Inactivating mutations of proapoptotic Bad gene in human colon cancers

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**Evidence exists that deregulation of apoptosis is involved in the mechanisms of cancer development, and the somatic mutations of apoptosis-related genes have been reported in human cancers. Bcl-XL/Bcl-2-associated death promoter (Bad), a proapoptotic member of Bcl-2 family, plays an important role in the intrinsic apoptosis pathway. To explore the possibility that the genetic alterations of Bad might be involved in the development of human cancers, we analyzed the entire coding region and all splice sites of human Bad gene in 47 colon adenocarcinomas. Overall, we detected two somatic missense mutations (4.3%) in Bad gene. Interestingly, both of the Bad mutations were detected in the gene sequences encoding the Bcl-2 homology3 domain of Bad, which has a crucial role in inducing cell death. Transfection study revealed that both of the tumor-derived Bad mutants had decreased apoptosis activities compared with the wild-type Bad, indicating that the Bad mutations reduced the cell death function of Bad. Co-immunoprecipitation assay revealed that binding of one of the tumor-derived Bad mutants with Bcl-2 and Bcl-XL is reduced. This is the first report on Bad gene mutation in human malignancies, and our data suggest that Bad gene is occasionally mutated in colon cancers and that somatic mutation of Bad may contribute to the development of colon cancers.**

**Introduction**

Apoptosis is a fundamental biochemical cell death pathway essential for normal tissue homeostasis, cellular differentiation and development (1). Although many pathways for inducing apoptosis may exist, only two, the intrinsic pathway and the extrinsic pathway, have been demonstrated in detail (1,2). The extrinsic pathway can be induced by members of the tumor necrosis factor receptor family, such as Fas and tumor necrosis factor-related apoptosis-inducing ligand receptors (1). The intrinsic pathway is induced by various apoptotic stimuli, including growth factor deprivation, hypoxia, radiation and anticancer drugs, and regulated by Bcl-2 family proteins, which operate immediately upstream of mitochondria. Both pro- and anti-apoptotic Bcl-2 family proteins exist, and many Bcl-2 family proteins bind each other, forming a complex network of homo- and heterodimers (1). Alterations of Bcl-2 family proteins have been associated with a variety of pathological conditions, including development and progression of human cancers (3–5). In mammals, Bcl-2 has at least 20 relatives all of which share more than one conserved Bcl-2 homology (BH) domains (1,6). A subset of Bcl-2 family proteins appears to have in common only the presence of the Bcl-2 homology3 (BH3) domain—an interacting domain that is both necessary and sufficient for the killing action. The ‘BH3-only’ proteins, including Bcl-XL/Bcl-2-associated death promoter (Bad), Bik, Bim, Hrk and Noxa, are all pro-apoptotic in their function, and their cell death-inducing activity depends on their ability to dimerize with anti-apoptotic Bcl-2 family members, typically functioning as trans-dominant inhibitors of proteins such as Bcl-2 and Bcl-XL (1,6). Bcl-XL/Bad, a death-promoting BH3-only member of the Bcl-2 family, is regulated primarily by rapid changes in phosphorylation that modulate its protein–protein interactions and cellular location (7–10).

Deregulation of apoptosis may be directly involved in several human diseases, including degenerative diseases, neoplasia and AIDS (1). Cells from human cancers have a reduced capability to undergo apoptosis in response to some physiological stimuli (2). It is now believed that clonal expansion and tumor growth are the results of the deregulation of intrinsic proliferation (cell division) and cell death (apoptosis) (2). Failure of apoptosis could allow the survival of transformed cells that are prone to undergo further genetic damage and play an important role in the pathogenesis of tumors. Either inactivation of pro-apoptotic pathway or activation of anti-apoptotic pathway results in failure of apoptosis, thereby promoting tumor cell survival. All the anti-apoptotic Bcl-2 members are likely to be oncoproteins. For example, Bcl-2 over-expression by chromosomal translocation gave rise to follicular lymphoma (4). Conversely, pro-apoptotic Bcl-2 members are likely to be tumor suppressors. Bax, Bak and Bik are somatically mutated in several types of human cancers, and loss of Bax increases tumorigenicity (11–15). Recently, Ranger et al. (16) observed that Bad-deficient mice spontaneously develop tumors, which were mainly B cell lymphomas, strongly indicating the role of Bad in tumor suppression. This observation raised a question whether the Bad gene is somatically mutated in human cancers, and whether the mutations inactivate the proapoptotic function of Bad. Regulation of Bcl-2 homolog expression, including Bad, in normal colonic crypt cells plays a pivotal role in either survival or apoptosis of the cells (17,18). However, to date, there have been no reports on somatic mutation of Bad in human cancer. In the present study, to explore the possibility that the alterations of the Bad gene might play a role in the development of human cancers, we investigated the
presence of Bad gene mutations in colon cancers that occur with a high incidence and is one of the leading causes of cancer death in the world.

