation status was analyzed by bisulfite genomic sequencing of the CpG islands. The fragment covering 15 CpG sites from BTG4 promoter region was amplified from bisulfite-modified DNA. The primers used were 5′-TTTTCGAGGGGTATAAGGAGAGTTTATTTT-3′ (sense) and 5′-CCAAAACTCTAACTCCTAAATAAACA-3′ (antisense). Amplified products were cloned into pMD18-T simple vector (Takara), 6 independent clones were sequenced.

Semiquantitative polymerase chain reaction. Total RNA was extracted from gastric cancer or normal tissues using TRIZOL

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(Invitrogen). Reverse transcription was carried out using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega). The polymerase chain reaction was performed using SYBR Green kit following the manufacture's protocol (Takara). Primers used in RT-PCR: BTG4-F: 5′-GTTTCTCTTTCTGATCTAGCAGGA-3′; BTG4-R: 5′-TCAGAGTGCCAGTGA CTTCTGTA-3′. Actin-F: 5′-AGATGT GGATCAGCAAGCGGAGT-3′; Actin-R: 5′-GCAATCAAAGTCCTCGG CCACATT-3′. A 7900HT Fast Real-time PCR System (Applied Biosystems) was used for testing. Following the protocol of the manufacturer, the amount of BTG4 expression, normalized to a human actin endogenous reference is given by: $2^{-\Delta\Delta Ct}$ Real-time RT-PCR was repeated at least three times for each specimen, and mean was obtained.

DNA extraction from paraffin block. The DNA extraction was performed as previously described [7]. Briefly, formalin-fixed, paraffin wax-embedded tissues were cut into 10 mm thick sections. Before DNA extraction, the sections were placed on slides and stained with hematoxylin–eosin to evaluate the admixture of non-tumorous tissues. Areas corresponding to tumor or surrounding normal gastric tissue were microdissected separately. Microdissected tissues were collected in 15 ml centrifuge tubes, and deparaffinized overnight at 63 °C in xylene. After centrifugation at full speed for 5 min, the supernatant was removed. Ethanol was added to the pellet to remove residual xylene, and then removed by centrifugation. The genomic DNA was isolated using DNeasy Tissue Kit according to the manufacturer's instruction.

Methylation-specific PCR (MSP). To study BTG4 promoter hypermethylation, we also performed MSP as described previously. For the BTG4 promoter methylation study, we used two sets of primers that could amplify the modified DNA of either the methylated or unmethylated alleles separately. For the methylated allele (128 bp), the sense primer was 5′-ATTCGTTTCGTTTCGCGTTCGTT TC-3′ and the antisense primer was 5′-TTTTTATTTGTTTTGTTTTG TGTTTGTTTTG-3′. For the unmethylated allele (134 bp), the sense primer was 5′-CCTAAAACTAACTCTCTC AACCCCA-3′ and the antisense primer was 5′-AATACTAAAATACAACATCAC CTCCA-3′. The annealing temperatures for the methylated and unmethylated DNA were 67 °C and 61 °C, respectively, for 40 s each. Hot-start PCR with a total cycle number of 30 was used in all MSP DNA amplifications. Denaturation and extension cycles were maintained for 30 and 45 s, respectively. The methylation status of the p16 promoter was determined by MSP as described previously [8]. For the methylated allele, the sense primer was 5′-TTATTA-GAGGGTGGGGCGGATCGC-3′ and the antisense primer was 5′-GACCCCGAACCGCGACCGTAA-3′. For the unmethylated allele, the sense primer was 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ and the antisense primer was 5′-CCACCTAAATCAACCTCCAACCA-3′.

Construction of plasmids and stable cell line generation. For construction of pCMV4-flag-BTG4, the BTG4 cDNA was generated by reverse transcription PCR using BTG4 forward primer (5′-AAAG CCTTATGAGAGATGAAATTGCAACAA-3′) and reverse primer (5′-GG ATCCCTACAGTTTTGTTCTTTCCA-3′). The sequence was confirmed by DNA sequencing, and ligated into the HindIII and BamHI sites of pCMV-flag vector (Invitrogen). For transfection experiments, BCG-823 and MKN45 cells were plated into 6-well plates 24 h before transfection. The cells were transfected with 5 μg/well of empty pCMV-flag or pCMV4-flag-BTG4 using Superfect (Qiagen, Germany) according to manufacturer's instructions. For 48 h after transfection, the cells were passaged at 1:5 and cultured in medium supplemented with G418 at 500 μg/ml for 4 weeks. One BCG-823 clone and one MKN45 clone reexpressing BTG4 (BCG-823/BTG4, MKN45/BTG4) were selected for further study, with

