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Advances in Brief

Mutations of the *bak* Gene in Human Gastric and Colorectal Cancers

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Abstract

The Bcl-2 homologue Bak is a potent inducer of apoptosis. We performed PCR-based single-strand conformational polymorphism and sequencing analysis of the entire coding region of the *bak* gene (exons 2–6) in 24 primary gastric cancers (6 early-stage and 18 advanced-stage cancers) and 20 primary colorectal cancers (6 early-stage and 14 advanced-stage cancers). The data herein demonstrate, for the first time, the mutation of the *bak* gene in gastric and colorectal cancers. Missense *bak* gene mutations were observed in 3 of 24 (12.5%) gastric cancers and 2 of 20 (10.0%) colorectal cancers. Sequence alterations without amino acid alteration were observed 1 of 24 (4.2%) gastric cancers and 2 of 20 (10.0%) colorectal cancers. Mutations in the *bak* gene were observed only in advanced-stage gastrointestinal cancers but not in early-stage cancers. Our observations suggest that mutations in this gene predispose bearers to the development of gastrointestinal malignancies in at least a subset of the cases.

Introduction

Homeostasis of gastrointestinal mucosa is maintained by a balance between growth and apoptosis of mucosal cells. The Bcl-2 family of proteins plays a central role in the regulation of apoptotic cell death, which is induced by a wide variety of stimuli (1). Recently, *bak* (a Bcl-2 homologous antagonist/killer) has been cloned as a bcl-2-related gene (2–4). The *bak* gene encodes a 211-amino acid protein with a relative molecular weight (M_r) of 23,400. Gene transfer-mediated elevations in Bak protein levels accelerate apoptosis induced by growth factor deprivation in murine lymphoid, neuronal, and fibroblastic cells (2–4), suggesting that Bak functions primarily as a promoter of apoptosis.

Bak expression has been reported in normal gastrointestinal epithelium (2, 3). Strong Bak immunoreactivity has been shown to be present in the gastric epithelial cells lining the gastric pits and parietal cells, whereas the self-renewing mucous cells located below the gastric pits in the gastric neck region are immunonegative (5). In the colon, Bak immunoreactivity has been shown to exist in the form of a gradient along the crypt-villus axis. The epithelial cells in the apical portions of the crypts, which are destined for programmed cell death, are intensely immunopositive, whereas the self-renewing population of cells, which are located in the base of the crypts, show a weaker immunostaining (5, 6). Therefore, in gastrointestinal epithelial tissues, the up-regulation of Bak expression during differentiation may help to ensure that cell turnover occurs in a normal fashion. Recently, apoptosis in gastric epithelial cells induced by *Helicobacter pylori* has been shown to be accompanied by an increased level of Bak expression (7). It has also been shown that induction of apoptosis by sulindac sulfide in the rat normal small intestine cell line IEC 18 is accompanied by increased Bak expression (6). Furthermore, in studies using the human colon cancer cell line HT 29, sodium butyrate-induced apoptosis has been shown to be associated with increased Bak levels (6, 8). These observations suggest that Bak plays an important role in modulating apoptosis in gastrointestinal epithelial cells.

Apoptosis is critical not only for tissue homeostasis but also in the pathogenesis of a variety of diseases, including cancer. The transformation of colorectal epithelial tissue to carcinomas has been shown to be associated with the progressive inhibition of apoptosis (6, 9). It has been reported that gastric and colorectal tumors have reduced Bak levels compared with normal mucosa, suggesting that a perturbation of bak-mediated apoptosis may contribute to the pathogenesis of gastric and colorectal cancers (10, 11). However, mutations of the *bak* gene have not been examined in the case of tumors, including gastrointestinal cancers. Therefore, to clarify any possible relationship between the mutation of the *bak* gene and the occurrence of gastrointestinal cancers, we carried out a SSCP[2] analysis of bak in 24 primary gastric cancers (6 early-stage cancers and 18 advanced-stage cancers) and 20 primary colorectal cancers (6 early-stage cancers and 14 advanced-stage cancers).

