ANTICANCER RESEARCH *39*: 5361-5367 (2019)
doi:10.21873/anticanres.13729

Promoter Methylation Down-regulates B-cell Translocation Gene 1 Expression in Breast Carcinoma

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Abstract. *Background/Aim: The mechanism responsible for B-cell translocation gene 1 (BTG1) down-regulation in breast carcinoma remains unknown. We examined the BTG1 expression status in breast carcinoma cells and investigated the mechanism underlying the observed alterations. Materials and Methods: Four breast carcinoma cell lines (SK-BR-3, MDA-MB-231, T-47D, and MCF-7), and one normal mammary epithelial cell line (MCF-10A) were analyzed. BTG1 expression was examined using quantitative reverse transcription polymerase chain reaction (PCR) and western blot. Methylation status of the BTG1 promoter was analyzed using methylation-specific PCR (MSP). To investigate the effect of methylation on BTG1, the cells were treated with a demethylating agent. Results: The carcinoma cells expressed significantly lower levels of BTG1 mRNA and protein than normal cells. The BTG1 promoter was highly methylated in the carcinoma cells. 5-aza-2-deoxycytidine significantly restored BTG1 expression. Conclusion: Down-regulation of BTG1 expression through epigenetic repression is involved in mammary carcinogenesis. BTG1 is a potential diagnostic marker and therapeutic target for breast carcinoma.*

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Key Words: Breast carcinoma, B-cell translocation gene 1, promoter methylation.

Breast carcinoma is one of the most common malignancies among women, accounting for approximately 25% of female cancer patients worldwide. In the Republic of Korea, breast carcinoma is the second most common cancer among women, after thyroid carcinoma. The number of newly diagnosed breast cancer patients has steadily increased in the Republic of Korea in the past decade (1).

B-cell translocation gene 1 (*BTG1*) was first identified in B-lymphoblastic leukemia, and is a member of the antiproliferative gene family (2, 3). Proteins encoded by members of this gene family are involved in the regulation of cell growth and differentiation in various types of cells and tissues (4). Overexpression of *BTG1* has been shown to block erythroid differentiation of primary murine bone marrow cells, and to induce cell cycle arrest or apoptosis in murine fibroblasts, myoblasts, and microglia (5-8).

Tumor development and progression are based on uncontrolled cellular proliferation and reduced cell death. As a tumor suppressor gene, *BTG1* can inhibit cellular proliferation, regulate the cell cycle, and induce cell death. Recent studies have demonstrated that decreased *BTG1* expression is associated with aggressive biological behaviors and worse prognosis of several human malignancies (9-16). The tumor-suppressive role of *BTG1* has also been identified in breast carcinoma. *BTG1* inhibits cellular growth through induction of cell cycle arrest and apoptosis in breast carcinoma cells (17).

However, the mechanism responsible for the down-regulation of *BTG1* expression is not well established. Although a previous study in gastric carcinoma has reported that *BTG1* expression is partially regulated by methylation of its promoter (18), this has not been investigated in breast carcinoma. In this study, we first examined the expression status of BTG1 mRNA and protein in breast carcinoma cells and normal mammary epithelial cells. We then investigated the effects of methylation on *BTG1* expression, by treating the cells with a demethylating agent. This study could further our understanding of the mechanisms regulating *BTG1*

expression and the potential role of BTG1 as a diagnostic marker and therapeutic target for breast carcinoma.

Materials and Methods

Cell culture and treatment. Four human breast carcinoma cell lines, SK-BR-3, MDA-MB-231, T-47D, and MCF-7, and one human normal mammary epithelial cell line, MCF-10A, were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium or Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Gibco Life Technologies, Grand Island, NY, USA). All cell lines were cultured at 37˚C in a humidified atmosphere with 5% carbon dioxide. The demethylating agent, 5-aza-2-deoxycytidine (5-aza-CdR; Sigma-Aldrich, St. Louis, MO, USA), was dissolved in phosphate-buffered saline (PBS) to a concentration of 50 mg/ml as a stock solution and stored at –20˚C until use. Immediately before use, the 5-aza-CdR stock solution was diluted in RPMI 1640 medium without FBS. The cells were seeded in 6-well plates at a density of 5×10^5 cells/ml and treated with 5-aza-CdR (final concentration, 1 μM). The 5-aza-CdR was replaced with freshly prepared solution every 24 h, and the cells were harvested 96 h after the initial treatment. Control cultures were treated under identical experimental conditions in the absence of 5-aza-CdR (PBS only).

