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Analysis of Apoptosis Protein Expression in Early-Stage Colorectal Cancer Suggests Opportunities for New Prognostic Biomarkers

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Abstract

Purpose: Although most stage II colon cancers are potentially curable by surgery alone, ~20% of patients relapse, suggesting a need for establishing prognostic markers that can identify patients who may benefit from adjuvant chemotherapy. We tested the hypothesis that differences in expression of apoptosis-regulating proteins account for differences in clinical outcome among patients with early-stage colorectal cancer.

Experimental Design: Tissue microarray technology was employed to assay the expression of apoptosis-regulating proteins by immunohistochemistry in 106 archival stage II colorectal cancers, making correlations with disease-specific survival. The influence of microsatellite instability (MSI), tumor location (left versus right side), patient age, and gender was also examined.

Results: Elevated expression of several apoptosis regulators significantly correlated with either shorter (cIAP2; TUCAN) or longer (Apaf1; Bcl-2) overall survival in univariate and multivariate analyses. These biomarkers retained prognostic significance when adjusting for MSI, tumor location, patient age, and gender. Moreover, certain combinations of apoptosis biomarkers were highly predictive of death risk from cancer. For example, 97% of patients with favorable tumor phenotype of cIAP2^{low} plus TUCAN^{low} were alive at 5 years compared with 60% of other patients ($P = 0.00003$). In contrast, only 37% of patients with adverse biomarkers (Apaf1^{low} plus TUCAN^{high}) survived compared with 83% of others at 5 years after diagnosis ($P < 0.0001$).

Conclusions: Immunohistochemical assays directed at detection of certain combinations of apoptosis proteins may provide prognostic information for patients with early-stage colorectal cancer, and therefore could help to identify patients who might benefit from adjuvant chemotherapy or who should be spared it.

Colorectal cancer is the second leading cause of cancer-related death in the United States and most industrialized countries and the fourth leading cause of cancer-related death worldwide (1). Currently, selecting patients for postoperative adjuvant chemotherapy relies mainly on pathologic and clinical staging but inadequately addresses the heterogeneity in survival among similarly staged patients. Although stage II (Dukes' B) colon cancer is potentially curable by surgery alone, ~20% of patients relapse (reviewed in refs. 2, 3). Clinical guidelines for management of patients with no histologic evidence of lymph node invasion are controversial (4, 5), primarily because it is not currently possible to discriminate which patients have micrometastatic disease at the time of diagnosis. A need therefore exists for establishing prognostic markers that can identify patients who may benefit from adjuvant chemotherapy.

Adenocarcinoma of the colon results from a multistep process involving changes in the expression and function of genes relevant to cell division, cell migration, and programmed cell death (apoptosis; reviewed in ref. 6). In normal colonic mucosa, a self-renewing population of stem cells located in the crypt region of the colonic villi gives rise to progeny which differentiate into surface epithelial cells, and migrate to the villus tip where they eventually die by programmed cell death and are discarded into the lumen of the bowel. Defects in intrinsic cell suicide mechanism thus can contribute to cell accumulation in the colon, promoting malignancy. Apoptosis also figures prominently in the cytotoxic action of chemotherapeutic drugs and ionizing radiation thus making defects in apoptosis mechanisms relevant to problems of chemoresistance and radioresistance. Moreover, defects in apoptosis pathways can contribute to metastasis, allowing epithelial cells to survive in a suspended state, thereby promoting their hematogenous or lymphatic dissemination (7).

The human genome contains >120 genes directly involved in apoptosis regulation, representing core components of or direct

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inputs into the cell death machinery (http://www.apoptosis-db.org; ref. 8). We tested the hypothesis that differences in the expression of specific apoptosis-regulating proteins could account for differences in clinical outcome among patients with early-stage epithelial malignancies, focusing on colorectal cancer as a test case. Accordingly, monospecific antisera were generated against several apoptosis proteins and applied for immunohistochemical analysis of archival tumor specimens. Tissue microarray technology was used to increase the throughput of this analysis, permitting rapid testing of multiple antibodies on replicate sections of arrays of archival colorectal cancer specimens derived from patients with stage II disease treated by surgery with curative intent. The findings reveal examples of altered regulation of apoptosis protein expression in stage II colorectal cancer and suggest that simple immunohistochemical assays directed at detection of certain combinations of apoptosis proteins could have potential utility as prognostic biomarkers for patients with early-stage cancer. If confirmed in additional cohorts, selected apoptosis biomarkers identified here may help to distinguish early-stage patients who may benefit from adjuvant chemotherapy.

Materials and Methods

Patient specimens. Colorectal cancer specimens were obtained from the Department of Pathology, Yonsei University, Seoul, Korea, under the Institutional Review Board approval. Tissue samples included 106 primary tumors derived from patients with stage II colorectal cancer (as defined by American Joint Committee on Cancer and Union Internationale Contre le Cancer criteria) who had undergone curative surgical resection between 1986 and 1996 and for whom follow-up information was available. Cases without postoperative adjuvant chemotherapy were collected, whereby 63 patients survived without recurrence, seven patients had recurrent disease, and 36 patients died from colorectal cancer. Thus, while not an unbiased sequential case series, the survival profile of this cohort closely resembles that of a random population of stage II colorectal cancer patients, with 72.5% of individuals alive at 5 years (data not shown). Clinical data represent a median follow up of 66 months. The following demographic characterized the investigated cohort: females (40 of 106, 40%), males (66 of 106, 62%); younger patients (dichotomized at median age, 55 years; 59 of 106, 56%), older population (47 of 106, 44%). Fresh snap-frozen samples obtained by cryofractionation methods were used for analysis of microsatellite instability (MSI), whereas formalin-fixed paraffin-embedded samples were used for tissue microarray and immunohistochemical analysis. Patient data were collected retrospectively from hospital records and the Korean National Statistics Office. Patients with double primary malignancy in other organs and a case of familial adenomatous polyposis coli were excluded from the analysis. Only patients whose primary cause of death was due to colorectal cancer were eligible for inclusion. Individuals whose tumors had positive circumferential margins were not included in the study. We defined right-sided tumors as those originating proximal to the splenic flexure and left-sided as those arising distal to this site.

Antibodies. Polyclonal antisera for Survivin (AR-26), Apaf1 (AR-25), XIAP (AR-27A), and SMAC (AR-50B) were generated in New Zealand rabbits using recombinant protein immunogens. Survivin and SMAC (full-length proteins) and Apaf1 (residues 1–420) were produced as glutathione *S*-transferase fusion proteins and affinity-purified as described previously (9). Affinity-purified His6-tagged-XIAP (BIR2) recombinant protein was produced as described previously (10) and was used as an immunogen for producing XIAP-specific antiserum. An additional anti-Apaf1 antibody was generated in rabbits using a synthetic peptide (NH2-CGPKYVVP-VESSLGKEKGLE-amide) corresponding to residues 264 to 282 of human (hu) Apaf1, which was synthesized with an NH_2-terminal cysteine appended to permit conjugation to maleimide-activated carrier proteins KLH and OVA (Pierce, Rockford, IL, Inc.; ref. 11) and used to generate a rabbit polyclonal antiserum (AR-23). An anti-AIF serum was produced in rabbits using a synthetic peptide corresponding to residues 151 to 170 of human AIF. The generation of Bcl-2, Bcl-X_L, Bax, Bid, and TUCAN-specific antisera has been described elsewhere (12–14). Anti-cIAP1 and cIAP2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and R&D Systems, Inc. (Minneapolis, MN), respectively; β-Catenin antiserum from BD Transduction Laboratories (Lexington, KY); and antibodies to p53 (clone DO-7), MIB-1, and BAG1 (clone KS-6C8) from DAKO (Carpinteria, CA). The monospecificity of all antibodies for their intended protein targets was confirmed by SDS-PAGE/immunoblot analysis. Only those antibodies showing selective immunoreactivity with target antigens were employed for subsequent immunohistochemical assays.

