Expression of Phosphorylated Ser\textsuperscript{70} of Bcl-2 Correlates with Malignancy in Human Colorectal Neoplasms

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Abstract

Purpase: Bcl-2 is a model apoptosis suppressor postulated to promote tumorigenesis. Recently, it has been reported that Bcl-2 undergoes phosphoregulation of its Ser\textsuperscript{70} to substantially alter its molecular function. Previous studies further suggest that such phospho-Bcl-2 regulation may influence tumor progression in colorectal and other cancers; however, phosphorylation status of the Ser\textsuperscript{70} of Bcl-2 (pSer\textsuperscript{70}) \textit{in vivo} in tumors remains obscure. To elucidate this question that may suggest the biological role, we molecularly screened a panel of human colorectal adenomas and adenocarcinomas for endogenous expression of pSer\textsuperscript{70} Bcl-2.

Experimental Design: An antibody specific against pSer\textsuperscript{70} Bcl-2 was generated for thorough immunohistochemical examination of paraffin-embedded tumor specimens, allowing detection of the endogenously expressed antigen among a range of Bcl-2-positive colorectal neoplasms, including 75 tubular adenomas, 114 adenocarcinomas, and 15 cases of cancer in adenomas.

Results: Loss of pSer\textsuperscript{70} Bcl-2 expression was observed in adenocarcinomas in a differentiation-dependent manner (positivities: well differentiated 63%, moderately differentiated 52%, and poorly differentiated 12%), whereas tubular adenomas maintained their expression (positivity 88%). Interestingly, an inverse correlation was found between expression of pSer\textsuperscript{70} Bcl-2 and Ki-67 antigen in those cases of cancer in adenoma (P < 0.01). It was further observed that loss of pSer\textsuperscript{70} Bcl-2 expression was associated with significantly shorter survival (P < 0.05) and correlated with clinical stages and lymph node metastasis (P < 0.05 and P < 0.05, respectively).

Conclusions: Loss of pSer\textsuperscript{70} Bcl-2 expression is closely linked to biological aggressiveness in colorectal tumors and represents a statistically significant molecular index for prognosis of patients with these tumors.

Bcl-2 has been identified as an important suppressor of apoptosis. Expression of this molecule is believed to enhance tumor progression by counteracting apoptosis triggers, such as hypoxia, viral infections, cytokone deprivation, and the proapoptotic action of Bcl-2 family members (1–4). Recent studies have used \textit{in vitro} assays to detect Bcl-2 phosphorylation activated by agents, including cytokines (e.g., interleukin-3), chemotherapy drugs [e.g., the microtubule-active drug paclitaxel (Taxol)], and serine/threonine kinases Cdc2, extracellular signal-regulated kinase, c-Jun NH\textsubscript{2}-terminal kinase, and Cdk6/v-cyclin complex; all of these agents are activated during the mitotic phase (5–9). These data collectively revealed intriguing functional aspects of Bcl-2 in relation to apoptosis.

It is known that Bcl-2 undergoes multisite phosphorylation centered at the loop region that includes Thr\textsuperscript{56}, Thr\textsuperscript{69}, Ser\textsuperscript{70}, Thr\textsuperscript{74}, and Ser\textsuperscript{87} (8, 10–12). Phosphorylation of Ser\textsuperscript{70} and Thr\textsuperscript{69} in this loop was observed by \textit{in vitro} assay using cell lines treated with the microtubule-active agent Taxol. c-Jun NH\textsubscript{2}-terminal kinase has been shown to phosphorylate Bcl-2 at Thr\textsuperscript{56}, Ser\textsuperscript{70}, Thr\textsuperscript{74}, and Ser\textsuperscript{87} in \textit{in vivo} assays of cell lines, and Thr\textsuperscript{69} may be involved in mitotic or meiotic events that are essential for cell cycle progression (10). These data suggest that the phosphorylated form of Bcl-2 may contribute to advancement of human neoplasms, to include colorectal cancer (13, 14). Despite this accumulating data, the mechanistic role of Bcl-2 phosphorylation in the tumor biology remains unclear (15). Results from Taxol-treated cell lines imply that phosphorylation inhibits Bcl-2 ant apoptotic function, although interleukin-3-induced phosphorylation of Bcl-2 increased this function in interleukin-3-dependent cell lines (7, 10). Other studies propose that Bcl-2 phosphorylation may be involved in mitotic or meiotic events that are independent of apoptosis (15–19). In view of these sometimes
conflicting results, an in vivo study of phospho-Bcl-2 expression would be valuable to the improved understanding of Bcl-2 in tumor progression. Here, we investigated the in vivo expression of phospho-Bcl-2, focusing on the phosphorylated Ser$^{70}$ (pSer$^{70}$) residue in colorectal neoplasms, including tubular adenomas and adenocarcinomas, to elucidate the molecular mechanism of Bcl-2 in the context of the actual disease setting.

