Altered Subcellular Localization of Suppressin, a Novel Inhibitor of Cell-Cycle Entry, Is an Independent Prognostic Factor in Colorectal Adenocarcinomas


ABSTRACT

Purpose: Suppressin (SPN), a novel inhibitor of the entry into the cell cycle, has properties of a tumor suppressor gene; however, its role in the development and progression of a human malignancy is not studied. Therefore, we evaluated the status of spn and its prognostic value in human colorectal adenocarcinoma (CRC).

Experimental Design: Inhibition of cell proliferation by exogenous/extracellular SPN was assessed by [3H]thymidine incorporation. The genetic status of spn in two colon cancer cell lines (LS180 and WiDr) and in a human CRC was determined using direct cDNA sequencing techniques. Phenotypic expression of SPN was evaluated in 105 CRC archival tissues using immunohistochemical methods. Univariate Kaplan-Meier and multivariate Cox proportional hazards models were used to determine the prognostic significance of SPN expression.

Results: Exogenous SPN inhibited the proliferation of the LS180 cell line, which also has a mutation in one allele of the spn gene. The spn gene was also mutated in the primary CRC. Expression of SPN was primarily cytoplasmic in non-mucinous CRCs and nuclear in mucinous CRCs. However, the evaluation of 85 nonmucinous CRCs demonstrated that nuclear localization of SPN, nuclear accumulation of p53, and nodal status were independent prognostic indicators with hazard ratios of 2.34, 2.33, and 3.04, respectively. Nuclear localization of SPN plus nuclear accumulation of p53 formed a stronger prognostic indicator (hazard ratios = 5.45) than local nodal status.

Conclusions: This is the first report of genetic alterations in the spn gene in a human malignancy and suggests that genetic alterations in spn and the resulting immunohistochemical phenotypes based on SPN subcellular localization in CRCs may be useful in determining prognosis of patients with subtypes of CRC.

INTRODUCTION

Although several genetic alterations in genes that regulate cell cycle progression and DNA damage checkpoint control are implicated in the progression of sporadic CRCs, there is some evidence implicating genetic alterations in genes that are associated with the regulatory functions of cells entering into the cell cycle (1). Several recent studies by LeBoeuf et al. (2–5) have identified spn as a novel negative regulator of the cell cycle and demonstrated that the exogenous/extracellular SPN inhibit the proliferation of several tumor cell lines by arresting cells in the G0 or G1 phase.

SPN is a monomeric protein with an apparent molecular mass of 63 kDa by SDS-PAGE analysis that has all of the characteristics of a global negative regulator of cell proliferation. Studies, specifically in lymphocytes, have demonstrated that SPN arrests cells in the G0/G1 phase of the cell cycle after reduction of their RNA, protein, and DNA synthesis (global), suggesting that SPN inhibits the process required for G0 transition to G1 (2, 3, 6, 7). Both rat and human spn cDNA have been cloned and sequenced (GenBank accession nos. RNU59659 and AF007165), and they show high sequence conservation. SPN is highly homologous to deformed epidermal regulatory factor-1. Sequence comparisons between SPN and deformed epidermal regulatory factor-1 gene in Drosophila (2). Recent studies by Zeng et al. (7) and Guldner et al. (8) reported sequence homology of SPN at the protein level with a glucocorticoid modulatory element binding factor-2 protein and with a splice variant of the nuclear dot-associated Sp100 protein.

The results of cell-cycle analysis and neutralization experiments indicate that SPN is a negative regulator of cell cycle entry (4). Many cell types secrete SPN and high levels of extracellular SPN inhibit cellular proliferation (2–5). Thus, somatic mutations in the spn gene resulting in functional dysregulation of SPN could confer a growth advantage to the mutated cell that may have clinical implications in the development and progression of neoplasia. The spn gene has been mapped to human chromosome 11p15.5, which is a telomeric region that is associated with loss of heterozygosity in a variety of human tumors, but the causative genes have not been identified; how-
ever, this region of the chromosome is not affected frequently in CRC (9–16).

In this study we report that genetic abnormalities in the spn gene may be clinically relevant. Specifically, we show that: (a) SPN is involved in regulating proliferation of colonic cells; (b) the spn gene is mutated in a colorectal tumor cell line and in a primary CRC; and (c) altered subcellular localization of SPN in CRCs is a prognostic factor that identifies a subgroup of patients with reduced survival. These functional roles of SPN are similar to the tumor suppressor gene p53. Therefore, we examined the association of the p53NAC plus altered SPN subcellular localization with clinical outcomes of patients particularly with nonmucinous CRCs. Moreover, the evaluation of multiple markers in human tumors provides increased ability to predict the patient prognosis.