Tissue microarrays. To construct colorectal cancer microarrays, two to four cylinders of 1-mm-diameter tissue were cored from representative areas of formalin-fixed tumors embedded in paraffin blocks and arrayed into a new recipient paraffin block with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD). Serial sections (4 μm) were applied to APES-coated slides (Sigma, St. Louis, MO).

Immunohistochemistry. Dewaxed tissue sections were immunostained using a diaminobenzidine-based detection method as described in detail, employing the Envision-Plus-Horseradish Peroxidase (HRP) system (DAKO) and using an automated immunostainer (DAKO Universal Staining System; ref. 15). Antisera specific for Survivin, XIAP, Apaf1, TUCAN, AIF, SMAC, Bax, and Bid were applied at 1:3,000 to 10,000 (v/v), whereas antibodies against Bcl-2 and Bcl-X_L at 1:2,000 (v/v). The dilutions of cIAP1, cIAP2, and β-Catenin antibodies were 1:600 (v/v); BAG1 and MIB-1 were 1:100; and p53 was at 1:50 (v/v). For all polyclonal antisera employed, the immunostaining procedure was done in parallel using preimmune serum to verify specificity of the results. Initial confirmations of antibody specificity also included experiments in which antiserum was preabsorbed with 5 to 10 μg/mL of either synthetic peptide or recombinant protein immunogens.

The scoring of tumor immunostaining was based on the percentage of immunopositive cells (0-100) multiplied by staining intensity score (0/1/2/3), yielding scores of 0 to 300. Results from triplicate or quadruplicate cores were averaged. A preliminary analysis of the data for TUCAN (13) and Bid (14) has been published previously.

Microsatellite instability. Specimens were analyzed for MSI by PCR amplification of microsatellite markers using DNA from either snap-frozen tumor specimens or from formalin-fixed paraffin blocks. The laser capture cryostat microdissection method using H&E-stained cryosections containing >80% of tumor cells was used for sample collection and extraction of DNA. Extracted DNAs from tumors and matched normal mucosae were PCR amplified at six microsatellite loci to evaluate the MSI. The markers included the National Cancer Institute–recommended panel of five markers, plus BAT40 (16).

The sampling of specimens from paraffin blocks was done on the 106 paraffin blocks using Beecher Inst. Arrayer. One millimeter in diameter tumor regions were cored from the paraffin blocks and the cylinders were cut in smaller pieces for the DNA isolation described above. The tumors were analyzed for MSI by PCR amplification of BAT26 (17). Analysis exclusively of tumor was adequate for MSI determination as the shorter *BAT26* allele associated to Africans is absent in the Korean population (not found in over 500 individuals analyzed). PCR products were separated in 6% polyacrylamide gels containing 5.6 mol/L urea followed by autoradiography. MSI was determined by the mobility shift of the PCR products. In tumors with MSI, additional bands were found relative to the normal alleles. MSI in three or more markers of which more than two mononucleotide repeat markers were included was classified as MSI-H whereas all others were

classified as microsatellite stable. Only 1 of the 39 cases classified as MSI-H because of positivity for BAT25 and D2S123 was negative for the commonly used marker, BAT26.

Immunoblotting. Colon cancer specimens (n = 10) with high ratios of cancer cells relative to stroma (>70%) were selected for immunoblotting analysis. The protein lysates were prepared without additional microdissection or fractionation. The tumor lysates and the samples of the normal mucosa from the same patients were prepared using modified radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.25% Na-deoxycholate, 1% NP40, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride] containing complete protease inhibitor cocktail (Sigma), Pan-Caspase inhibitors z-Asp-2,6-dichlorobenzoyloxymethylketone (Bachem, Torrance, CA), and zVAD-fmk (Calbiochem, La Jolla, CA). Samples were normalized for total protein content (100 μg), resolved by SDS-PAGE (12-15% gels), and proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia, Piscataway, NJ). After blocking with 5% skim milk in TBST [50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 0.05% Tween 20] for 2 hours, blots were incubated overnight with specific antisera at 1:1,000 to 1:10,000 (v/v) dilutions at 4°C. After incubation with HRPase-conjugated secondary goat anti-rabbit (either Bio-Rad, Hercules, CA or Santa Cruz Biotechnology) antibody at room temperature for 1 hour, immunodetection was accomplished by an enhanced chemoluminescence method (Amersham), with exposure to X-ray film (Kodak/XAR). Densitometry was done to quantify the intensity of bands, using Image-pro Plus software.

Statistical analysis. Data were analyzed using the STATISTICA software package (StatSoft, Tulsa, OK). Overall survival (OS), defined as the time from study entry to death, was determined in univariate survival analysis using the Kaplan-Meier method. Log-rank test was used for correlation of immunostaining data with the patient survival. Multivariate Cox proportional hazards models were fitted to the data to assess which biomarkers were independently associated with OS. All factors that had prognostic significance when considered alone ($P \leq 0.05$) were entered into a multiple regression analysis whereby hazard ratios (HR) and significance levels were estimated. In backward selection, a factor that was not statistically significant was removed from the model until all remaining factors were significant. The 95% confidence interval (95% CI) for HR was calculated by a formula $\exp[\beta \pm 1.960\mathrm{SE}(\beta)]$.

Results

Immunohistochemical analysis of apoptosis biomarkers in normal colonic mucosa and colorectal cancer. A tissue microarray was constructed using primary tumor specimens derived from a cohort of 106 patients presenting with stage II disease to a single institution, who were treated by surgical resection with curative intent. Microarrays were immunostained using antibodies specific for IAP family proteins (Survivin, XIAP, cIAP1, and cIAP2), Bcl-2 family proteins (Bcl-2, Bcl-X_L, Bax, and Bid), mitochondrial proteins (SMAC and AIF), and other apoptosis regulators such as Apaf1, TUCAN, and BAG1. In addition, because previous studies showed prognostic significance in colon cancer (18–20), immunostaining was done for β-Catenin, p53, and the cell proliferation marker MIB-1. All immunostaining results were quantified according the percentage of immunopositive tumor cells (0-100%) and immunointensity (on a 0-3 scale with reference to internal control cells), and then an immunoscore was calculated from the product of the percentage immunopositivity and immunointensity (0-300). Several of the 106 tumor specimens on the array (~65%) contained adjacent normal colonic mucosa (59-70), depending on the particular slide, permitting side-by-side comparisons of immunostaining results for normal versus malignant epithelium. In addition, four specimens of normal colon derived from individuals who were not diagnosed with colon cancer were stained separately.