Materials and Methods

Materials. Matching normal and tumor tissues were obtained at the time of surgical resection or endoscopic biopsy from 24 patients with gastric cancers and 20 patients with colorectal cancers. The gastric and colorectal cancers were staged by the tumor-node-metastasis (TNM) system (early stage, stage I; advanced stage, stage II and III).

DNA Extraction. Genomic DNA was extracted from a single 5-μm microdissected paraffin-embedded section using DEXPAT (Takara Shuzo, Kyoto, Japan).

SSCP and DNA Sequencing. Primers located within the intronic sequences were used to amplify the entire coding sequence of bak in five fragments of $<$262 bp each and including all of the intron/exon boundaries (Table 1; Ref. 12). PCR amplification of each exon was performed under standard conditions in a 10-μl reaction mixture containing 0.5 μl of template DNA, 0.5 μM each primer, 200 μM each deoxynucleotide triphosphate, 2.0 mM $MgCl_2$, 0.25 unit of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT), and 2 μl of 10$\times$ PCR buffer. The reaction mixture was denatured for 5 min at 95°C and then incubated for 40 cycles (denaturing at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min). PCR products were diluted with 90 μl of loading solution containing 90% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue; denatured at 90°C for 5 min; and applied to 15% polyacrylamide gels containing 1$\times$ Tris-glycine buffer. Electrophoresis was performed at 300 V for 3 h at 10°C. The resulting gels were stained with CYBR Gold Nucleic Acid Gel Stain (Molecular Probes, Eugene, OR) and analyzed on a Fluor Imager 595 (Molecular Dynamics Japan, Tokyo, Japan). DNA fragments of the mobility-shifted bands were extracted from the gels by SSCP analysis and reamplified. The resulting PCR products were sequenced directly by the Amplicycle Sequencing Kit (Perkin-Elmer, Branchburg, NJ) using the same primers used for PCR amplification. All of the

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[2] The abbreviations used are: SSCP, single-strand conformational polymorphism; BH, Bcl-2 homology domain.

Table 1 *Primers used for amplification of bak coding region exon 2–6*

Primer	Sequence	Product size (bp)
BAK/2F	TGGTTATGGGATGGGTGAGG	165
BAK/2R	CTGCTTTTTCTCGCCCTTCC	
BAK/3F	TGCCTCCCTGAAGATGTCCT	204
BAK/3R	TGACTCCCAGCTTTGATCCT	
BAK/4F	GGCAGGGTATGGTATGGTTG	262
BAK/4R	TCCCGACTGCCTGGTTACTG	
BAK/5F	CCAACAGCAGGGAGGACATT	247
BAK/5R	CAATGCTATGGGATGCTCTG	
BAK/6F	GCAAGGGAACAGAGAAGGCA	210
BAK/6R	TGACCACCTTGTTTCTCCCG	