Materials and Methods

Tissues and cell lines. Paraffin-embedded tissues of colorectal tumors were selected based on the diagnostic records of the Department of Clinical Pathology at Okayama University Hospital and Division of Pathology of Iwate Medical University (Morioka, Japan). In total, 209 colorectal tumors were examined, which included 75 cases of tubular adenomas, 119 cases of adenocarcinomas, and 15 cases of cancer in adenomas; all tissues were surgically excised between 1993 and 2002, representing clinically untreated primary tumors. Histologic typing of the adenocarcinomas was done according to the WHO International Histological Classifications (20) and the corresponding clinical data were retrospectively compiled. Other clinicopathologic diagnosis for cases of the adenocarcinomas was based on tumor-node-metastasis system (21). A human colorectal adenocarcinoma cell line, SW620 (American Type Culture Collection, Manassas, VA), was cultured in RPMI 1640 with 10% fetal bovine serum (FBS). Cells were seeded on the tissue culture slides and treated with 200 nmol/L Taxol [Bristol-Myers Squibb K.K., Tokyo, Japan] for 8 hours. Samples were sequentially fixed with 4% paraformaldehyde in PBS for immunofluorescence. Baby hamster kidney (BHK) cells used for DNA transfection assay were also cultured in DMEM supplemented with 10% FBS.

All the patient-derived materials in the present study were used following the guideline of Japanese Society of Pathology.

Antibodies. Rabbit serum against human pSer$^{70}$ Bcl-2 was raised by 3-fold immunization with a synthetic phosphorylated oligopeptide encoding the 15 amino acids (RDPVARTpSPLQTPAA) at the loop region of human Bcl-2. After ELISA tier measurement, serum was collected and purified through an IgG affinity column.

For the preabsorption assay, phosphopeptide (10 μg; sc-16647p; Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with 1 μg anti-pSer$^{70}$ Bcl-2 polyclonal antibody for 24 hours at 4°C, and the mixture was applied as primary antibody solution at the immunohistochemistry step to establish the specificity of the anti-pSer$^{70}$ Bcl-2 antibody. A mixture of 1 μg anti-pSer$^{70}$ Bcl-2 antibody and 10 μg recombinant truncated Bcl-2 protein (sc-4096; Santa Cruz Biotechnology) was also used in parallel to confirm the specific reactivity of the pSer$^{70}$ antibody. The following antibodies were used for immunohistochemistry: anti-Bcl-2 polyclonal antibody (sc-783; Santa Cruz Biotechnology) and anti-Ki-67 monoclonal antibody (mAb; MIB-1; DakoCytomation A/S, Glostrup, Denmark).

