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Methylation-Mediated Downregulation of the B-Cell Translocation Gene 3 (*BTG3*) in Breast Cancer Cells

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The incidence of solid tumors is low in individuals with Down syndrome (trisomy 21), suggesting the presence of one or more tumor suppressor genes on chromosome 21. Consistent with this finding, previous work has demonstrated frequent loss of heterozygosity (LOH) of a small (<5 Mb) region of chromosome 21, particularly in breast cancer, indicating that a tumor suppressor gene(s) may be located in this region. We investigated the expression of *BTG3*, a gene in the LOH region on chromosome 21, in breast cancer cell lines. BTG3 has been shown to be a negative regulator of SRC tyrosine kinase, and BTG3 is a target of p53 and inhibits the activity of the E2F1 transcription factor. Here we demonstrate that in a wide variety of human breast cancer cell lines, *BTG3* expression is markedly reduced in the absence of detectable mutations in the *BTG3* promoter and coding region. In these cell lines, the promoter region of the *BTG3* gene is hypermethylated when compared to normal breast cell lines. *BTG3* gene expression can be restored by treatment with 5′-aza-deoxycytidine, an inhibitor of DNA methylation. These data support the hypothesis that BTG3 may act to suppress tumorigenesis and that hypermethylation is an important mechanism for inactivation of *BTG3* and perhaps other tumor suppressor genes. The findings are consistent with a role for an additional copy of *BTG3* in the reduced incidence of breast cancer in individuals with Down syndrome.

Key words: *BTG3*; DNA methylation; Breast cancer; Tumor suppressor gene

INTRODUCTION

Breast cancer is the most common cancer among women, and its incidence (132.5 per 100,000 per year for 1992 to 2001) is increasing, possibly due to improved detection. In contrast, breast cancer mortality declined by 2.3% per year from 1990 through 2001, which is likely due to multiple factors, including improved (and earlier) detection, and novel and more effective treatments. However, breast cancer remains the second leading cause of cancer deaths (28.8 per 100,000 per year for 1992 to 2001) among women, after lung cancer (1). The etiology of breast cancer appears to involve multiple risk factors, including age, economic status, geographic location, reproductive events, exogenous hormones, lifestyle risk factors, familial history, etc. In addition, genetics must also play an important role, but the genetic contribution to breast cancer, in particular sporadic breast cancer, is poorly understood.

We investigated the expression of the *BTG3* (B-cell translocation gene 3) [also known as abundant in neural epithelium area (*ANA*) and antiproliferative protein 4 (*APRO4*)] gene, located on chromosome 21, in breast cancer cell lines to test the hypothesis that this gene is a breast cancer tumor suppressor. This hypothesis is based upon a number of observations.

First, it has been known for some time that cancer

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incidence in individuals with Down syndrome (DS) is significantly different from that in the general population (10). The incidence of leukemia is much higher, while the incidence of solid tumors is greatly reduced, and the occurrence of breast cancer is essentially nonexistent. Individuals with DS have a complete or partial extra chromosome 21 (trisomy 21), which strongly suggests that there are genes on chromosome 21 that suppress these types of tumors in a dosage-dependent manner. *BTG3* is one of only a few genes (8%) on chromosome 21, in which expression levels are elevated in both lymphoblastoid cell lines and fibroblasts from individuals with DS, at a level quantitatively consistent with the increase of the gene copy number in the individuals (22,28).

Second, loss of heterozygosity (LOH), the loss of a normal wild-type allele at a heterozygous locus, is the most common somatic alteration in primary human breast tumors (3). Frequent LOH in a genomic region strongly implies that a tumor suppressor gene, or a gene related to tumor pathogenesis, is located in that region (16). A small region (<5 Mb) on chromosome 21, which shows frequent LOH in various cancers, including lung and breast, has been identified (9,15,18,19,24). This region contains only a few genes, one of which is *BTG3*.

Third, there is currently evidence from animal models suggesting that another member of the *BTG* gene family (including *PC3/TIS21/BTG2, BTG1, TOB, TOB2, BTG4*, and others), *BTG1*, may be a tumor suppressor. Mice in which the *BTG1* gene has been inactivated by targeted mutagenesis are prone to spontaneous tumors and also to chemically induced tumors (29). Such activity is consistent with the putative role of tumor suppressors. Members of this gene family act to inhibit cell proliferation, a common function of tumor suppressors (17).