Immunohistochemical analysis of tumor tissues on the microarray revealed several examples of cancer-specific alterations in the expression of apoptosis-regulatory proteins. Figure 1 shows some examples of the immunostaining results and Fig. 2 summarizes the data. The mean intensity of immunostaining was significantly higher in the invasive cancer compared with normal colonic epithelium for all investigated proteins (Fig. 1C, E-F, KM, O, P) with the exception of Bcl-2, Bax, and AIF (Fig. 1G and H). Moreover, whereas immunostaining results varied widely among specimens examined, the immunoscores for a portion of the cancer specimens clustered into groups displaying clear elevations in immunoreactivity when compared with normal specimens, including cIAP1, cIAP2, XIAP, Survivin, Bcl-X_L, BAG1, p53, and MIB-1 (Fig. 2).

Immunoblot analysis of apoptosis regulators in colon carcinoma. To corroborate the immunohistochemical data, five frozen colon cancer specimens from the same cohort were identified that had sufficient amounts of both adjacent normal (N) and tumor (T) tissue for immunoblot analysis using antibodies specific for IAPs, Apaf1, and other proteins. Detergent lysates of these tissue specimens were prepared and normalized for total protein content before SDS-PAGE/immunoblot analysis (Fig. 3A). Densitometry analysis was also done to quantify band intensities, and the results from the loading control blot were used to normalize all data (Fig. 3B). Higher levels of cIAP2, XIAP, Survivin, and Apaf1 were detected in every specimen evaluated compared with case-matched normal tissue. Levels of cIAP1 protein, as well as the antiapoptotic protein TUCAN, were elevated in some tumor specimens compared with normal but not others. These (Fig. 3A) and other (Fig. 3C) immunoblot data also confirmed the specificity of the antibody reagents. Although done on small number of samples, the data presented here and elsewhere (21) confirmed monospecifity of the applied antibodies and corroborated the immunohistochemical differences seen in normal and malignant colon.

Relation of apoptosis biomarkers to microsatellite instability, tumor location, and other patient information. In several prior studies of early-stage colon cancer, MSI negativity, male sex, left-sided tumors, and older patient age were associated with unfavorable outcome or inadequate response to adjuvant chemotherapy (22–24). Therefore, in the investigated cohort, we compared the expression of apoptosis biomarkers with these tumor and patient characteristics to preliminarily explore, if differences in expression of these proteins might be related to the known differences in the clinical behavior of colon cancers.

Comparison of MSI (n = 22, 21%) versus microsatellite stable (n = 83, 79%) tumors revealed statistically significant differences in the immunopercentage data for cIAP1, cIAP2, TUCAN, and p53 staining (Fig. 4A) but not Apaf1, XIAP, Survivin, AIF, SMAC, Bcl-2, Bax, Bcl-X_L, Bid, BAG1, β-Catenin, or MIB-1, using an unpaired t test and a threshold of $P \leq 0.05$ without correction for multiple comparisons. Significant differences in immunoscore data were also observed for cIAP1, cIAP2, XIAP, TUCAN, Bax, and β-Catenin (Fig. 4B), using a $P \leq 0.05$ as a cutoff for unpaired t tests, again without correction for multiple comparisons.