Double-labeled immunofluorescence. Immunofluorescence stainings used anti-pSer$^{70}$ Bcl-2 polyclonal antibody along with an anti-heat shock protein 60 (Hsp60) mAb (BD Biosciences, San Jose, CA), anti-Ki-67 mAb, or anti-Myc tag mAb (9E10; American Type Culture Collection) on paraffin-embedded tissue sections or cytosmeared sections of human bcl-2-transfected BHK cells or SW620 cells. Primary antibodies were employed in combination with mAb (anti-Hsp60 and anti-Ki-67) and a rabbit polyclonal antibody (anti-pSer$^{70}$ Bcl-2). For secondary antibodies, Cy3-labeled goat anti-mouse IgG was used to label anti-Hsp60 and anti-Myc tag mAb, and FITC-labeled goat anti-rabbit IgG was employed for anti-pSer$^{70}$ Bcl-2 polyclonal antibody. For double immunofluorescence detecting Myc-tagged Bcl-2 and Ki-67 antigen, specimens were first stained with anti-Ki-67 mAb (second antibody: FITC-goat anti-mouse IgG), blocked with normal mouse serum (DakoCytomation), and sequentially moved to staining with Cy3-conjugated 9E10 (Sigma-Aldrich, St. Louis, MO). Immunofluorescence was detected with a confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Immunohistochemistry. Enzymatic immunohistochemistry was done by standard indirect staining. For heat-induced antigen retrieval to detect endogenous pSer$^{70}$ Bcl-2 or Ki-67, deparaffinized slides were boiled in 10 mmol/L citrate buffer (pH 6.4) in a microwave oven for 10 minutes at 600 W. Endogenous peroxidase activity was blocked by incubation in 3% H$_2$O$_2$ for 20 minutes followed by incubation with goat serum for 10 minutes at room temperature. Samples were incubated with primary antibody overnight at 4°C, and signals were visualized using the Dako EnVision kit at the secondary immunostaining step (EnVision+, DakoCytomation) according to the manufacturer’s instruction. Counterstaining was in Mayer’s hematoxylin.

Expression of phosphorylated Ser$^{70}$ Bcl-2 in colorectal tumors. For colorectal adenocarcinomas, the cells expressing pSer$^{70}$ Bcl-2 were distributed among three major categories. The group in which the pSer$^{70}$-positive segment covered >50% of the cancerous area was classified as “diffuse positive,” whereas the group characterized by <20% coverage was termed “very localized.” An intermediate group was termed “localized.” These classifications are shown in Table 1. Expression of total Bcl-2 was evaluated as either positive or negative, because it was observed diffusely and uniformly with almost the same intensity in the positive cases. Results from immunostainings of both pSer$^{70}$ Bcl-2 and total Bcl-2 were independently evaluated by five pathologists before being categorized.

MIB-1 index. The MIB-1 index, a measure of Ki-67 staining, was assessed as the percentage of Ki-67-positive tumor cell nuclei among all tumor cell nuclei. More than 1,000 nuclei were evaluated from each section.

Apoptotic cell assay. Triplicate samples of 1 × 10⁵ SW620 cells transfected by 1 μg phosphomimetic mutants of Ser$^{70}$ Bcl-2 were assayed for the number of apoptotic cells based on morphologic shrinkage, nuclear fragmentation, or chromatin condensation with Hoechst dye staining 24 hours after transfection.

DNA transfections. BHK cells (1 × 10⁵) were transfected with 1 μg pcDNA3-Myc epitope–tagged human bcl-2 lacking the transmembrane domain (ΔTM-Bcl-2) or with pcDNA3-Myc-Ser$^{70}$Ala-ΔTM-bcl-2 in which Ser$^{70}$ was substituted with alanine by site-directed mutagenesis (Ser$^{70}$Ala-ΔTM-Bcl-2); transfection was by the calcium phosphate method (22). Sixteen hours after transfection, the cells were treated with 200 nmol/L Taxol and collected for further assays as shown in Fig. 1. To examine effect of phosphomimicking Ser$^{70}$ Bcl-2 expression on colon adenocarcinoma cells, 1 × 10⁵ SW620 cells were transfected with either 1 μg pcDNA3-Myc-bcl-2 (wild-type), Myc-Ser$^{70}$Ala-bcl-2, or Myc-Ser$^{70}$Asp-bcl-2 (Ser$^{70}$ substituted with aspartate by site-directed mutagenesis) by lipofection (LipofectAMINE 2000, Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, the cells were stained by the immunofluorescence method described above using 9E10 mAb and Cy3-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for apoptosis assay.

Immunoblotting. Immunoblotting was used to determine the specificity of affinity-purified rabbit anti-pSer$^{70}$ Bcl-2 polyclonal antibody. Briefly, lysis of BHK cells expressing ΔTM-Bcl-2 or Ser$^{70}$ ΔTM-Bcl-2 were resolved by 12% SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane and probed with the antibodies anti-Myc tag, anti-human Bcl-2, anti-pSer$^{70}$ Bcl-2, and anti-actin. Signals were developed by chemiluminescence detection using SuperSignal (Pierce Chemical Co., Rockford, IL).

