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Original Article

BTG3 gene alterations in colorectal carcinoma

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Abstract: Aims: To investigate whether the *BTG3* gene is involved in colorectal carcinogenesis. Methods and results: we have examined the genetic alterations, including somatic mutations and promoter hypermethylation of the *BTG3* gene in 78 sporadic colorectal carcinomas. Results revealed that no mutation was detected in the coding region of the *BTG3* gene and promoter hypermethylation was detected in 5 colorectal carcinoma samples. The expression of BTG3 proteins was also examined via immunohistochemistry in 78 colorectal carcinomas. There was loss or reduced expression of BTG3 in the 43 (55.1%) of the 78 colorectal carcinomas. Statistically, altered expression of BTG3 was not associated with clinicopathological parameters, including tumor differentiation, location, and lymph node metastasis ($P>0.05$). Conclusion: Our results suggest that genetic, epigenetic, and protein expression pattern alterations of the *BTG3* gene might play a minor role in the development or progression of colorectal carcinomas.

Keywords: *BTG3,* colorectal carcinoma, mutation, methylation, immunohistochemistry

Introduction

Colorectal carcinoma (CRC) is the third leading cause of cancer death worldwide with a dismal outcome [1]. A rising incidence and mortality from CRC have recently been observed in China. However, little is known about the molecular genetic events in the development and progression of CRC [2]. Allelotype studies have shown that specific regions of the genome are frequently deleted in many tumor types. In addition, frequent loss of heterozygosity (LOH) at certain chromosomal regions is the hallmark of the presence of a tumor suppressor gene. Therefore, the identification of consistent areas of chromosomal deletion in the DNA of tumors may point to the regions harboring a tumor suppressor gene that is associated with carcinogenesis of a specific tissue type. Allelotype studies have reported frequent LOH on 21q in several human cancers, including lung, breast, oral cancer [3-7].

Src nonreceptor tyrosine kinase plays an important role in multiple signaling pathways that regulate several cellular functions including proliferation, differentiation and transformation [7]. Interestingly, *BTG3*, a member of the anti-proliferative *BTG* (B-cell translocation gene)/*Tob* (Transducer of ErbB2) gene family, is localized at chromosome 21q11.2-q21.1 and found to associate with and inhibit Src tyrosine kinase through its C-terminal domain in PC12 cells [6, 8]. Recently, BTG3 was identified as a transcriptional target of p53 and its anti-proliferative action was clarified through inhibition of E2F1 [7]. The transcription factor E2F1 controls the cell cycle by activating genes important for G1/S progression: these include the genes for cyclin E, PCNA, DNA polymerase a, Cdc6, dihydrofolate reductase (DHFR) and others [8, 9]. BTG3 binds to and inhibits E2F1 through an N-terminal domain including the conserved box A [9]. However, no genetic alterations of the *BTG3* gene have been reported on human sporadic colorectal carcinoma. Therefore, we hypothesized that *BTG3* might function as a tumor suppressor gene in the development and/or progression of colorectal carcinoma.

In order to determine whether genetic and epigenetic alterations and expression patterns of the *BTG3* gene are involved in the development or progression of CRC, somatic mutations, hypermethylation and protein expression of the *BTG3* gene were analyzed in colorectal carcinomas.

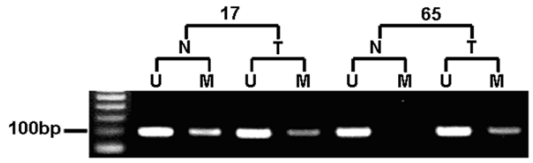

Figure 1. Methylation-specific PCR results of the *BTG3* gene in colorectal carcinoma tissues and corresponding non-cancerous tissue. The tumor tissue from case no. 65 showed both the methylated and unmethylated PCR products, whereas the corresponding non-neoplastic DNA demonstrated only the unmethylated PCR product. Case no. 17 showed unmethylated and methylated DNA in both corresponding non-neoplastic and cancer tissues. (N, non-neoplastic DNA, T, tumor DNA. U, unmethylated DNA, M, methylated DNA).

Materials and methods

Tissue samples

A total of 78 CRC samples were examined. Informed consent was obtained from all patients. No patient had a family history. Their ages ranged from 35 to 85 years, with an average of 52 years. The male to female ratio was 47 to 31. Two pathologists independently reviewed one 6 μm section stained with Hematoxylin & Eosin. Approval was obtained from the institutional review board at the Binzhou Medical University of Shandong.