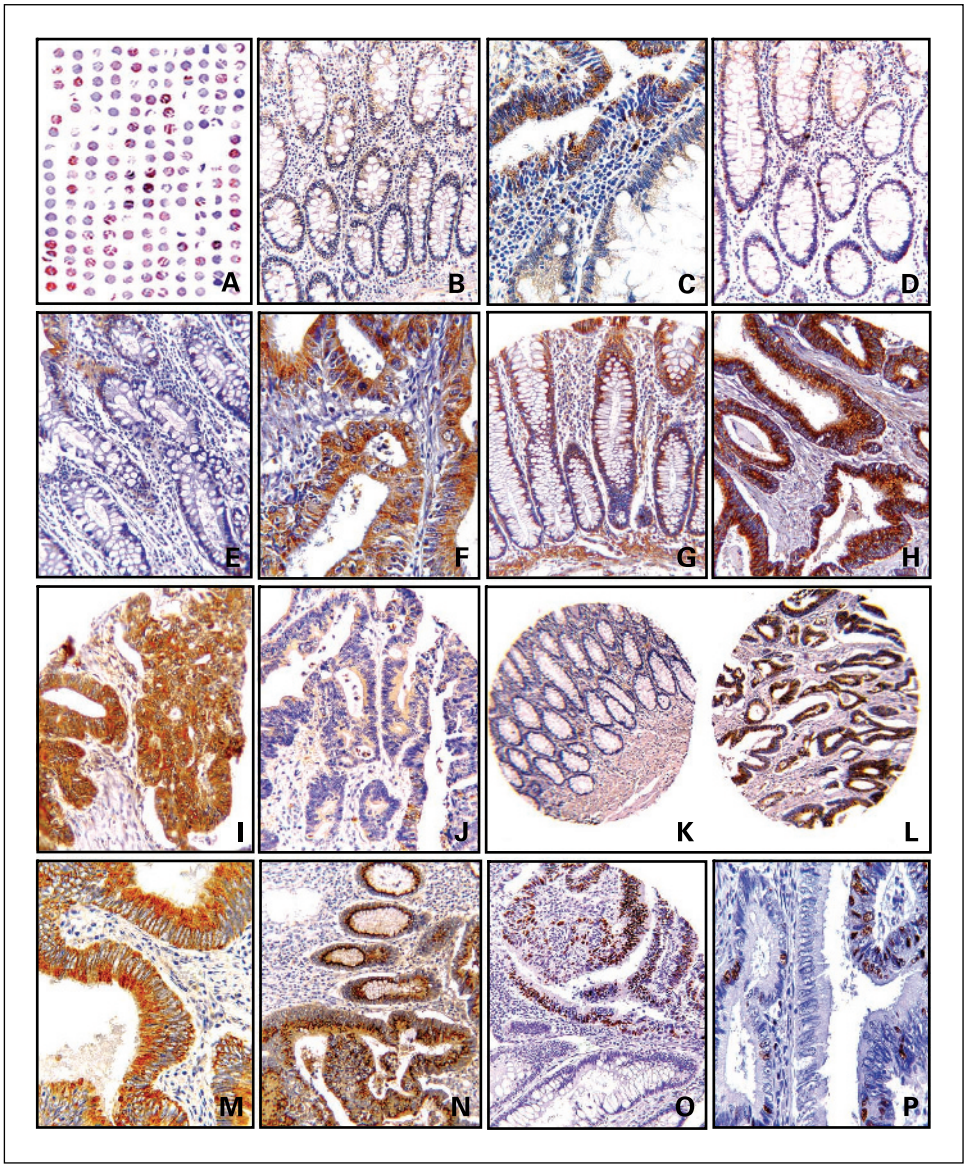

Fig. 1. Examples of immunohistochemical analysis of apoptosis proteins expression in normal and malignant colon. Tissue microarray blocks were prepared using 1-mm-diameter punches from 106 colorectal carcinoma specimens. Tissue sections were immunostained using various antisera followed by detection using a HRPase-based method with diaminobenzidine calorimetric substrate (*brown*). Nuclei were counterstained with hematoxylin (*blue*). Representative data: A, colorectal cancer microarray slide stained for cIAP2. Magnification, ×5. Examples of immunostaining of normal colonic epithelium are presented for cIAP1 (*B*, ×100), Survivin (*D*, ×150), SMAC (*E*, ×150), AIF (*G*, ×150), and TUCAN (*K*, ×20). Immunostaining results in regions of invasive cancer are shown for SMAC (*F*; ×400), AIF (*H*, ×250), Apaf1 (*I-J*, ×200), TUCAN (*L*, ×20; *M*, ×400), and Bcl-2 (*N*, ×150). Examples of malignant and the adjacent normal colonic epithelium are presented for cIAP2 (*C*, ×40), p53 (*O*, ×150), and MIB-1 (*P*, ×400). The specificity of these immunostaining results was confirmed by control stainings done using either preimmune serum or immune antisera, which had been preabsorbed with the relevant immunogens (data not shown).

A similar comparison was done for left-sided (n = 76, 72%) versus right-sided (n = 30, 28%) tumors, because tumors arising in these different anatomic locations are thought to arise via different mechanisms (23). In the investigated cohort, immunopercentage data were significantly higher in left-sided compared with right-sided tumors for TUCAN and p53 (Fig. 4A), the latter observation consistent with prior reports (25). Immunoscore data were significantly higher for Bcl-X_L in left-sided compared with right-sided tumors, whereas Apaf1 was significantly lower in left-sided compared with right-sided tumors (Fig. 4B).

Correlations of apoptosis biomarkers with patient survival. To correlate apoptosis biomarkers with patient survival, we empirically dichotomized immunostaining at the median, comparing the survival of patients whose tumor immunoscores were above the median with those below the median. A traditional method for dichotomizing p53 data was used, where immunopercentage of >20 % is considered positive (26), but similar results were obtained by using values between 5% and 25% (data not shown). Note that increases in p53 immunostaining can be due to several causes, including mutations that stabilize the p53 protein, loss of Mdm2 expression, alterations in p53 phosphorylation, and other events (reviewed in ref. 27). For univariate survival analysis, the Kaplan-Meier method was applied, and the differences between survival curves were assessed by the log-rank test.

In the investigated cohort, significant correlations were observed between shorter OS and immunoscores indicative of higher TUCAN expression ($P < 0.0001$), higher cIAP2 (P = 0.003), lower Apaf1 (P = 0.0003), lower Bcl-2 (P = 0.001), and lower SMAC (P = 0.04; Fig. 5A). Univariate analysis also showed significant correlations of shorter OS with immunopercentage data indicative of higher TUCAN ($P < 0.0001$), higher cIAP2 (P = 0.0002), higher p53 (P = 0.03), lower Apaf1 (P = 0.01), and lower Bcl-2 (P = 0.0004) immunopositivity (Fig. 5B). In contrast, significant correlations with survival were not found for biomarkers cIAP1, XIAP, Survivin, AIF, Bcl-X_L, Bax, Bid, BAG1, β-Catenin, or MIB-1 (data not shown).

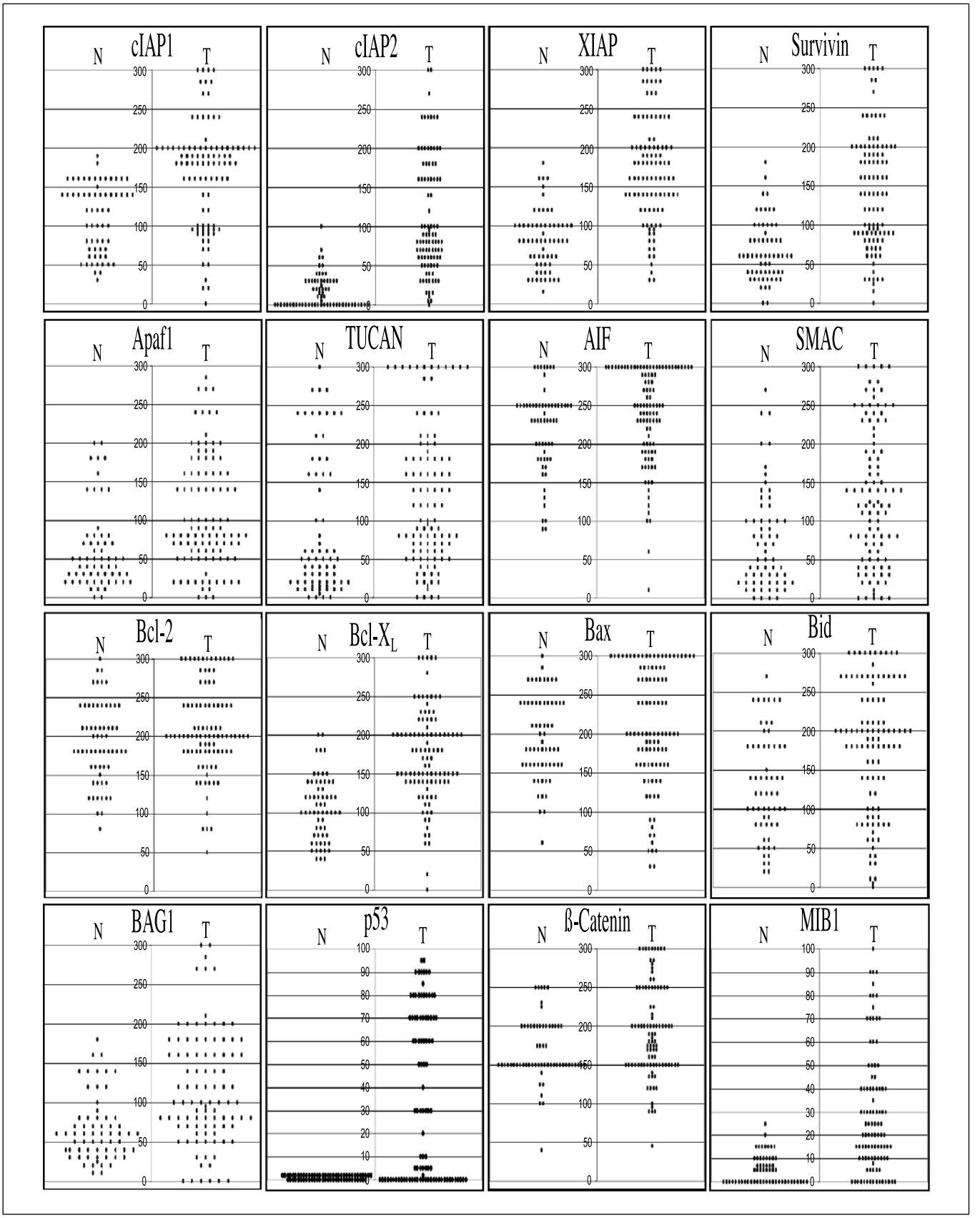

Fig. 2. Comparison of immunoscores for normal and malignant colon tissue. Immunoscores (*y-axis*) for normal and malignant colon epithelium are summarized as dot histograms.