Materials and methods

Tissue samples and plasmids

Formalin-fixed tissues of 47 human colon adenocarcinomas were obtained from surgically treated patients. The carcinomas originated from cecum (n = 5), ascending colon (n = 7), descending colon (n = 7), sigmoid colon (n = 10) and rectum (n = 18). The samples consisted of 47 primary colon cancers and their corresponding metastatic tumors of the same patients. The patients had never been pre-treated before the surgery. Approval was obtained from the Catholic University of Korea, College of Medicine’s institutional review board for this study. Informed consent was provided according to the Declaration of Helsinki. Normal cells and malignant cells from the same patients were selectively procured from hematoxylin and eosin-stained slides using a 30G/2 hypodermic needle (Becton Dickinson, Franklin Lakes, NJ) affixed to a micromanipulator, as described previously (19). DNA extraction was performed by a modified single-step DNA extraction method, as described previously (19). The mammalian expression plasmids encoding human wild-type Bad, Bcl-2 and Bcl-XI have been described previously (20).

Single strand conformation polymorphism (SSCP) analysis and DNA sequencing

Genomic DNAs from tumor cells and corresponding normal cells were amplified with six primer pairs covering the entire coding region (three exons) of human Bad gene. Oligonucleotide primers were designed with the program Oligo (National Biosciences, Plymouth, MN) using sequences obtained from Genbank (accession no. NT_033241) (Table I). Numbering of cDNA of Bad was done in respect to the ATG start codon (Genbank accession no. NM_004322). Radioisotope was incorporated into the polymerase chain reaction (PCR) products for detection by autoradiogram. The PCR reaction mixture was denatured for 1 min at 94°C and incubated for 30 cycles (denaturing for 30 s at 94°C, annealing for 30 s at 58°C and extending for 30 s at 72°C). Other procedures of PCR and SSCP analysis were performed as described previously (21–23). After SSCP, DNAs showing mobility shifts were cut out from the dried gel, and re-amplified for 30 cycles using the same primer sets. Sequencing of the PCR products was carried out using the cyclic sequencing kit (PerkinElmer, Foster City, CA) according to the manufacturer’s recommendation.

Site-directed mutagenesis

Site-directed mutagenesis was performed using a Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. To change a base, a plasmid that contained the Bad gene in pcDNA3.1-HA was used as a template. The nucleotide sequences of the mutagenized plasmids were confirmed (data not shown).

Co-immunoprecipitations and immunoblotting assay

Human embryonic kidney 293T cells in log phase were transfected in 6-well plates with expression plasmids using Lipofectamine Plus transfection reagent (Invitrogen, Carlsbad, CA). Cells were harvested 1 day later and lysed in ice-cold Nonidet P-40 lysis buffer supplemented with 1× protease inhibitor mix (Roche Molecular Biochemicals, Mannheim, Germany). Cell lysates were subjected to immunoprecipitation using agarose-conjugated anti-Flag M2 antibodies (Sigma, St Louis, MO). Immunocomplexes were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The resulting blots were incubated with anti-HA (1:1000 v/v, Roche Molecular Biochemicals) followed by horseradish peroxidase-conjugated secondary antibodies and detection by an enhanced chemiluminescence method (ECL, Amersham Pharmacia Biotec, Buckinghamshire, UK). Alternatively, lysates were analyzed directly by immunoblotting after normalization for total protein content.

Apoptosis assay

293T cells in 6-well plates were transfected by Lipofectamine Plus transfection reagent (Invitrogen, Carlsbad, CA) with various combinations of expression plasmids (total 1.3 μg) and 0.2 μg of green fluorescence protein (GFP) marker...
Twenty-four hours after transfection, cells were fixed in 10% methanol for 15 min, and stained with 1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 15 min, and the percentage of GFP-positive cells with nuclear apoptotic morphology was examined by fluorescence microscopy.

**Immunohistochemistry**

Antibody for human Bad (Pharmingen, San Diego, CA; dilution 1/50) was used to detect Bad protein on tissue sections. Immunohistochemical procedures were performed as described previously (21,22). Tumors were interpreted as positive by immunohistochemistry when at least weak to moderate cytosolic staining was seen in >30% of the neoplastic cells. The results were reviewed independently by two pathologists.

