Inhibition of Apoptosis by Survivin Predicts Shorter Survival Rates in Colorectal Cancer

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Abstract

Deregulated inhibition of apoptosis (programmed cell death) may facilitate the insurgence of neoplasia, but whether it also influences the outcome of common cancers has remained controversial. In this study, we investigated the expression of a novel inhibitor of apoptosis, survivin, in colorectal cancer and its relationship with tumor cell apoptosis and overall prognosis. By immunohistochemistry, survivin was expressed in 91 of 171 (53.2%) cases of colorectal carcinomas of histological stages 0 to IV. In contrast, normal colon epithelium did not express survivin. Although survivin expression did not correlate with p53 abnormalities (46.5% versus 58.0%; P = 0.18), survivin-positive cases were strongly associated with bcl-2 expression (72.5% versus 27.4%; P < 0.0001) and reduced apoptotic index (0.76% ± 0.39% versus 1.17% ± 0.62%; P < 0.0001). Expression of survivin alone in bcl-2-negative (discordant) cases also resulted in reduced apoptotic index (0.82% ± 0.57% versus 1.16% ± 0.66%; P = 0.0046). When analyzed for prognostic significance, patients with low apoptotic index (<0.97%) had worse survival rates than the group with high apoptosis (P < 0.001), and a multivariate Cox proportional hazard model identified reduced apoptosis as an independent predictive factor for overall survival (P < 0.0001). These data demonstrate that apoptosis inhibition by survivin, alone or in cooperation with bcl-2, is an important predictive/prognostic parameter of poor outcome in colorectal carcinoma and identify survivin as a new diagnostic/therapeutic target in cancer.

Introduction

Dysregulation of apoptosis (programmed cell death) is thought to contribute to cancer by aberrantly extending cell viability and favoring the accumulation of transforming mutations (1). This is typically exemplified by the overexpression of antiapoptosis bcl-2 protein in follicular lymphoma and its critical role in multidrug resistance and disease progression (2). In addition to the bcl-2 family of regulators of apoptosis, a second gene family of IAP³ has been identified recently (3). Highly evolutionarily conserved from viruses to mammals, IAP proteins contain two/three Cys/His baculovirus IAP repeat(s) and a COOH-terminal RING finger (3). These molecules are thought to block an evolutionarily conserved step in apoptosis downstream of bcl-2 (4), potentially involving direct inhibition of terminal effector caspase-3 and -7 (5), through a direct inhibition of terminal effector caspase-3 and -7 (5), through a

Materials and Methods

Patients and Samples. A total of 171 cases of colorectal carcinoma was involved in this study. The patients with colorectal carcinoma, who had undergone potentially curative tumor resection at Osaka Medical College Hospital from 1988 to 1991, had received neither chemotherapy nor radiation therapy before surgery. Clinicopathological factors, tumor histologies, and disease stage were assigned according to the General Rules for Clinical and Pathological Studies on Cancer of Colon, Rectum, and Anus (11). Materials were composed of 6 cases of stage 0, 27 cases of stage I, 64 cases of stage II, 64 cases of stage III, and 10 cases of stage IV. There were 97 males and 74 females, and the mean age of the patients was 59.7 years (SD, 12.0 years; range, 20–88 years). Routinely processed formalin-fixed, paraffin-embedded blocks of containing principal tumor were selected. Serial sections of 2–4 μm were prepared from the cut surface of blocks at the maximum cross-section of the tumor.

Immunohistochemical Staining for Survivin and Scoring Method for Its Expression. The method of immunohistochemical staining for survivin antigen was conducted by the standard avidin-biotin-peroxidase complex technique using L.V. Dako LSAB kit (DAKO A/S, Carpinteria, CA) and of antigen retrieval by pressure cooking using mAb 8E2 raised against purified recombinant survivin and characterized in previous studies (10). Briefly, before labeling with primary antibody, deparaffinized and rehydrated sections were immersed in a 10⁻² M sodium citrate buffer (pH 6.0) and boiled for 20 min in a pressure cooker while maintaining the pressure and cooled and washed in PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl). Primary antibody (diluted at 1:5) was added to the slides after quenching in 3% hydrogen peroxide and blocking for 5 min each, incubated overnight at 4°C, and washed. Biotinylated anti-mouse immunoglobulin and streptavidin conjugated to horse radish peroxidase were applied subsequently for 30 min at room temperature each. Finally, 3,3'-diaminobenzidine was used for color development and hematoxylin for counterstaining. Negative control slides in the absence of primary antibody were included for each staining. Scoring method for survival expression was modified from that of bcl-2 expression described by Sincirope et al. (12). Positive tumor cells were quantified by two independent observers, and a mean percentage of positive tumor cells was determined in at least five areas at ×400 and assigned to one of five categories: (a) 0, <5%; (b) 1, 5–25%; (c) 2, 25–50%; (d) 3, 50–75%; and (e) 4, >75%. The intensity of survivin immunostaining was scored as: (a) weak, 1+; (b) moderate, 2+; and (c) intense, 3+. For tumors showing heterogeneous staining, the predominant pattern was taken into account for scoring. The staining intensity of infiltrated lymphocytes to bcl-2 was defined as 3+ of relative intensity of survivin staining. The percentage of positive tumor cells and staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores <1 were defined as negative; otherwise were defined as positive.