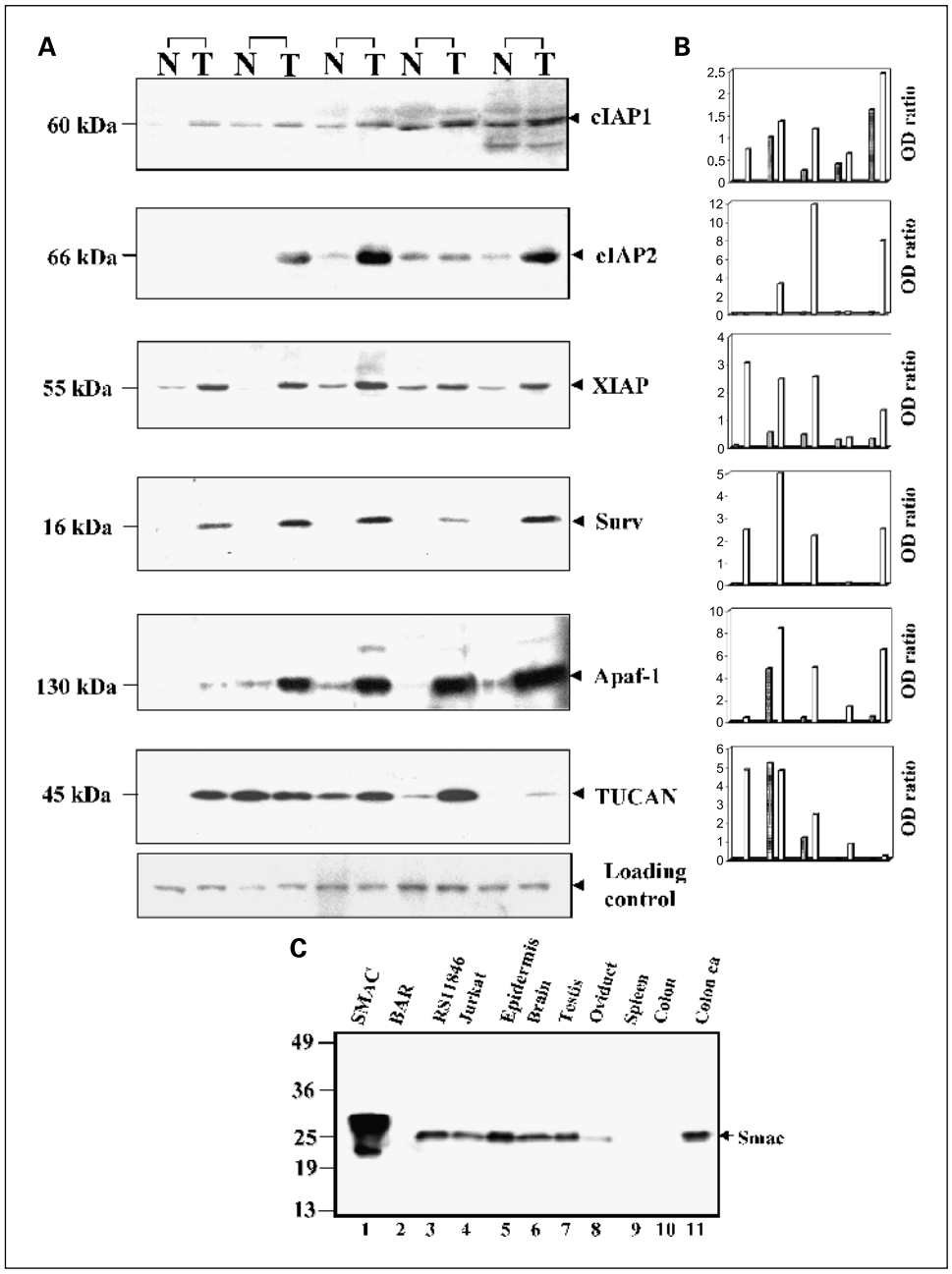

Fig. 3. Analysis of apoptosis protein expression in colon carcinoma by immunoblotting. *A*, lysates from five matched pairs of colon carcinoma (*T*) and normal colonic mucosa (*N*) specimens were normalized for total protein content (100 μg per lane) and subjected to SDS-PAGE/immunoblot analysis, using the antisera specific for c-IAP1, c-IAP2, XIAP, Survivin, Apaf-1, and TUCAN. *B*, immunoblot data were quantified by scanning-densitometry using Pro-Image software. Data are expressed as arbitrary densitometric units. Data was transformed to percentages of the densitometric levels observed on scans from loading control. A nonspecific band obtained during preblocking procedure with a secondary ECL antibody (Bio-Rad), served as a loading control. *C*, an example is provided of the analysis done to verify specificity of antibodies. Incubation with SMAC antiserum detected only SMAC *in vitro* – translated protein. Detergent lysates were prepared from various human tissues, normalized for total protein content (50 μg), and subjected to SDS-PAGE/immunoblot assay using antiserum specific for SMAC. Molecular weight markers are indicated in kilodaltons. Antisera were raised against recombinant proteins and synthetic peptides for immunodetection of various apoptosis-relevant proteins.

Furthermore, MSI positivity, right-sided tumor location, and younger age were associated with significantly better overall survival (Fig. 5C), consistent with prior reports (28, 29).

Calculation of HRs (with 95% CI) showed a relative risk of death due to colorectal cancer for patients with immunoscore (apoptosis markers) or immunopercentage (p53) data above the median ("high") in this cohort as follows: TUCAN (HR, 6.57; 95% CI, 2.71-15.96), cIAP2 (HR, 3.19; 95% CI, 1.48-6.85), p53 (HR, 2.28; 95% CI, 1.09-4.77), Apaf1 (HR, 0.26; 95% CI, 0.12-0.56), Bcl-2 (HR, 0.28; 95% CI, 0.14-0.58), and SMAC (HR, 0.46; 95% CI, 0.22-0.97; Table 1). Thus, of the 16 biomarkers evaluated, six showed correlations with patient survival in this cohort of stage II colorectal cancer patients treated with surgery alone.

Similar correlations with survival were obtained for TUCAN, cIAP2, Apaf1, and Bcl-2 when MSI-positive tumors were excluded from the analysis (data not shown) thus excluding bias that might arise from representation of tumors that arise through an alternative pathway compared with most colon cancers. Similarly, when the analysis was limited to left-sided tumors, TUCAN, cIAP2, Apaf1, and Bcl-2 remained significantly associated with OS (data not shown).

Multivariate analysis of apoptosis biomarker association with patient survival. A Cox proportional hazards model was used to evaluate whether apoptosis biomarkers showed independent prognostic significance when MSI status, tumor location, gender, and patient age were included as variables (Table 1). In multivariate analysis, TUCAN, cIAP2, Apaf1, and Bcl-2 maintained independent prognostic significance ($P < 0.0001$,

P = 0.009, P = 0.003, and P = 0.0009, respectively) in the investigated cohort. HR calculations indicated a relative risk of death from colorectal cancer at 7.7 times higher for high TUCAN tumors and 2.9 times higher for high cIAP2 compared with a reduced hazard rate of ~75% for high Apaf1 and high Bcl-2 tumors (Table 1).

