Proliferation, Apoptosis, and Survival in High-Level Microsatellite Instability Sporadic Colorectal Cancer[1]

Julie M. Michael-Robinson, Lynne E. Reid, David M. Purdie, A-E. Biemer-Hüttmann, Michael D. Walsh, Nirmala Pandeya, Lisa A. Simms, Joanne P. Young, Barbara A. Leggett, Jeremy R. Jass, and Graham L. Radford-Smith[2]

Conjoint Gastroenterology Laboratory, Royal Brisbane Hospital Foundation Clinical Research Centre, Herston [J. M. M-R., L. A. S., J. P. Y., B. A. L., G. L. R-S.]; Population and Clinical Sciences Division, Queensland Institute of Medical Research, Brisbane [D. M. P., N. P.]; and Department of Pathology, University of Queensland Medical School, Brisbane [L. E. R., A-E. B-H., M. D. W., J. R. J.], Queensland 4029, Australia

ABSTRACT

Sporadic colorectal cancer (CRC) characterized by high-level DNA microsatellite instability (MSI-H) has a favorable prognosis. The reason for this MSI-H survival advantage is not known. The aim of this study was to correlate proliferation, apoptosis, and prognosis in CRC stratified by MSI status. The proliferative index (PI) was measured by immunohistochemical staining with the Ki-67 antibody in a selected series of 100 sporadic colorectal cancers classified according to the level of MSI as 31 MSI-H, 29 MSI-Low (MSI-L), and 40 microsatellite stable (MSS). The Ki-67 index was significantly higher in MSI-H cancers ($P < 0.0001$) in which the PI was 90.1 ± 1.2% (mean ± SE) compared with 69.5 ± 3.1% and 69.5 ± 2.3% in MSI-L and MSS subgroups, respectively. There was a positive linear correlation between the apoptotic index (AI) and PI ($r = 0.51$; $P < 0.001$), with MSI-H cancers demonstrating an increased AI:PI ratio indicative of a lower index of cell production. A high PI showed a trend toward predicting improved survival within MSI-H cancers ($P = 0.09$) but did not predict survival in MSI-L or MSS cancers. The AI was not associated with survival in any MSI subgroup. In conclusion, this is the first study to show that sporadic MSI-H cancers are characterized by a higher AI:PI ratio and increased proliferative activity compared with MSI-L and MSS cancers, and that an elevated PI may confer a survival advantage within the MSI-H subset.

INTRODUCTION

On the basis of the level of DNA MSI,[3] three distinct subgroups of CRC have been identified (1–3). The majority of sporadic CRCs (70–80%) show allelic loss (LOH) with no MSI, and these are defined as MSS. The other two CRC subgroups defined as MSI-L and MSI-H, each comprising 10–15% of sporadic CRC, develop small insertion and deletion mutations at an accelerated rate in repetitive DNA sequences. Genotypically, MSI-L cancers are distinguished by low-level DNA instability at <40% of microsatellite loci, chromosomal instability (allelic loss), and mutation of the tumor suppressor genes *APC* and *TP53* and the oncogene K-*ras* (3). In contrast, the MSI-H subgroup characterized by MSI at >40% of microsatellite loci rarely shows allelic loss (3–6). However, because of defective DNA mismatch repair caused by hypermethylation-induced "silencing" of the promoter region of one of the mismatch repair genes, *hMLH1* (7), sporadic MSI-H cancers often acquire frameshift mutations in the repeat regions of genes implicated in tumor progression, including *TGF*-β *RII* (8), *BAX* (6, 9), and insulin-like growth factor type II receptor (10). Phenotypically, MSI-H cancers are predominantly right-sided (located in the proximal colon) and more likely to be poorly differentiated, mucinous, and larger at presentation (2, 4, 11). In addition, we have shown recently that >70% of sporadic MSI-H cancers are characterized by the presence of TILs, and these TILs are predominantly $CD8^+$ T cells (12).

MSI-H in sporadic CRC is associated with a less aggressive phenotype and improved survival when compared with stage-matched MSS and MSI-L cancers (13–16). Proliferative activity also has prognostic significance in various types of solid tumors including CRC (17–26). In one study, a higher PI correlated with the presence of lymph node metastases in CRC (25). To date, the reason for the association between MSI-H and favorable clinical outcome remains unclear, and it is not known whether lower proliferative activity explains the MSI-H survival advantage, because previous studies assessing proliferative activity in CRC did not subdivide tumors according to the level of MSI. In the current study, we measured the PI by immunohistochemical examination with an anti-Ki-67 antibody in sporadic CRC classified according to MSI status. The Ki-67 antibody detects a nuclear matrix-associated antigen that is present only

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[2] To whom requests for reprints should be addressed, at Department of Gastroenterology, Ward 1E, Royal Brisbane Hospital, Herston, Queensland 4029, Australia. Phone: 61-7-3636-1321; Fax: 61-7-3636-7283; E-mail: smithgr@health.qld.gov.au.

