Inhibitory effects of B-cell translocation gene 2 on skin cancer cells via the Wnt/β-catenin signaling pathway

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Abstract. B-cell translocation gene 2 (BTG2), a tumor suppressor gene, is downregulated in several types of human cancer cell. However, its function in skin cancer cells has not been fully elucidated. Therefore, the present study investigated the expression and function of BTG2 in skin cancer cells, and investigated the underlying molecular mechanism. The results indicated that BTG2 expression was downregulated in skin cancer cell lines. Overexpression of BTG2 significantly inhibited cell proliferation, cell cycle progression, and the invasion and migration of skin cancer cells. Furthermore, it was determined that overexpression of BTG2 significantly decreased the protein expression levels of β-catenin, cyclin D1 and v-myc avian myelocytomatosis viral oncogene homolog in skin cancer cells. This suggests that BTG2 may function as a tumor suppressor by interfering with the Wnt/β-catenin signaling pathway in skin cancer cells. Thus, novel therapeutic strategies and agents targeting BTG2 may be potential treatments for skin cancer.

Introduction

As the largest organ of the body, the skin is frequently exposed to sunlight and ultraviolet rays. The function of skin is to protect internal organs from environmental harm and to maintain homeostasis (1), thus, the skin requires protection from frequent exposure to ultraviolet radiation, which may cause skin cancer. Skin cancer, the most common type of cancer worldwide, is a global public health problem and a burden on healthcare expenditures (2,3). Therefore, more effective therapeutic strategies are required for its treatment.

B-cell translocation gene 2 (BTG2) is an important member of the BTG/TOB family (4,5). Located on band 2, region 3 of the long arm of chromosome 1, BTG2 encodes a 158-amino acid protein and structural deletions or changes often result in the occurrence of various tumors in humans (6-8). Previous studies have determined that BTG2 is a tumor suppressor of various cancer cells (4,5). Zhang *et al* (9) reported that BTG2 suppressed proliferation and invasion of MDA-MB-231 triple-negative breast cancer cells. Additionally, BTG2 inhibited bladder cancer invasion via suppression of DNA methyltransferase 1 (10). BTG2 may also induce cellular differentiation, counteract cellular transformation and promote activity of pro-apoptotic stimuli (11-13). Additionally, BTG2 expression is frequently downregulated or blocked in various human tumors, including gastric and breast cancer (14,15). However, the function of BTG2 in skin cancer remains unclear.

The Wnt/β-catenin signaling pathway is important in the progression of tumors, including skin cancer (16-18). It was reported that mouse skin tumors demonstrated cytoplasmic and nuclear accumulation of β-catenin, and upregulation of β-catenin/Tcf target genes, including v-myc avian myelocytomatosis viral oncogene homolog and c-jun (19). Thus, inhibiting the Wnt/β-catenin signaling pathway may prevent the progression of skin cancer.

The present study investigated the expression and function of BTG2 in skin cancer cells, as well as the underlying molecular mechanism. The results demonstrated that BTG2 was downregulated in skin cancer cells lines. Furthermore, overexpression of BTG2 significantly inhibited cell proliferation, cell cycle progression, cell invasion and migration of skin cancer cells via the Wnt/β-catenin signaling pathway.

Materials and methods

Cell lines and culture. The human skin cancer cell lines (A431 and SCC13) were obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All the cells were cultured as monolayers to 80% confluence in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 μg/ml penicillin-streptomycin (GE Healthcare, Logan, UT, USA) in a humidified incubator with 5% CO_2 at 37˚C.