**Results**

**Bad gene mutations and allelic status**

Through the microdissection technique, we selectively procured tumor cells from histological sections (Figure 1). Genomic DNA was isolated and analyzed for potential mutations in all three exons in the coding region of the Bad gene by PCR–SSCP analysis. Enrichment and direct sequence analysis of aberrantly migrating bands led to the identification of two mutations in the 47 samples (4.3%) (Table II and Figure 2). None of the corresponding normal samples showed evidence of mutations by SSCP (Figure 2), indicating the mutations had

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**Table II. Summary of Bad mutations identified in the colon cancers**

<table>
<thead>
<tr>
<th>Case no. (site)</th>
<th>Mutation exon</th>
<th>Mutation domain</th>
<th>Nucleotide change</th>
<th>Predicted amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (rectum)</td>
<td>Exon 2</td>
<td>BH3</td>
<td>340 C to T</td>
<td>Leu (L) → Phe (F)</td>
</tr>
<tr>
<td>27 (rectum)</td>
<td>Exon 2</td>
<td>BH3</td>
<td>337 G to A</td>
<td>Glu (E) → Lys (K)</td>
</tr>
</tbody>
</table>

*Numbering of cDNA of Bad was done with respect to the ATG start codon.

plasmid pEGFP N2 (Clontech, Palo Alto, CA). Twenty-four hours after transfection, cell were fixed in 10% methanol for 15 min, and stained with 1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI) for 15 min, and the percentage of GFP-positive cells with nuclear apoptotic morphology was examined by fluorescence microscopy.

**Fig. 2.** Somatic mutations of Bad gene in colon cancers SSCP (A and B) and sequencing analysis (C and D) of normal tissues from two separated sites (Lane N) and colon cancers (T). (A) The exon 2 was amplified by PCR using a primer set. SSCP of DNA from colon cancer (T) of case 4 shows only two aberrant bands (arrows) without any wild-type bands as compared with SSCP from the normal tissues (N). (B) The exon 2 was amplified by PCR using a primer set. SSCP of DNA from colon cancer (T) of case 27 shows wild-type bands and additional aberrant bands (arrows) as compared with SSCP from the normal tissues (N). (C) Cyclic sequencing analysis from the aberrant band of case 4. There is a C-to-T transition at nucleotide 340 (arrow) in cancer tissue (T) as compared with normal tissue (N). (D) Cyclic-sequencing analysis from the aberrant band of case 27. There is a G to A transition at nucleotide 337 (arrow) in cancer tissue (T) as compared with normal tissue (N).
risen somatically. The corresponding metastatic tumors of the same patients also showed the same mutations. Both mutations were missense mutations and identified in the exon 2 of Bad gene, which encodes the BH3 domain of Bad protein. One mutation (case 27) showed a G-to-A transition at the nucleotide 337, which would result in the substitution of E by K at the amino acid residue 113. The other mutation (case 4) showed a C-to-T transition at the nucleotide 340, which would result in the substitution of L by F at the amino acid residue 114 (Table II). Because the BH3 domain of Bad protein is localized at the amino acids 109–122 (7), the two mutations detected would change the amino acids in the BH3 domain of the Bad protein. We repeated the experiments three times, including tissue microdissection, PCR, SSCP and DNA sequencing analysis to ensure the specificity of the results, and found that the data were consistent (data not shown).

**Reduced death-promoting activities of the Bad mutants**

The BH3 domain of Bad protein is required for cell death induction by Bad. To determine whether the mutant forms of Bad are functionally defective, we transfected 293T cells either with HA-pcDNA3-Bad wild-type or HA-pcDNA3-Bad-E113K or HA-pcDNA3-Bad-L114F, and found that both mutants showed a significant defect in apoptosis function compared with the wild-type (Figure 3; Dunnett’s multiple comparison test, \( P < 0.001 \)). The trend of the apoptosis decreases by the Bad mutants was consistent by changing the amount of the vectors transfected (Figure 3; Dunnett’s multiple comparison test, \( P < 0.001 \)).

**The Bad mutants showed reduced binding with Bcl-2 and Bcl-X\(_L\)**

Because the BH3 domain of the Bad protein is required for heterodimerization with Bcl-2 and Bcl-X\(_L\) (7), we tested the possibility that these interactions are hampered by the mutations detected in the BH3 domain. For these experiments, 293T cells were transiently transfected with expression plasmids encoding Flag-Bcl-X\(_L\) either with HA-pcDNA3-Bad-wild-type or HA-pcDNA3-Bad-E113K or HA-pcDNA3-Bad-L114F. Immunoprecipitations were then performed with anti-Flag antibody, and the resulting immunocomplexes were analyzed by immunoblotting using anti-HA antibody. Figure 4A shows Flag-Bcl-2 co-immunoprecipitated with HA-Bad-L114F, which was different from that of HA-Bad wild-type with Bcl-X\(_L\) and Bcl-2 (Figure 4A and B).