[3] The abbreviations used are: MSI, microsatellite instability; MSI-H, high-level MSI; MSI-L, low-level MSI; MSS, microsatellite stable; CRC, colorectal cancer; LOH, loss of heterozygosity; TGF-β RII, transforming growth factor β receptor II; TIL, tumor-infiltrating lymphocyte; AI, apoptotic index; PI, proliferative index; TBS, Tris-buffered saline.

in proliferating cells (27), and Ki-67 immunoreactivity correlates with tumor proliferative activity (27, 28).

CRC growth is regulated by the balance between proliferation and apoptosis (29, 30). We have shown recently that MSI-H cancers have an increased AI within the tumor cell population compared with MSS cancers, whereas MSI-L cancers are intermediate (12). The aim of this study was to evaluate PI and AI:PI ratio in the same series of 100 sporadic CRCs of known MSI status to determine the relationship between proliferative and apoptotic activities and to clarify the prognostic impact of these biological properties on patients with MSI-H CRC.

MATERIALS AND METHODS

Tumor Samples. This study was carried out on a selected series of 100 sporadic colorectal adenocarcinomas surgically resected from 99 patients at the Royal Brisbane Hospital between 1989 and 1999. Of these, 91 tumors were derived from a cohort of 303 cancers characterized previously for clinicopathological and molecular features including microsatellite instability (2), and 9 MSI-H tumors were added to increase the statistical power of comparison within this group. Microsatellite instability was originally defined by PCR at six microsatellite loci including *MYCL, AT3, D2S123, F13B, BAT-26,* and *BAT-40* (2). Analysis at additional loci *ACTC, BAT-25, BAT-34C4, D5S346, D10S197, D17S250,* and *D18S55* from the National Cancer Institute panel (31) has not altered the classification of any of these tumors. Altogether, 31 cancers were MSI-H (with bandshifts in at least three of six markers), 29 were MSI-L (with bandshifts in one or two microsatellite markers), and 40 were microsatellite stable with no bandshifts.

Clinical Follow-up. The mean postoperative follow-up in this study was 42 months (range, 5–60 months). The vital status of each patient was determined at the date of last follow-up or at the end of a 5-year follow-up period, and if deceased, the cause of death was ascertained from the medical record and/or death certificate. The cause of death was classified as secondary to, or unrelated to, colon carcinoma. Only CRC deaths were considered as outcome events; all other end points were considered as censored.

Immunohistochemistry. Immunohistochemistry was performed on archival tissue obtained from the Department of Pathology, Royal Brisbane Hospital. Paraffin sections (4 μm) were pretreated with DAKO high pH antigen retrieval system (DAKO, Carpinteria, CA) using a domestic 600 kW microwave oven. Endogenous peroxidase activity was quenched in 1% H_2O_2, 0.1% sodium azide in TBS. Nonspecific antibody binding was blocked by incubating sections in 4% commercial skim milk powder followed by 10% nonimmune goat serum (Zymed Corp., San Francisco, CA). Primary antibody (DAKO polyclonal anti-Ki-67) was applied at a 1:75 dilution overnight at room temperature. Sections were then incubated with prediluted goat antirabbit secondary antibody (Zymed Corp.) for 30 min. Ki-67 localization was demonstrated with prediluted streptavidin-horseradish peroxidase (Zymed) and 0.05% 3,3′-diaminobenzidine in TBS, with H_2O_2 as the substrate. All sections were lightly counterstained with hematoxylin. A negative control, omitting primary antibody, was included in all staining runs.

Apoptosis staining using the novel M30 CytoDEATH antibody was described in detail previously (12, 32). Briefly, dewaxed sections were pretreated by microwave heating in 0.01 M citrate buffer (pH 6.0), and M30 CytoDEATH monoclonal antibody (Boehringer Mannheim, Mannheim, Germany) was applied overnight at a 1:200 dilution. Prediluted goat antimouse secondary antibody (Zymed) followed by streptavidin-horseradish peroxidase (Zymed) was used, and the product was developed in 0.05% 3,3′-diaminobenzidine in the presence of hydrogen peroxide as described above for Ki-67 staining.

We confirmed previously that all 31 MSI-H sporadic CRCs in this series showed loss of hMLH1 expression by immunohistochemistry (34).[4]

Quantification of Proliferation and Apoptosis. Ki-67 scoring was performed by two independent observers (J. M. M-R., L. E. R.) with differences resolved using a conference microscope. The PI was defined as the percentage of tumor nuclei showing Ki-67 staining per total of 1000 neoplastic cells counted in five fields of 200 tumor cells. Consistent with previous reports (22, 25), considerable intratumor heterogeneity was observed in the distribution of Ki-67-stained neoplastic cells in each lesion. Ki-67-positive tumor cells were therefore scored in areas with the highest proliferative activity. Tumor cells were considered positive for Ki-67 whenever diffuse or punctate brown nuclear staining could be identified.