Fourth, recent evidence indicates that BTG3 interacts with and negatively regulates SRC tyrosine kinase activity (23). Several recent studies implicate Src activity as a factor in breast cancer growth, migration, and invasiveness (11). Thus, decreased BTG3 activity would be expected to play a role in these aspects of breast cancer. Moreover, BTG3 appears to be a target for p53 and is an inhibitor of the transcription factor E2F1 (20). These observations are beginning to provide a mechanistic basis for the possible tumor suppressor activity of *BTG3*.

MATERIALS AND METHODS

Cell Lines

Nine breast cancer cell lines were analyzed, including MDA-MB-231, DU4475, MDA-MB-330, T47D, ZR-75-1 (from the University of Colorado Comprehensive Cancer Center Tissue Culture/Monoclonal Antibody Core), and MCF7, HCC1143, HCC1599, and HCC2157 (from the American Type Culture Collection).

Control cell lines include: human nontumor mammary epithelial cell lines MCF-10A and MCF-12A (from the University of Colorado Comprehensive Cancer Center Tissue Culture/Monoclonal Antibody Core), normal human lymphoblast cell lines GM03714 and GM03657, and human DS lymphoblast cell lines AG10098 and GM04927 (from Coriell Cell Repositories).

The cells were cultured according to the providers' protocols. Cell line DNA was prepared using the PureGene kit (Gentra Systems) and RNA was prepared using Trizol (InVitrogen), following the manufacturer's recommended procedure.

Annotation of the CpG Island and Promoter Region of the BTG3 *Gene*

The CpG island and the promoter region of the *BTG3* gene was identified, annotated, and verified using analysis programs, including CpG Island Searcher (http://ccnt.hsc.usc.edu/cpgislands/), CpGplot (http://www.ebi.ac.uk/emboss/cpgplot/), PromoterInspector (http://www.genomatix.de), MatInspector (http://www.genomatix.de), and TESS (http://www.cbil.upenn.edu/tess/).

Gene Expression Analysis of the BTG3 *Gene*

Expression of the *BTG3* gene was measured by real-time RT-PCR with two specifically designed primer pairs that cross exons 3 and 4 and exons 4 and 5 of the gene, respectively. Reverse transcription reactions were performed using the ImProm-II Reverse Transcription System with oligo (dT) primer (Promega) and 400 ng of total RNA in a 20-μl reaction. Real-time PCR was performed on LightCycler system using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics). Thermal cycling consisted of a preincubation step of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 5 s, and elongation at 72°C for 9 s. At the end of PCR, melting curve analyses were performed to validate the generation of the expected specific PCR product. Each reaction was repeated twice, and the *BTG3* mRNA level in each cancer line (measured as *x*-fold relative to the average level in the nontumor breast cell lines MCF-10A and MCF-12A) were calculated and normalized using a published approach (21). The standard curve was established using normal human mammary gland RNA (BD Biosciences) and reference genes TATA binding

protein (*TBP*) and RNA polymerase II (*RPII*). The PCR primers are listed in Table 1.

Mutation Analysis of the BTG3 *Gene*

We designed 11 pairs of primers to amplify the CpG island, including the promoter, and all exons of the *BTG3* gene for mutation analysis (Table 1). The FailSafe PCR System (Epicentre) was used for the PCR reactions. Each amplified DNA fragment was analyzed for mutations using denaturing HPLC (DHPLC) technology. Briefly, each amplified cancer DNA fragment was hybridized to its corresponding fragment from a normal control sample and analyzed by the WAVE nucleic acid fragment analysis system (Transgenomic) according to the manufacturer's instructions. If a cancer fragment contains a mutation, it will form a hybrid molecule with the normal fragment, which will be detected by the system.

DNA Methylation Analysis

Bisulfite and Sequencing-Based DNA Methylation Analysis. Genomic DNA was modified with bisulfite using the CpGenome DNA Modification Kit (Intergen). The modified DNA was then amplified by PCR in a 25-μl volume containing 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-Cl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.2 mM dNTPs, 1 μM each primer, 5% DMSO, and 1 U Taq DNA polymerase (Eppendorf). Five sets of primers were specifically designed for amplifying the modified *BTG3* CpG island region (Table 1). PCR was performed with 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 10 min. The PCR products were sequenced using an Applied Biosystems 3730 DNA analyzer with a manufacturer recommended sequencing kit (Applied Biosystems).