Plasmids and transfection assays. A431 and SCC13 cell lines were transfected with the cDNA of the BTG2 gene. For stable

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transfection, pcDNA3.1 expression vector (Thermo Fisher Scientific, Inc.) was used for insertion of BTG2 cDNA between the *Kpn*I and *Bam*HI sites. The orientation of the insertion was confirmed via restriction digestion and DNA sequencing. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect the pcDNA3.1-BTG2 expression vector or the empty pcDNA3.1 vector into A431 and SCC13 cells, in accordance with the manufacturer's protocol. The cells were then cultured in DMEM containing 800 μg/ml G418 for antibiotic selection. After 28 days, positive clones were collected from the A431 and SCC13 cell cultures in the pcDNA3.1-BTG2 vector transfection group (A431-BTG2 and SCC13-BTG2) and the empty pcDNA3.1 vector transfection group (A431-PC and SCC13-PC). Western blot analysis was performed in order to detect BTG2 protein expression in the A431-BTG2, A431-PC, SCC13-BTG2 and SCC13-PC cell groups.

Cell proliferation assay. Transfected and untreated cells were seeded into 96-well culture plates (Corning Life Sciences, MA, USA) at a density of 1×10^4 cells/well and cultured for 24 h with saturated humidity and 5% CO_2 at 37°C to form a cell monolayer. MTT solution (100 μl; Nalge Nunc International, Roskilde, Denmark) was aliquoted per well and incubated with 5% CO_2 at 37°C for 4 h. Next, the DMEM was discarded and 150 μl dimethyl sulfoxide was added to each well. The absorbance was measured at 570 nm with a microplate reader (BD Biosciences, San Jose, CA, USA).

Cell cycle assay. When the cells reached 70-90% confluence, a serum-free DMEM was applied for synchronization. The cells were cultured for 24 h, then trypsinized (Sigma-Aldrich, St. Louis, MO, USA) and fixed with 100% ethanol overnight, followed by propidium iodide staining. Cell cycle analysis was performed after 30 min using a flow cytometer (BioVision, Inc., CA, USA). Each experiment was replicated three times.

Cell invasion and migration assays. Twenty-four well Transwell chambers (8 μm; Corning Life Sciences) were used to detect the invasive and migratory ability of the skin cancer cell lines. For the invasion assay, the upper chamber was washed with serum-free DMEM three times, filled with 20 μl diluted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and incubated for 30 min at 37°C to form the artificial basement membrane. Subsequently, all the cells were suspended in the serum-free DMEM and added to the upper chamber. The lower chamber was filled with DMEM containing 10% FBS. The cells were incubated for 24 h and those remaining in the upper chamber surface of the basement membrane were removed with cotton swabs, cells invading the lower chamber surface were stained with crystal violet. The number of cells crossing the polycarbonate membrane was counted under a microscope (CX22; Olympus Corporation, Tokyo, Japan), at a magnification of x400. The migration assay was performed according to the above-mentioned procedure, except that Matrigel was not used, instead the Transwell chamber was used alone.

Western blotting. The cells were lysed using a lysis buffer (Sigma-Aldrich). The total protein (30 μg) was separated by 10% SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and transferred onto polyvinylidene fluoride membranes (Thermo Fisher Scientific, Inc.). The membrane was blocked at room temperature for 1 h with Tris-buffered saline (TBS) containing 5% non-fat milk and then incubated overnight at 4°C with the mouse primary antibodies (all from Santa Cruz Biotechnology, Dallas, TX, USA) against BTG2 (1:2,500; cat. no. sc-517187), β-catenin (1:2,000; cat. no. sc-53484), cyclin D1 (1:3,000; cat. no. sc-20044), v-myc avian myelocytomatosis viral oncogene homolog (c-Myc; 1:2,500; cat. no. sc-47694) and β-actin (1:1,500; cat. no. sc-130300). After washing with TBS Tween-20 (TBST), the membrane was incubated for 1 h at 37°C with horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (1:3,000; Santa Cruz Biotechnology, Inc.; cat. no. 395760). Subsequent to another wash with TBST, protein expression was detected using an enhanced chemiluminescence kit (Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analysis of experimental data was performed with SPSS 17.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA). The Student's t-test was conducted for the comparison of two groups and one way analysis of variance was performed for multiple comparisons. Data are presented as the mean ± standard deviation and P<0.05 was considered to indicate a statistically significant difference.