M30 CytoDEATH scoring was also performed by two independent observers (J. M. M-R., A-E. B-H.) as described previously (12). The AI was defined as the mean percentage of cells expressing cytoplasmic M30 staining per 2000 neoplastic epithelial cells counted in 10 randomly selected fields of 200 tumor cells.

Mutation and LOH Analyses. DNA extraction from fresh tumor samples was described previously (2). *BAX* and *TP53* mutations were detected previously in this cohort using SSCP analysis (6). Determination of *TP53* LOH on chromosome 17p was described and analyzed previously (3). Mutations in repeat sequences of the *TGF-β RII* of MSI-H cancers were identified as originally described by Markowitz *et al.* (8). Detection of point mutations in codons 12 and 13 of the K-*ras* proto-oncogene using nonradioactive PCR-RFLP was also described and analyzed previously in this laboratory (33).

Statistical Analysis. Continuous variables are summarized in terms of mean ± SE. Nonparametric methods were used for bivariate analyses, because the primary variables of interest (Ki-67 and apoptosis:Ki-67 ratio) were not normally distributed (Fig. 2). In general this type of analysis is more conservative than equivalent parametric approaches. Mean and median PI and AI:PI were compared across MSI levels using a Kruskal-Wallis nonparametric ANOVA. Multiple linear regression was used to compare PI and AI:PI across MSI levels after adjusting for potential confounding by other variables such as age, tumor stage, and grade. Spearman's nonparametric correlation coefficient was used to measure the degree of association between PI and tumor size.

The effect of PI and AI:PI on patient survival was assessed

[4] Unpublished observations.

Table 1 Relationship between clinicopathologic findings and Ki-67 PI

	n	Mean PI	SE	Krukas-Wallis (Wilcoxon test) *P*	Multiple linear regression − adjusted *P*[a]
Age					
≤55	11	73.15	3.06		
55–65	13	68.32	5.82		
65–75	46	77.61	2.32		
>75	30	77.45	3.08	0.32	0.91
Sex					
Female	56	75.69	2.34		
Male	44	76.09	2.27	0.92	0.152
MSI					
MSI-H	31	90.11	1.19		
MSI-L	29	69.47	3.12		
MSS	40	69.46	2.32	<0.0001	0.0004
Side					
Left	51	71.17	2.32		
Right	48	80.84	2.18	0.002	0.705
TIL					
Negative	62	71.23	2.08		
Positive	38	83.43	2.2	<0.0001	0.618
Dukes' stage					
A	14	76.08	5.09		
B	47	77.71	2.34		
C	22	75.35	3.02		
D	15	68.38	4.57	0.26	0.054
Grade					
Moderate/Well	70	73.32	1.98		
Poorly differentiated	30	81.80	2.92	0.04	0.34
Type					
Adenocarcinoma	79	73.36	1.89		
Mucinous	21	84.89	2.52	0.004	0.748
TP53 LOH[b]					
Negative	41	77.29	2.69		
Positive	38	69.24	2.56	0.015	0.322
K-*ras* mutation[b]					
Negative	60	74.22	2.43		
Positive	17	68.99	3.71	0.21	
*TGF*β *RII* mutation[c]					
Negative	11	90.77	7.50		
Positive	20	89.75	5.89	0.39	
BAX mutation[c]					
Negative	15	89.97	1.35		
Positive	16	90.24	1.97	0.46	

[a] Linear regression model was applied to the squared transformation of Ki-67 percentage that was closer to the normal distribution. Variables included in the model are patient age and sex, MSI, TIL status, stage, tumor site, stage, grade, type, and *TP53* LOH.

[b] Not all tumors were available for mutation testing.

[c] Only MSI-H cancers were tested for mutations in repetitive DNA sequences of these genes.

in a number of ways. The subjects were split based on the median Ki-67 score, and a Kaplan-Meier survival analysis was conducted to compare the patient survival in each group. A similar analysis was also conducted for the AI:PI ratio. The effects of continuous PI and AI:PI scores on patient survival were assessed using Cox's Proportional Hazard Modeling, which also allowed adjustment for confounding by age, stage, and MSI status. The results of the survival analysis are presented as hazard ratios along with 95% confidence intervals.

RESULTS

MSI-H Cancers Are Characterized by a Higher Proliferative Activity Than MSI-L or MSS Cancers. Clinicopathological data from the selected series of 100 primary CRC are shown in Table 1. Patients' ages ranged from 27 to 87 years with a mean of 69.7 ± 10.3 years. Patients with MSI-H cancer were older (72.8 ± 5.9 years; range, 59–87 years) than patients with MSI-L or MSS cancer (68.2 ± 11.5 years; range, 27–87 years). Patients with any family history suggestive of hereditary nonpolyposis colon cancer syndrome were excluded from this study.