Methylation-Sensitive PCR. Target DNA was modified with bisulfite and amplified individually with MSP-M (methylation-specific) and MSP-U (unmethylation-specific) primer pairs (Table 1). MSP-M1 and MSP-U1 primer pairs were designed to test the methylation status of the #3, #4, #5, #13, and #14 CpG sites of the *BTG3* CpG island. The MSP-M1 primer pair amplifies an allele on which all these sites are methylated; when these sites are unmethylated, the allele will be amplified by the MSP-U1 primer pair. If the methylation of these sites is not uniform (e.g., some are methylated and the others are not in an al-

TABLE 1
PCR PRIMERS FOR THE *BTG3* GENE ANALYSIS

Primer	Forward	Reverse
Mutation screening		
BE1	cccgccagtcctctcaac	ctcccccgatacccacag
BE2	cagagcttcatcttcccagttt	gggttgatcagcctctgct
BE3	tccattaacgtaactccactttg	tccagcatggtcatcagttc
BE4	accactgtgcccggctaat	ccacgaagtatcactcagtcactt
BE5	tgctgatgtgacttcaagattt	aatccctgcacatcccttta
BE6-1	acttaatgtgttctcttccctacag	tctcaacatgacaccaacacaa
BE6-2	gaatgcattgtgaccggaat	ccaatattaaaaacttaggcacttga
BE6-3	tagatgggccaaaccatca	ttcacacaattctcttaaacaacga
Bpro1	agcacacaagcgtccaca	gacacaccctcgccctac
Bpro2	ggtcccacaggccttcag	cgccatgtctgcctttcc
Bpro3	ggggaaaggcagacatgg	cctccccgacaacatcct
Methylation detection		
BM1	tataggaggtaggttttgttgtgag	aattctaaacccaactctctaaacc
BM2	ttygttttgagggtgttaagtgtag	cctcrccctaccctaaacctaac
BM3-1	gyggggttttataggtttttag	acraataaacrcaaccccaaaac
BM3-2	gtygttttggggttgygtttatt	tatcctaaccraaaactaaaaactcc
BM4	ggagtttttagttttyggttaggata	aaacccatacaacctaattccatc
BM5	gatggaattaggttgtatgggttt	ccaacaaacaaacraaactccaa
MSP-M1	cggttgtacggttaacgtgc	gaacttaatcctttcgactatctcga
MSP-U1	tagtagggtggttgtatggttaatgtgt	acacaaacttaatcctttcaactatctca
MSP-M2	cgtttattcgtgtgcgcgt	cgaccgaaaattcgacgac
MSP-U2	tggggttgtgtttatttgtgtgt	actcttcaaccaaaaattcaacaac
Real-time PCR		
BTG3-RT-1	gtgaaacccagttcggtgac	caaatggaacaggaggagga
BTG3-RT-2	ttgtatagtgacctgggcttgcca	tcaccgaactgggtttcacttcca
TBP-RT	gaatataatcccaagcggtttg	acttcacatcacagctcccc
RPII-RT	gcaccacgtccaatgacat	gtgcggctgcttccataa

lele), the allele may not be amplified by either of the primer pairs. Similarly, the MSP-M2 and MSP-U2 primer pairs were designed to test the methylation status of the #68, #69, #96, and #97 CpG sites of the CpG island.

5′-aza-2′-Deoxycytidine (5′-aza-CdR) Treatment

Cells were cultured in medium supplemented with 10 μM 5′-aza-2′-deoxycytidine (Sigma) for 96 h (medium was changed at 48 h) and tested for *BTG3* expression as described above.

RESULTS

Genomic Annotation of the BTG3 *Gene*

The *BTG3* gene spans approximately 20 kb of nucleotides on the long arm of chromosome 21 at 21q21. It contains a 1224-bp typical 5′ CpG island that spans from −567 to +657 bp of the gene, and includes exon 1. The 5′ CpG island has a high GC content (71.3%) with an obs_{CpG}/exp_{CpG} ratio of 0.94 [calculation based on previously described criteria (26)], and contains a putative promoter region from −500 to +100 (Fig. 1). No discernible TATA box was identified; however, a CCATT box (−375) and several SP1 binding sites are located in the promoter region. In addition, putative binding sites for various transcription factors, such as GABP, AP-2, CREB, MAZ, CDE, ZBP-89, ETS-1, and E2F, etc., were also identified (data not shown). These features are commonly found in genes lacking a TATA box (12).

BTG3 *Expression in Breast Cancer Cell Lines*

To test the hypothesis that *BTG3* is a tumor suppressor active in the suppression of breast cancer, we examined transcription (as a measurement of expression) of the gene in nine breast cancer cell lines (Fig. 2). Seven of these lines show reduction in *BTG3* expression compared to the nontumor breast cell lines. In particular, the expression level was extremely low in cancer line MCF7, and undetectable in cancer line T47D. The remaining two, HCC1599 and HCC1143, showed approximately 1.1-fold and 2.0-fold expression compared to the nontumor lines, respectively.