MATERIALS AND METHODS

SPN Preparation and Purification. SPN was extracted and affinity purified to >95% purity from frozen bovine pituitaries (Pel Freeze) using a monospecific anti-SPN polyclonal antibody as described previously (6). Purity of SPN preparations was assessed by SDS-PAGE followed by visualization with silver staining, and the purity of SPN preparations was >95%.

Cell Lines and Proliferation Assays. To identify the functional role of SPN in tumors with wild-type and mutated p53 gene, we selected one cell line from each category. The cell line WiDr is known to express abnormal p53 protein and exhibit missense point mutation in the p53 gene at codon 273, whereas the cell line LS180 expresses wild-type p53. These human colorectal tumor cell lines, LS180 and WiDr, were obtained from American Type Culture Collection (Rockville, MD) and maintained in the recommended medium and supplements. Proliferation assays (3H-thymidine incorporation) were performed in 96-well microtiter tissue culture plates (Falcon Plastics, Madison, SD) with and without purified SPN as described previously (2). Three to six replicates were determined for each treatment, and the reduction in proliferation was expressed as a percentage of the mean control [3H]thymidine incorporation. SEs from experimental control and treatment replicates ranged from 8 to 15% of the mean obtained for a replicate.

RNA Extraction, PCR, and Direct Sequencing of SPN cDNAs. Total cytoplasmic RNA was extracted from colorectal cell lines and tissues with a RNAeasy kit (Qiagen, Chatsworth, CA). Total RNA (2–5 μg) was used as template in oligo (dT)-primed first strand cDNA synthesis with SuperScript RT (Life Technologies, Inc.) as described previously (17). All of the PCR was performed using the proofreading thermostable DNA polymerase Tth XL (Perkin-Elmer, Norwalk, CT). Reaction products were resolved by agarose gel electrophoresis and purified using a Qiaquick gel extraction kit. Gel-purified reaction products were used as template in cycle-sequencing reactions (Perkin-Elmer), and sequencing reaction products were resolved on an ABI Prism 307 Automated DNA sequencer (Iowa State University Sequencing Facility, Ames, Iowa). Seven spn-specific sequencing primers were used to obtain the complete nucleotide sequence of both strands of the entire human spn coding sequencing of the human spn cDNA. SPN cDNA sequence compilation and analysis were performed with the Ge-nepro program (Riverside Scientific, Brainbridge Island, WI). Nucleic acid changes in the spn cDNA sequence were confirmed by sequencing of the opposite strand. All of the spn cDNA sequences have been submitted to the GenBank/EBI Data Bank and are identified as follows: spn cDNA from LS180, AF068892 (contains stop codon), and AF068893 (codes for full-length SPN), spn cDNA from WiDr AF068894 and AF068895 (both code for full-length SPN), spn cDNA from human unmucinous colorectal mucosal tissue AF068897; and spn cDNA from human colorectal tumor tissue and unmucinous mucosal tissue AF068896.

Patients and Tissue Specimens. Formalin-fixed, paraffin-embedded, archival tissue blocks of CRCs were selected randomly from the University of Alabama at Birmingham Hospital and the affiliated Birmingham Veterans Administration Hospital. All of the patients had been diagnosed with primary CRCs (with or without distant metastasis), had undergone surgery between 1982 and 1993, and this sample contained only patients with “first primary” CRCs, the paraffin tissue blocks and the follow-up information of which were available. Survival or cause of death (whether it was attributed to CRC or not) was collected from hospital charts or tumor registries. H&E stained slides of all of the cases were reviewed for pathological stage, differentiation, and tumor type (mucinous or nonmucinous). Tumor-Node-Metastasis tumor staging was performed according to the American Joint Committee on Cancer criteria (18). Histological grade was coded as low, moderate, and high. Tumor typing was performed according to the WHO classification system (19). As per the WHO definition, mucinous CRCs were categorized as tumors ≥50% of cells as mucin producing. The anatomical location of tumors was identified using International Classification of Diseases for Oncology codes (20). The anatomical locations of tumors were grouped into proximal (cecum to the right two-thirds of transverse colon) and distal (left one-third of transverse colon to the rectum) as described earlier (21–24). The evaluation of SPN localization was performed blind to clinical outcome.

The number of mucinous tumors available was inadequate to correlate with clinicopathological parameters or to analyze the prognostic significance of SPN in mucinous tumors separately. In addition, mucinous tumors had a distinct pattern of SPN phenotypic expression; thus, the analysis of clinical outcome was restricted to nonmucinous CRC.