Ki-67 nuclear protein expression was quantified by immunohistochemistry (Fig. 1) and analyzed with respect to patient age and sex, tumor MSI status, tumor site, presence of TILs, clinicopathological stage, level of differentiation, tumor type, allelic loss at *TP53*, proliferation, point mutation in codon 12 or 13 of K-*ras*, and frameshift mutations in the *TGF*-β *RII* receptor or *BAX* genes (Table 1). The Ki-67 index was significantly associated with MSI status, with MSI-H tumors exhibiting a higher mean Ki-67 labeling index (90.1 ± 1.2%) than the MSI-L (69.5 ± 3.1%) or MSS

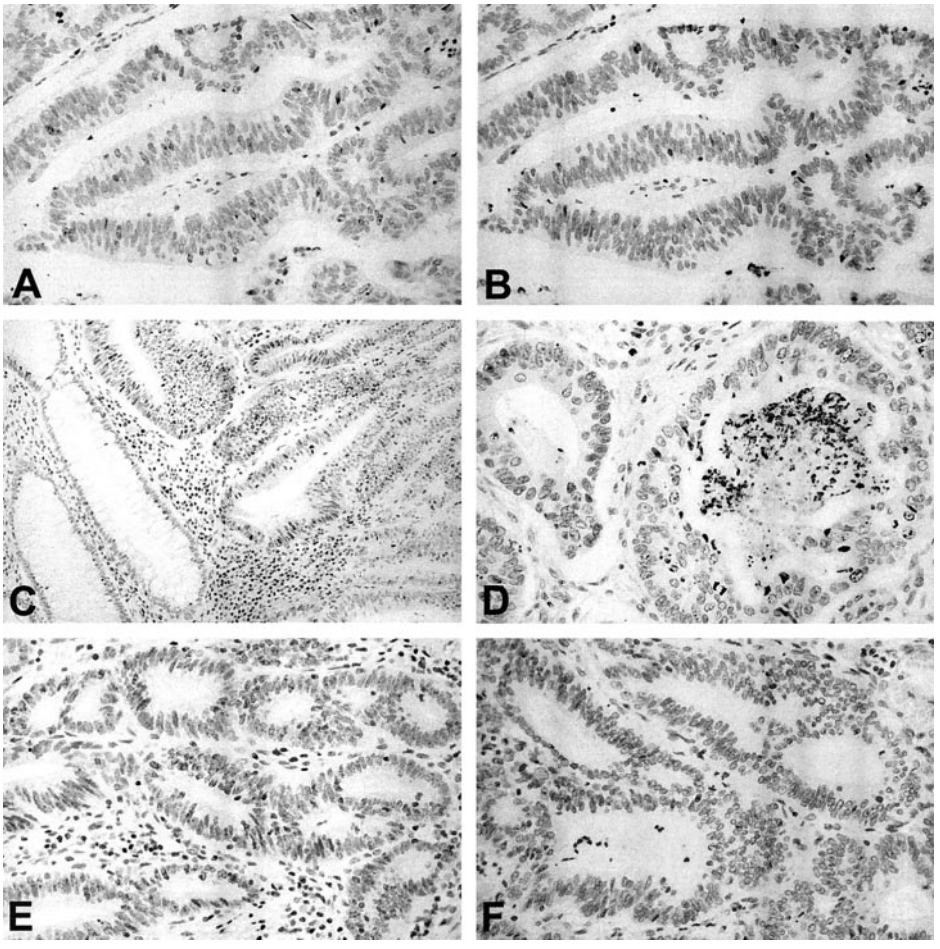

Fig. 1 Ki-67 immunohistochemistry. *A*, nuclear staining of a moderately differentiated MSI-L colorectal adenocarcinoma. *B*, serial section of the same cancer shown in *A*, where omission of the primary antibody served as a negative control (×120). *C*, Ki-67 is overexpressed in colorectal carcinoma relative to adjacent normal crypts (×60). *D*, moderately differentiated MSS cancer with an average Ki-67 labeling index (68.1%). *E*, MSI-H cancer with abundant Ki-67 staining (99.0% in some areas). *F*, MSI-L cancer with a low Ki-67 proliferation index (48.1%). *D–F*, ×120.

(69.5 ± 2.2%) subgroup, which had similar Ki-67 indices ($P < 0.0001$; Table 1 and Fig. 1). In addition, a high Ki-67 index was significantly associated with proximal (right-sided) location, TIL positivity, poor differentiation, mucinous histology, and absence of *TP53* LOH ($P < 0.05$), all of which are characteristic features of MSI-H cancers. When multiple linear regression was performed adjusting for all variables (age, sex, MSI, side, stage, TIL status, tumor grade, type, and *TP53* status), only MSI status remained significant (Table 1), indicating that MSI-H is the only variable independently associated with a high proliferative activity in CRC. There was a trend toward lower proliferative activity in Dukes' stage D (metastatic) tumors (Fig. 1*F*), and this negative association between Ki-67 expression and tumor stage approached significance when corrected for all other variables in the multivariant analysis (Table 1). The PI, as determined by Ki-67 staining, was not related to patient age or sex, or K-*ras* status, in any MSI subgroup. Although both high PI and frameshift mutations in *TGF*-β *RII* and *BAX* are associated with MSI-H cancers, the PI was not associated with *TGF*-β *RII* or *BAX* status within the MSI-H subgroup (Table 1). There was a trend toward increased Ki-67 staining with larger tumor size, although this did not reach significance (Spearman correlation coefficient for tumor size *versus* Ki-67 index was 0.136; $P = 0.18$). Furthermore, the extent of Ki-67 staining was not related to the site of the tumor *per se*. In all three MSI subgroups, left-sided tumors (located distal to the splenic flexure) had similar Ki-67 scores to right-sided (proximal) tumors (Table 2).