Mutation Analysis of the BTG3 *Gene*

We searched for genomic mutations that may be associated with decreased *BTG3* expression in the coding and promoter regions of the gene. However, no mutations were detected in all cell lines used in this study, similar to previous findings, in which the *BTG3* gene typically was not mutated in human lung carcinoma (15).

Methylation Status of the BTG3 *CpG Island*

Because DNA mutation cannot explain the decrease in *BTG3* expression in most of the cancer lines, we investigated the methylation status of the CpG island of the *BTG3* gene using bisulfate and sequencing-based analysis. The CpG island contains 146 CpG sites, and 73 (#4 to #76) are located in the putative promoter region. All the CpG sites, except

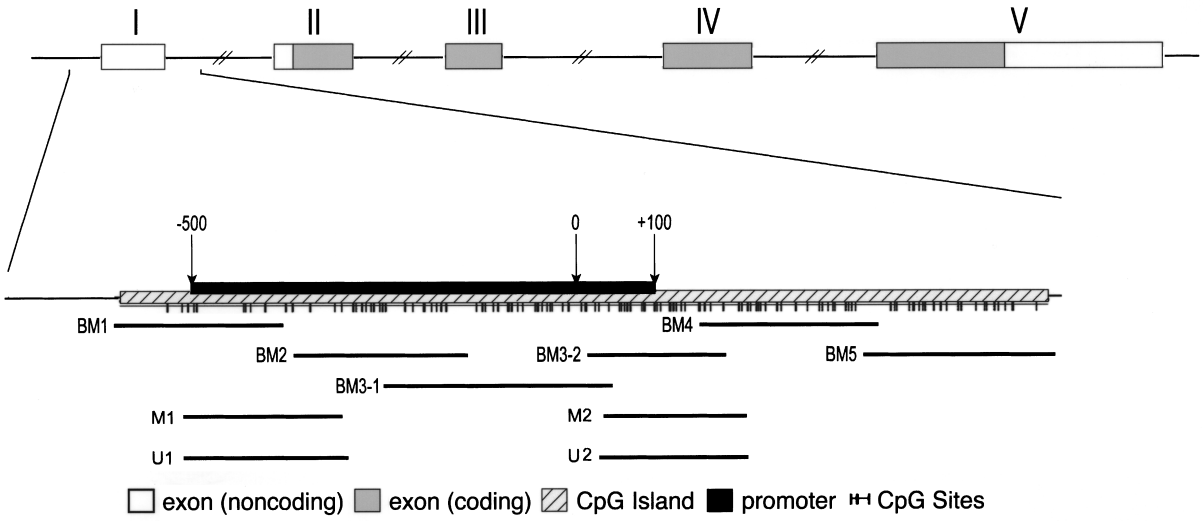

Figure 1. Diagram of the structure of the *BTG3* gene. The exon/intron structure is indicated in the upper portion of the figure. The lower portion of the figure shows the promoter region, CpG island, and CpG sites, as well as the regions amplified by primers used for bisulfite and sequencing-based DNA methylation analysis (BM1, BM2, BM3-1, BM3-2, BM4, and BM5), and by primers used for MSP analysis (M1, M2, U1, and U2).