Results

BTG2 expression in A431 and SCC13 cell lines and their transfectants. BTG2 protein expression in A431 and SCC13 cells and their transfectants was detected by western blot analysis. BTG2 protein expression was observed in A431-BTG2 and SCC13-BTG2 cells; however, it was not detected in A431, A431-PC, SCC13 and SCC13-PC cells (Fig. 1).

Effects of BTG2 overexpression on cell proliferation. The proliferation of A431-BTG2 cells was significantly reduced in comparison with the A431-PC and untreated A431 cells (P<0.05). No significant difference was identified between the control groups (A431 and A431-PC; Fig. 2A). Cell proliferation in the SCC13-BTG2 group was significantly reduced when compared with the control groups (P<0.05; Fig. 2B).

Effects of BTG2 overexpression on cell cycle progression. Cell cycle analysis of A431 cells in each group was performed using flow cytometry. BTG2 overexpression significantly increased the number of A431 and SCC13 cells in the G_0/G_1 phase; however, it decreased the number of A431 and SCC13 cells in the G_2/M phase (P<0.05; Fig. 3). These results suggested that cell cycle distribution in A431 and SCC13 cells was affected by increased BTG2 expression levels and resulted in cell cycle arrest in the G_0/G_1 phase in A431 and SCC13 cells.

Effects of BTG2 overexpression on cell invasion and migration. The effects of BTG2 overexpression on invasion and migration of A431 and SCC13 cells were investigated. As shown in Fig. 4A and B, the invasive and migratory abilities of A431-BTG2 cells were significantly reduced when compared with A431-PC and untreated A431 cells (P<0.05). The effects
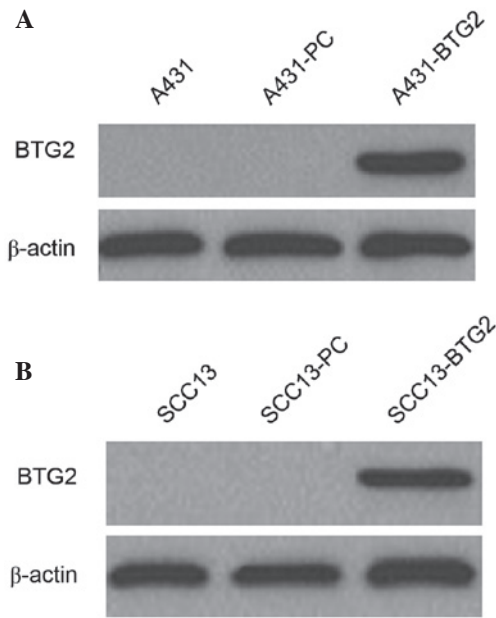
Figure 1. BTG2 expression in A431 and SCC13 cell lines and their transfectants. Western blot results of BTG2 in (A) A431, A431-PC, and A431-BTG2 and (B) SCC13, SCC13-PC and SCC13-BTG2 cells. BTG2, B-cell translocation gene 2; PC, empty vector.
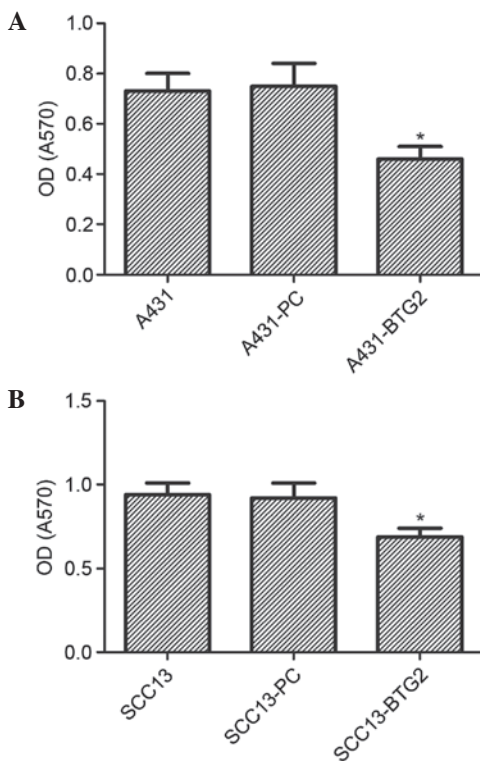