Immunohistochemical Analysis. Selected paraffin blocks representative of normal mucosa and invasive adenocarcinoma were sectioned at a 5-μm thickness and were mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). Tissue sections were cut 1–2 days before immunostaining to avoid potential problems in antigen recognition attributable to storage of cut tissue sections on glass slides (25). Immunohistochemical analysis was performed using a standard staining method described elsewhere (21, 26). SPN localization and accumulation were detected using the anti-SPN immunoglobulin M monoclonal antibody, 3F10 at 4 μg/ml, of which the specificity has been demonstrated and reported earlier (27). Preimmune goat serum was used to test for nonspecific staining and reactivity of the secondary detection system with the tissue (deletes or negative control). The remainder of the staining procedure was carried out using the BioGenex (San Ramon,
CA) Super Sensitive Biotin-Streptavidin Horseradish Peroxidase Detection kit (BioGenex) as described earlier (21). Positive control slides were CRC sections known to stain positively for SPN\textsuperscript{NAC} and p53\textsuperscript{NAC}. No differences were observed in the level of intensity or number of cells expressing SPN in colorectal tissues with or without antigen retrieval methods; therefore, antigen enhancement techniques were not used in this study. The data on p53\textsuperscript{NAC} was from our recent study (21). Antigen enhancement techniques were not used for p53, because potential problems have been reported in their use in CRC (21, 28).

Assessment of Expression of SPN and p53\textsuperscript{NAC}. Evaluation of the immunostaining was performed jointly by three observers (U. M., W. E. G., and A. R. F.) to limit bias. A semi-quantitative ISS for SPN expression was obtained as described previously (21). Immunostaining intensity of individual cells was scored on a scale from 0 (no staining) to +4 (strongest staining), and each observer estimated the proportion of cells stained at each intensity. For the SPN\textsuperscript{CYT}, the percentage of cells at each intensity was multiplied by the corresponding intensity value to obtain an ISS that ranged from 0 to 4. The combined scores of each observer were used to obtain an overall average ISS. An ISS of \(\geq 1.5\) was chosen as a cutoff value of positivity for SPN\textsuperscript{CYT}, and SPN\textsuperscript{NAC} was considered positive if \(\geq 25\%\) of tumor cell nuclei were immunostained at or above a low level of intensity. Cutoff values were chosen based on the SPN staining levels in histologically normal colorectal tissues. Tumors with only very weak (just detectable) staining intensity in any number of cells were considered to be negative.

Tumor cells with distinct nuclear immunostaining for p53 were considered positive, and the tumors were considered positive only if p53\textsuperscript{NAC} was \(\geq 10\%\) of all of the malignant cells in a section (21, 22).

Statistical Analysis. The \(\chi^2\) test was used to assess the univariate association of clinicopathological characteristics with SPN localization. The period from the date of surgery to the date of death or last contact (if alive) was used to perform survival analysis. Deaths because of unknown causes or of causes other than CRC were treated as censored observations at the time of death. The log-rank test was used to compare Kaplan-Meier univariate survival curves (29). Multivariate Cox regression survival analysis (30) was used to assess the prognostic significance of SPN\textsuperscript{NAC}, or SPN\textsuperscript{CYT} and p53\textsuperscript{NAC} with adjustments for other confounding clinical variables (age, sex, tumor location, tumor size, differentiation, and pT, pN, and M components of Tumor-Node-Metastasis stage). A stepwise model-building procedure was used to determine the significant factors in predicting colorectal cancer survival. HRs and 95% CIs were calculated for each significant factor in the final model. \(P\)s of \(< 0.05\) were considered to indicate statistical significance.

RESULTS
SPN Inhibits the Proliferation of Human Colorectal Tumor Cell Lines
We showed previously that exogenous SPN inhibits the proliferation of a variety of rat, human, and mouse tumor cell lines (6). To determine whether SPN affected the proliferation of human colorectal cancer cell lines, we added exogenous SPN to cultures of the LS180 and WiDr. The results of these experiments showed that inhibition of proliferation differed between these two cell lines (Fig. 1). Specifically, the LS180 cell line was much more sensitive (87% inhibition) to exogenous SPN, whereas the proliferation of WiDr cells was essentially unaffected except at the highest concentration of SPN.