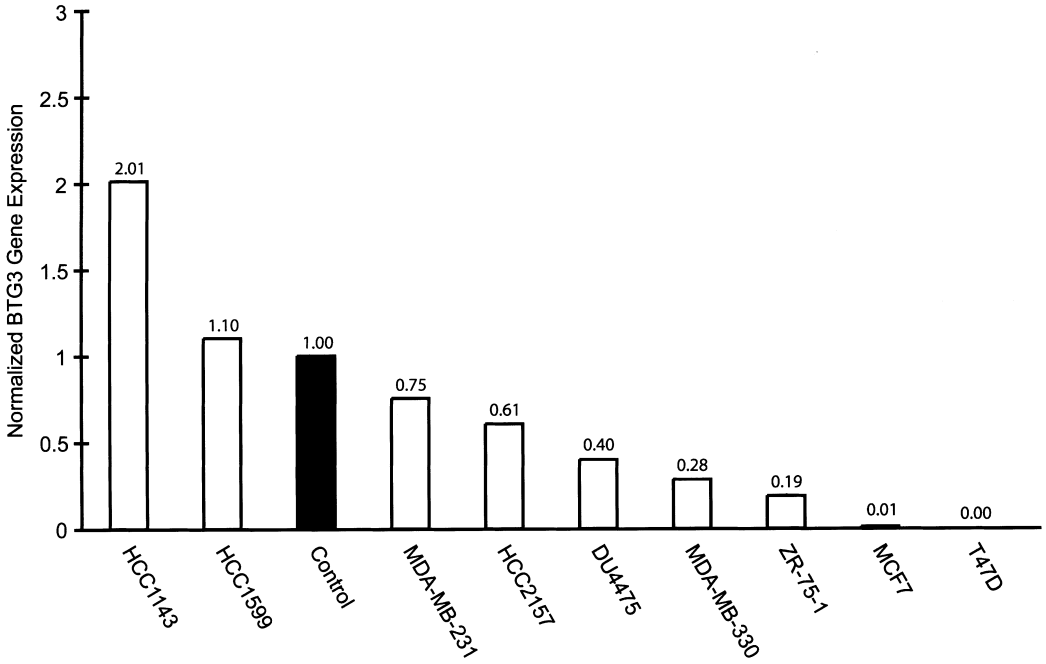

Figure 2. Normalized *BTG3* transcription levels. Values are expressed relative to nontumor breast cell lines (set at 1).

for #10 and #11 (due to technical difficulties), were analyzed in the breast cancer lines and the control cell lines, including nontumor breast cell lines, normal lymphoblast cell lines, and DS lymphoblast cell lines. The methylation status of the first 65 CpG sites is shown in Figure 3. In the nontumor breast cell lines, only partial methylation was detected in the first 30 CpG sites; the remaining sites were mostly unmethylated. In contrast, all seven cancer lines with reduced *BTG3* expression showed hypermethylation at the first 30 CpG sites and the hypermethylation extended further into the downstream sites in six lines. The greatest increase in methylation was detected in the cancer line T47D. Of 144 CpG sites analyzed in this line, 138 were fully methylated, 6 were partially methylated, and no unmethylated sites were detected. Correspondingly, this cancer line showed no detectable *BTG3* expression. Indeed, there appears to be a correlation between increased methylation and decreased *BTG3* expression. Consistent with this correlation, hypermethylation was not detected in two cancer lines (HCC1599 and HCC1143) that did not show decrease in *BTG3* expression. The methylation in the normal lymphoblast cell lines was very similar to what was seen in the nontumor mammary epithelial cell lines and there were no significant differences between normal and Down syndrome cell lines (Fig. 3), suggesting that the methylation pattern seen in the nontumor lines is representative of the methylation state in other noncancer tissues.

The methylation in selected CpG sites in each cell line was further verified by methylation-sensitive PCR (MSP) analysis and the results are shown in Figure 4. The CpG sites #3, #4, #5, #13, and #14 of the *BTG3* CpG island were partially methylated in normal and DS cell lines, nontumor mammary epithelial cell lines, and cancer cell lines HCC1143, HCC1599, HCC2157, and MDA-MB-330; the same sites were fully methylated in the remaining 6 cancer lines. In contrast, the CpG sites #68, #69, #96, and #97 were unmethylated in all cell lines, except for T47D, in which these sites were fully methylated. These results are consistent with the findings of bisulfate and sequencing-based analysis (Fig. 3). Importantly, the MSP results also suggest that methylation occurs in *cis* on homologue alleles (e.g., methylated CpG sites are located on one chromosome homologue and unmethylated sites are located on the other one in partially methylated cells; otherwise, no band would be amplified by the MSP primers).

Rescue of BTG3 *Expression*

Because the reduction in *BTG3* expression appeared to be associated with hypermethylation in the CpG island, we tested if *BTG3* expression could be "rescued" by treating three cancer lines with 5′-aza-CdR, a DNA methylation inhibitor. Noticeably, the *BTG3* expression was restored in T47D and increased remarkably in ZR-75-1 and MCF7 cancer lines (Fig.