Immunohistochemical Staining for bcl-2 and p53. Deparaffinized and rehydrated sections were immunostained using the same technique as for

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³ The abbreviations used are: IAP, inhibitor of apoptosis; mAb, monoclonal antibody; AI, apoptotic index.

5071

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SURVIVIN EXPRESSION IN COLORECTAL CARCINOMAS

Survivin antigen staining. Monoclonal mouse antibody against bcl-2 oncoprotein (clone 124, diluted at 1:20; DAKO, Copenhagen, Denmark) and a mouse antihuman p53 antibody (DO7, diluted at 1:50; DAKO) were used as primary antibodies for bcl-2 and p53 immunostaining. Before the addition of the primary antibody, deparaffinized sections were immersed in a 10−3 M sodium citrate buffer (pH 6.0) and heated in a microwave three times at 900 W for a total of 15 min. The other staining procedures were the same as for survivin. Scoring criteria for bcl-2 were the same as for survivin, and cases with weighted scores < 1 were judged as negative. For p53 expression, cases with <5% positively stained tumor cells were defined as negative; otherwise, they were defined as positive.

Histochemical Detection of Apoptosis and Determination of the AI. To identify apoptotic cells and apoptotic bodies, we used the Apop Tag in situ detection kit (S7101-KIT; Oncor, Gaithersburg, MD). Briefly, deparaffinized and rehydrated sections were digested with proteinase K (20 µg/ml in PBS; Wako, Osaka, Japan) for 20 min at room temperature and washed. After quenching in 3% hydrogen peroxide for 5 min, washing with PBS, and adding the equilibration buffer for 10 min, terminal deoxynucleotidyl transferase enzyme was pipetted onto the sections, which were then incubated at 37°C for 1 h. After stopping the reaction by putting sections in stop/wash buffer and washing, anti-digoxigenin-peroxidase was added to the slides. Finally, slides were washed with PBS, stained with diaminobenzene (DAKO A/S, Glostrup, Denmark) substrate, and counterstained with methyl green. A positive control was prepared by nicking DNA with DNase I (0.7 µg/ml; Stratagene Co., La Jolla, CA) for the first staining procedure. A specimen known to be positive for apoptotic cells was used as positive control for subsequent staining. Substitution of terminal deoxynucleotidyl transferase with distilled water was used as a negative control. The AI was expressed as the ratio of positively stained tumor cells and bodies to all tumor cells, given as a percentage for each case, and determined according to the criteria described previously (13). Briefly, a minimum of 3000 cells was counted under a 400-fold magnification. Positively staining tumor cells with the morphological characteristics of apoptosis were identified using standard criteria, including chromatin condensation, nuclear disintegration, and formation of crescentic caps of condensed chromatin at the nuclear periphery.

Statistical Analysis. All statistical analysis was performed by the SPSS 6.1 J software package for Macintosh (SPSS Inc., Chicago, IL). Variables associated with survivin expression as well as the correlation between survivin and p53 or bcl-2 expression were analyzed by χ2 test. Differences in tumor cell AI for groups dichotomized according to survivin expression were checked by independent Wilcoxon method, and the correlation between AI and weighted score of survivin on a per case basis was further analyzed by Pearson’s correlation coefficients test. The survival curves were plotted according to Kaplan-Meier method and checked by log-rank test. A value of P < 0.05 was considered statistically significant.

Results

Expression of Survivin in Colorectal Carcinomas. By immunohistochemistry, anti-survivin mAb 8E2 exclusively reacted with colorectal carcinoma cells, with positive staining of the cytoplasm of cancer cells. In contrast, no expression of survivin in neighboring normal tissues was observed (Fig. 1A). The intensity of survivin staining was usually homogeneous within a case tested, but the number of tumor cells positively stained by the anti-survivin mAb ranged between 20 and 100%, depending on the case examined. After multiplying the weighted survivin score, 91 cases of colorectal carcinoma in the present series were defined as positive (53.2%), with weighted survivin scores from 1 to 12.

Correlation between Expression of Survivin and Clinicopathological Factors. A clinicopathological analysis of survivin-positive cases is shown in Table 1A. As shown, none of the prognostic parameters analyzed in survivin-positive cases, including different
tumor size, tumor depth, lymphatic or venous invasion, lymph node metastasis, histological stage, Dukes' classification, and recurrence, reached statistical significance.

**Correlation of Expression between Survivin and bcl-2 or p53.** A positive cytoplasmic immunoreactivity for bcl-2 was detected in 98 of 171 cases (57.3%; Fig. 1B). Expression of survivin clearly segregated with bcl-2-positive tumors as compared with bcl-2-negative cases (71 of 98, 72.5% versus 20 of 73, 27.4%) with high statistical significance ($P < 0.0001$). In contrast, nuclear accumulation of p53 was demonstrated in 71 of 171 cases (41.5%; Fig. 1C), without statistically significant stage differences of tumors, but there was no correlation between survivin and p53 expression ($P = 0.183$).