Analysis of the SPN Transcript in LS180 and WiDr Cells
Differences in the sensitivity of tumor cell lines to exogenous SPN may indicate that one cell line produces more endogenous SPN than the other. Alternatively, it may be that one cell line contains a mutated or functionally inactive form of the \( spn \) gene, and the other does not, or there may be other types of dysregulation in the pathways related to SPN. To test this hypothesis, we analyzed the expressed transcripts of \( spn \) in LS180 and WiDr cells by direct DNA sequence analysis to permit the simultaneous sequence analysis of both expressed \( spn \) alleles if present. The results of DNA sequence analysis of the complete coding region of \( spn \) showed that, similar to our results on \( spn \) gene expression in other human cells and cell lines, both alleles of the \( spn \) gene were expressed in these two tumor cell lines. Both expressed \( spn \) transcripts in the WiDr cell line contained the appropriate SPN open reading frame to yield the entire deduced SPN amino acid sequence. However, in the
LS180 cell line, one transcript coded for the complete deduced SPN amino acid sequence, but the other transcript coded for a deduced truncated SPN protein. This SPN transcript had a mutation that produced a stop codon at Glu295 in the SPN amino acid sequence. Thus, the results of the molecular analysis of the spn gene in these two colorectal tumor cell lines indicated that differences in sensitivity to exogenous SPN correlated with lesions in the spn gene.

**SPN Subcellular Location and Extent of Accumulation in Normal and Malignant Colorectal Tissues**

**Benign Colorectal Mucosa.** Histologically normal appearing colonic epithelium (uninvolved mucosa) from patients with tumors had varying patterns of subcellular localization of SPN. In the basal areas of the crypts, in the bottom one third of the mucosal layer, staining ranged from faint to absent in either the cytoplasm (SPNCYT) or nuclei (SPNNAC) of most cells, and only a very few cells (<5%) demonstrated SPNNAC (Fig. 2A). In the upper one-third of colonic crypts the staining intensity for SPN and proportion of cells staining was increased compared with the lower one-third of the mucosa with ~20% of cells exhibiting SPNCYT and SPNNAC staining at moderate intensity (Fig. 2B). The strongest immunostaining for SPN in all of the areas of the mucosa was found in the superficial luminal epithelium (Fig. 2A) in which both SPNCYT and SPNNAC are present in ~40% of cells. Additionally, for a majority of cases examined, SPNNAC was greater than SPNCYT in these superficial epithelial cells. The non-neoplastic epithelium (“transitional mucosa”) adjoining adenocarcinomas had the same pattern of increased SPN staining from lower to higher layers of the epithelium (Fig. 2C).

In general, the pattern of subcellular localization of SPN in colonic epithelia correlates with proliferation. In the lower third of the mucosa where there are more cycling cells, most cells have low amounts of intracellular SPN in both the cytoplasm and the nucleus. In contrast, in cells in the upper third of the mucosa where there are fewer proliferating cells, there is greater accumulation of SPN in both the nucleus and cytoplasm with the SPNNAC greater than SPNCYT in most cells.

**SPN Subcellular Location and Extent of Accumulation in Colorectal Adenomas**

Tissue samples from 10 patients that contained adenomatous components contiguous with adenocarcinomas were examined separately to determine whether changes in SPN subcellular location and extent of accumulation of SPN in adenomatous lesions, which may be considered as precursors of CRCs. If there were no changes in the extent of accumulation and/or...
subcellular localization of SPN in adenomas compared with uninvolved epithelium, then we anticipated that adenomas should show reductions in both the extent of subcellular accumulation of SPN and the number of cells containing intracellular SPN because of the increased proliferation in adenomas compared with cells in uninvolved mucosa. This was not the case; the results of this analysis showed that 100% of the adenomatous components showed increased accumulation of both SPN\textsuperscript{NAC} and SPN\textsuperscript{CYT} and an increased proportion of cells with this pattern (50–60%) compared with uninvolved colorectal mucosa. Additionally, the proportion of SPN-positive cells and the intensity of SPN staining (accumulation) were both marginally higher in all 10 of the adenomatous components (approximately 50–60%) compared with matched contiguous CRC (approximately 35–45%). Lastly, in all of the adenomatous components evaluated, SPN\textsuperscript{NAC} and SPN\textsuperscript{CYT} were essentially equivalent.