**Expression of Bad**

We analyzed Bad protein expressions in the two tumors with Bad gene mutations and 20 tumors without Bad gene mutation...
by immunohistochemistry. Both of the two tumors with the mutations showed immunoreactivity for Bad (Figure 5A). Also, Bad was expressed in 19 of the 20 (95%) colon tumors without Bad mutation (Figure 5B). The intensities of the immunostaining between the tumor groups were similar.

Discussion

The aim of the current study was to address whether human colon cancer has Bad gene mutations. If so, the next aim was to address whether the mutations inactivate the proapoptotic activity of Bad protein. We found that in colon cancers, the Bad gene is occasionally mutated. Moreover, the functional studies revealed that all of the cancer-derived Bad mutants showed a reduced apoptosis function. These data together with the earlier reports on the inactivating mutations of proapoptosis genes in human cancers (11-15,21-23), indicate that somatic mutation of pro-apoptosis genes, including Bad, may be a frequent event in the pathogenesis of human cancers, and suggest that these mutations may play an important role in the apoptosis resistance of cancer cells in vivo.

Although the precise mechanisms Bad protein used to induce cell death is still unknown, deletion analysis demonstrated the importance of BH3 domain in mediating both its heterodimerization with Bcl-2 and Bcl-XL, and its death-promoting activity (24). Previously, Zha et al. (24) have made artificial mutants of mouse Bad, and found that substitution of leucine within the BH3 domain, which is conserved among most of the Bcl-2 family members, abrogated the heterodimerization of the mutant with Bcl-2 and Bcl-XL, and its proapoptotic activity. Interestingly, substitution of the same leucine at the BH3 domain of human Bad gene (mutant Bad-L114F) was detected in the current study (case 4). In agreement with this previous study (24), the mutant Bad-L114F showed decreased apoptotic activity than the wild-type Bad. However, still the L114F mutant had somewhat high apoptogenic activity. The apoptogenic activity of the L114F mutant could be explained by the findings that the L114F mutation abrogated the binding of Bad to Bcl-2 but only decreased the Bad/Bcl-XL association (Figure 3 and 4). Although another Bad mutant (Bad-E113K; case 27) within the BH3 domain also significantly reduced apoptosis activity compared with the wild-type Bad (Figure 3), the heterodimerization of this mutant with Bcl-2 and Bcl-XL, was not different from that of the wild-type Bad (Figure 4). It can be hypothesized that either the difference of apoptosis activity between wild-type Bad and Bad-E113K could not be detected by co-immunoprecipitation analysis (below the detection levels of the analysis), or there might be another apoptosis-promoting mechanism, which could be inactivated by the Bad-E113K mutant, besides heterodimerization with Bcl-2 and Bcl-XL.

Under suitable conditions SSCP is capable of detecting over 90% of mutations occurring within any sequence, and the sensitivity of PCR-SSCP is generally believed to be high if the fragments are shorter than 200 bp (25). It is possible that some mutational changes may not be detected by SSCP in this study. However, since we have analyzed the samples by SSCP three times and used primers shorter than 200 bp, it can be thought that the missing of Bad mutations, if any, would be very rare in this study.

The functions of Bcl-2 family proteins are often regulated by more than one mechanism. For example, loss-of-function mutations have been identified in the Bax gene of human tumors (13-15). And expression of Bax gene is also transcriptionally regulated by p53 (26). So far, the best-known mechanism of Bad inactivation is phosphorylation of Bad protein (8-10). Replacement of an amino acid of Bad (D119G) within the BH3 domain impaired the phosphorylation-mediated regulation, and as a result of disrupted phosphorylation Bad D119G could not interact with 14-3-3 (27). The influence of phosphorylation-mediated regulation by the E113K and L114F mutation should be analyzed in future studies.

Prior to this study, the report on the occurrence of lymphomas in Bad-deficient mice (16) led us to analyze the somatic mutations of Bad in human lymphomas, but we could not detect any Bad gene mutation in 100 samples (unpublished data). Because the Bad-deficient mice also produced solid tumors (16), we analyzed Bad mutation in colon cancers and found Bad mutations in them. To clarify that Bad mutation occurs widely in human cancers, clearly studies are now needed that attempt a widened survey for Bad gene mutations in human cancers.
Supplementary Material

Supplementary Material can be found at: http://www.carcin.oupjournals.org/.

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References