DNA extraction

Malignant cells were selectively obtained from the hematoxylin and eosin-stained slides using a laser microdissection device. The genomic DNA was prepared using a procedure described previously [10].

Mutational analysis

Genomic DNAs from the cancer cells and corresponding non-cancerous tissue were amplified with four sets of primers covering the entire coding region (4 exons) of the *BTG3* gene. The primers used were the following: for exon 1: 5'-AATGAAGAATGAAATTGCTG-3' (sense primer) and 5'-TGCTTAATCCAATCTAAT-3' (antisense primer). For exon 2: 5'-GGCAAGTATGAATAGATATTAC-3' (sense primer) and 5'-ATCAGTTCA GCCTCTAAC-3 (antisense primer). For exon 3: 5'-AATTTTATTCTT AGGTATGGAG-3' (sense primer) and 5'-TTTTCATGGTTACCTGG-3' (antisense primer), and for exon 4: 5'-CCCTACAGATTCAGAACTTAT-3' (sense primer) and 5'-AACACAATCAAAAACGAAG-3' (antisense primer). Numbering of the DNA of the *BTG3* gene was done with respect to the ATG start codon according to the genomic sequence of Genbank, accession no. NM006806. Each polymerase chain reaction (PCR) was performed under standard conditions in a 10 μl reaction mixture containing 20 ng of template DNA, 0.5 μM of each primer, 0.2 μM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.4 unit of Taq polymerase, 0.5 μCi of [^{32}P]dCTP (Amersham, Buckinghamshire, UK), and 1 μl of 10× buffer. The reaction mixture was denatured for 12 min at 94°C and incubated for 35 cycles (denaturing for 40 s at 94°C, annealing for 40 s at 52-55°C, and extension for 40 s at 72°C). The final extension was continued for 5 min at 72°C. After amplification, the PCR products were denatured for 5 min at 95°C at a 1:1 dilution of sample buffer containing 98% formamide/5 mmol/L NaOH and were loaded onto a single-strand conformation polymorphism (SSCP) gel (Mutation Detection Enhancement, FMC BioProducts, Rockland, ME, USA) with 10% glycerol. After electrophoresis, the gels were transferred to 3 MM Whatman paper and dried; autoradiography was performed using Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). For the detection of mutations, DNAs showing mobility shifts were cut out from the dried gel and reamplified for 30 cycles using the same primer sets. Sequencing of the PCR products was carried out using cyclic sequencing kits (Perkin-Elmer, Foster City, CA, USA) according to the manufacturer's recommendations.

Methylation-specific PCR (MSP)

The methylation status of the promoter region of the *BTG3* gene was determined using sodium bisulfite treatment of the DNA followed by MSP, as described in the literature with slight modification [11, 12]. Briefly, DNA was incubated in 0.2 M NaOH at 42°C for 30 min in a total volume of 50 μl. After adding 350 μl of 3.6 M sodium bisulfite containing 1 mM hydroquinone at pH 5.0, the samples were incubated for 16 h at 55°C in the dark. The modified DNA was recovered with 5 μl glassmilk (BIO 101, Irvine, CA, USA) and 800 μl of 6 M NaCl. The glassmilk catching the modified DNA was washed three times with 70% ethanol at room temperature,