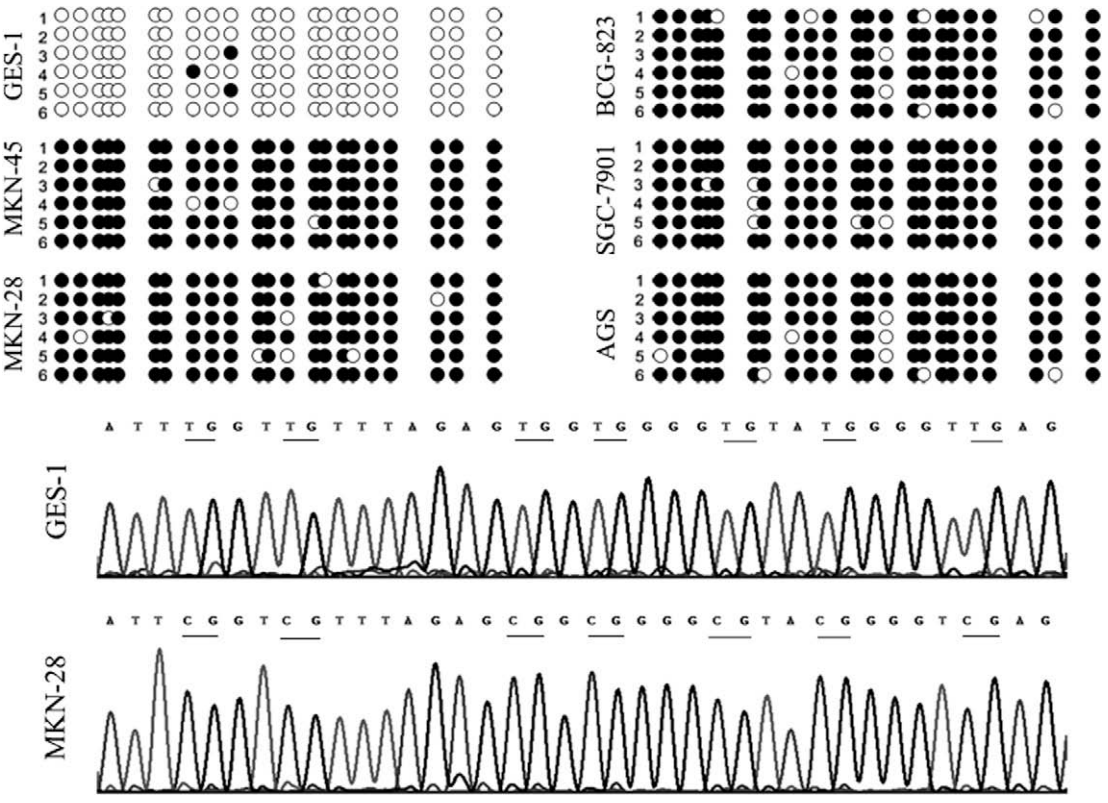

Fig. 1. Demonstration of BTG4 promoter methylation by sequencing of sodium bisulfite–modified DNA from GES-1 and the indicated gastric cancer cell lines. Methylated and unmethylated CpG dinucleotides are shown by closed and open circles, respectively. Each line of circles represents analysis of a single cloned allele. An illustrative fragment of the sequencing electropherogram is shown for GES-1 and MKN-28, the CpG sites are underlined.

BTG4 expression verified by western blot. As a control group, cells stably transfected with an empty vector pCMV-flag were also generated (BCG-823/vector, MKN45/vector).

Western blotting. Cells were harvested and samples (20 μg) of the cell lysate were subjected to 10% SDS–PAGE gel electrophoresis, after which the resolved proteins were transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were then blocked with 5% non-fat milk and 0.1% Tween 20 in Tris-buffered saline and probed with anti-flag antibody (Sigma), after which the blots were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Evaluation of apoptosis. Apoptosis was detected by flow cytometric analysis of annexin V staining. Annexin V–FITC versus PI assay was performed as previously reported. Briefly, adherent cells were harvested and suspended in the Annexin-binding buffer (1×10^6 cells/ml). Thereafter, cells were incubated with Annexin V–FITC and PI for 15 min at room temperature in the dark and immediately analyzed by flow-cytometry. The data are presented as bi-parametric dot plots showing Annexin V–FITC green fluorescence versus PI red fluorescence.