**SPN Subcellular Location and Extent of Accumulation in CRC**

Separation of CRCs into mucinous and nonmucinous types revealed a striking difference in patterns of subcellular localization between these two histological types of CRCs (Figs. 2 and 3). Moreover, both patterns of subcellular accumulation of SPN based on the extent of proliferation in these cells relative to uninvolved histologically normal mucosa were different from the pattern of subcellular accumulation in proliferating cells located in the uninvolved epithelium. Specifically, in nonmucinous tumors, SPN subcellular accumulation was primarily cytoplasmic, and the staining intensity ranged from moderate to strong (Fig. 2 C–F). In contrast, in mucinous adenocarcinomas SPN subcellular accumulation was nuclear, and staining was consistently strong (Fig. 3, A, B, and D). The proportion of nonmucinous tumors demonstrating SPN\textsuperscript{CYT} was 69% (59 of 85), whereas, 55% (11 of 20) of mucinous tumors demonstrated SPN\textsuperscript{CYT} (Table 1). In contrast, 70% (14 of 20) of mucinous tumors demonstrated SPN\textsuperscript{NAC}, but only 31% (26 of 85) of nonmucinous tumors showed SPN\textsuperscript{NAC} (Table 1). The small number of mucinous colorectal tumor samples (n = 20) available for examination precluded additional analyses and, therefore, the remaining nonmucinous CRCs (n = 85) were studied in more detail.

**Subcellular Localization of SPN in Nonmucinous CRCs Is Associated with Tumor Location**

The subcellular localization of SPN relative to other clinicopathological variables (e.g., race, sex, age, tumor location, and so forth) in nonmucinous adenocarcinomas was analyzed to determine whether there was an association between these variables and SPN subcellular localization. The results of this analysis showed that SPN\textsuperscript{NAC} but not SPN\textsuperscript{CYT} had a significantly positive association with location of the CRC. Specifically, proximal CRC were more positive for SPN\textsuperscript{NAC} (44%, 15 of 34) than were distal CRCs (22%, 11 of 51; P = 0.027). No other

<table>
<thead>
<tr>
<th>Variables</th>
<th>Nonmucinous adenocarcinomas</th>
<th>Mucinous adenocarcinomas</th>
<th>P\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear SPN</td>
<td>26 (31)</td>
<td>14 (70)</td>
<td>0.001</td>
</tr>
<tr>
<td>Cytoplasmic SPN</td>
<td>59 (69)</td>
<td>11 (55)</td>
<td>0.219</td>
</tr>
<tr>
<td>p53\textsuperscript{NAC}</td>
<td>42 (49)</td>
<td>6 (30)</td>
<td>0.117</td>
</tr>
</tbody>
</table>

\textsuperscript{a} \chi^2 test P.

![Fig. 3 Immunohistochemical localization of SPN in mucinous CRCs. A, in an island of invasive malignant cells, surrounded by mucin (curved arrow), SPN staining was observed primarily in the nuclei at moderate to strong intensity (straight arrow), and a low intensity of staining for SPN was detected in the cytoplasm. Magnification \times 100. B, in some tissue sections of a mucinous adenocarcinoma, a faint to low intensity of staining for nuclear SPN (straight arrow) was observed in malignant cells scattered in the intratumor mucin (curved arrow). Magnification, \times 60. C, varying intensities of nuclear and cytoplasmic staining for SPN was noted in some mucinous tumors. Magnification \times 100. D, strong localization of nuclear SPN was demonstrated in a mucinous CRC. Magnification, \times 160.](http://clincancerres.aacrjournals.org)
patient variable was significantly associated with SPN NAC or SPN CYT.

**p53 NAC in CRCs**

Nuclear accumulation of the tumor suppressor p53 NAC indicates either a mutation in the p53 gene or dysregulation of p53. We have examined p53 NAC in a previous study (21), and the results of this study demonstrated that the malignant cells of CRCs have a distinct pattern of p53 NAC, but the cells in uninvolved mucosa do not. The overall incidence of p53 NAC was 49% and 30% in nonmucinous and mucinous CRCs, respectively (Table 1).

**SPN NAC and p53 NAC in both Mucinous and Nonmucinous CRCs**

Although the number of mucinous tumors precluded additional detailed analysis, there were sufficient numbers to determine whether changes in the p53 gene covaried with tumor type and subcellular accumulation of SPN. The results of this analysis showed that there was an association between p53 NAC and SPN NAC and that this association differed between mucinous and nonmucinous CRCs (Table 2). In nonmucinous CRC only 24% of the tumors with p53 NAC had SPN NAC, whereas in contrast, all of the mucinous adenocarcinomas with p53 NAC showed SPN NAC. These results suggest that changes in the subcellular localization of SPN cosegregate with p53 NAC in a specific group of CRCs. In contrast to the association of p53 NAC with SPN NAC, there was not a significant association between p53 NAC and SPN CYT in both histological groups examined (Table 2).