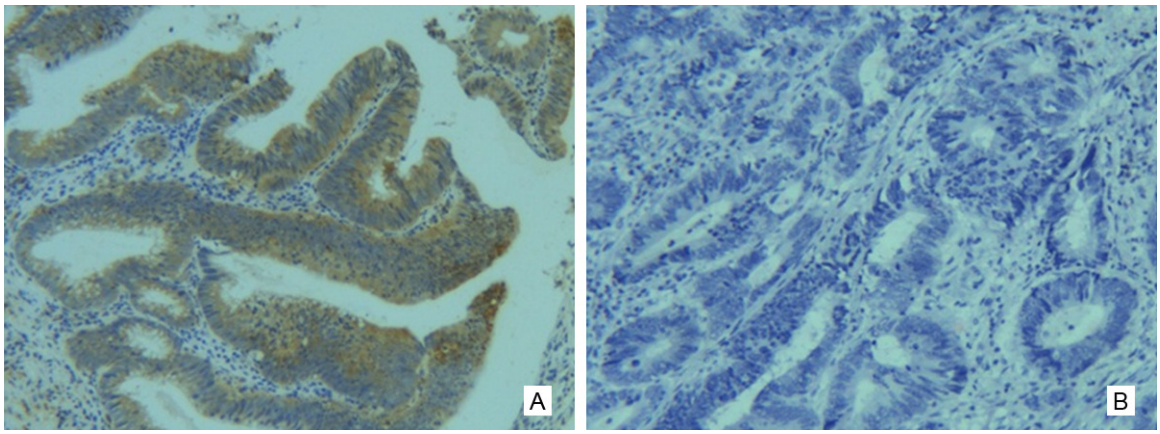

Figure 2. BTG3 expression in colorectal cancers. Colorectal cancers exhibited cytoplasmic positive staining for BTG3 (A). Loss of BTG3 expression in colorectal cancers (B). (Original magnification, ×200).

treated with 0.3 M NaOH/90% ethanol, and washed twice with 90% ethanol. The DNA was finally eluted from the dried pellet with 30 μl 1 mM Tris-HCl (pH 8.0) for 15 min at 55°C; 4 μl of the bisulfite-modified DNA was subjected to MSP using two sets of primers for the methylated and unmethylated *BTG3*. The primers used were the following: for methylation primer, 5'-TTGGATTTTTTTTGGGTTTTATTTAC-3' (sense primer) and 5'-ATAACGTCATCCTAAAACCTCGTC-3' (antisense primer); for unmethylation primer: 5'-TGGATTTTTTTTGGGTTTTATTTAT-3' (sense primer) and 5'-TAACATCATCCTAAAACCTCATC-3' (antisense primer). The PCR was performed in a total volume of 30 μl, containing 5 μl of the template DNA, 0.5 μM of each primer, 0.2 μM of each dNTP, 1.5 mM $MgCl_2$, 0.4 unit of Ampli Taq gold polymerase (Perkin-Elmer, Foster City, CA, USA) and 3 μl of 10× buffer. The reaction solution was initially denatured for 1 min at 95°C. Amplification was carried out for 40 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, followed by a final 5 min extension at 72°C. Each PCR product was loaded directly onto 2% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

Immunohistochemistry for BTG3

For the immunohistochemical analysis, Heat-induced epitope retrieval was performed by immersing the slides in Coplin jars filled with 10 mmol/L citrate buffer (pH 6.0) and boiling the buffer for 30 min in a pressure cooker inside of a microwave oven at 700 W, the jars were then cooled for 20 min. For the latter, the Renaissance TSA indirect kit (NEN Life Science, Boston, MA, USA), which included streptavidin-peroxidase and biotinylated tyramide, was used. After rinsing with PBS, the slides were treated with 1% H_2O_2 in PBS for 20 min at room temperature. After washing with TNT buffer (0.1 mol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl and 0.05% Tween 20) for 20 min, the slides were treated with TNB buffer (0.1 mol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl and 0.5% blocking reagent). The sections were incubated overnight at 4°C with BTG3 antibodies (1/100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection was carried out using biotinylated goat anti-rabbit antibody (Sigma, St. Louis, MO, USA), followed by incubation with a peroxidase-linked avidin-biotin complex. Diaminobenzidine was used as a chromogen, and the slide was counterstained with Mayer's hematoxylin. For the negative controls, the slide had the primary antibody replaced with non-immune serum. Staining for the BTG3 antigen was considered positive when >5% of the cytoplasm stained positively. The results were reviewed independently by two pathologists.

Results

Mutations of the BTG3 gene

The presence of mutations, a possible sign of inactivation of a tumor suppressor gene, in all of transcription region of the *BTG3* gene was investigated using PCR-based SSCP and sequencing analysis. There was no aberrant SSCP pattern identified in this region, which suggests that there was no mutation of the

Table 1. Correlation of BTG3 protein expression with clinicopathological Parameters in the colorectal cancers

Parameters	BTG3 protein expression		
	+	-	*P* value
Differentiation			0.7717
Well	13	13	
Moderately	12	15	
Poorly	10	15	
L/N metastasis			0.9189
+	29	36	
-	6	7	
Stage			0.5633
A	2	1	
B	6	4	
C	18	23	
D	9	15	
Side			0.3174
Right	5	10	
Left	30	33	
Size			0.1936
<5 cm	19	17	
≥5 cm	16	26	
Sex			0.1506
Female	17	14	
Male	18	29	

BTG3 gene in the colorectal carcinoma. The experiments, including PCR and SSCP analysis, were repeated three times to ensure the reliability of the results.