Anchorage-independent growth. For anchorage-independent growth assay, 2×10^4 cells were plated in 0.3% low melting point agar/growth medium onto six-well plates with a 0.6% agar underlay. After 3 weeks, colonies that were >1 mm in diameter were counted.

Tumorigenecity assay. The tumors were established by s.c. injection of 1×10^6 BCG-823 cells, BCG-823/vector, BCG-823/BTG4 into BALB/c nude mice.10 mice were included in each group in all experiments. Tumors sizes were estimated by using the equation $V = 4/3\pi \times L/2 \times (W/2)^2$, where L is the mid-axis length, and W is the mid-axis width.

Statistical analysis. Pearson Chi-Square test and one-ANOVA were used for statistical analysis of group differences. Pearson Chi-Square tests were performed to evaluate the significance of

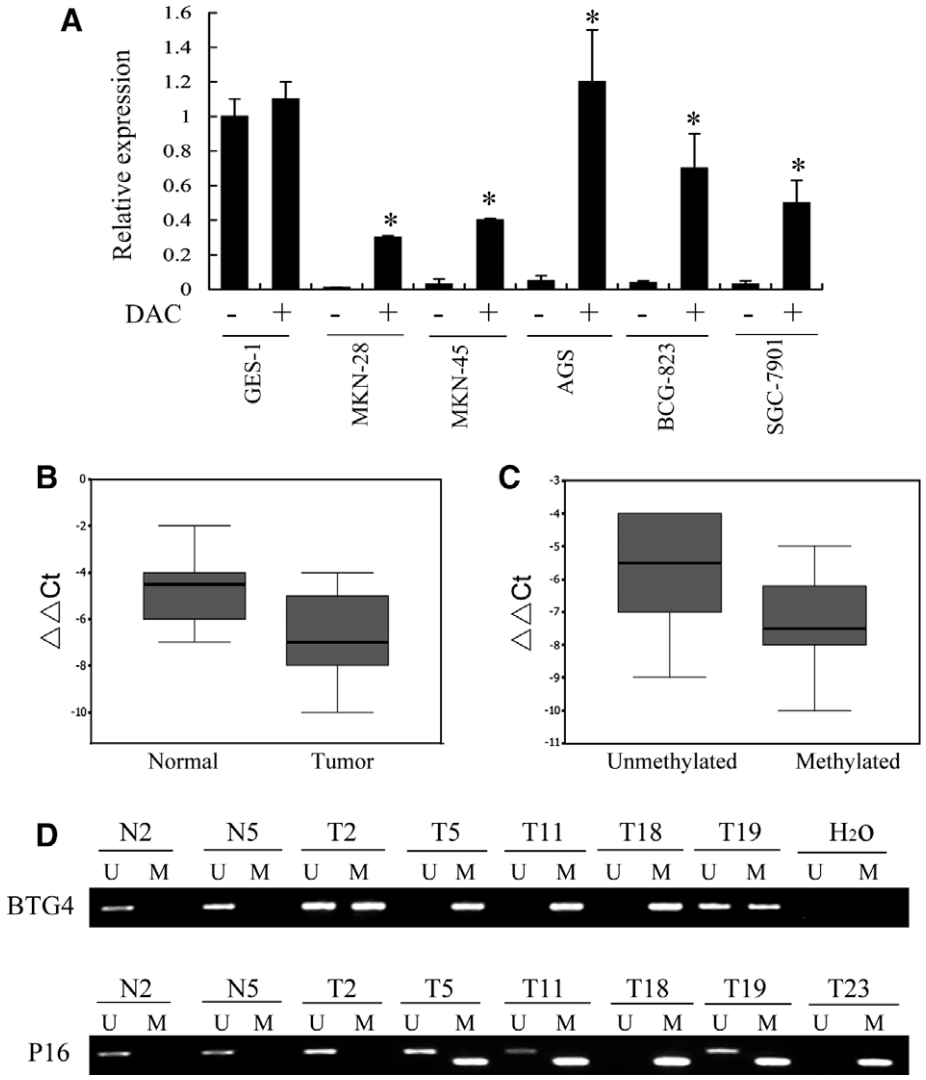

Fig. 2. (A) Real-time RT-PCR results for BTG4 expression in the gastric cancer cell lines, with or without DAC treatment. Results are shown relative to a value of 1 assigned to mock-treated GES-1 cells after normalized to actin expression. $^*P < 0.01$. (B) Real-time RT-PCR analysis of BTG4 was carried out on 38 gastric cancers, 15 normal mucous samples. For each sample, the relative mRNA level of BTG4 was normalized to the β-actin level. The line within each box represents the median $\Delta\Delta C_t$ value; the upper and lower edges of each box represent the 75th and 25th percentile, respectively; the upper and lower bars indicate the highest and lowest values determined, respectively. $P = 0.002$, two-sided t test. (C) Relative BTG4 mRNA expression in gastric cancer stratified by BTG4 promoter methylation. BTG4 mRNA levels and promoter methylation were determined by real-time RT-PCR and MSP, respectively. Depending on each tumor's promoter methylation status, the cases were subdivided into two groups, unmethylated ($n = 10$), methylated ($n = 28$). Note significantly lower mean BTG4 mRNA expression in the tumors with promoter methylation ($P = 0.012$, two-sided t test). (D) MSP for the BTG4 and p16 genes was performed using unmethylation-specific (*U*) and methylation-specific (*M*) primer sets. Twenty microliters of PCR product was run on 2% metaphore agarose gel, stained with ethidium bromide and visualized under UV illumination. The numbers shown are sample identification numbers.

the differences between the frequencies of BTG4 promoter hypermethylation status of the various tissue categories and comparisons with clinical characteristics. *P* values less than 0.05 was considered significant.

Results

Bisulfite sequencing analysis