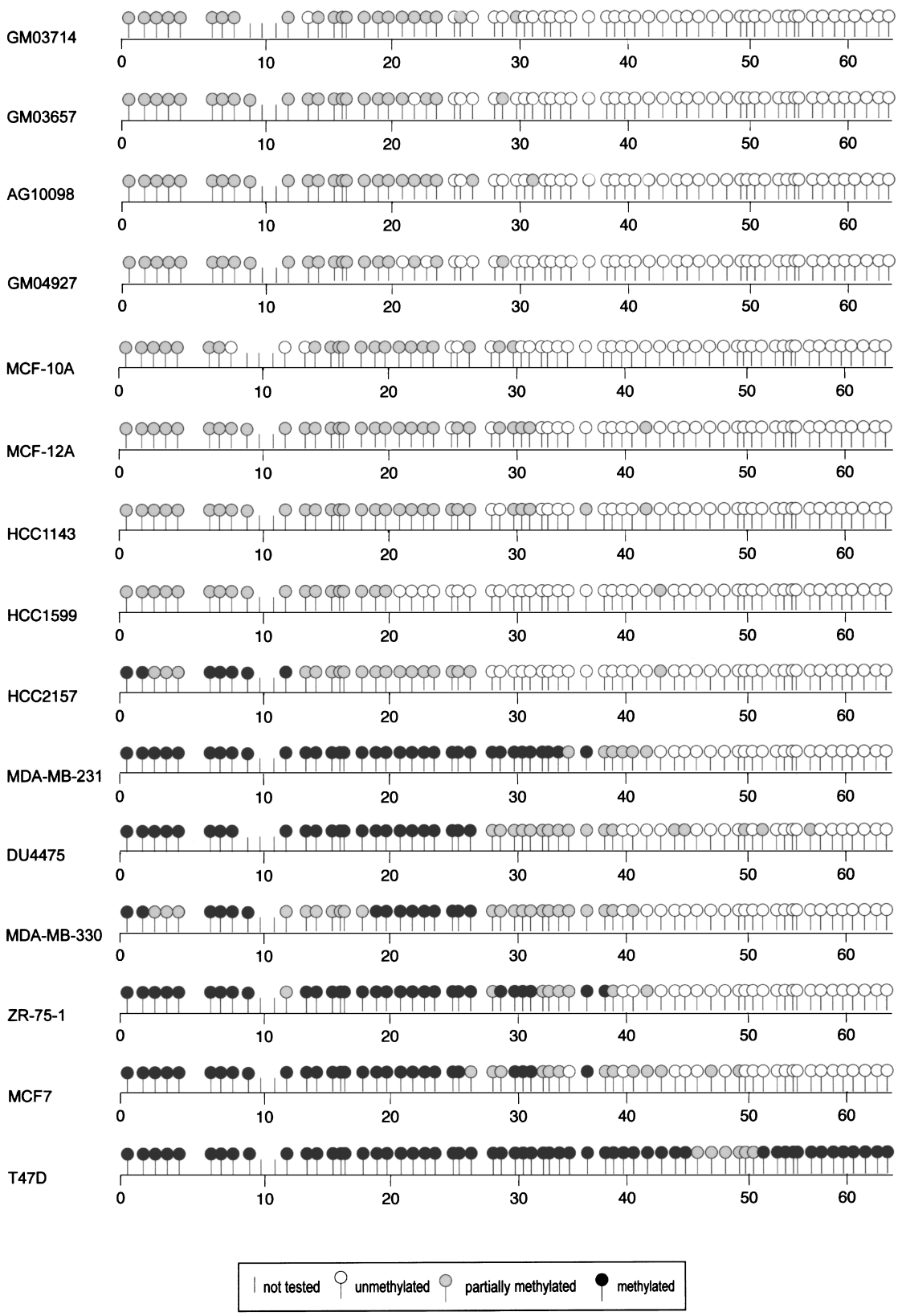

Figure 3. Diagram indicating *BTG3* promoter methylation in the cell lines. *BTG3* promoter methylation status was determined using the bisulfate and sequencing-based method as described in Materials and Methods.