**Localization of SPN and Prognosis**

**Univariate Analysis.** We used univariate survival analysis (Kaplan-Meier) to determine whether subcellular localization of SPN in nonmucinous CRCs was associated with patient survival. The results of this analysis found different 5-year survival rates for these two groups (60% SPN NAC negative; 47% SPN NAC positive), but the difference was not statistically significant (log-rank, \( P = 0.465 \); Fig. 4A). The survival rates of patients with tumors with varying SPN CYT were essentially identical (log-rank, \( P = 0.945 \); data not shown).

Because we observed that p53 NAC and SPN NAC showed an association in a subgroup of CRCs, we performed survival analysis based on both p53 status and subcellular localization of SPN. As observed previously, survival analyses indicated a trend of reduced survival for patients of which the tumors demonstrated both SPN NAC plus p53 NAC; however, this association with clinical outcome was not statistically significant (log-rank, \( P = 0.066 \), Fig. 4B). Decreased survival was also reflected in 5-year survival comparisons with the lowest patient survival group being p53 NAC SPN NAC (32%); ~40% of the patients in this group died in the first year after surgery compared with 10% in the groups without this pattern. No other combinations of the subcellular localization of SPN and presence or absence of p53 NAC approached statistical significance. Moreover, the 3-year survival for all of the remaining groups was at least 20% greater than the group with p53 NAC + SPN NAC.

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**Table 2**  Association between localization of SPN and p53 NAC with mucinous and nonmucinous CRCs

<table>
<thead>
<tr>
<th>Variables</th>
<th>Nonmucinous adenocarcinomas</th>
<th>Mucinous adenocarcinomas</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No. of positive (%)</td>
<td>No. of positive (%)</td>
</tr>
<tr>
<td>p53 NAC +</td>
<td>10 (24)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>Nuclear SPN +</td>
<td>28 (67)</td>
<td>3 (50)</td>
</tr>
<tr>
<td>Cytoplasmic SPN +</td>
<td>16 (37)</td>
<td>8 (57)</td>
</tr>
<tr>
<td>p53 NAC –</td>
<td>31 (72)</td>
<td>8 (57)</td>
</tr>
</tbody>
</table>

* Fisher exact test \( P \).
# \( \chi^2 \) test \( P \).
of this hypothesis, we used direct sequence analysis to examine lesions and may not accurately reflect molecular events that many years are likely to have accumulated numerous molecular

Multivariate Analysis

The results of univariate analyses indicated that the combination of p53NAC + SPN NAC might provide more meaningful insight into patient survival than either marker alone. To additionally investigate this relationship and more accurately define this subgroup within the nonmucinous CRC population, we used multivariate Cox regression survival analysis to evaluate the influence of subcellular localization of SPN and p53NAC with other potentially confounding variables. The results of the multivariate Cox regression analysis showed only SPN NAC (HR = 2.34; 95% CI: 1.05–5.25; P = 0.038), p53NAC (HR = 2.33; 95% CI: 1.09–4.96; P = 0.029), and regional lymph-node invasion (pN1 versus pN0; HR = 3.04; 95% CI: 2.10–4.42; P = 0.0001) were significant prognostic variables (Table 3). The subgroup of patients with p53NAC + SPN NAC were 5.45 times (95% CI: 1.82–16.36) more likely to die from colorectal cancer than patients with tumors that were negative for both variables; this suggests that the combined effects of p53NAC plus SPN NAC can be very useful in predicting clinical outcome.

Primary Human Nonmucinous CRC Contain a Mutated SPN Gene

An underlying assumption of the hypothesis that altered subcellular localization of SPN in CRCs is of prognostic importance is that these changes in the subcellular accumulation of SPN reflect either underlying mutations in the spn gene or dysregulation of SPN synthesis and localization. Identification of a mutated form of the spn gene in the LS180 cell line provides support for this hypothesis and suggests that mutations in the spn gene may affect the development and progression of CRC. However, tumor cell lines that have been in culture for many years are likely to have accumulated numerous molecular lesions and may not accurately reflect molecular events that occur in primary tumors. Therefore, to provide a more direct test of this hypothesis, we used direct sequence analysis to examine the spn transcript in tissue samples of uninvolved histologically normal colonic mucosa 8 cm from the tumor and from a CRC collected from the same patient that demonstrated strong immunostaining for cytoplasmic SPN. The results of this analysis demonstrated that in the cells from the histologically normal appearing colonic epithelium, both alleles of the spn gene were expressed (GenBank accession no. AF068896 and AF068897), whereas in the cells from the CRC only one allele was present indicating a loss of expression of one spn allele in the tumor cells (GenBank accession no. AF068897). These results provide strong supporting evidence for the hypothesis that immunostaining changes observed in the subcellular localization of SPN reflect underlying molecular changes in the spn gene. Unfortunately, fresh tissues from a patient with SPN NAC or SPN NAC plus p53NAC were not readily available for analysis so that all molecular changes in the spn gene could not be defined. We anticipate that these groups may show additional changes in the spn gene than those revealed by the present analysis.