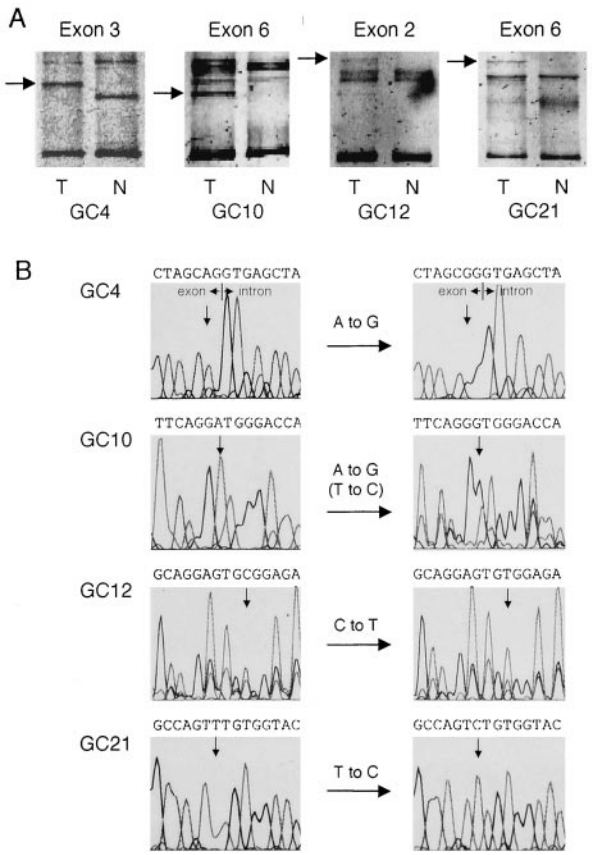

Fig. 1. Mutations of the *bak* gene in gastric cancer specimens. *A*, PCR-SSCP analysis. PCR products from tumor DNA (*T*) were run alongside products from normal DNA (*N*) isolated from nonneoplastic mucosa from the same individuals. *Arrows* indicate mobility shift bands. *B*, sequence analysis. Nucleotide changes are apparent when compared with normal DNA samples from the same individuals. For sample GC10, histograms for sequencing in the reverse direction are shown. *Arrows* indicate sequence alterations.

mutations were verified by repeated PCR and gel analyses under different SSCP conditions.

Results and Discussion

Fragments showing abnormal mobilities, which are indicative of sequence alteration, were observed in 4 of 24 gastric cancers (Fig. 1). In sample GC4T, an A to G transversion occurred in exon 3, leading to a Ser→Gly substitution at codon 69; sample GC10T contained a T to C transition in exon 6, leading to a Ile→Thr substitution at codon 188; and sample GC21T contained a T to C transition in exon 6, leading to a Phe→Ser substitution at codon 203 (Table 2). Sample GC12T contained a C to T transition in exon 6, with no alteration in the amino acid sequence.

In 4 of 20 colorectal cancers, fragments with altered mobilities were identified (Fig. 2). In sample CRC7T, an A to T transversion occurred in exon 2, leading to a Gln→Leu substitution at codon 5; sample CRC12T contained a T to C transition in exon 6, leading to a Ile→Thr substitution at codon 188. Samples CRC15T (which had a C to T transition in exon 2) and CRC23T (which had a C to T transition in exon 2) had sequence alterations but no amino acid amino acid alterations. Additional studies showed that no sequence alteration was present in matched nonneoplastic tissue samples of gastric or colorectal cancer patients. This is the first report of mutations in the *bak* gene in any type of human malignancy. Bak, a Bcl-2 family protein, contains three conserved regions termed BH1, BH2 and BH3 and the membrane-anchoring region (13). Bak has been shown to increase apoptosis as a result of binding with Bcl-x_L, which inhibits the antiapoptotic effect of Bcl-x_L (13). The BH3 region is critical for the ability of Bak to promote apoptosis and bind to the Bcl-x_L protein (13, 14). However, in this study, there was no evidence of sequence

Table 2 *Summary of bak sequence alterations in gastric and colorectal cancers*

Tumor	Stage[a]	Exon	Codon	Sequence changes	Predicted effect
Gastric cancers					
GC4T	Advanced	3	69	AGC→GGC	Ser→Gly
GC10T	Advanced	6	188	ATC→ACC	Ile→Thr
GC12T	Advanced	2	14	TGC→TGT	Silent
GC21T	Advanced	6	203	TTT→TCT	Phe→Ser
Colorectal cancers					
CRC7T	Advanced	2	5	CAA→CTA	Gln→Leu
CRC12T	Advanced	6	188	ATC→ACC	Ile→Thr
CRC15T	Advanced	2	14	TGC→TGT	Silent
CRC19T	Advanced	2	6	GGC→GGT	Silent

[a] Gastric and colorectal cancers were staged by the tumor-node-metastasis (TNM) system (early stage, stage I; advanced stage, stage II and III).