Recently, promoter methylation of BTG4 gene and the resultant gene suppression have been shown in colorectal cancer, these findings prompted us to examine whether such methylation is also present in gastric cancer. We determined the BTG4 CpG island methylation status in GES-1 and gastric cancer cell lines by bisulfite genomic sequencing. The PCR products were cloned into a plasmid vector and six independent clones were sequenced. As shown in Fig. 1, most CpG dinucleotides were methylated in gastric cancer cell lines, the methylation was absent in GES-1 cells.

Association of the BTG4 promoter methylation with transcriptional gene silencing

To elucidate whether the aberrant methylation of BTG4 is associated with loss of BTG4, We examined the BTG4 mRNA expression in gastric cancer cell lines using real-time RT-PCR. Abundant levels of BTG4 mRNA were observed in normal gastric epithelia cell line (GES-1), in contrast, BTG4 expression was significantly reduced in a majority of gastric cell lines (Fig. 2A).

To confirm that this loss of expression was because of the BTG4 promoter methylation, gastric cancer cell lines were treated with 5-aza-dC (an inhibitor of the methylase enzyme, which can re-activate mRNA expression suppressed by methylation) for 48 h and performed real-time RT-PCR to detect BTG4 expression (Fig. 2A). After treatment with 5-aza-dC, we found the levels of BTG4 mRNA were induced in these gastric cancer cell lines, suggesting that the BTG4 gene silencing is accounted for by hypermethylation.

Expression and methylation analysis of BTG4 in primary gastric cancers

BTG4 mRNA expression levels in each of the 38 gastric cancer tissues were assessed relative to the normal tissues using real-time RT-PCR analysis. The results showed that BTG4 was significantly down-regulated in tumor tissues compared to normal tissues ($P = 0.002$) (Fig. 2B). To determine whether the hypermethylation of CpG islands in primary gastric cancer samples was correlated with loss of BTG4 expression, we also performed MSP analysis. The result of MSP revealed that the BTG4 was methylated in 28 of 38 primary gastric cancers tested, no methylation was detected in normal gastric samples. Correlation of the promoter methylation data with BTG4 mRNA expression revealed a significantly lower mean expression level in the 28 tumors with promoter methylation when compared with the tumors without promoter methylation ($P = 0.012$) (Fig. 2C). In total, these data suggested that the frequent mechanism of transcriptional downregulation of BTG4

Table 1
Clinicopathological characteristics and hypermethylation status of BTG4 in gastric cancer patients.