Phosphorylation assay. Phosphorylation assay of Bcl-2 was done as described in previous studies (19, 23). Briefly, BHK cells expressing truncated Bcl-2 (Myc epitope–tagged ΔTM-Bcl-2) were first treated with 1 μmol/L A23187 (Calbiochem, San Diego, CA) or 250 ng/mL Taxol for 1 hour and then metabolically labeled with 0.5 mCi/mL ($^{32}$P) orthophosphate for 4 hours. The labeled cells were lysed in a 0.5% Triton X-100 lysis buffer containing protease inhibitors and phosphatase inhibitors. At this step, the same volume of the lysates from these three samples were aliquoted for immunoblotting probed with anti-actin mAb (Chemicon, Temecula, CA) to show the amount of total protein from each sample used for immunoprecipitation. Cellular lysates were
then incubated with anti-Myc mAb-coated protein A beads to precipitate Myc-D\textsuperscript{TM}-Bcl-2. The eluted proteins were fractionated by 12.5% SDS-PAGE; sequentially, the gel was analyzed by a phosphorimager (Bioimage Analyzer BAS2000-II, Fuji Photo Film Co., Honshu, Japan). Signal from the imager analysis was normalized against the actin levels obtained in the parallel immunoblot probed with anti-actin mAb.

Statistical analyses. Probabilities of survivals were calculated using the Kaplan-Meier method (24), and statistical significance of the differences between pSer\textsuperscript{70} Bcl-2-positive and Bcl-2-negative groups was evaluated by the log-rank test. Comparisons between pSer\textsuperscript{70} Bcl-2 expression and each clinical variable was calculated by nonparametric Mann-Whitney U test (age, sex, and differentiation; ref. 25) and by Spearman’s rank-correlation test (wall penetration, lymph node metastasis, and stage) using ranks adjusted for multiple comparisons because the data did not show a normal distribution. Statistics were done by StatView J4.5 statistical analysis software (Hulinks, Tokyo, Japan). Two-sided \( P < 0.05 \) was the threshold for significance.

Results

Characterization of the newly generated antibody against phosphorylated Ser\textsuperscript{70} Bcl-2. We first examined the specificity of the pSer\textsuperscript{70} Bcl-2 antibody by immunoblotting and \(^{32}\text{P}\) phosphorylation assay using Taxol or ionomycin (A23187) – treated BHK cells expressing D\textsuperscript{TM}-Bcl-2 based on the finding that Taxol, an inhibitor of microtubule assembly, induces hyperphosphorylation of Bcl-2 (5, 7, 11, 26, 27), whereas ionomycin was shown to dephosphorylate Bcl-2 (22). Immunoblots probed with anti-Myc-tag mAb revealed that BHK cells transfected with Myc epitope – tagged D\textsuperscript{TM}-Bcl-2 showed a D\textsuperscript{TM}-Bcl-2 mobility up-shift in response to Taxol treatment, whereas this mobility was shifted downward for cells treated with ionomycin (Fig. 1A, left). The same mobility shifts were detected in immunoblots probed with rabbit anti-Bcl-2 polyclonal antibody. The phosphorylation assay employing \[^{32}\text{P}\]dATP\textgamma labeling showed that this mobility shift was caused by Taxol-induced phosphorylation of Myc-D\textsuperscript{TM}-Bcl-2 (Fig. 1A, right, Taxol\textsuperscript{+}). In immunoblots probed with anti-pSer\textsuperscript{70} Bcl-2 antibody, the single band corresponding to up-shifted Myc-D\textsuperscript{TM}-Bcl-2 was distinctly observed only in the sample treated with Taxol, and no band was observed in any sample derived from the cells expressing Myc-Ser\textsuperscript{70}Ala-D\textsuperscript{TM}-Bcl-2, which substituted Ser 70 with alanine (Fig. 1A, right).

