Tumour infiltrating lymphocytes and apoptosis are independent features in colorectal cancer stratified according to microsatellite instability status

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Abstract

***Background*—The presence of high level DNA microsatellite instability (MSI-H) in colorectal cancer is associated with an improved prognosis, as is the presence of tumour infiltrating lymphocytes (TILs). It is not clear if TILs contribute directly to the survival advantage associated with MSI-H cancers through activation of an antitumour immune response.**

***Aims*—To correlate TIL and apoptosis rates in colorectal cancer stratified by MSI status.**

***Methods*—The distribution of TILs was characterised and quantified in a selected series of 102 sporadic colorectal cancers classified according to levels of MSI as 32 MSI-H, 30 MSI-low (MSI-L), and 40 microsatellite stable (MSS). Archival blocks were immunostained using the T cell markers CD3 and CD8, and the B cell marker CD20. Apoptosis of malignant epithelial cells was quantified by immunohistochemistry with the M30 CytoDEATH antibody.**

***Results*—Positive staining with anti-CD3 and negative staining with anti-CD20 identified virtually all TILs as T cells. The majority of $CD3^+$ TILs (>75%) also stained with anti-CD8. TILs were most abundant in MSI-H colorectal cancers in which 23/32 (72%) scored as TIL positive. Only 5/40 (12.5%) MSS tumours and 9/30 (30%) MSI-L cancers were TIL positive ($p<0.0001$). MSI-H cancers showed a two-fold higher rate of apoptosis (mean (SD) 3.52 (0.34)%) than the MSS cancers (1.53 (0.23)%) while the MSI-L subgroup had an intermediate level (2.52 (0.35)%) ($p<0.0001$). Overall, there was a small ($r=0.347$) but significant linear correlation between $CD3^+$ and $M30^+$ random apoptosis counts ($p<0.001$). However, TILs and apoptosis showed little colocalisation.**

***Conclusions*—While TILs might be expected to explain the increased apoptotic rate and improved prognosis of MSI-H cancers, it is likely that TILs and apoptosis are independent characteristics of MSI-H cancers.**

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Keywords: colorectal cancer; DNA microsatellite instability; tumour infiltrating lymphocytes; apoptosis

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In recent years it has become apparent that there are three genetically distinct subgroups of sporadic colorectal cancer (CRC) based on the level of DNA microsatellite instability (MSI).[1–3] The majority of sporadic CRCs (70–80%) show allelic losses with no MSI and are defined as microsatellite stable (MSS). The other two subgroups defined as MSI-low (MSI-L) and MSI-high (MSI-H), each comprising 10–15% of sporadic CRC, develop small insertion and deletion mutations in repetitive DNA.[1–3] Genotypically, MSI-L cancers are distinguished by both low level DNA instability at <40% of microsatellite loci and inactivation of the tumour suppressors APC and *p53*, with frequent activation of the K-*ras* oncogene.[3] In contrast, the MSI-H subgroup has a low level of mutation in APC, *p53*, and K-*ras* genes.[3–6] However, due to the high level of DNA instability at >40% of microsatellite loci, MSI-H cancers often acquire frameshift mutations in the repeat regions of genes implicated in tumour progression, including proapoptotic BAX[6 7] and the receptors for transforming growth factor β[8] and insulin-like growth factor.[9] MSI-H cancers are predominantly right sided (located in the proximal colon), poorly differentiated, mucinous, and larger at presentation.[2 4 10]

Furthermore, the presence of high level MSI is associated with an improvement in survival[5 10 11] and there is evidence that, unlike MSS cancers, MSI positive cancers are characterised by the presence of an inflammatory reaction in the form of tumour infiltrating lymphocytes (TILs).[2 4 12] TILs also appear to be an important prognostic indicator in CRC.[13 14] Earlier studies of TILs did not subdivide CRC according to the level of MSI. Therefore, the aim of this study was to correlate TIL and apoptosis rates in colorectal cancers stratified by MSI status.