Relationship between Proliferation and Apoptosis in MSI Colorectal Cancer. When assessed by immunohistochemistry, tumors exhibited a wide range of Ki-67 expression from 31.0 to 99.0% (Fig. 2*A*), indicative of a large variation in proliferative activity. Overall, the median and mean (± SE) proliferative indices were 78.6% and 69.7% ± 10.3% respectively. Apoptosis in the same set of tumors was assessed previously by immunohistochemistry in this laboratory (12), using the M30 CytoDEATH antibody that binds to the caspase-cleaved fragment of cytokeratin-18 during the early stages of epithelial cell apoptosis. Median and mean apoptotic indices were 1.9% and 2.5% ± 2.0%, respectively, and ranged from 0.2 to 8.2% (Fig. 2*B*). The PI and AI were strongly correlated with a Spearman rank coefficient of 0.51 ($P < 0.001$).

The AI:PI ratio was also examined in relation to the same clinicopathological variables tested against PI in Table 1. In both univariate and multivariate regression analysis, MSI status was significantly correlated with the AI:PI ratio (Table 3). The AI:PI ratio was not associated with any other variable tested including patient age, sex, tumor location, TIL status, stage, tumor grade and type, *TP53* LOH, K-*ras*, *TGF*-β *RII* or *BAX* status (Table 3 and data not shown).

Table 2 Univariate analysis of the relationship between MSI status, tumor site, and Ki-67 PI

Ki-67% according to MSI status and tumor site[a]	*n*	Mean	SE	Krukas-Wallis (Wilcoxon test) *P*
MSI-H				
Left side	4	92.45	4.56	
Right side	27	89.77	1.22	0.19
MSI-L				
Left side	21	68.41	3.79	
Right side	7	71.59	6.49	0.75
MSS				
Left side	26	70.11	2.89	
Right side	14	68.24	4.01	0.72

[a] Left side represents tumors found distal to the splenic flexure, and right side represents tumors located proximal to the splenic flexure.

A High Proliferative Index Is Associated with Longer Survival in MSI-H Sporadic CRC. To determine whether the PI is related to prognosis in any MSI subgroup, we compared patient survival and tumor PI as determined by Ki-67 staining. A survival advantage, which approached significance ($P = 0.09$), was associated with higher Ki-67 expression among MSI-H cancers but not MSI-L or MSS cancers (Table 4). Using the median Ki-67 score of 78.6% as a cut point, neither MSI-L nor MSS tumors showed a trend toward reduced survival in cancers with a higher Ki-67 index (data not shown). The AI as assessed by staining with the M30 CytoDEATH antibody was not associated with survival either overall ($P = 0.41$) or within MSI subgroups (data not shown).

Because the AI:PI ratio gives an indication of net cell production, we postulated that the AI:PI might be more informative than either index alone. Overall, a low AI:PI ratio of <0.26 (using the median value as the cutoff) was associated with a more favorable prognosis, compared with a high AI:PI ratio of >0.26 (Fig. 3), and this difference approached significance ($P = 0.087$). This survival difference did not appear to be particular to any one of the three MSI subgroups.

DISCUSSION

Immunostaining with the Ki-67 antibody is widely accepted as a reliable and reproducible method for evaluating proliferative activity in human tumors. Although the PI estimated by Ki-67 staining has been shown to produce a consistent overestimate of the growth fraction of tumors and is therefore not an exact measurement of tumor growth (35), the Ki-67 index correlates well with the predicted growth fraction (27, 35, 36). In another independent study, the Ki-67 index was significantly associated with the mitotic activity in tumor tissue (21), and there is good evidence that Ki-67 immunoreactivity correlates well with other markers of cell proliferation (37–39).

In this study, we found that MSI-H cancers exhibit a significantly higher Ki-67 index ($90.1 \pm 1.2\%$; $P < 0.0001$) than MSI-L or MSS cancers that had similar mean Ki-67 indices ($69.5 \pm 3.1\%$ and $69.5 \pm 2.2\%$, respectively). To our knowledge, this is the first study to show that the PI is significantly correlated with MSI status in sporadic CRC. Cycling cells are more likely to be undifferentiated, and MSI-H cancers are more likely to show poor differentiation. This could explain the higher proportion of cycling cells in the MSI-H subset. Consistent with our findings, Aoki *et al.* (25) reported recently that mucinous or poorly differentiated CRCs were characterized by an increased PI, both these features being characteristic of MSI-H cancers (2). Similarly, in the present study a high Ki-67 index was significantly associated with mucinous lakes, poor differentiation, proximal location, the presence of TILs, and wild-type *TP53*, all characteristics of MSI-H cancers. None of these associations remained significant after multiple linear regression analysis, indicating that MSI status was the primary predictor of PI in this series of cancers.