Combinations of biomarkers. Because certain apoptosis proteins showed significant prognostic value, we asked whether combining pairs of biomarkers more accurately identified subgroups of patients with distinct survival characteristics compared with use of only a single biomarker.

Accordingly, we compared patients with two favorable variables (e.g., low cIAP2 and high Apaf1) versus all other patients in this cohort. Of the 94 patient samples successfully analyzed for cIAP2 and Apaf1, 25 (27%) had both low cIAP2 and high Apaf1 immunoscores. All (100%) of these patients were alive at 5 years after diagnosis compared with only 60% alive among the other patients (P = 0.00001; Fig. 5D). Combining the two favorable variables of low cIAP2 and low TUCAN produced similar results. Among patients with a combination of low cIAP2 and low TUCAN, 97% were alive, compared with only 60% alive among the other patients (P = 0.00003; Fig. 5D). Likewise, 3% of the patients whose tumors expressed a combination of TUCAN^{low} and Bcl-2^{high} (two favorable biomarkers) died of colorectal cancer compared with 45% of the others. However, when patients with two adverse biomarkers (low Apaf1 and high TUCAN) were compared with other patients, only 37% of patients with this combination of proteins remained alive compared with 83% of others at 5 years after diagnosis (P < 0.0001; Fig. 5D). The discrepancy was even larger at 8 years of follow-up, with 0% versus 82% overall survival for the Apaf1^{low} plus TUCAN^{high} group compared with other patients. Examination of combination data for another pair of adverse biomarkers (cIAP2^{high} plus Bcl-2^{low}) revealed that 17% of the patients were alive in this group 5 years after surgery compared with 76% of others (P = 0.002; Fig. 5D). Thus, pairwise combinations of certain apoptosis biomarkers identified groups of patients at extremely high or low risk of relapse within this cohort of patients with early-stage colorectal cancer.

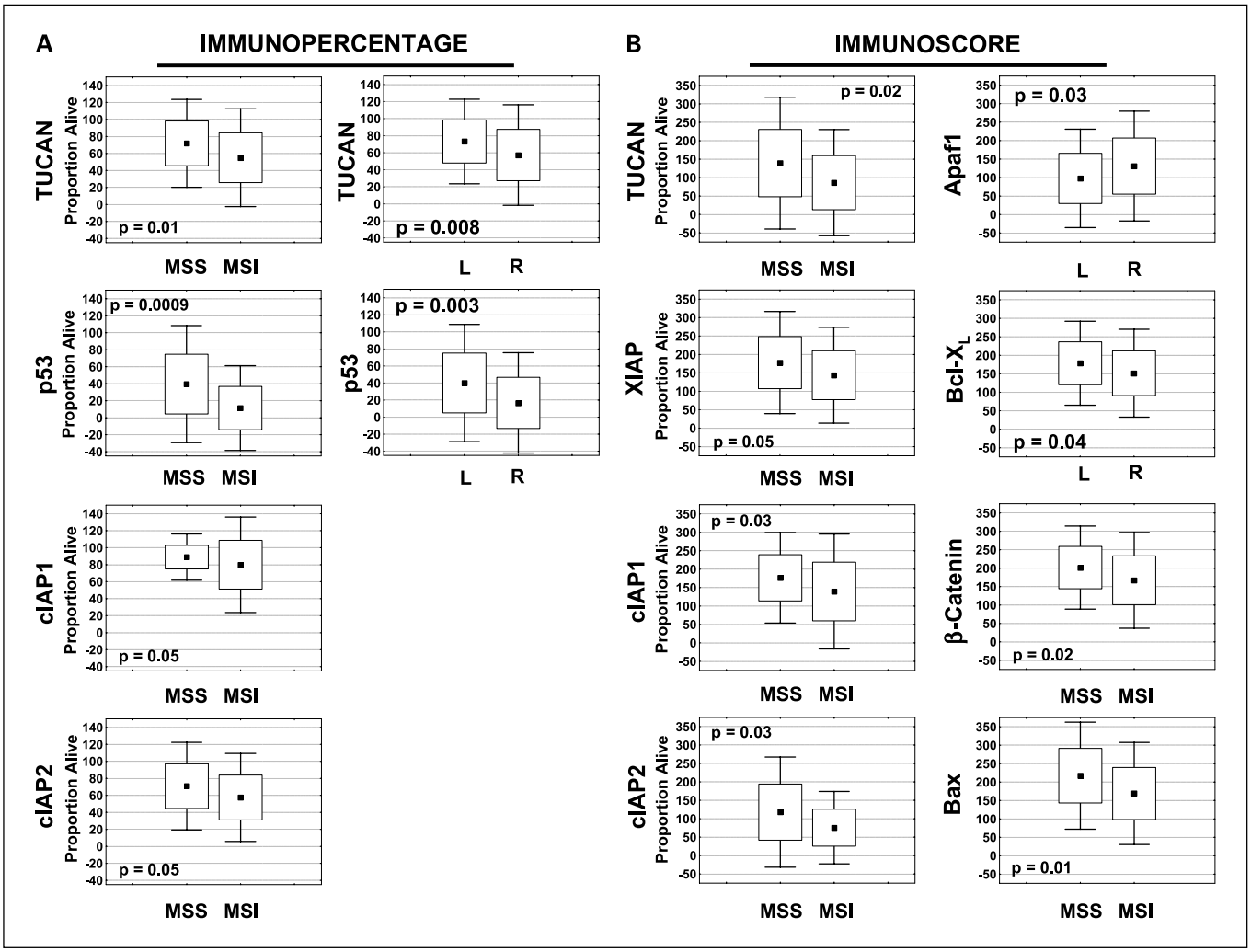

Fig. 4. Correlations of protein immunopercentage (*A*) and immunoscore (*B*) with MSI status and anatomic location of colorectal carcinomas. Box and whisker plots display the distribution of immunopercentage (*A*) and immunoscore (*B*) data for apoptosis-relevant proteins expressed in colorectal tumors. The mean immunopercentage/immunoscore is plotted as a middle point. *Box*, ±SE; *whiskers*, ±SD. The ANOVA test was used for statistical comparison of immunostaining data in MSI versus microsatellite stable (*MSS*) or in left-sided (*L*) versus right-sided (*R*) tumors.