Materials and methods

TUMOUR SAMPLES

The study comprised a selected series of 102 sporadic colorectal adenocarcinomas obtained from 101 patients undergoing surgery at the Royal Brisbane Hospital between 1989 and 1999. Of these, 93 cases were derived from a cohort of 303 cancers previously characterised for clinicopathological and molecular features,

Abbreviations used in this paper: CRC, colorectal cancer; MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, MSI-low; MSI-H, MSI-high; TILs, tumour infiltrating lymphocytes; TBS, Tris buffered saline; DAB, 3,3'-diaminobenzidine; IEL, intraepithelial lymphocytes.

including MSI,[2] and nine MSI-H cancers were added to this series to increase the statistical power of comparison with this group. Altogether, 32 were MSI-H cancers (with band shifts in at least three of six markers), 30 were MSI-L (with band shifts in one or two microsatellite markers), and 40 were MSI-null or MSS. The study was approved by the Royal Brisbane Hospital Ethics Committee.

IMMUNOHISTOCHEMISTRY

Routinely processed paraffin embedded blocks were obtained from the Department of Pathology, Royal Brisbane Hospital. Tumour sections (4 μm) were affixed to adhesive slides (Menzelgläser, Braunschweig, Germany) and air dried overnight at 37°C. Sections were dewaxed in xylene and rehydrated through descending grades of alcohol to water, then transferred to Tris buffered saline (TBS), pH 7.4. Heat based antigen retrieval was performed using the technique of Shi and colleagues.[15] Sections were boiled twice for five minutes each in 0.01 M citric acid buffer, pH 6.0, in a domestic 600 kW microwave oven, then cooled to room temperature, prior to washing in three changes of TBS for five minutes each. Endogenous peroxidase activity was quenched in 1% H_2O_2, 0.1% sodium azide in TBS. After washing three times for five minutes in TBS, non-specific antibody binding was blocked by incubating the sections in 4% commercial skim milk powder for 15 minutes. After a brief rinse in TBS the sections were placed in a humidified chamber and incubated for 20 minutes in non-immune goat serum (Zymed Corporation, San Francisco, California, USA). Primary antibody was applied at the appropriate titre and sections were incubated overnight at room temperature. The following antibodies were diluted in TBS: anti-CD3 (rabbit polyclonal, 1:300), anti-CD8 (murine monoclonal, clone C8/144B, 1:30), and anti-CD20 (clone L26, 1:50) from Dako (Carpinteria, California, USA); and CytoDEATH (clone M30, 1:200) from Boehringer Mannheim (Mannheim, Germany). Sections were then thoroughly washed in three changes of TBS for five minutes each (with inclusion of 0.5% v/v Triton X-100 in the first M30 wash). The CD3 sections were incubated with prediluted goat antirabbit secondary antibody (Zymed), while the CD8, CD20, and M30 CytoDEATH sections were incubated with prediluted goat antimouse secondary antibody (Zymed) for 30 minutes. Sections were washed in three changes of TBS and prediluted streptavidin-horseradish peroxidase (Zymed) was applied for 15 minutes. After three washes in TBS, antigenic sites were revealed using 0.05% 3,3'-diaminobenzidine (DAB) in TBS with H_2O_2 as substrate. Sections were lightly counterstained in Mayer's haematoxylin, dehydrated, cleared, and mounted using DePeX (BDH Gurr, Poole, UK). Appropriate negative and positive controls (normal human colon for M30 and lymph node for lymphocyte markers) were included in all staining runs.

For M30 CytoDEATH/CD3 double staining, M30 staining was performed to the DAB step, as described above. Sections were then reblocked with 4% skim milk powder and goat serum and incubated with anti-CD3 (1:200) for two hours at room temperature. Following standard goat antirabbit and streptavidin incubations, purple VECTOR VIP (Vector Laboratories Inc., Burlingame, California, USA) was applied for 3–4 minutes and the slides were washed well in water before counterstaining with methyl green (Vector Laboratories) for three minutes at 60°C.