Group	BTG4 methylation		*P* value
	U	*M*	
Normal tissues	15	0	
Cancer tissues	1 0	2 8	$P < 0.001$[a]
Differentiation	4	1	
Well	3	9	
Moderate			
Poor	3	18	$P = 0.011$
Metastasis	2	17	
Yes			
No	8	11	$P = 0.027$
Size (cm)	3	16	
<2	7	12	$P = 0.141$
⩾2			
p16 Methylation	6	5	
Yes	4	23	$P = 0.012$
No			

[a] Statistically significant when compared with the normal tissues.

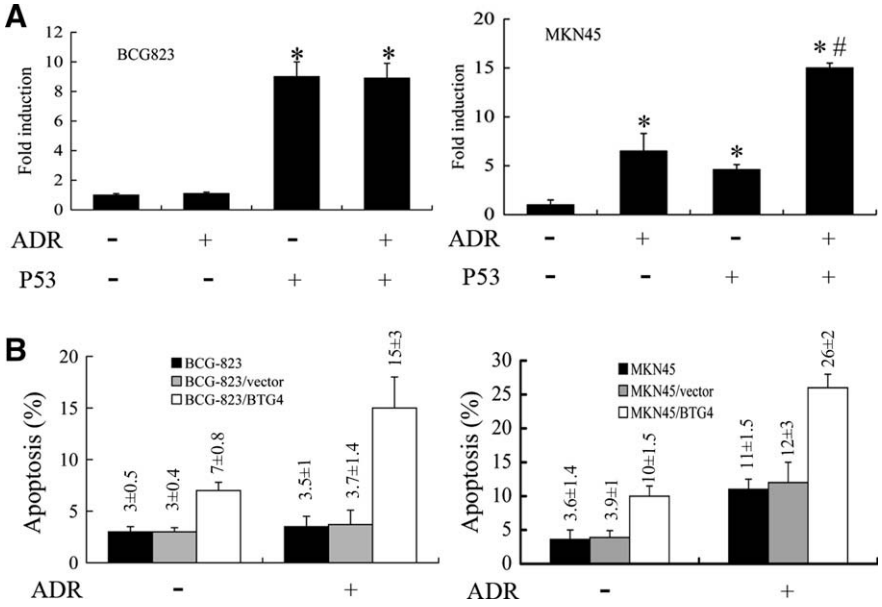

Fig. 3. (A) Real-time RT-PCR results for BTG4 expression in BCG-823 and MKN45 cells treated with ADR or/and p53 expression plasmid. Results are shown relative to a value of 1 assigned to mock-treated BCG-823 or MKN45 cells after normalized to actin expression. $^{*}P < 0.01$ versus ADR-/p53-group; $^{\#}P < 0.01$ versus ADR+ or p53+ group. (B) The apoptotic percentage of the cells with or without ADR treatment was detected by flow cytometric analysis. The values above each bar represent the fraction of annexin V+/PI−.

in gastric cancers was BTG4 promoter hypermethylation. Representative examples of the gel analysis of MSP was shown in Fig. 2D.

The association between BTG4 methylation and the clinicopathological features of patients are listed in Table 1. The prevalence of BTG4 methylation was significantly different between normal and cancerous samples ($P < 0.001$). There was no significant correlation between hypermethylation of BTG4 and tumor size ($P = 0.141$), but not the type of cell differentiation ($P = 0.011$) and metastasis ($P = 0.027$). There was no obvious difference between male and female patients in this study (data not shown).

The relationship between BTG4 and p16 methylation was analyzed for further understanding the role of BTG4 in the tumorigenesis of gastric cancer, since the biological and clinical significance of p16 in gastric cancers has been studied intensively and is known well [9,10]. We found that p16 methylation status was statistically significantly associated with BTG4 hypermethylation in gastric cancers ($P = 0.012$).

P53 regulates BTG4 expression

Since BTG2, BTG3 were targets of p53, we decided to examine whether BTG4 was regulated by p53 as well. We treated BCG-823 and MKN-45 cells with ADR, a DNA damaging agent known to induce endogenous p53 expression. In contrast to MKN-45, ADR could not up-regulate BTG4 expression in BCG-823 cells (Fig. 3A). MKN-45 cells were selected because of the expression of wild-type p53 [6]. To further validate the role of p53 in the regulation of BTG4, we transiently transfected p53 expression plasmid, pcDNA3(−)–p53, into BCG-823 cells. We observed that BTG4 expression was dramatically induced by exogenous p53 ($P < 0.05$) (Fig. 3A). We also observed synergistic effect with the combination of pcDNA3(−)–p53 and ADR.