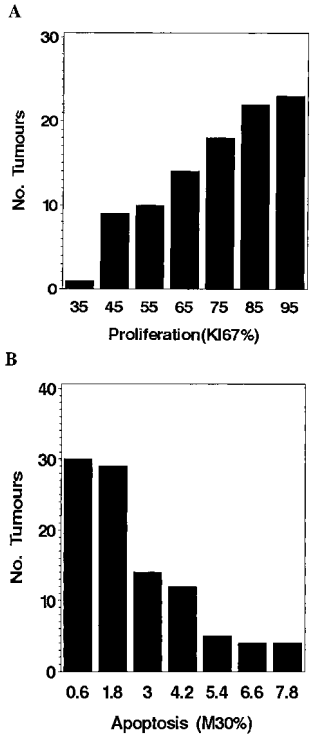

Fig. 2 Frequency distribution of PIs (*A*) and AIs (*B*) in sporadic CRC ($n = 100$).

Consistent with previous reports, no correlation was found between the level of Ki-67 staining and age or sex (36, 37, 40, 41). Interestingly, there was a negative association between Ki-67 expression and stage that approached significance when corrected for all other significant variables in the multivariate analysis ($P = 0.054$). This trend suggesting that a lower PI is associated with Dukes' stage D colorectal tumors is in contrast to previous findings by Kyzer and Gordon (42), who found a positive correlation between the Ki-67 proliferative index and stage D tumors; however, only 4 of 30 patients had metastatic disease. Consistent with our findings, the Kyzer study found no correlation between Ki-67 score and Dukes' B or C cancers. Conversely, an earlier study by Suzuki *et al.* (24) that included 58 colorectal carcinomas found that the mean Ki-67 labeling index was higher in Dukes' B and Dukes' C tumors than in Dukes' A tumors. Similarly, Aoki *et al.* (25) reported recently a significant association between lymph node metastases (Dukes'

Table 3 Relationship between clinicopathological variables and apoptosis:Ki-67 ratio

	n	Ratio of apoptosis:Ki-67 (Mean ± SD)	Krukas-Wallis (Wilcoxon test) *P*	Multiple linear regression − adjusted *P*[a]
MSI				
MSI-H	31	0.04 ± 0.004		
MSI-L	29	0.04 ± 0.004		
MSS	40	0.02 ± 0.003	<0.001	0.004
Side				
Left	51	0.03 ± 0.003		
Right	48	0.03 ± 0.003	0.43	0.36
TIL				
Negative	62	0.03 ± 0.003		
Positive	38	0.04 ± 0.004	0.08	0.88
Stage				
A	14	0.03 ± 0.005		
B	47	0.03 ± 0.004		
C	22	0.03 ± 0.005		
D	15	0.04 ± 0.006	0.77	0.93
Grade				
Moderate/Well	70	0.03 ± 0.003		
Poorly differentiated	30	0.03 ± 0.003	0.26	0.71
Type				
Adenocarcinoma	79	0.03 ± 0.002		
Mucinous	21	0.04 ± 0.006	0.35	0.91
TP53[b]				
Negative	41	0.03 ± 0.004		
Positive	38	0.03 ± 0.004	0.72	0.16
K-*ras* mutation[c]				
Negative	60	0.03 ± 0.003		
Positive	17	0.02 ± 0.003	0.19	

[a] Linear regression model was applied to the squared transformation of Ki-67 percentage that was closer to the normal distribution. Variables included in the model are patient age and sex, MSI, TIL status, stage, tumor site, stage, grade, type, and *TP53* LOH.
[b] Analysis is based on results from 19 MSI-H, 22 MSI-L, and 38 MSS cancers.
[c] Analysis is based on results from 19 MSI-H, 20 MSI-L, and 38 MSS cancers.

Table 4 Association between tumor PI and patient survival within MSI subgroups, showing a trend toward improved survival in association with increased proliferation within the MSI-H subgroup

Within	Mean PI (%)	Hazard ratio (95% CI)[a]	*P*
MSI-H	90.11	0.90 (0.82–1.01)	0.09
MSI-L	69.47	0.99 (0.95–1.02)	0.40
MSS	69.46	1.01 (0.97–1.05)	0.67

[a] Analysis was adjusted for age, sex, tumor stage, grade, and MSI status. CI, confidence interval.