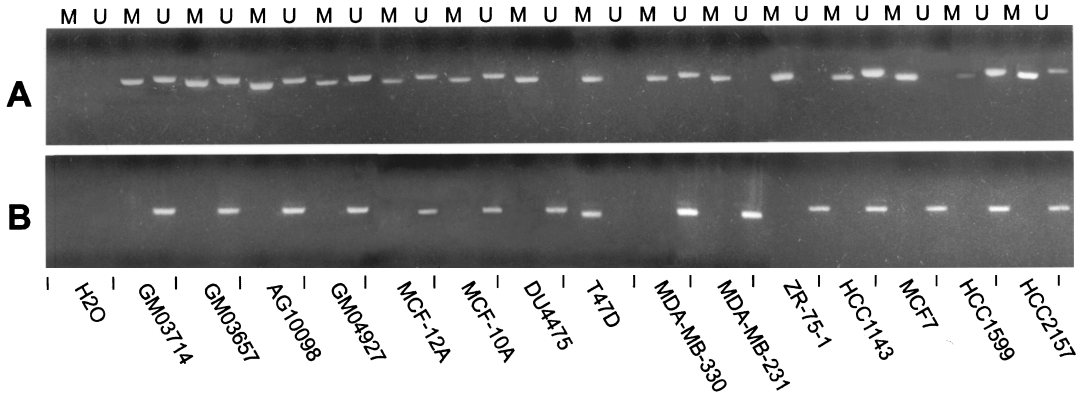

Figure 4. Methylation-sensitive PCR (MSP) analysis of the *BTG3* promoter region. (A) MSP with MSP-M1 and MSP-U1 primer pairs to evaluate the methylation status of CpG sites #3, #4, #5, #13, and #14 of the *BTG3* CpG island. (B) MSP with MSP-M2 and MSP-U2 primer pairs to evaluate the methylation status of CpG sites #68, #69, #96, and #97. In both (A) and (B), an M lane band indicates the presence of a methylated allele, while a U lane band indicates an unmethylated allele.

5), suggesting that BTG3 expression is inhibited by DNA methylation in these cell lines. The findings of this study strongly imply that CpG island methylation plays a role in the regulation of *BTG3* expression in breast cancer cells.

DISCUSSION

Our results demonstrate that *BTG3* expression is reduced or eliminated in many breast cancer cell lines compared to noncancerous breast cell lines. This implies that *BTG3* expression inhibits or suppresses tumorigenesis or tumor cell proliferation in breast cancer. This finding is consistent with recent information on the function of BTG3. Specifically, Rahmani (23) has shown that BTG3 (called APRO4 in that manuscript) interacts with and downregulates SRC tyrosine kinase. Moreover, downregulation of endogenous *BTG3* in PC12 cells induces the activation of SRC and the concomitant spontaneous formation of neurons (23). Similarly, treatment with histone deacetylase inhibitors increases expression of *BTG3* associated with neuronal differentiation in adult rat forebrain precursor cells, indicating a possible role for epigenetic mechanisms in regulation of *BTG3* and a role for *BTG3* in determining cell differentiation pathways (25).

BTG3 has also been shown to inhibit the transcription factor E2F1 (20). These authors hypothesize that

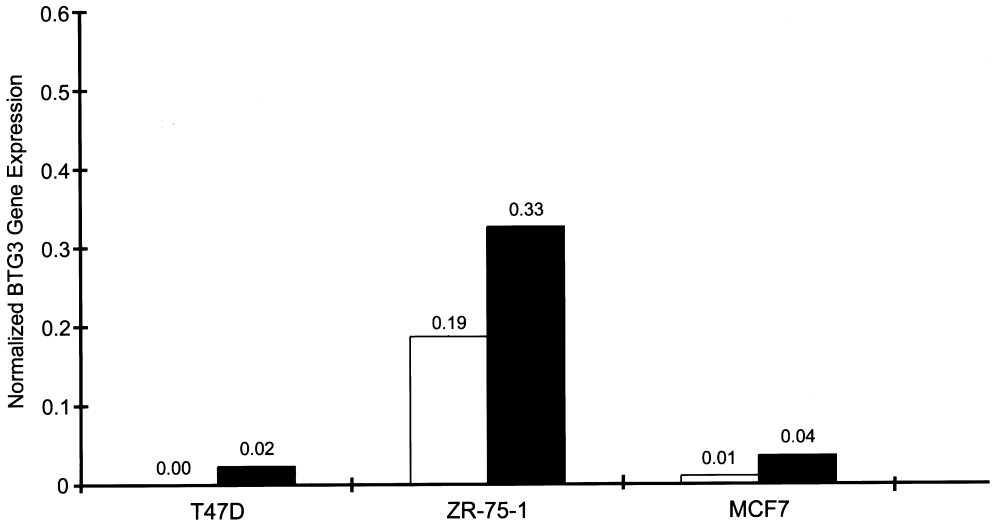

Figure 5. Treatment of three breast cancer lines with 5′-aza-CdR, a DNA methylation inhibitor. The open columns indicate native *BTG3* expression (relative to nontumor breast cell lines), while the shaded columns indicate expression after treatment with 5′-aza-CdR.

ablation of *BTG3* expression due to "oncogenic stress" would be equivalent to oncogene activation and would promote tumor cell proliferation. In this case, increasing *BTG3* expression may be an approach to cancer treatment.