Complementary DNA (cDNA) synthesis. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). RNase-free DNase I (Thermo Fisher Scientific, Waltham, MA, USA) treatment was performed to remove contaminating genomic DNA from total RNA purified from the cell lines. Isolated total RNA was diluted to 1 mg/ml with sterile diethylpyrocarbonate (DEPC)-treated water, and 2.5 ml were added to reactions containing 1× DNase I buffer and 1 U DNase I (final volume, 10 ml). The mixtures were incubated at 37˚C for 30 min, followed by incubation at 70˚C for 10 min to stop the reactions. DNase I-treated RNA was reverse-transcribed into first-strand cDNA using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Briefly, 1 μg of DNase I-treated RNA and 250 ng of random primers were mixed in a 0.5-ml polymerase chain reaction (PCR) tube and brought to 11 ml with sterile DEPC-treated water, heated at 65˚C for 5 min, and chilled quickly on ice. Other reagents were then added to the 20 ml reaction volume at the following final concentrations: 1× First-Strand Buffer, 10 mM dithiothreitol, 0.5 mM each dNTP, and 200 U Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Reactions were incubated at 42˚C for 1 h, heated to 70˚C for 10 min, and the products stored at –20˚C. The amount of cDNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Quantitative reverse transcription PCR. The cDNA was used for PCR analysis using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). PCR was carried out in a 20 μl reaction containing 0.5 μM of each primer, 1× Thunderbird SYBR qPCR Mix (Toyobo), and 2 μl of template cDNA. PCR for BTG1 was performed in a C1000 Thermal Cycler (Bio-Rad Laboratories) using the following reaction protocol: polymerase activation at 98˚C for 2 min, followed by 40 cycles at 98˚C for 2 sec, 60˚C for 5 sec, and 75˚C for 10 sec. Amplification patterns were analyzed and threshold cycle numbers (Ct) for each sample were determined using CFX Manager Software (Bio-Rad Laboratories). The primer sequences used for *BTG1* were: forward: 5'-CAA GGG ATC GGG TTA CCG TTG T-3'; reverse: 5'-AGC CAT CCT CTC CAA TTC TGT AGG-3' (19, 20). The ΔΔCt method was used to calculate relative *BTG1* expression after normalization to expression of *β-actin* (*ACTB*) (21). Amplification of *BTG1* was confirmed by a melting-curve analysis, and the target amplicon size was confirmed by agarose gel electrophoresis. Each sample was assayed in triplicate.

Western blot. Protein extracts were prepared using RIPA Buffer (Thermo Fisher Scientific) containing freshly added Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). The concentrations of the total cell lysates were measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Twenty micrograms of total protein were mixed with 5× sample buffer and heated at 95˚C for 5 min. The samples were loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis and then transferred to a polyvinylidene fluoride membrane (EMD Millipore, Burlington, MA, USA) *via* a Transblot apparatus (Bio-Rad Laboratories). After a 1 h incubation in blocking solution [5% non-fat milk in Tris-buffered saline with Tween 20 (TBS-T)], the membranes were exposed to the following primary antibodies overnight at 4˚C: anti-BTG1 (polyclonal, 1:200; Abcam, Cambridge, MA, USA) and anti-β-actin (clone C4, 1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed three times in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA) for 1 h at room temperature. Protein bands were visualized using the Clarity Western ECL Substrate (Bio-Rad Laboratories). The intensities of protein bands were quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Methylation-specific PCR (MSP). Genomic DNA was extracted from cultured cells using a NucleoSpin Tissue Kit (Macherey-Nagel, Duren, Germany) and quantified using a DropSense96 multichannel spectrophotometer (Trinean, Gentbrugge, Belgium). Bisulfite treatment was performed on 2 μg of DNA using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). The bisulfite-modified DNA was then subjected to PCR using primer pairs that specifically amplify either methylated or unmethylated sequences of *BTG1*. The following methylated *BTG1*-specific primers were used (19, 20): MSP1 (–149 to –289), 5'-GTT TTT AAG TTA AAA GGA AGG AAG TC-3' (sense) and 5'-ATA TCA AAA AAT ATT AAA AAT CAC GCA-3' (antisense); MSP2 (–517 to –645), 5'-TTT GAG GAG TTA GTT ATC GAG ATT C-3' (sense) and 5'-AAA TAA ATA AAA ACC GCC TAA CG-3' (antisense). The following unmethylated *BTG1*-specific primers were used: USP1 (–149 to –289), 5'-GTT TTT AAG TTA AAA GGA AGG AAG TTG T-3' (sense) and 5'-ATA TCA AAA ATA TTA AAA ATC ACA CA-3' (antisense); USP2 (–517 to –645), 5'-TGA GGA GTT AGT TAT TGA GAT TTG G-3' (sense) and 5'-AAA TAA ATA AAA ACC ACC TAA CAC A-3' (antisense). MSP was performed in 20 μl mixtures for 40 cycles using HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). The same unconverted genomic DNAs were used in the PCR assays as negative controls.