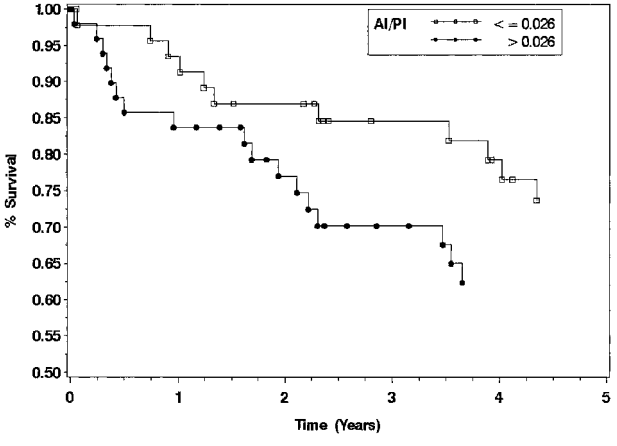

Fig. 3 Comparison between overall patient survival and apoptosis: Ki-67 ratio ($P = 0.087$). Analysis was based on data from 97 tumors including 40 MSS, 29 MSI-L, and 28 MSI-H cancers.

stage C) and a higher Ki-67 index. In apparent contradiction, five additional studies found no correlation between the Ki-67 index and tumor stage (36, 37, 40, 42, 43). One reason for these discrepancies could be differences in tumor sampling. The sample size in these studies ranged from 30 to 255 tumors. Our observations are consistent with others that noted marked heterogeneity of Ki-67 expression within different tumor stages (22). Taken together, these results suggest that the Ki-67 index is not a reliable indicator of tumor stage.

In agreement with previous reports, our data suggest that Ki-67 staining is not dependent upon tumor site (22, 36, 42, 44). Therefore, the higher PI associated with MSI-H cancers was not related to the predilection for proximal location. In fact, four left-sided MSI-H tumors in this series had a slightly higher mean PI than the 27 right-sided tumors. Left- and right-sided MSS and MSI-L tumors also had similar Ki-67 scores. Therefore, the increased proliferative activity in MSI-H cancers is probably explained by the high level of MSI, although the mechanism is unclear. Similarly, we found that the level of apoptosis did not differ according to tumor site in the same

Table 5 Summary of studies assessing the relationship between proliferation, apoptosis, and prognosis in sporadic CRCs

Author and year	*n*	Tumor stage	MSI analysis	Multivariant analysis	Proliferation measure	PI as a prognostic indicator	Apoptosis measure	AI as a prognostic indicator	Ref.
Schutte *et al.*, 1987	279	All	No	Yes	Flow cytometry	Yes (stage C)	ND[a]	ND	(51)
Harlow *et al.*, 1991	69	C only	No	Yes	Flow cytometry	Yes	ND	ND	(49)
Witzig *et al.*, 1991	694	B_2/C	No	Yes	Flow cytometry	Yes	ND	ND	(50)
Barreton *et al.*, 1996	95	A/B	No	Yes	Ki-67 immuno	No	TUNEL	No	(45)
Kyzer *et al.*, 1997	30	B/C/D	No	No	Ki-67 immuno	Yes (stage D)	ND	ND	(42)
Jansson *et al.*, 1997	255	All	No	No	Ki-67 immuno	No	ND	ND	(43)
Sinicrope *et al.*, 1999	154	A/B	No	Yes	Flow cytometry	No	H&E stain	No	(44)

[a] ND, not done; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; immuno, immunohistochemistry.

series (12). Indeed, MSI-H cancers are characterized by both increased proliferation and apoptosis, indicative of higher cell turnover, compared with their MSI-L and MSS sporadic counterparts.

There was a significant correlation (Spearman rank coefficient of 0.51; $P < 0.001$) between PI and AI, suggesting that some degree of coordinated regulation remains. This finding is in agreement with another study by Baretton *et al.* (45) that found an elevated Ki-67 index was associated with a high AI. In contrast, two other recent reports found no significant correlation between tumor PIs and AIs (44, 46). However, this discrepancy may be attributable to methodology differences. One of these studies assessed proliferation by flow cytometry, which included only S-phase and G_2-M phases of the cell cycle in the proliferating fraction (44), whereas the Ki-67 antibody binds to nuclei in all phases of the cell cycle except G_0 and early G_1 (27). In addition, both studies assessed the level of apoptosis by morphological analysis on H&E-stained sections. We have shown that immunohistochemical assessment with the M30 antibody is more sensitive than morphological measurement (12). We also observed a wide range of Ki-67 expression (31.0–99.0%) and M30 CytoDEATH staining (0.2–8.2%), reflecting marked heterogeneity in both PIs and AIs, respectively. Tumor stage is the most important prognostic variable in CRC (47), but stage-independent variability in clinical outcome is still observed, and this may be a consequence of altered rates of apoptosis and cell proliferation (44). Our findings reinforce the need to stratify according to MSI in studies of CRC.

Another report published recently by Bocker Edmonston *et al.* (48) included a comparison of Ki-67 staining and microsatellite status in CRC. However, their MSI-H subgroup comprised a majority (84%) of hereditary nonpolyposis cancers that are genetically different and may have quite different growth characteristics than their sporadic counterparts. Furthermore, Ki-67 staining was analyzed semiquantitatively. Taken together, these differences may explain why the Bocker Edmonston study found no significant correlation between the Ki-67 PI and MSI status (48).