Considerable recent evidence strongly suggests a role for SRC activity in growth factor and anchorage-independent growth, motility, and invasiveness, including breast cancer (2,11). Indeed, SRC inhibitors are now in clinical trials. Therefore, we hypothesize that one role for BTG3 may be inactivation of SRC, leading to breast cancer suppression. Elevated expression of *BTG3* in DS might then explain, at least in part, the rare occurrence of breast cancer in DS. Importantly, *BTG3* gene methylation is not increased in DS, and *BTG3* gene expression is increased in lymphoblasts and fibroblasts by 1.42- to 1.82-fold (22,28). In fact, *BTG3* is one of only 8% of chromosome 21 genes of which the level of expression in DS and diploid individuals has virtually no overlap (22).

Our finding of reduced *BTG3* expression in breast cancer cell lines reveals a positive functional association between the *BTG3* gene and breast cancer. It also suggests that instead of DNA mutation, CpG island methylation may play a critical role in regulating *BTG3* expression. Compelling evidence demonstrates that hypermethylation of CpG island promoter regions silences tumor suppressor genes, including *MLH1*, *BRCA1*, and many others, resulting in cancer development and/or progression (6). Therefore, such hypermethylation is an example of "epimutation," functionally equivalent to an inactivating mutation. Epimutation is a common phenomenon that may occur in germline or somatic cells and has also been reported in constitutional genetic conditions in humans, plants, and animals (5,7). It has been suggested that Knudson's "Two Hit" Hypothesis for cancer development (14) should be modified to include gene inactivation via epigenetic mechanisms [e.g., methylation (13)].

It is noteworthy that hypermethylation appears to occur mostly in the first 30 CpG sites in the CpG island of the *BTG3* gene, and 27 of these sites are located in the beginning of the promoter region (Figs. 1 and 3). This suggests that methylation of the CpG sites in this particular promoter region is most critical to the regulation of *BTG3* expression. These findings are similar to the results from analysis of regulation of *MLH1* gene expression (4). These critical methylation sites show a "partial methylation" status with both methylated and unmethylated forms in nontumor cells, and they become fully methylated in most breast cancer cell lines. Further extension of methylation to the remaining CpG sites in the CpG island seems to result in full inactivation of the gene as seen in the T47D cell line.

Partial methylation is a common finding in methylation studies, but the mechanism and genomic features of this phenomenon are unknown. Although LOH of the region involving the *BTG3* gene on chromosome 21 is a common finding in breast cancer, loss of a copy of the *BTG3* region was found only in some cells in one cancer line (MDA-MB-231); in fact, most of the lines show gain of additional (1 to 4) copies of chromosome 21 according to the karyotypes from the providers and our FISH analysis (data not shown). We hypothesize that the partial methylation of the *BTG3* gene in nontumor cells may result from allelic methylation differences—one allele is methylated and the other allele is not (supported by our MSP results). Two changes may occur in cancer cells: duplication or amplification of the methylated chromosome homologue/allele and loss or deletion of the unmethylated chromosome homologue/allele. As a result of these changes, the cancer cells would show decreased *BTG3* expression, an appearance of LOH of the *BTG3* gene region, and gain of additional chromosome 21 homologues.

It should be kept in mind that *BTG3* (or any putative tumor suppressor gene) might have different functions in different cell types and may be regulated by multiple independent pathways. Thus, *BTG3* function appears to be important for neuronal differentiation (23,25). Interestingly, BTG3 has recently been identified as a novel prognostic marker for acute lymphoblastic leukemia (ALL), its expression being elevated 1.6-fold in T-cell ALL patients with a high likelihood of an adverse outcome (8). Individuals with DS are at an increased risk of developing ALL, although the incidence of T-cell ALL may not be as elevated as that of B-cell ALL (27). Thus, BTG3 may suppress some malignancies but enhance others. Our work in conjunction with published work on *BTG3* expression shows that the *BTG3* gene expression can be regulated by gene copy number, DNA methylation, and histone methylation (22,25). It seems likely that other mechanisms for regulation of *BTG3* will be identified.

One approach to testing the hypothesis that BTG3 is a tumor suppressor would be through production of mice in which the expression of *BTG3* is altered. As discussed above, mice in which *BTG1* has been inactivated by targeted mutagenesis have increased susceptibility to spontaneous and chemically induced tumors (29). Similar studies with *BTG3* are straightforward, as is the possibility of production of mice overexpressing *BTG3*, which might be expected to

have a decreased risk of experimentally induced breast (and perhaps other) tumors.

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