Figure 2. Effects of BTG2 overexpression on cell proliferation. MTT assay was performed to determine the effects of BTG2 overexpression in skin cancer cell proliferation. (A) Cell proliferation of A431-BTG2 cells was significantly decreased in comparison with A431 and A431-PC cells. (B) Cell proliferation of SCC13-BTG2 cells was significantly decreased when compared with SCC13 and SCC13-PC cells. *P<0.05 vs. the control group. BTG2, B-cell translocation gene 2; PC, empty vector; OD, optical density.

of BTG2 overexpression on the invasion and migration of SCC13 cells were also determined. As shown in Fig. 4C and D,
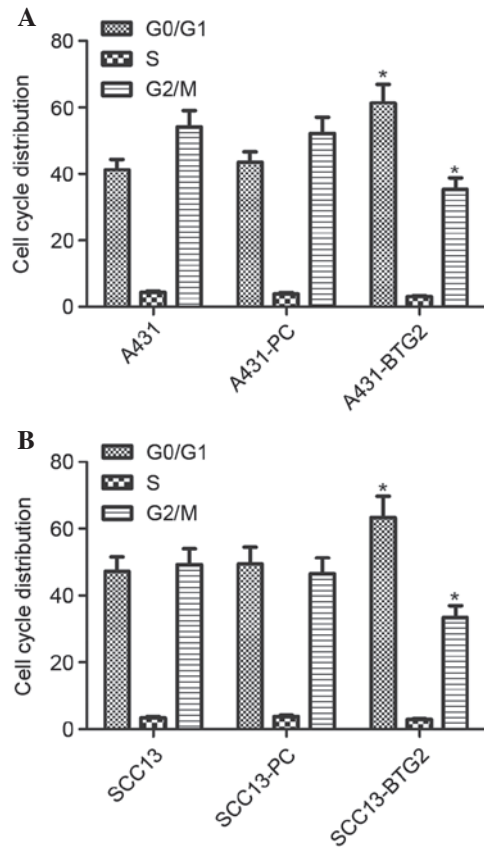

Figure 3. Effects of BTG2 overexpression on cell cycle progression. Flow cytometry was used to analyze the cell cycle distribution of A431 and SCC13 cells in each group. BTG2 overexpression increased the number of (A) A431-BTG2 cells at the G_0/G_1 phase, however, decreased cells in the G_2/M phase, in comparison with A431 and A431-PC cells. Similar increases and decreases were found in the number of (B) SCC13-BTG2 cells at the G_0/G_1 and G_2/M phase, respectively in comparison with SCC13 and SCC13-PC cells. *P<0.05 vs. the control group. BTG2, B-cell translocation gene 2; PC, empty vector.

invasive and migratory abilities of SCC13-BTG2 cells were significantly reduced when compared with SCC13-PC and untreated SCC13 cells (P<0.05).

Effects of BTG2 overexpression on β-catenin, cyclin D1 and c-Myc expression levels. Western blot analysis was performed to detect the effects of BTG2 overexpression on β-catenin, cyclin D1 and c-Myc. A significant decrease in the expression levels of β-catenin, cyclin D1 and c-Myc in A431-BTG2 cells when compared with A431-PC and untreated A431 cells (P<0.05; Fig. 5).