Specificity of the anti-pSer\textsuperscript{70} Bcl-2 antibody was further examined by immunofluorescence of the Taxol-treated BHK cells transfected with Myc-D\textsuperscript{TM}-Bcl-2. Double immunofluorescence using both anti-pSer\textsuperscript{70} Bcl-2 polyclonal antibody and anti-Myc-tag mAb (9E10) revealed that these cells were strongly positive for anti-pSer\textsuperscript{70} Bcl-2 antibody only in the presence of Taxol (Fig. 1B, top, Taxol\textsuperscript{+}/C0 and Taxol\textsuperscript{+}). In the preabsorption assay, the cells lacked significant signal only when the anti-pSer\textsuperscript{70} antibody was absorbed with an excess amount of the pSer\textsuperscript{70} oligopeptide before staining (Fig. 1C, bottom). We noted that, with respect to background activity, neither anti-total Bcl-2 nor anti-pSer\textsuperscript{70} Bcl-2 reacted to endogenous Bcl-2 in BHK (Fig. 1A and B).

Taken together, the results in Fig. 1A and B show that anti-pSer\textsuperscript{70} Bcl-2 antibody specifically recognizes the form of Bcl-2 phosphorylated at Ser\textsuperscript{70}. We then asked whether this antibody could detect endogenous pSer\textsuperscript{70} Bcl-2, particularly as the intracellularly localized molecule, in the tubular adenoma cases using double-labeling immunofluorescence with combined anti-pSer\textsuperscript{70} antibody and Hsp60. Hsp60 is a mitochondrial chaperone protein that localizes to mitochondrial outer membrane (28). The double immunofluorescence detected by confocal laser scanning microscopy showed that endogenous pSer\textsuperscript{70} Bcl-2 mainly colocalized with Hsp60 at mitochondria (Fig. 1C) and, to a lesser extent, in the cytoplasm and Golgi apparatus (detailed data not shown).

Table 1.

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<th>B. pSer\textsuperscript{70} - Bcl-2 expression in colorectal tumors</th>
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<td>C. pSer\textsuperscript{70} expression of cancer in adenoma</td>
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* \( P < 0.01 \)
Expression of phosphorylated Ser^{70} Bcl-2 in colorectal adenomas and adenocarcinomas. The anti-pSer^{70} Bcl-2-specific antibody was used to examine expression of the form of Bcl-2 phosphorylated at Ser^{70} in colorectal neoplasms. Standard immunohistochemistry was employed to examine tumors, including 75 cases of tubular adenomas and 119 cases of adenocarcinomas at various stages (I-IV); all tumors were primary, lacking any pretreatment, and surgically removed between 1993 and 2002 (Table 1A). Expression of total Bcl-2 was first determined in these tumors by employing anti-Bcl-2 polyclonal antibody, which can detect both dephosphorylated and phosphorylated forms of Bcl-2 as shown in the immunoblot of Fig. 1A. As indicated in Table 1, all cases of tubular adenomas (75 of 75) were strongly positive for total Bcl-2. Similarly, >90% of the cases of adenocarcinomas (114 of 119) were positive for total Bcl-2, even the cases showing weak staining (Table 1A). Based on the immunohistochemical results from the adenocarcinomas, total Bcl-2 expression seems to be independent of the stage of differentiation (Table 1A). The expression of pSer^{70} Bcl-2 was further investigated among the total Bcl-2-positive tumors by using the anti-pSer^{70} Bcl-2 antibody. The expression pattern of pSer^{70} Bcl-2 as detected by immunohistochemistry varied with each case of adenocarcinoma, whereas that of total Bcl-2 expression was uniform among the cancer cells in each case. Therefore, the patterns of pSer^{70} Bcl-2 expression were classified into four groups, including Bcl-2-negative cases, according to the proportion of pSer^{70}-positive segment in the total Bcl-2-positive area. With respect to frequency of positives, ~76% of the cases of tubular adenomas diffusely and uniformly expressed pSer^{70} Bcl-2 (Table 1B; Fig. 2A). In the adenocarcinomas, frequency of pSer^{70} Bcl-2 expression seemed to depend on the stage of differentiation. Specifically, 63% of the well-differentiated cases were positive for the anti-pSer^{70} antibody, whereas <12% of the poorly differentiated adenocarcinomas cases expressed pSer^{70} Bcl-2 (Table 1B; Fig. 2B and D). The moderately differentiated adenocarcinomas showed an intermediate frequency (52%; Table 1B; Fig. 2C). The difference between tubular adenoma and
adenocarcinoma with respect to pSer\textsuperscript{70} Bcl-2 expression was found to be statistical significant (\(P < 0.0001\)). The difference between well-differentiated and poorly differentiated types was also found to be statistically significant (\(P < 0.0001\)). Distribution analysis revealed that over half the cases of well-differentiated adenocarcinomas showed diffuse or localized expression of pSer\textsuperscript{70} Bcl-2. By contrast, only a small portion of cancer cells expressed pSer\textsuperscript{70} Bcl-2 throughout the Bcl-2-positive cases of poorly differentiated adenocarcinomas (3 of 26 cases; Table 1B). These results indicate that well-differentiated adenocarcinomas express pSer\textsuperscript{70} Bcl-2 with a higher frequency and over a broader area in comparison with poorly differentiated adenocarcinomas. To equalize the effects of the passage of time, we selected all of the cases of adenomas and adenocarcinomas derived from the same year (1995) and reexamined pSer\textsuperscript{70} expression within this subset (data not shown); these results were in excellent agreement with the findings for the 1993 to 2002 range. Nonneoplastic epithelial cells at crypts of colorectal mucosa were also broadly positive for pSer\textsuperscript{70} Bcl-2 and it seemed mainly accumulated in the mitochondria and Golgi apparatus, much as the tubular adenoma cells (Fig. 2E).