BTG4 reexpression sensitizes gastric cancer cells to proapoptotic stimuli and reduces colony formation in vitro and tumor growth in mice

From a functional standpoint, we next wanted to examine whether BTG4 could induce apoptosis as target of p53. We established BCG-823 and MKN45 stable transfectants with empty vector and BTG4 expression plasmid. The expression of BTG4 in BCG-823/BTG4 and MKN45/BTG4 cells were confirmed by Western analysis (data not shown). We treated BCG-823, BCG-823/vector, BCG-823/BTG4, MKN45, MKN45/vector, and MKN45/BTG4 cells with ADR for 24 h. BCG-823/BTG4, MKN45, MKN45/vector, and MKN45/BTG4 cells undergo apoptosis in response to the DNA damage by ADR. In contrast, ADR was not able to induce apoptosis in BCG-823, BCG-823/vector (Fig. 3B). We further showed the growth inhibitory features of BTG4 reintroduction in colony formation assay and nude models. We observed that BTG4 reexpression revealed tumor suppressor activity, there was lower colony formation compared to control vector ($P < 0.05$) (Fig. 4A). We examined the ability of BCG-823/BTG4 cells to form tumors compared to BCG-823/vector. All mice injected with BCG-823/vector cells and three of ten mice received injection of BCG-823/BTG4 cells developed palpable tumors. Tumors derived from BCG-823/vector cells were 5–6 times larger than that derived from BCG-823/BTG4 cells ($P < 0.001$) (Fig. 4B).

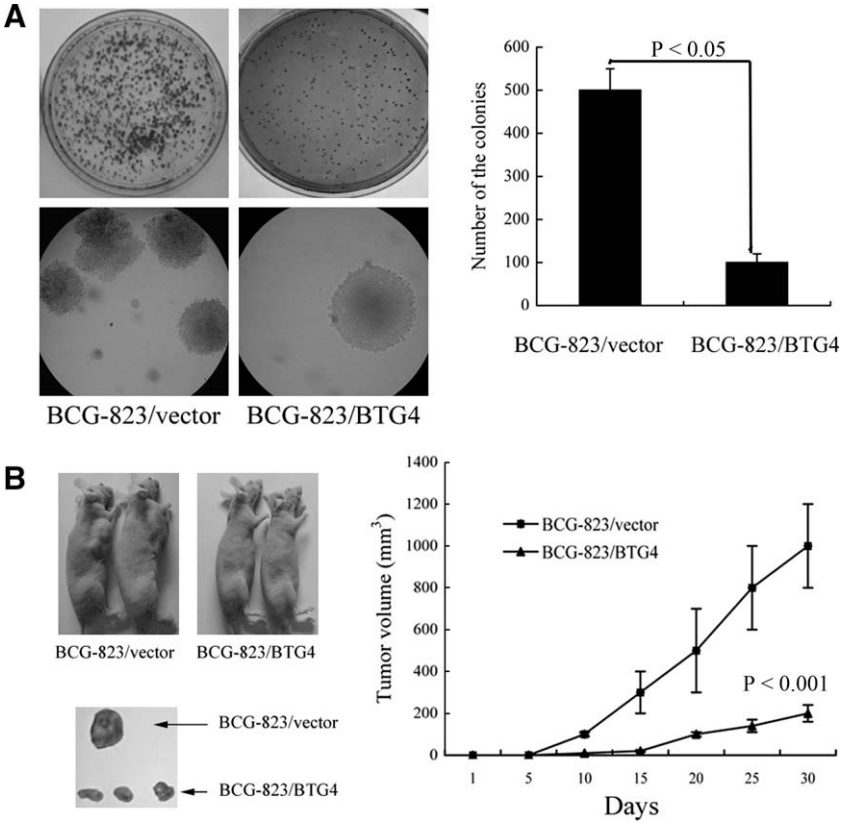

Fig. 4. (A) Examples of colony assay after 4-week selection. Bottom, detailed images from the top panels. Cells were stained with Giemsa, colonies were counted. Graph represents means of three independent experiments ± standard deviations; $P < 0.05$ compared with vector. (B) Effect of BTG4 on the growth of BCG-823 cells in nude mice. Shown are nude mice 30 d after injection of 10^6 BCG-823/vector or BCG-823/BTG4 cells. The large tumor, corresponding to vector cells; the small ones, corresponding to BTG4 cells (bottom). Tumors were monitored every 5 days, each points represent the mean tumor size (left panel).