Discussion

BTG2, a transient early response protein, has been reported to be a potential tumor suppressor gene (6,12,20,21). Previous studies have highlighted the importance of BTG2 in tumor incidence, development, metastasis and invasion (14,22,23). BTG2 overexpression may suppress proliferation of prostate cancer cells (24,25). Additionally, BTG2 has frequently been downregulated or dysfunctional in previous studies of cancerous tumors (6,26,27). Takahashi *et al* (23) determined that low expression levels of BTG2 in breast cancer may inhibit
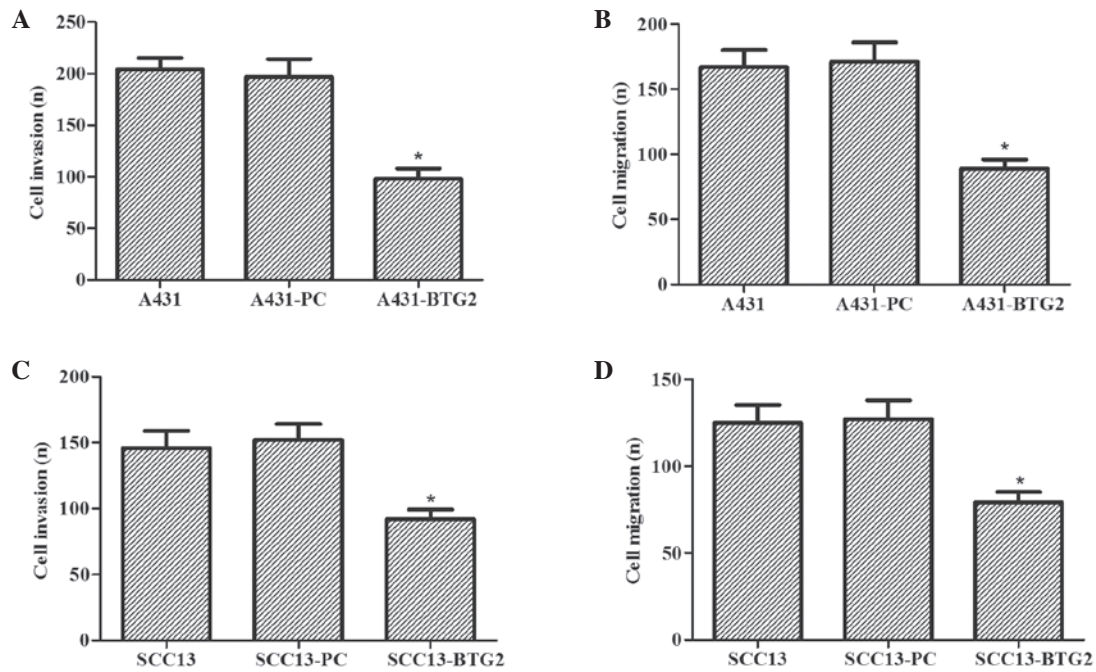
Figure 4. Effects of BTG2 overexpression on cell invasion and migration. Transwell chambers were used to determine the effect of BTG2 overexpression on invasion and migration of A431 and SCC13 cells. BTG2 overexpression decreased the quantity of A431-BTG2 cells (A) invading and (B) migrating through the membrane, in comparison with A431 and A431-PC cells. SCC13-BTG2 cell (C) invasion and (D) migration through the membrane compared with SCC13 and SCC13-PC cells. *P<0.05 vs. the control group. BTG2, B-cell translocation gene 2; PC, empty vector.
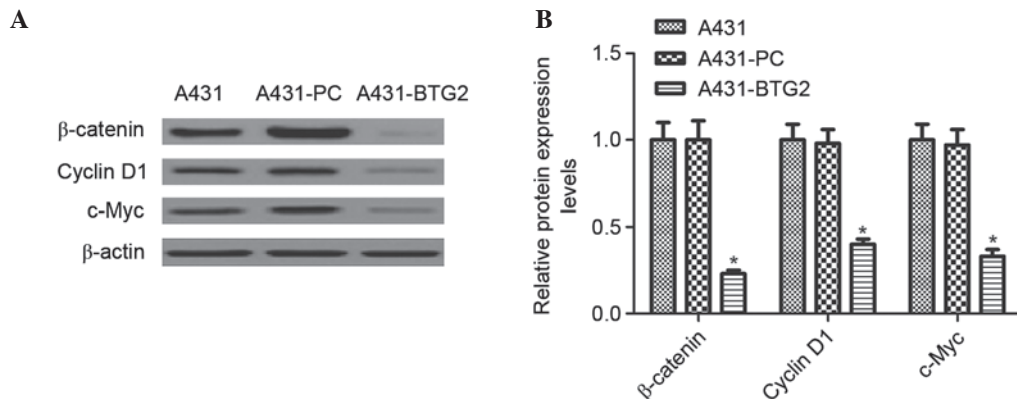