Correlation between expression of phosphorylated Ser\textsuperscript{70} Bcl-2 and Ki-67 in cancer in adenoma. Because a distinct difference in the expression of pSer\textsuperscript{70} Bcl-2 was recognized between tubular

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**Fig. 2.** Immunohistochemistry of pSer\textsuperscript{70} (pS70) Bcl-2 in colorectal tubular adenomas and adenocarcinomas. *Left,* H&E stainings; *middle,* total Bcl-2 expression; *right,* pSer\textsuperscript{70} Bcl-2 expression. Representative stainings of tubular adenoma (*A*), well-differentiated adenocarcinoma (*B*), moderately differentiated adenocarcinoma (*C*), and poorly differentiated adenocarcinoma (*D*). Original magnification, \(\times 10\). Nonneoplastic epithelial cells at colonic crypts (*E*). Original magnification, \(\times 50\).
adenomas and adenocarcinomas, the cases of cancer in adenomas were subjected to further investigation to determine whether the phenomenon is also observed between a benign region and a malignant region originating from the same background. Among 15 cases of cancer in adenoma obtained from polypectomies, all showed strong pSer\textsuperscript{70} Bcl-2 expression in the areas of the adenomas. Interestingly, however, the areas of adenocarcinomas arising from the same cases lost substantial expression in approximately half of these instances. This result was statistically significant (\(P < 0.01\); Table 1C). Although 7 of 15 (47%) cases were positive for the anti-pSer\textsuperscript{70} antibody, pSer\textsuperscript{70} Bcl-2 expression was not diffused over the entire cancerous area but was restricted to a localized region of 20\% to 40\% of total cancerous area (Table 1C). Based on this finding, we sought to determine if any biological markers correlated to pSer\textsuperscript{70} Bcl-2 expression by employing these cancer in adenoma cases. Consequently, the expression of the Ki-67 antigen detected by MIB-1 mAb showed a definite inverse correlation with pSer\textsuperscript{70} Bcl-2 expression. The cancerous pSer\textsuperscript{70} Bcl-2-negative areas were predominantly positive for Ki-67 (MIB-1 index = 54.9), whereas both adenomatous areas and pSer\textsuperscript{70} positive cancerous areas showed dramatically reduced Ki-67 expression (MIB-1 indices = 24.0 and 22.8, respectively; Fig. 3A and B). This observation was confirmed by double immunofluorescence using both pSer\textsuperscript{70} Bcl-2 antibody (FITC-labeled) and MIB-1 mAb (Cy3-labeled; Fig. 3C) and was supported by statistical analysis as shown in Table 1 (Fig. 3A). We also noted that the inverse correlation between expression of pSer\textsuperscript{70} Bcl-2 and that of Ki-67 antigen was seen at sites of vascular invasion in several cases of pSer\textsuperscript{70} positive adenocarcinomas. In these cases, the foci of vascular invasion showed reduced or abrogated phospho-Bcl-2 expression and substantial Ki-67 antigen levels, although pSer\textsuperscript{70} Bcl-2 was strongly expressed at the primary cancerous site (Fig. 3D).