Statistical analysis. Data are expressed as mean±standard deviation of three independent experiments, each performed in triplicate, and presented relative to the control. The Wilcoxon rank sum test was used to compare the promoter methylation status and *BTG1* mRNA

and protein expression levels between the normal and carcinoma cells. We used the Wilcoxon signed-rank test to compare the expression levels of *BTG1* mRNA and protein before and after 5-aza-CdR treatment in each cell line examined. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 20 (IBM Corporation, Armonk, NY, USA). A p-value <0.05 was considered statistically significant.

Results

BTG1 expression in human breast carcinoma cells. We observed varying degrees of BTG1 mRNA and protein expression among the different cell lines. MDA-MB-231 (normalized expression ratio=0.727; $p<0.001$), T-47D (0.578; $p<0.001$), and SK-BR-3 (0.403; $p<0.001$) demonstrated significantly reduced BTG1 mRNA expression compared to MCF-10A (1.000; Figure 1A). In line with these results, western blot analysis revealed that the expression levels of BTG1 protein in MDA-MB-231 (normalized expression ratio=0.176; $p<0.001$), T-47D (0.111; $p<0.001$), and SK-BR-3 (0.080; $p<0.001$) were significantly lower than that of MCF-10A (1.000; Figure 1B). Although MCF-7 (normalized expression ratio=1.040) showed similar *BTG1* mRNA expression levels to those of MCF-10A ($p=0.199$), the BTG1 protein expression levels (normalized expression ratio=0.158) were substantially decreased in MCF-7 compared to MCF-10A ($p<0.001$).

Mechanisms contributing to down-regulation of BTG1 expression in human breast carcinoma cells. Similar to a previous study, we have recently observed that BTG1 down-regulation is mediated by *BTG1* promoter methylation and BTG1 expression is restored after treatment with a demethylating agent in ovarian and colorectal carcinoma cells (19, 20). Based on this knowledge, we hypothesize that promoter methylation might play a role in the regulation of *BTG1* expression in breast carcinoma cells. Increased methylation of the BTG1 promoter region was observed (MSP1; –149 to –289) in MDA-MB-231, T-47D, MCF-7, and SK-BR-3 cells (Figure 1C), indicating promoter methylation as a possible mechanism for the reduced BTG1 expression. There was no difference in the methylation status of MSP2 (–517 to –645) between the carcinoma and normal epithelial cells. To further investigate the effect of promoter methylation on BTG1 protein down-regulation, the cells were treated with the demethylating agent, 5-aza-CdR. Treatment with the 5-aza-CdR significantly restored *BTG1* mRNA expression in the cells, with a 1.291 fold increase in MDA-MB-231 ($p=0.044$), 1.671 fold increase in T-47D ($p<0.001$), 1.247 fold increase in MCF-7 ($p=0.012$), and 1.814 fold increase in SK-BR-3 ($p=0.004$), compared to the respective pre-treatment levels (Figure 1D). The restorative effect of 5-aza-CdR on *BTG1* mRNA expression was confirmed at the protein level (Figure 1E). MDA-MB-231, T-47D, MCF-7, and SK-BR-3 showed a 1.461 ($p<0.001$), 1.349 ($p=0.017$), 2.358 ($p<0.001$), and 1.220-fold ($p=0.033$) increase in BTG1 protein expression after 5-aza-CdR treatment, respectively. These findings indicate that promoter methylation is responsible for the decreased BTG1 expression in breast carcinoma cells.