Figure 5. Effects of BTG2 overexpression on β-catenin, cyclin D1 and c-Myc. (A) Western blotting visualization of protein expression of β-catenin, cyclin D1 and c-Myc in A431, A431-PC and A431-BTG2 cells. (B) Quantification of relative protein expression of β-catenin, cyclin D1 and c-Myc in A431, A431-PC and A431-BTG2 cells. *P<0.05 vs. the control group. BTG2, B-cell translocation gene 2; c-Myc, v-myc avian myelocytomatosis viral oncogene homolog.

tumor metastasis (23). These studies indicated the association between BTG2 and tumor progression, and the importance of BTG2 in tumor incidence and proliferation. However, whether BTG2 is expressed in skin cancer and the effects BTG2 exerts on skin cancer remained unclear.

The present study investigated the expression and function of BTG2 in skin cancer progression and BTG2 expression was not observed in the skin cancer cell lines. Additionally, upregulation of BTG2 markedly inhibited cell proliferation, cell cycle progression, cell invasion and migration of skin cancer cells.

The cell cycle assay performed in the current study indicated that BTG2 overexpression may significantly increase the number of cells in the G_0/G_1 phase and decrease those in the S and G_2 phases. This was consistent with the results of the cell

proliferation assay performed in the present study, indicating that BTG2 exerted an inhibitory effect on cell proliferation. These results demonstrated the possible association between BTG2 overexpression and cell proliferation inhibition.

The effects of BTG2 on invasion and migration of skin cancer cells was examined using a Transwell assay. There was a significant decrease in cells crossing the polycarbonate membrane of the Transwell chamber, suggesting that BTG2 overexpression may be associated with the inhibition of skin cancer cell invasion and migration.

The present study demonstrated that overexpression of BTG2 significantly decreased the expression levels of β-catenin, cyclin D1 and c-Myc. The Wnt signaling pathway is fundamental for the determination of cell fate, polarity, proliferation and death (28). Additionally, environmental and

genetic perturbations of the Wnt signaling pathway may lead to a variety of human diseases including various types of cancer (29). β-catenin, and its accumulation within the nucleus and cytoplasm, is associated with various types of cancer and is important in the successful functioning of Wnt signaling (30). In the current study, β-catenin was highly expressed in skin cancer cells and BTG2 overexpression significantly inhibited its expression levels. As target genes of β-catenin, cyclin D1 and c-Myc are powerful proto-oncogenes with similar downstream effects (31) and the two are associated with cell cycle control (32,33). The present study identified a significant decrease in their expression levels, which may explain the inductive effect of BTG2 on the cell cycle arrest at the G_0/G_1 phase in skin cancer cells.

In conclusion, the present study investigated the effects of BTG2 overexpression on skin cancer cells and evaluated the underlying molecular mechanism. The results demonstrated that BTG2 was not expressed in skin cancer cells lines and its upregulation may suppress cell proliferation, cell cycle progression, cell invasion and migration of skin cancer cells via the Wnt/β-catenin signaling pathway. Therefore, BTG2 may serve as a potential target for the treatment of skin cancer.

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