Hypermethylation of the BTG3 gene

Since no *BTG3* gene mutations were detected, this study focused on the possible role of epigenetic mechanisms for *BTG3* gene inactivation. Of 78 corresponding non-cancerous tissue, 55 and 23 showed only unmethylated, both methylated and unmethylated PCR products, respectively. Interestingly, five of the 23 showed both the methylated and unmethylated PCR products, whereas the corresponding non-neoplastic DNA demonstrated only the unmethylated PCR product, which indicates hypermethylation of the *BTG3* gene (**Figure 1**).

Expression patterns of the BTG3

In immunohistochemistry, a moderate-to-strong immunopositivity for BTG3 was clearly marked on the cytoplasm of normal colorectal mucosa and cancer cells (**Figure 2**). Reduced or loss of BTG3 expression was detected in 43 (55.1%) of 78 colorectal cancers. Statistically, there was no significant relationship between altered expression of BTG3 protein and clinicopathologic parameters, including tumor differentiation, location, and lymph node metastasis (Chi-Square test, $P>0.05$) (**Table 1**).

Discussion

In Down syndrome with trisomy 21, the incidence of solid tumors including colorectal carcinoma is considerably lower than that of general population [13-15]. The low risk of colorectal carcinoma in individuals with Down syndrome may be related to the gene-dosage effect of the extrachromosome 21. This suggests that tumor suppressor genes playing a role in the pathogenesis of colorectal carcinoma may be present on chromosome 21. Several cytogenetic studies have reported that the chromosome region 21q11.2-q21.1 is a commonly deleted region for solid tumors such as lung cancer, breast cancer oral carcinoma [3, 5, 8]. The *BTG3* tumor suppressor gene, which is located at 21q11.2-q21.1, plays an important role in the negative control of the cell cycle. In addition, BTG3 is directly associated with and positively regulates the tumor suppressor p53 by inhibiting E2F1 [7]. Although we cannot completely rule out the possibility that other unknown genes in this region may be the target of frequent LOH, the findings to date suggest that *BTG3* is a candidate tumor suppressor gene associated with the development of colorectal carcinomas.

In the present study, we found the absence of mutations of the *BTG3* gene. Because many tumor suppressor genes are known to be inactivated by DNA promoter methylation, we examined promoter hypermethylation of the *BTG3* gene. Unexpectedly, there was a low frequency of hypermethylation in the promoter region of the *BTG3* gene. The immunohistochemical studies showed that loss of BTG3 protein expression was detected in 43 (55.1%) of 78 colorectal cancers and was not associated with clinicopathologic parameters, including tumor differentiation, location and lymph node metastasis. Therefore, we concluded that the *BTG3* gene might play a minor role in colorectal carcinogenesis. Although our results may underestimate the prevalence of the *BTG3* somatic

mutation in colorectal carcinoma, the chance that we missed a mutation is very low, since we repeated the experiments three times.

Absence of *BTG3* inactivation in tumors where LOH occurs at 21q11.2-q21.1 has been reported in several cancers [4-6]. Apart from arguing against a pathogenetic role for the gene in the tumorigenesis of lung cancer, it could also signify the existence of other tumor-suppressor genes at this locus. However transcriptional mapping of chromosome 21 has revealed that 21q11.1-q21.1 is a gene poor region, and the gene density of the region has been estimated as being less than 1 per Mb [16, 17]. Another gene, Ubiquitin specific protease, has been mapped to 21q11.2-q21.1, but it does not appear to be an attractive candidate as a lung cancer tumor-suppressor gene [18]. Large-scale sequencing must be awaited to rule out completely the presence of any other tumor suppressor genes which could explain deletions in this region in some neoplasia.

In conclusion, the results of this study showed no mutations in the *BTG3* gene, low frequency of hypermethylation in the promoter region of the *BTG3* gene. Thus, we concluded that the *BTG3* gene may a minor role in the development or progression of colorectal carcinoma. Further studies are required to identify the target gene at 21q11.2-q21.1 responsible for the development of colorectal carcinoma.

Acknowledgements

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Disclosure of conflict of interest

None.

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