Discussion

In this study, we investigated the expression of *BTG1* in human breast carcinoma cells. All four carcinoma cell lines examined demonstrated significantly lower levels of BTG1 expression than a normal mammary epithelial cell line. In particular, western blot analysis revealed that BTG1 protein expression in the carcinoma cells was obviously reduced compared to normal cells. BTG1 protein was readily detected in MCF-10A cells, but was at negligible levels in MDA-MB-231, T-47D, MCF-7, and SK-BR-3 cells. These findings are consistent with observations from our previous studies indicating significantly reduced *BTG1* mRNA and protein expression in ovarian and colorectal carcinoma cells (19, 20). The significant differences in *BTG1* expression between breast carcinoma cells and normal mammary epithelial cells suggest that BTG1 down-regulation is associated with the development of breast carcinoma. We are currently conducting immunohistochemical analyses in breast carcinoma tissues to determine whether BTG1 protein expression is a potential diagnostic biomarker for breast carcinoma.

DNA methylation is one of the major epigenetic mechanisms of gene silencing, along with histone modifications (22, 23). DNA methylation plays a critical role not only in tumorigenesis, but also in normal development such as X chromosome inactivation and genomic imprinting (24-27). In the mammalian genome, methylation takes place only at cytosine bases located at a CpG dinucleotide (28-31). Over the past decade, it has become apparent that aberrant hyper- and hypomethylation of DNA occurs during tumor development and progression in many types of carcinoma (22, 23). There is a critical difference between sequence mutation and epigenetic gene silencing: the former is irreversible but the latter is potentially reversible. Consequently, the epigenetic changes provide new opportunities for the clinical management of carcinoma. These potentially reversible alterations are very stable and exert a significant impact on the regulation of gene expression. Changes in methylation of the promoter may mimic the effect of mutations of various tumor suppressor genes. Based on previous data (18-20), we hypothesized that promoter methylation could be one of the mechanisms underlying down-regulation of *BTG1* gene expression in the breast carcinoma cells examined in this study. Indeed, our results show that treatment with a demethylating agent significantly restored *BTG1* mRNA and protein expression in all four carcinoma cell lines, suggesting that promoter