DISCUSSION

Inhibition of proliferation of human colonic tumor cell lines by exogenous SPN is consistent with earlier observations on SPN effects on other tumor cell lines (3). The disparity in the inhibitory effect of exogenous SPN between the WiDr and LS180 cell lines was anticipated, because we do not expect every tumor to show mutations or dysregulation in SPN; moreover, we have observed similar effects with exogenous SPN on lymphocytic tumor cell lines (3). These results are also consistent with our hypothesis that differences in the sensitivity of tumor cell lines to exogenous SPN reflect differences in the extent and/or type of defects in the spn gene or the regulation of SPN. Our molecular analysis for cDNAs of spn in LS180 and WiDr cells provides strong support for this hypothesis, because the cell line that was sensitive to exogenous SPN (LS180) contained an altered spn allele, which should result in a reduced amount of endogenous SPN available to inhibit cell cycle entry, whereas both alleles of the spn gene were apparently functional in the SPN-insensitive tumor cell line (WiDr).

The most compelling evidence for a role of SPN in regulating proliferation of colorectal epithelial cells and in the development of neoplasia is that alterations in the spn gene can be observed in the neoplastic phenotype of CRCs. These results suggest a role for SPN in maintaining tissue homeostasis in terms of growth of normal colorectal epithelial cells.

Earlier studies established SPN as a secreted molecule, which acts as an inhibitor of cell cycle entry in lymphocytes; specifically, neutralization of endogenous SPN in cultures of lymphocyte resulted in increased proliferation in the absence of exogenous growth factors (4). Additionally, SPN inhibits mitogen and growth factor stimulated proliferation in human and murine primary lymphocytes and lymphocytic cell lines (2, 3, 5, 6). We have demonstrated that a variety of cells from several tissues contain intracellular SPN, and the changes in the subcellular localization of SPN correlated with the proliferative status of cells (17). For example, in highly proliferating cells in the crypts of the rat intestine, low amounts of SPN were predominant, whereas in the nonproliferating cells near the tips of

Table 3. Cox proportional hazards regression analysis to determine prognostic significance of SPN localization, p53NAC and other clinicopathological parameters in patients with nonmucinous CRCs.

<table>
<thead>
<tr>
<th>Prognostic variable</th>
<th>Indicator of poor prognosis</th>
<th>HR (95% CI)</th>
<th>P</th>
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<tr>
<td>Nuclear localization of SPN</td>
<td>p53NAC vs. p53</td>
<td>2.33 (1.09–4.96)</td>
<td>0.029</td>
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<td>pN component of stage (regional lymph node invasion)</td>
<td>pN1 vs. pN0</td>
<td>3.04 (2.10–4.42)</td>
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<td>pN2 vs. pN0</td>
<td>9.25 (4.39–19.52)</td>
<td>0.0001</td>
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<tr>
<td>pN3 vs. pN0</td>
<td>28.16 (9.20–86.31)</td>
<td>0.0001</td>
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microvilli, greater amounts of SPN^{NAC} are found. The results of studies on human uninvolved colonic epithelial cells support this general association of SPN accumulation and subcellular localization with proliferation.

The results of immunohistochemical analyses on the extent of SPN accumulation and subcellular localization in both mucinous and nonmucinous sporadic CRCs demonstrated that in tumor cells the association of SPN subcellular localization with proliferative status was altered compared with uninvolved colorectal epithelial cells. In CRCs exhibiting high proliferative activity, the extent of SPN accumulation in tumor cells was much higher than in uninvolved cells, and SPN was localized usually in the cytoplasm and sometimes in both the cytoplasm and nucleus of nonmucinous CRCs (Figs. 2 and 3). Mucinous tumor cells had primarily SPN^{NAC} (70% versus 31% in nonmucinous), whereas nonmucinous tumors displayed primarily SPN^{CYT} (69% versus 55% in mucinous). Molecular analysis of the spn gene in one nonmucinous tumor showed that the cytoplasmic accumulation detected by immunohistochemical analysis was associated with a loss of expression of one of the spn alleles. This result and the effects of SPN on the LS180 cell line suggest that changes in the functional activity of the spn gene may be a characteristic of a tumor phenotype. Furthermore, molecular analyses will establish the mutation spectrum of spn in human CRCs.