Given the strong correlation between the AI and PI, we examined possible correlations with the AI:PI ratio. These included patient age, sex, MSI status, tumor stage, site, grade and type, *TP53*, K-*ras*, *TGF*-β *RII* and *BAX* mutation status. Unlike the Ki-67 univariate analysis, only MSI status was significantly associated with the AI:PI ratio, with MSI-H and MSI-L cancers showing a 2-fold higher ratio than MSS cancers This analysis confirms that MSI status is the only variable that independently predicts apoptosis and proliferative activity in CRC.

Ki-67 was identified previously as an independent prognostic marker for patients with CRC in some studies (42, 49–51) but not others (Refs. 43–45; Table 5). In the current study, a high Ki-67 index was associated with improved survival in MSI-H cancers ($P = 0.09$) but not MSI-L or MSS cancers. Stage D MSS/MSI-L tumors in this series tended to have lower proliferative activity (Ki-67, <50%). It is noteworthy that in the four CRC studies that found a positive correlation between a high PI and poor prognosis, the correlations were limited to particular stages (Refs. 42, 49–51; Table 5). Two studies that measured proliferation by Ki-67 immunostaining in a large CRC series not stratified by MSI found no significant correlation between PI and survival (43, 45). Significantly, diploidy (49–51) and right-sided location (49) independently predicted better survival in CRC, especially diploidy in proximal cancers (44). These findings are consistent with results obtained in our laboratory, given that MSI-H cancers are predominantly right-sided and diploid (2). It is relevant that none of the previously mentioned studies assessed MSI status (Refs. 42–45, 49–51; Table 5). Therefore, the data from the 10 to 15% MSI-H cancers present in those unselected series would have been masked by the results of the other CRC subgroups.

The increased level of spontaneous apoptosis noted previously in MSI-H cancers indicates that apoptosis signaling does not appear to be especially defective or deregulated in sporadic MSI-H cancers (12, 52). Despite the higher mean AI and better prognosis in MSI-H cancers, we found that the AI was not significantly associated with survival in CRC, either overall or within MSI subgroups. We also found that a low AI:PI ratio across the entire series, indicating a higher index of cell production, was associated with a favorable prognosis with borderline significance ($P = 0.087$). The explanation for this finding is not clear, and the relationship was not observed when tumors were divided into subgroups according to MSI status. To our knowledge, the only other CRC study to compare the relationship between AI:PI ratio and patient survival did not find an association (44). However, that study included only lymph node-negative (Dukes' stage A and B) cancers and measured apoptosis by the less sensitive morphological assessment in H&E-stained sections.

Most patients with CRC die from metastatic disease rather than local tumor recurrence (53), and there is no relationship between tumor size and lymph node metastases (25). Consistent

with our findings, the Ki-67 index did not correlate with tumor size in any study to date (24, 25, 36, 43). This study provides further evidence that tumor size is not necessarily related to tumor aggression or invasive potential. MSI-H cancers are larger at presentation (2), yet are less aggressive than their MSI-L and MSS counterparts, rarely progressing to stage D disease with distant metastases (13–16).

MSI-H cancers are characterized by a higher PI that appears to be associated with an improved survival within this subset. The latter finding may be explained, at least in part, by biological heterogeneity within MSI-H cancers. MSI-H cancers with a low PI may be less typical or show more overlap with non-MSI-H cancers. Furthermore, the higher PI in MSI-H cancers is more than offset by the higher AI, giving a reduced index of cell production compared with MSS but not MSI-L cancers. This implies a greater disruption of apoptosis in MSS *versus* MSI-H cancers. The intermediate AI in MSI-L cancers is not easily explained because TP53 alterations (mutation, LOH, and protein expression) are similar in MSI-L and MSS cancers (3, 6, 33).

In conclusion, this study is the first to show that sporadic MSI-H cancers are characterized by a significantly increased Ki-67 proliferative index. MSI cancers also show an increased AI:PI ratio. Consistent with previous reports (22, 36, 43, 44), the PI was not related to the site of the tumor *per se*. Studies from this laboratory have also shown that the AI is significantly higher in MSI-H cancers (12). Apoptosis and proliferative indices were positively correlated in this series of sporadic CRC. In contrast to recent studies in lung, bladder, and breast cancer (17, 18, 20), as well as some studies in unselected CRC series (42, 49–51), but in agreement with several other CRC studies (43–45), we did not find that a high mean PI was a poor prognostic indicator. Rather, our survival analysis suggests that a high PI may be associated with the MSI-H survival advantage, whereas in MSI-L and MSS cancers, the PI did not correlate with survival. The increased PI in MSI-H cancers may be explained by the higher proportion of MSI-H cancers that are undifferentiated or poorly differentiated. This finding does not conflict with the known behavior of MSI-H cancer because the apparent increased growth fraction is more than offset by the higher AI. Additionally, an increased PI need not indicate increased tumor aggression because the latter is more directly explained by mechanisms underlying invasiveness and metastasis.

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