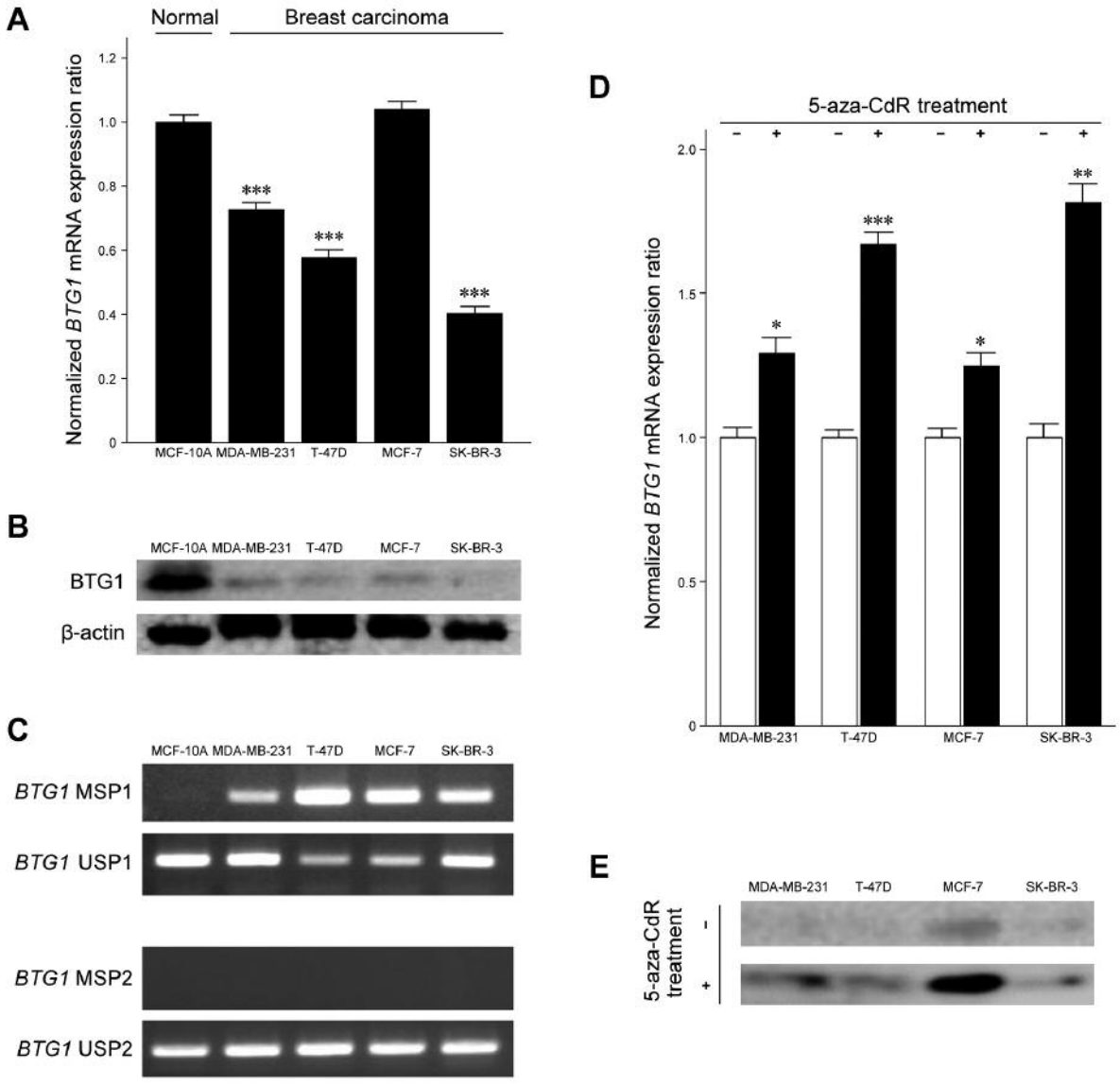

Figure 1. *Expression of B-cell translocation gene 1 (BTG1) in breast carcinoma and normal mammary epithelial cells and the effect of demethylation. A: BTG1 mRNA expression measured by quantitative reverse transcription polymerase chain reaction (PCR). Three of the four carcinoma cell lines show significantly lower expression levels than the normal epithelial cell line. B: BTG1 protein expression analyzed by western blot. The expression levels of BTG1 protein were negligible in all four carcinoma cell lines. C: Methylation status of two BTG1 promoter regions (MSP1: –149 to –289; MSP2: –517 to –645) analyzed by methylation-specific PCR. Significantly increased methylation at one of the BTG1 promoter regions (MSP1) was noted in all four carcinoma cell lines. D and E: BTG1 mRNA (D) and protein (E) expression before and after treatment with a demethylating agent. 5-aza-2-deoxycytidine (5-aza-CdR) treatment significantly restored BTG1 mRNA and protein expression in all four carcinoma cell lines. *p<0.05; **p<0.01; ***p<0.001.*

methylation contributes to decreased BTG1 expression. In addition, we analyzed the DNA methylation status of the *BTG1* gene promoter region in the breast carcinoma cell lines and found that down-regulation of BTG1 expression was associated with augmented promoter methylation.

The relationship between promoter hypermethylation and down-regulation of *BTG1* expression has been observed in various types of carcinoma. We have reported this association in ovarian and colorectal carcinoma cells (19, 20). Zheng *et al*. have observed that *BTG1* mRNA and protein expression levels were reduced in gastric carcinoma cells, and BTG1 was down-regulated due to promoter hypermethylation (18). They have also showed that treatment with 5-aza-CdR reduced *BTG1* promoter methylation and

restored mRNA expression in the gastric carcinoma cells. Taken together, these results provide novel insights into the altered BTG1 expression associated with breast carcinogenesis. We suggest that BTG1 is a potential target for the treatment of breast carcinoma.