Phosphomimetic Ser\textsuperscript{70} Bcl-2 mutant affected cell cycle control and induced apoptosis to colon adenocarcinoma cells. For attempt to disclose the potential role of altered phosphorylation

**Figure 3.** Inverse correlation between pSer\textsuperscript{70} (pS70) Bcl-2 and Ki-67 expression in the cases of cancer in adenoma (A–C) and in the cases of adenocarcinomas with vascular invasion (D). A, statistical significance of MIB-1 index was shown in comparisons between the pSer\textsuperscript{70}-negative area and the positive area. Note that the MIB-1 index of pSer\textsuperscript{70}-positive area showed over two times higher than that of the pSer\textsuperscript{70}-negative area. B, immunohistochemistry of pSer\textsuperscript{70} Bcl-2 and Ki-67 in a case of cancer in adenoma. Abbreviations: Ca, cancerous lesion; Ad, adenomatous lesion. Original magnification, \(\times 20\). C, indirect double immunofluorescence of pSer\textsuperscript{70} Bcl-2 (FITC labeled) and Ki-67 antigen (Cy3 labeled) in cancer in adenoma (confocal laser microscopy). D, loss of expression of pSer\textsuperscript{70} Bcl-2 at a focus of vascular invasion in the case of pSer\textsuperscript{70} Bcl-2–positive adenocarcinoma. The primary tumor was comprised of a pSer\textsuperscript{70}-positive well-differentiated adenocarcinoma (left), whereas the focus of vascular invasion lacked expression of the phosphorylated form of Bcl-2. Original magnification, \(\times 20\).
of Ser$^{70}$ Bcl-2 in colorectal cancers, we did transfection assay on the SW620 colon adenocarcinoma cell line using phosphomimetic mutants of Ser$^{70}$ Bcl-2. Interestingly, significant differences in cell cycle regulation and induction of apoptosis were observed between cells overexpressing phosphomimetic mutant Ser$^{70}$Asp-Bcl-2 and those overexpressing wild-type Bcl-2 or dephosphomimetic mutant Ser$^{70}$Ala-Bcl-2. The Ser$^{70}$Ala-overexpressing SW620 cells were highly proliferative; MIB-1 index was 88% at 12 hours after transfection. In contrast, MIB-1 index of the Ser$^{70}$Asp-overexpressing cells was 55%, which was even lower than that of the wild-type transfected cells (Fig. 4A, left, and B, left). Noticeably, in the Ser$^{70}$Asp-overexpressing cells, over half of them entered apoptosis at 24 hours after transfection, whereas majority of the cells expressing Ser$^{70}$Ala Bcl-2 or wild-type Bcl-2 were nonapoptotic (Fig. 4A, right, and B, right).

Specific correlation between phosphorylated Ser$^{70}$ Bcl-2 expression and clinicopathologic factors and influence of its expression on survival of patients with colorectal cancers. Phospho-Bcl-2 expression was statistically analyzed among patients subgroups defined by clinical and pathologic variables (age, sex, histologic differentiation of adenocarcinoma, wall penetration, lymph node metastasis, and clinical stage). First, a positive correlation with pSer$^{70}$ expression was observed in cancer cell differentiation by Mann-Whitney U test ($P < 0.001$; Table 2). Moreover, its expression was significantly correlated with both lymph node metastasis and clinical stages from Spearman’s rank correlation test, whereas no specific correlation was found between pSer$^{70}$ expression and age, sex, or wall penetration (Table 2). Following on these results, we next investigated whether the pSer$^{70}$ Bcl-2 expression would show prognostic value in patients with colorectal adenocarcinomas. Univariate analysis (log-rank test) revealed that the survival of the pSer$^{70}$-negative patients (total of 61 cases) was significantly shorter than that of the pSer$^{70}$-positive patients (total of 58 cases; Fig. 4C). Two- and 5-year overall survival rates were 50.5% and 39.3% in the pSer$^{70}$-negative subgroup, respectively, whereas those rates were 65.9% and 52.4% in the pSer$^{70}$-positive subgroup.