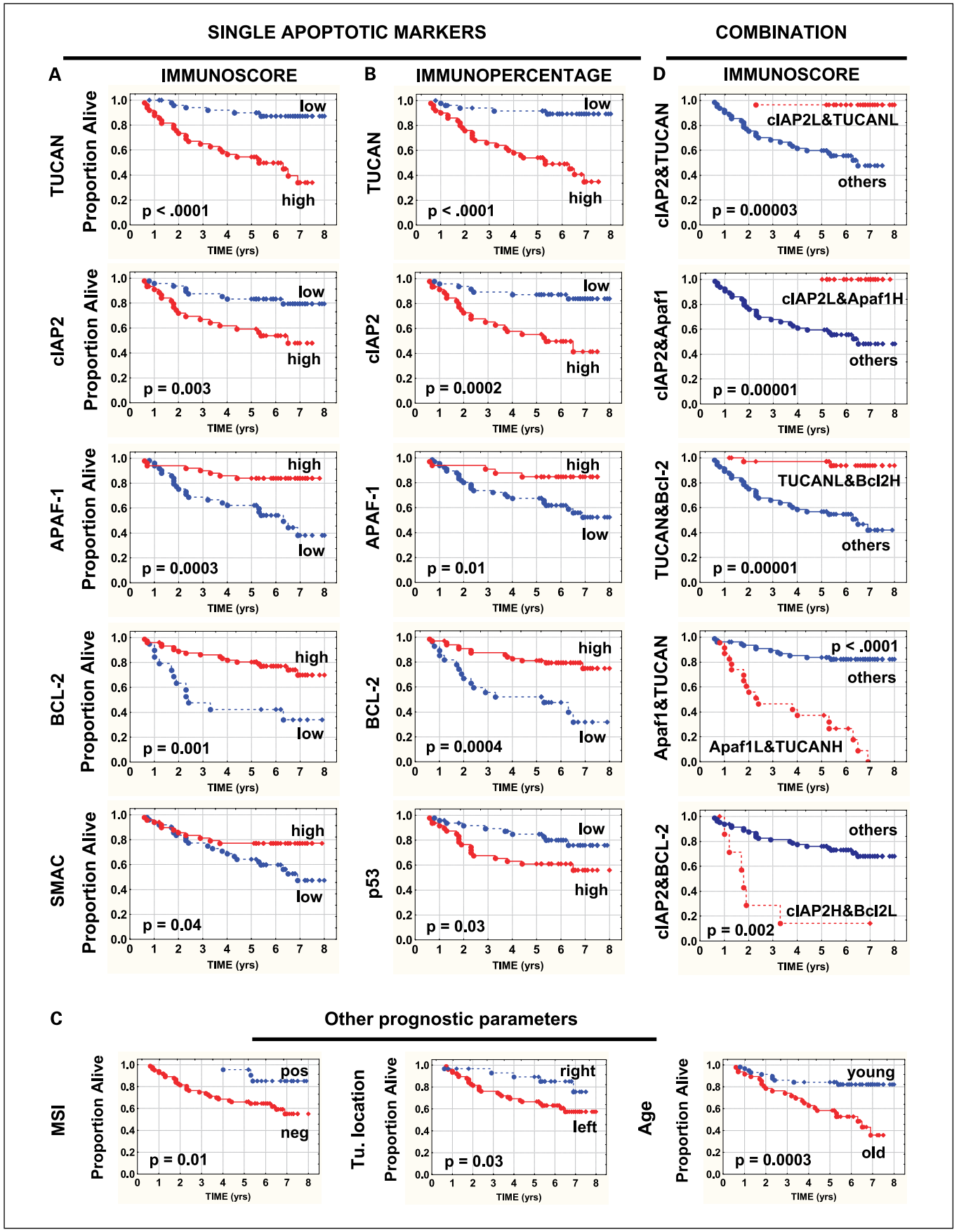

Table 1. Univariate and multivariate Cox proportional hazards analysis of patients with stage II colorectal cancer managed without adjuvant chemotherapy

Cox proportional hazards analyses					
Univariate			Multivariate		
Factor	HR (95% CI)	*P*	Factor	HR (95% CI)	*P*
MSS	3.749 (1.132-12.417)	0.03			
Age	3.671 (1.749-7.705)	0.0006			
Left side	2.622 (1.013-6.782)	0.05			
TUCAN	6.570 (2.706-15.956)	0.00003	TUCAN	7.658 (3.073-19.911)	<0.0001
cIAP2	3.188 (1.483-6.853)	0.003	cIAP2	2.908 (1.298-6.516)	0.009
p53	2.278 (1.088-4.768)	0.03			
Apaf1	0.255 (0.116-0.560)	0.0007	Apaf1	0.284 (0.125-0.647)	0.003
Bcl-2	0.282 (0.136-0.584)	0.0007	Bcl-2	0.251 (0.111-0.567)	0.0009
SMAC	0.464 (0.222-0.970)	0.04			

NOTE: Only those variables showing statistical significance ($P \leq 0.05$) are presented. All factors that showed prognostic significance in the univariate Cox analysis were included in the multivariate Cox proportional hazards model. Only significant results ($P \leq 0.05$) are presented.

Discussion

Differences in long-term survival of patients with early-stage, resectable colon cancer are most often ascribed to the presence or absence of micrometastases that are undetectable at the time of diagnosis. Defects in apoptosis can contribute to a wide variety of aggressive tumor phenotypes, including conferring an ability of tumor cells to survive after detachment from extracellular matrix and thus facilitating metastasis (reviewed in ref. 30). Tumor cells with resistance to apoptosis have also recently been shown to survive (and grow) intravascularly, when adherent to endothelial cells in distal capillary beds (31). For these reasons, we examined the expression of apoptosis-regulatory proteins in early-stage colorectal cancers, making associations with patient survival.

The purpose of this study was to determine whether alterations in the expression of apoptosis-regulatory proteins occur in colorectal cancers and to explore whether changes in apoptosis gene expression alone or in conjunction with other biomarkers, such as p53, β-Catenin, and MIB-1, might be predictive of survival in stage II colorectal cancer patients.

We found tumor-associated alterations in the expression of multiple apoptosis-relevant proteins in colorectal tumors, based on comparisons with adjacent morphologically normal colonic epithelium. One caveat in interpreting such comparisons of transformed and normal mucosa, however, is that genetic or epigenetic lesions could be present in adjacent epithelium, which seems normal in morphology (32). Nevertheless, our findings suggest that alterations in the expression of several IAP family proteins (XIAP, cIAP1, and cIAP2) as well as Apaf1 occur commonly in colon cancers, providing insights into the pathogenesis of this disease. It should be recognized however that the activity of many of the proteins evaluated here can be influenced by posttranslational modifications; thus, the levels of protein do not necessarily correlate with protein activity.

The mechanisms responsible for these alterations are a matter for speculation, but recent data suggest some possibilities. For example, the transcription factor nuclear factor-κB has been reported to induce expression of cIAP1, cIAP2, and XIAP in some types of cells (33), suggesting that tumor-associated increases in nuclear factor-κB activity could play a role in dysregulating expression of several IAP family genes. Indeed, the gene encoding cIAP2 contains several consensus binding sites for nuclear factor-κB in its promoter region, and induction of cIAP2 expression following nuclear factor-κB activation is consistently observed in multiple cell types. For Apaf1, it has been shown that retinoblastoma tumor suppressor protein (pRB) and E2F regulate the expression of this important proapoptotic protein (34). The Apaf1 gene promoter contains E2F-binding sites and is a direct transcriptional target of certain E2F family transcription factors (34). Because E2F family proteins are suppressed by pRB (reviewed in ref. 35), our finding that Apaf1 protein levels are elevated (compared with normal colon) in roughly two thirds of colorectal carcinomas raises the possibility that the pRB pathway may be functionally inactivated in these tumors thus resulting in deregulation of E2F and overexpression of Apaf1. However, in contrast to increased Apaf1 protein levels observed in our study, loss of heterozygosity of the chromosomal region encompassing the *APAF1* gene at 12q23 has been reported during progression of colorectal cancer, in association with reduced levels of Apaf1 mRNa (36). Thus, *APAF1* expression may be influenced by multiple mechanisms in colorectal cancers.