Emerging evidence on BTG1 expression in other types of carcinomas supports the idea that BTG1 serves as a tumor suppressor. Sun *et al*. have reported that BTG1 protein expression is substantially lower in renal cell carcinoma tissues than in normal kidney tissue (32). In their study, BTG1-overexpressing renal cell carcinoma cells, obtained by stable transfection of *BTG1* cDNA sequences, exhibited a significantly lower survival rate, higher apoptotic rate, and lower invasion capability than control cells, accompanied by reduced expression of cyclin D1, B-cell lymphoma-2 (Bcl-2), and matrix metalloproteinase 9 (MMP9). These findings indicate that BTG1 may be involved in cell-cycle regulation; BTG1 may inhibit cellular proliferation and modulate invasion and metastasis by down-regulating Bcl-2 and MMP9 expression, respectively. Similar results have been found in hepatocellular carcinoma, non-small cell lung carcinoma, and nasopharyngeal carcinoma (11, 14, 32), where *BTG1*-transfected cells had increased cell cycle arrest, higher apoptotic rates, and significantly lower invasiveness than the corresponding untransfected cells. Moreover, Zhu *et al*. have demonstrated that *BTG1*-transfected breast carcinoma cell xenografts had significantly smaller tumor sizes, reduced cell density, and more extensive tumor necrosis than untransfected xenografts in control mice (17), indicating that BTG1 over-expression mediates the inhibition of xenograft formation and breast carcinoma cell growth *in vivo*.

Many previous studies have reported an association between the reduction or loss of BTG1 expression and aggressive behaviors such as lymph node metastasis, worse histological grade, and advanced clinical stage, as well as worse overall survival in carcinomas of the thyroid, esophagus, nasopharynx, lung, liver, and breast (11-14, 16, 33). Notably, Zheng *et al*. have reported that reduced BTG1 expression correlates with advanced tumor stage, lymphovascular invasion, and lymph node metastasis in gastric carcinoma, emphasizing that BTG1 might be a candidate target for gastric carcinoma treatment (18). Similarly, Zhao *et al*. have shown that *BTG1* mRNA expression was lower in advanced-stage than in early-stage ovarian carcinomas (9). These results suggest that BTG1 down-regulation may play a crucial role in tumor progression and metastasis, and that the measurement of BTG1 expression may provide valuable clues to predict prognosis and select treatment options for patients.

Nevertheless, our observations of BTG1 down-regulation in breast carcinoma cells are not in agreement with some previous reports. Kanda *et al*. have reported that in gastric carcinoma cells and tissues, decreased *BTG1* mRNA expression was observed regardless of promoter methylation status (10). Even though down-regulation of *BTG1* mRNA in gastric carcinomas was significantly associated with shorter disease-specific and recurrence-free survival, no promoter hypermethylation events were detected. Possible explanations for this inconsistency include the differences in organs and cell types, the type and nature of malignancies, and the methods for analyzing the methylation status (*i.e*., PCR, MSP, and sequencing) (31, 34, 35), as well as alternative molecular regulatory mechanisms, *i.e*., microRNAs (36-39). For example, over-expression of miR-454-3p by transfection inhibited BTG1 expression and enhanced the radiosensitivity of renal carcinoma cells (37). In prostate carcinoma cells, miR-19a has been found to regulate proliferation and apoptosis by directly targeting *BTG1* (38). Taken together, these observations indicate that promoter hypermethylation may be responsible, at least in part, for repressed BTG1 expression.

In conclusion, we demonstrated that BTG1 expression is significantly down-regulated in breast carcinoma cells compared to normal mammary epithelial cells, suggesting that BTG1 may serve as a potential diagnostic biomarker for breast carcinoma. Moreover, one of the two studied CpG sites of the *BTG1* promoter was highly methylated, and treatment with a demethylating agent significantly restored BTG1 expression in all the carcinoma cells examined. Our data strongly suggest that methylation-dependent mechanisms influence the transcription of the *BTG1* gene. This study is the first to explore the expression status of BTG1 in breast carcinoma cell lines and show that promoter hypermethylation is one of the mechanisms involved in the regulation of BTG1 expression. Further studies on the role of BTG1 in breast carcinoma could shed light on its potential use as a therapeutic target.

Conflicts of Interest

None of the Authors have any conflicts of interest to declare regarding this study.

Author's Contributions

All Authors were responsible for: substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data, drafting the manuscript, revising the manuscript critically for important intellectual content, and final approval of the version to be published.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Korea government (MSIT) (2018R1D1A1B07047640 and 2018R1C1B5043725).

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Received August 27, 2019
Revised September 9, 2019
Accepted September 10, 2019