Earlier studies (21–24) have demonstrated that the phenotypic expression of several molecules including p53^{NAC}, Bcl-2, MUC1, and MUC2 was distinctly different in different histological types of CRC. Similarly, results of the current study demonstrate a distinct pattern of subcellular accumulation of SPN in mucinous and nonmucinous CRCs. These findings suggest that the altered pattern of SPN subcellular accumulation detected by immunohistochemical analysis could be used to differentiate different histological types of CRC segregated previously on basing content.

The consequences of alterations of the p53 gene and the phenotypic expression have been well established, in addition to their role in cell cycle regulation in several human malignancies, particularly in colorectal neoplasia. It is reasonable to correlate SPN with p53 because of their functional similarities. Moreover, the evaluation of multiple markers in human tumors provides increased ability to predict the patient prognosis. Therefore, in the current studies, we analyzed the patient survival using the concomitant expression nuclear pattern of SPN and p53^{NAC}.

Multivariate analysis revealed a statistically significant association between subcellular localization of SPN in nonmucinous CRCs and patient survival and clarified the prognostic significance of SPN. The results of this analysis showed that SPN^{NAC}, p53^{NAC} and regional lymph node metastasis were each independent prognostic indicators of poor overall survival with HRs of 2.34, 2.33, and 3.04, respectively. The combination of SPN^{NAC} plus p53^{NAC} identified a subgroup of patients with the worse survival outcome (545% greater risk of death attributable to CRC). Additionally, the HR of the combination of SPN^{NAC} plus p53^{NAC}, 5.45, was stronger than local node status alone in predicting clinical outcomes. This result provides an explanation for the poor survival of this group (SPN^{NAC} plus p53^{NAC}) seen in univariate survival curves (Fig. 4B). It should be noted that the predominant localization of SPN in mucinous CRCs was nuclear, and the results from nonmucinous tumors with SPN^{NAC} may predict that a significant proportion of mucinous tumors also may have greatly decreased survival.

These initial studies are providing several avenues to additionally explore the biological roles of alterations in spn in colorectal pathogenesis and to understand the underlying mechanism of action of abnormal SPN in cell proliferation, differentiation, or apoptosis. Results of the current study also warrant additional studies demonstrating a link between the expression and subcellular localization of SPN and the p53 status of neoplastic cells. To use SPN as a molecular marker to predict the patient outcome and as a marker of tumor progression in CRCs as well as in other human malignancies, additional studies are needed to demonstrate correlations between immunohistochemical evaluation and mutational analysis of SPN in a larger patient population.

We have been investigating molecular markers that can be used in CRCs to predict disease progression and consequently to aid in determining clinical outcomes. Recently, we reported that p53^{NAC} (indicative of dysregulation of p53) in CRC was an independent prognostic indicator of poorer survival in Caucasian patients with proximal colonic tumors (22), and expression of Bcl-2 in CRCs was a valuable indicator of good prognosis in both Caucasians and African-Americans when tumors are located in the distal colorectum (23). Furthermore, our recent study suggested that expression of MUC1, a mucin antigen, was an indicator of poor prognosis of Caucasians but not African-American patients with CRC (24). These earlier studies and findings of the current study suggest that in future studies evaluating the importance of the expression of SPN in predicting patient prognosis of CRC, researchers should consider the ethnicity of the patient, the anatomical location, and histological status of CRCs.

Our studies show that the synthesis of immunohistochemical and molecular analyses on SPN in nonmucinous CRCs has identified a new molecular marker with prognostic significance in this malignancy. The results from the analysis of SPN in adenomatous components of CRCs indicate that altered subcellular localization of SPN, unlike p53 status and nodal involvement, may serve as an early marker to predict the progression of early colorectal neoplastic lesions. Additional studies in benign and preinvasive lesions of the colorectum will establish utility of SPN as an early molecular marker in preinvasive colorectal lesions. The distinct and characteristic changes in the accumulation and localization of SPN between cells in histopathologically normal colorectal mucosa, adenomatous components, and CRCs indicate that a clinical analysis of SPN is feasible; specifically, prognostic analyses stratify the patient population with nonmucinous CRCs into a subgroup with significantly decreased survival. Conversely, this analysis segregates a group of patients in which there is relatively good overall survival. Thus, our studies provide a rational basis for the inclusion of molecular analysis of SPN in diagnostic analysis and in prognostic models to aid in the identification of patients that may benefit most from alternative therapies.

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