Fig. 5. Correlations of single and combined apoptotic biomarkers, MSI, age, and tumor location with overall survival of colorectal cancer patients. Immunoscore (*A*) and immunopercentage (*B*) data for selected apoptotic proteins were dichotomized into high (*red*) versus low (*blue*) expression groups based on the median values; <20% of p53 positive cells were used as cut-off for analysis of p53 immunostaining. *C*, based on PCR analysis, tumor samples were scored as exhibiting high-frequency MSI or as microsatellite stable (*MSS*). Right-sided tumors were defined as those originating proximal to the splenic flexure, and left-sided as those arising distal to this site. Median age was used to dichotomize patients into older versus younger groups. The Kaplan-Meier method was applied to generate the survival curves, and univariate survival distributions were compared by the log-rank test. Pairwise combinations of apoptosis biomarkers were compared with OS for colorectal cancer patients (*D*).

In this study, we characterized the expression of apoptosis biomarkers in cohorts of early-stage colorectal cancer patients who did not receive standard 5-fluorouracil-based adjuvant chemotherapy after surgery. The survival benefit of chemotherapy in stage II disease is debated, ranging from reports claiming 25% to 30% reduction in mortality (37) to other studies negating significant survival gain (38). Currently, American Society of Clinical Oncology does not recommend the routine use of adjuvant chemotherapy for patients with stage II colon cancer (38). Nevertheless, Surveillance, Epidemiology, and End Results Medicare data suggest that 27% of stage II patients do receive adjuvant chemotherapy in the United States (39). It is difficult to know which early-stage patients benefit from 5-fluorouracil-based chemotherapy versus which patients may have been unnecessarily subjected to the toxicity of chemotherapy. A need therefore exists for practical laboratory tests that can be applied to early-stage colon cancers for dichotomizing individuals into low-risk versus high-risk groups, thereby providing a rational basis to the postoperative management of these patients. By retrospectively investigating a cohort of patients who did not receive adjuvant chemotherapy, we sought to gain insights into the relevance of certain biomarkers to clinical outcome, focusing on apoptosis as a pathway for interrogation.

Although only an exploratory study, we found that altered expression of cIAP2, TUCAN, Apaf1, and Bcl-2 exhibited the strongest correlations with OS in this cohort of patients. In univariate and multivariate analyses, patients with tumors displaying low levels of cIAP2 or TUCAN and high levels of Apaf1 or Bcl-2 enjoyed longer OS compared with those with $cIAP2^{high}$, $TUCAN^{high}$, $Apaf1^{low}$, or $Bcl\text{-}2^{low}$ tumor phenotype. The survival of these patients with favorable apoptosis biomarker profiles seems to have been similar regardless of their MSI status, tumor location, age, or gender.

Furthermore, analysis of a variety of pairwise combinations of apoptosis biomarkers suggested that combining a relatively small number of variables might be adequate to achieve strong prognostic power. For example, ~28% of the tumors analyzed had both high TUCAN and low Apaf1, two adverse biomarkers. Among patients whose tumors expressed $TUCAN^{high}$ and $Apaf1^{low}$, only 37% of patients remained alive compared with only 83% of others at 5 years after surgery. Thus, the combination of $TUCAN^{high}$ and $Apaf1^{low}$ may identify a subset of early-stage colorectal cancer patients at particularly high risk of relapse and thus proposed for adjuvant chemotherapy. Similarly, the combination of high Apaf1 and low cIAP2, two favorable biomarkers, revealed that none of the patients with this tumor characteristic relapsed after surgery (with 5-year median follow36-up) compared with 60% of others. Likewise, 3% of the patients whose tumors expressed a combination of low TUCAN and high Bcl-2 (two favorable biomarker results) died of colon cancer compared with 45% of the others. Thus, patients whose tumors display either an $Apaf1^{high}$ plus $cIAP^{low}$ or a $TUCAN^{low}$ plus $Bcl\text{-}2^{high}$ phenotype may not benefit from adjuvant chemotherapy.

It is interesting to put these results obtained for apoptosis biomarkers into the context of previous investigations of these and other biomarkers in early-stage colorectal cancer. For example, among IAP family proteins, higher levels of Survivin immunostaining were previously correlated with shorter survival in a study of patients with Dukes' stage B patients who did not undergo preoperative or postoperative chemotherapy (40). In contrast, we failed to observe a significant correlation of Survivin with patient outcome but used different methods for dichotomizing data. Several prior studies have examined the prognostic significance of Bcl-2, finding an association of this biomarker with favorable outcome (41–43). Association of this antiapoptotic protein with longer OS may be a reflection either of the ability of Bcl-2 to be converted from a protector to a killer through interactions with other proteins (44), or related to the unexplained role of Bcl-2 in suppressing cell cycle entry (45, 46). Of the other Bcl-2 family members assessed here (e.g., Bcl-X_L, Bax, and Bid), only Bax has been examined previously in stage II patients, where expression of this protein was found to be associated with higher risk of local relapse. The role of tumor suppressor p53 as an indicator of aggressive disease is well documented (reviewed in ref. 47), and our data support a role for immunohistochemical evaluation of p53 as a promising prognostic marker in early-stage colorectal cancer among patients who did not receive chemotherapy (48).

The other biomarkers evaluated here, SMAC and AIF, have not heretofore been studied in colorectal cancer patients.

Approximately one sixth of unselected colon tumors accumulate hundreds of thousands of somatic mutations in microsatellite sequences (49). MSI-positive tumors display a mutator phenotype characterized by an over-two-orders-of-magnitude higher mutation rate than normal cells (49, 50). Within our study population, patients with MSI-positive tumors had a significant survival advantage over individuals with MSI-stable cancers, thus confirming results reported by others (reviewed in ref. 51). The molecular basis for these differences in outcome for MSI-positive tumors is only partly understood. Interestingly, MSI positivity was associated in the investigated cohort with significantly lower expression of antiapoptotic proteins cIAP1, cIAP2, and TUCAN, and with lower expression of proapoptotic protein Bax. It remains to be determined whether differences in expression of these apoptosis proteins contribute to the superior survival of patients with MSI-positive tumors.

These findings based on retrospective analysis of non-randomized archival tumor material from a single Asian institution suggest that further studies are warranted to determine whether apoptosis biomarkers can discriminate populations of early-stage colorectal cancer patients benefiting from versus not requiring adjuvant chemotherapy in other settings. Although multiple variables undoubtedly contribute to clinical outcome for patients diagnosed with early-stage colorectal cancer, these preliminary findings indicate that cIAP2, Apaf1, TUCAN, and Bcl-2 deserve further evaluation as potential prognostic markers. If confirmed in prospective studies, such prognostic biomarkers may help to guide treatment decisions for colorectal cancer patients, particularly regarding use of adjuvant chemotherapy.

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