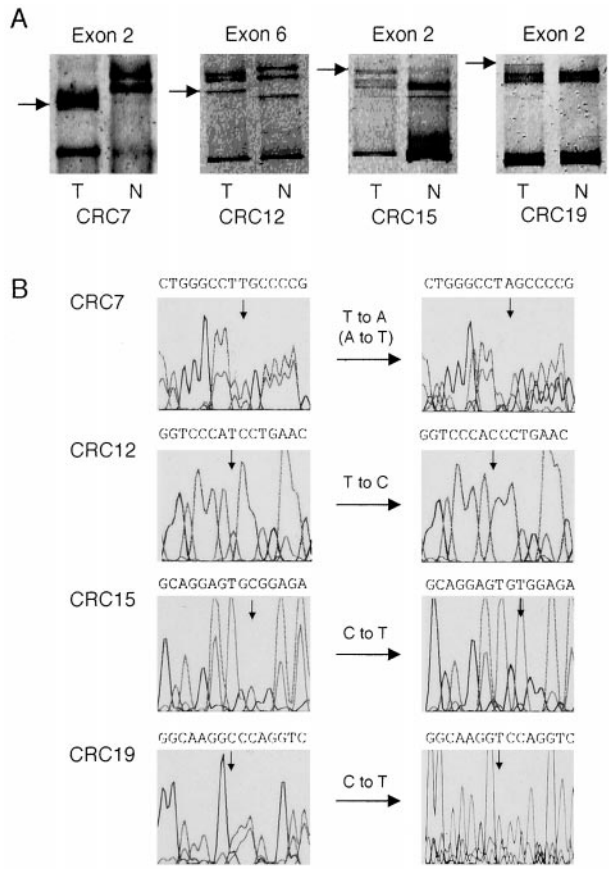

Fig. 2. Mutations of the *bak* gene in colorectal cancer specimens. *A*, PCR-SSCP analysis. *T* and *N*, tumor and surrounding nonneoplastic mucosa, respectively. *Arrows* indicate mobility shift bands. *B*, sequence analysis. For sample CRC7, histograms for sequencing in the reverse direction are shown. *Arrows* indicate sequence alterations.

alteration in exon 4, which encodes the BH3 region, in any of the gastrointestinal cancers examined. The membrane-anchoring region has also been shown to be important for the apoptotic activity of Bak. In studies using truncated Bak molecules, it has been reported that the truncated molecule, which includes BH3 but not the membrane-anchoring region, retains Bcl-x_L binding capacity but exhibits a reduced cell killing function due to altered subcellular localization (13). We identified two missense mutations in the membrane-anchoring region in gastric and colorectal cancers (samples GC10T and CRC12T, codon 188; sample GC21T, codon 203). Therefore, it is possible that in these cancers, the cell-killing activity of Bak is reduced by amino acid alterations in the membrane-anchoring region. Additional studies are needed to clarify the effects of *bak* missense mutations observed in this study on cell killing function and on subcellular localization.

It has been shown that the immunoreactivity of Bak is typically reduced in gastric carcinomas compared with normal foveolar cells, with no significant correlation of immunointensity with clinical stage (10). It has also been reported that in many colorectal adenomas and carcinomas, the intensity of immunostaining for Bak is reduced compared with that of normal mucosal epithelial cells (11). These observations suggest that reductions in Bak expression occur early in the progression of gastrointestinal tumors. However, in this study, *bak* gene alteration was not observed in early-stage gastric cancers ($n = 6$) or in early-stage colorectal cancers ($n = 6$). The missense mutation was observed only in advanced-stage cancers [3 of 18 (16.6%) advanced-stage gastric cancers and 2 of 14 (14.3%) advanced-stage colorectal cancers (Table 2)]. This suggests that mutation of the *bak* gene constitutes a late event in gastric and colorectal tumor progression.

Recently, it has been reported that an artificial mutation can be present when formalin-fixed specimens are used for DNA extraction (15). In this study, both tumor DNA and matched normal DNA were extracted from formalin-fixed specimens. However, *bak* sequence alterations were observed only in tumor DNA and not in normal DNA.

In conclusion, we have demonstrated several sequence alterations of the *bak* gene in gastrointestinal cancers. Missense *bak* gene alterations were observed in 3 of 24 (12.5%) gastric cancers and 2 of 20 (10.0%) colorectal cancers. This is the first report of missense mutations of this gene in any human malignant neoplasm and provides further support for the view that mutations in this gene serve to predispose bearers to the development of gastric and colorectal malignancies in at least a subset of cases. However, it is also possible that some or all of the sequence alterations we observed may represent polymorphisms.

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