Discussion

It is well known that hypermethylation of CpG islands in their promoter regions is an important mechanism for loss of function of several tumor suppressor genes, DNA repair genes and other genes in various types of human cancer. An increasing number of genes have been reported to undergo CpG island hypermethylation in gastric cancer. The promoters of ras association domain family 1A (RASSF1A), p16INK4a, adenomatous polyposis coli (APC), PKD1, MAL, and TAC1 are frequently methylated in gastric cancer [10–15]. These findings suggest that CpG island hypermethylation is an important molecular event in the development of gastric cancer.

BTG4 is a member of the BTG family which is characterized by antiproliferative properties. Of these BTGs, the BTG4 gene came into the focus of our interest in DNA methylation, because it was reported that BTG4 gene was completely absent or barely detectable in colorectal cancer cell lines, and overexpression of BTG4 could suppress colony formation in colorectal cancer cells. We assessed GES-1 and five gastric cancer cell lines for a potential epigenetic inactivation of BTG4 by aberrant CpG site methylation in the BTG4 promoter region. We treated these gastric cancer cell lines with DAC and found that BTG4 was up-regulated in all the cells tested.

Our findings showed that BTG4 is significantly down-regulated in gastric cancer compared with non-tumor tissues. Although promoter hypermethylation of BTG4 is significantly linked to cell differentiation, metastasis, there was no significant correlation with the other parameters, such as gender and tumor size.

Next, we found promoter hypermethylation was not the only mechanism that played role in regulating BTG4 expression. P53, which regulated BTG2, BTG3 expression, also was involved in the regulation of BTG4. We treated BCG-823 and MKN-45 cells with ADR to induce endogenous p53 expression. The BTG4 was induced in MKN-45 but not BCG-823 cells. Overexpression of p53 by transfected p53 expression plasmid could induce BTG4 in the BCG-823 cells. Finally, to test whether reexpression of BTG4 could induce apoptosis in gastric cancer cells, we performed FCM assays after treatment of BCG-823/BTG4 and MKN45/BTG4 cells with ADR. We found that reexpression of BTG4 sensitized the BCG-823 and MKN45 cells to the DNA damage signal.

Although much remains unknown about the function of BTG4, recent studies revealed that inactivation of BTG4 may contribute to the pathogenesis of diseases, such as chronic lymphocytic leukemia [16]. In addition, the BTG4 gene maps to 11q23, at which loss of heterozygosity (LOH) is frequent in several types of tumors of diverse cell origin, such as the breast cancer, gastric cancer, lung cancer, colorectal cancer, and malignant melanoma [17–22]. It was reported that LOH at 11q23 occurs in 30% of gastric cancers, suggesting that LOH is a potential mechanism for loss of BTG4 in gastric cancers [19].

In conclusion, our data is consistent with the hypothesis that BTG4 is a probable tumor suppressor gene in gastric cancer. We showed that BTG4 is epigenetically silenced in the majority of gastric cancers. Furthermore, BTG4 promoter methylation which prevailed in gastric cancer was significantly associated with clinicopathological characteristics, such as differentiation, metastasis and p16 methylation, suggesting that BTG4 promoter methylation could be used as a useful tumor biomarker to predict the aggressive behavior in patients with gastric cancer. Although significant induction of BTG4 was observed after demethylation in all of the gastric cancer cell lines tested, hypermethylation is not the only mechanism responsible for downregulation of BTG4 in gastric cancer. Similar to BTG2 and BTG3, BTG4 is also the target of p53. p53 transcriptionally regulates a number of genes that control apoptosis, BTG4 may participate in p53-mediated apoptotic pathway. Finally, reexpression of BTG4 reduces colony formation in vitro and tumor growth in mice, raising the promise that BTG4 methylation has its diagnostic and therapeutic values in gastric cancer.

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