**Discussion**

The expression pattern of phosphorylated Bcl-2 in vivo had been unexplored previously, and its biomedical significance has accordingly been controversial [15]. As one of the reasons, it is conceivable that the opposing results are strongly influenced by the different backgrounds of Bcl-2 family expression among the various cell lines. Our main purpose in the present study is to investigate in vivo expression of phospho-Bcl-2 in colorectal...
neoplastic tissues to reveal clues about its biological significance from the pathologic and clinical standpoints.

To this end, we investigated the expression of pSer70 in the loop region of Bcl-2, because that residue is the most frequently observed Bcl-2 phosphorylation site in human colorectal adenomas and adenocarcinomas. Consequently, differential expression of pSer70 Bcl-2 was observed between adenomas and adenocarcinomas. For tubular adenoma differentiated adenocarcinoma cases were positive for pSer70. Immunohistochemistry indicated that <12% of the poorly differentiated adenocarcinoma cases were positive for pSer70 Bcl-2, whereas ~60% of the well-differentiated adenocarcinoma cases were positive. Interestingly, loss of pSer70 expression was also observed at sites of vascular invasion in some cases of pSer70-positive adenocarcinomas.

From the cases of cancer in adenoma for detailed investigation, it was found that loss of pSer70 Bcl-2 expression was observed at mitotically active cancer area, whereas adenomatous area maintained the expression. Thus, Ki-67 antigen showed a distinct inverse correlation with pSer70 Bcl-2 in colorectal tumors in vivo. These immunohistochemical and statistical analyses supported the correlational significance of the absence of pSer70 Bcl-2, combined with the presence of Ki-67-positive neoplastic cells of high mitotic activity, whereas Ki-67-negative cells tended strongly to express pSer70 Bcl-2. In colon adenocarcinoma cell line as an in vitro model, transfection assays using phosphomimetic or dephospho-mimetic Ser70 bcl-2 mutants revealed that enhanced expression of phosphorylated form of Ser70 Bcl-2 (Ser70Asp-Bcl-2) seemed to promote cell cycle arrest that was proven by increasing MIB-1 index on Ser70 Bcl-2-expressing cells, and induction of apoptosis was also observed in these cells. At this moment, detailed molecular mechanism is still necessary to be investigated because bcl-2 has been reported to be multifunctional and biological effect of its phosphorylation still remains to be complexity (15, 19, 22, 26, 29). However, it seemed, at least, that change in numbers of the Ki-67-expressing cells and apoptotic cells by the mutative Ser70-Bcl-2 overexpression showed parallel behavior. Taking these findings together, it implies that phosphorylation at Ser70 of Bcl-2 might converge to suppression of colorectal tumor progression.

In the clinicopathologic study, clinical factors, such as degree of lymph nodal metastasis and tumor stage, were revealed to be associated with pSer70 Bcl-2 expression in addition to cellular differentiation of adenocarcinoma. Specifically, loss of the pSer70 expression was more frequently recognized in the cases with advanced stage of lymph nodal metastasis and clinical stages, which suggests its utility as a biological marker for the extent of malignancy in colorectal cancer. Moreover, the result of nonparametric analysis indicated the shorter survival of the pSer70 Bcl-2-negative patients in comparison with that of the pSer70-positive patients. Thus, from clinicopathologic viewpoint, expression of pSer70 Bcl-2 seems to be useful as a novel prognostic indicator for colorectal cancer patients. In the present study, we attempted to show the phosphorylation-regulation of Bcl-2 in colorectal tumors. Clearly, more investigation is needed to provide further insight on phospho-Bcl-2 molecular actions during cancer progression. However, we believe that our present work offers a logical framework for evaluating the specific molecular enhancement of malignancy and for integrating the increasing clinical knowledge with definitive in vivo and molecular studies of Bcl-2-contributed tumorigenesis.

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**References**


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Eisaku Kondo, Takayoshi Miyake, Masao Shibata, et al.


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