**Relationship between Tumor Cell Apoptosis and Expression of Survivin and bcl-2.** Apoptotic cells and apoptotic bodies were detected in all cases of colorectal carcinoma examined by in situ labeling (Fig. 1D). The relationships between AI and expression of survivin and bcl-2 were studied. The mean AI of 171 cases was 0.95% (SD, 0.55%; range, 0.15-3.69%) with a median value of 0.90%, and no significant association was observed between AI and tumor stage. The mean AI for survivin-positive tumors ($n = 91$) was 0.76% (SD, 0.39%), which was significantly lower than 1.17% (SD, 0.62%) observed in survivin-negative tumors ($n = 80$; $P < 0.0001$). The mean AI for bcl-2-positive tumors ($n = 98$) was significantly lower than that for bcl-2-negative tumors ($n = 73$; $P = 0.021$). Seventy-one of 91 cases that expressed survivin were also positive for bcl-2 expression, and the mean AI in these cases was 0.74% (SD, 0.33%). For 73 cases, which were negative for bcl-2 expression, the mean AI for 20 survivin-positive tumors was 0.82% (SD, 0.57%), which was significantly lower than those of 1.16% (SD, 0.66%) observed in 53 survivin-negative tumors ($P = 0.0046$).

**Analysis of Prognostic Factor in Patients with Colorectal Carcinoma.** Kaplan-Meier curves for patients with colorectal carcinoma categorized according to survivin expression was shown in Fig. 2A. Overall 5-year survival rates for patients with survivin-positive colorectal carcinomas (72.5%; 66 of 91) were less than those with survivin-negative tumors (82.5%; 66 of 80), but the difference was not statistically significant. For the study on correlation between AI and prognosis, the patients were dichotomized by the cutoff of 0.97% for AI, which provided a more sensitive parameter of survival differences in the present patient series. The overall 5-year survival rates for the group with high AI ($\geq 0.97$) and another group with low AI ($< 0.97$) were 89.6% (60 of 67) and 69.2% (72 of 104), respectively (Fig. 2B; $P = 0.001$). In addition, multivariate Cox proportional hazard model by use of the variables, including tumor depth, lymphatic or venous invasion, lymph node metastasis, peritoneal or hepatic metastasis, and AI, which were all identified as prognostically significant by univariate analysis, demonstrated that AI, as well as the development of peritoneal or hepatic metastasis after surgery, was an independent predictor for overall survival ($P < 0.0001$; Table 2).

**Discussion**

In this study, we have shown that apoptosis inhibition by survivin (7) is a new predictive indicator of poor prognosis and shorter survival rates in colorectal cancer. Relevant features of survivin expression in this series included its selective distribution in 20-90% of neoplastic cells but
not in normal colon epithelium, a higher incidence of positive cases than in gastric cancer (53.2% versus 34.5%; Ref. 10), and a highly significant coassociation with bcl-2 (P < 0.0001). Unlike gastric cancer (10), there was no significant correlation between survivin expression and p53 abnormalities in colorectal cancer. When compared with other series of colorectal cancer, the percentage of bcl-2-positive cases in our series (57.3%) was comparable with that of other investigators (14, 15).

As one of the most significant aspects of this study, the presence of survivin in colorectal cancer was strongly associated with expression of bcl-2 and with reduced AIs. A similar coassociation between survivin and bcl-2 was observed in neuroblastoma (9), gastric cancer (10), and high-grade non-Hodgkin’s lymphoma.4 The chromosomal location of survivin at 17q25 (16) is inconsistent with its association with tumors of low proliferative index. Apart from bcl-2 expression, reduced AIs have been correlated recently with more aggressive and invasive forms of colorectal cancer, but potential effector molecules responsible for the aberrantly increased viability have not been identified. Here, the low AI associated with survivin expression emerged as a compelling predictive/prognostic indicator of poor overall survival in colorectal cancer (P = 0.0001). This was independently confirmed by multivariate Cox proportional hazard model, which defined the AI as a highly significant independent predictor of overall survival (P = 0.0001), although the decrease in survival rates in survivin-positive patients did not reach statistical significance.

Although the bulk of previous studies has shown the involvement of other apoptosis regulators, including p53 and bcl-xl, in tumor progression (1), these data suggest that inhibition of apoptosis by survivin, alone or in combination with bcl-2, may significantly influence the outcome of colorectal cancer and translate into considerably shorter survival rates. This is consistent with the current hypothesis of molecular pathogenesis of colorectal cancer in which early somatic and inherited mutations of the adenomatous polyposis coli tumor suppressor gene may be potentially associated with reduced apoptotic cell death (20). Although targeted antagonists of survivin may be beneficial at increasing the susceptibility of transformed cells to apoptosis-based therapy, these data propose the determination of the AI as a highly sensitive predictive/prognostic indicator in colorectal cancer.

References

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