TIL and apoptosis scoring was performed by two independent observers (JMM-R and AEB-H). For each case, the number of intraepithelial $CD3^+$ and $CD8^+$ lymphocytes in the 10 most infiltrated fields of 200 tumour nuclei were counted and the five highest values were averaged to obtain the final lymphocyte score per 1000 neoplastic cells. To define TIL positive tumours, the cut off of 5% (that is, $\geqslant$50 $CD3^+$ lymphocytes per 1000 tumour nuclei) was chosen for statistical and pathological reasons. There was a natural separation in the bimodal distribution of $CD3^+$ lymphocytes at a score of 50/1000 tumour nuclei and a pathologist (JRJ) could not easily detect a CD3 count of less than 5% by routine haematoxylin and eosin staining. In addition, the ratio of intraepithelial lymphocytes (IEL) to epithelial cells in the normal human colon is approximately 5:100.[16] The distribution of apoptotic epithelial cells was graded in several areas of each tumour. Firstly, 10 randomly selected fields of 200 nuclei from within the middle of each tumour were counted and averaged to obtain a final score of random per cent apoptosis (random %). Secondly, apoptosis at the luminal surface and growing (advancing) edge of the tumour was scored as 0 (<1%), + (1–5%), ++ (5–10%), +++ (10–15%), or ++++ (>15%). Finally, epithelial cell apoptosis occurring within the glandular lumen was graded semiquantitatively as 0 (little or none), + (some), or ++ (widespread) according to the degree of M30 positive staining. M30 negative lumen are likely to contain apoptotic TILs, inflammatory cells, and necrotic debris. When the observers' scores differed by more than 1% for TIL counts or 2% for apoptosis scores, cases were discussed over a double headed microscope and the final grade was reached by consensus.

MUTATION ANALYSIS

P53 mutations were detected using single strand conformational polymorphism analysis as described previously.[6] Detection of mutations in codons 12 and 13 of the K-*ras* proto-oncogene using non-radioactive polymerase chain reaction-restriction fragment length polymorphism were described previously.[3] Genetic alterations in the coding 8-guanine repeat in the BAX gene were analysed as previously described.[7]

STATISTICAL ANALYSIS

A non-parametric approach to analysis was used as the primary variables of interest, that is, CD3, CD8, and random apoptosis counts were not normally distributed. This type of analysis

Table 1 Clinicopathological features of microsatellite instability-high (MSI-H), microsatellite instability-low (MSI-L), and microsatellite stable (MSS) colorectal cancers

Variables	*Total*	*MSI-H (n=32)*	*MSI-L (n=30)*	*MSS (n=40)*	*p Value*
Age (mean (SD)		72.6 (6.1)	69.0 (10.9)	67.9 (12.1)	0.280†
Sex*					
Male	44	11 (34.4%)	17 (56.7%)	16 (40.0%)	0.210‡
Female	57	20 (65.6%)	13 (43.3%)	24 (60.0%)	
Type					
Adenocarcinoma	79	15 (46.9%)	27 (90.0%)	37 (92.5%)	<0.0001‡
Mucinous	23	17 (53.1%)	3 (10.0%)	3 (7.5%)	
Site**					
Right colon	49	28 (87.5%)	7 (24.1%)	14 (35.0%)	<0.0001‡
Left colon	28	3 (9.4%)	10 (34.5%)	14 (35.0%)	
Rectum	25	1 (3.1%)	12 (41.4%)	12 (30.0%)	
Differentiation					
Well	5	2 (6.3%)	1 (3.3%)	2 (5.0%)	0.002‡
Moderate	66	12 (37.5%)	25 (83.3%)	29 (72.5%)	
Poor	31	18 (56.3%)	4 (13.3%)	9 (22.5%)	
Duke's stage***					
A	15	6 (19.4%)	4 (13.3%)	5 (12.5%)	0.071‡
B	51	18 (58.1%)	14 (46.7%)	19 (47.5%)	
C	20	5 (16.1%)	3 (10.0%)	12 (30.0%)	
D	16	2 (6.5%)	9 (30.0%)	4 (10.0%)	

†Kruskal-Wallis test.
‡χ^2 test.
*One MSI-H female patient had two metachronous tumours and therefore is included only once for the sex comparison.
**One MSI-L site not available.
***One MSI-H stage not available.

is also more conservative than a traditional parametric approach. Therefore, mean and median age, CD3, CD8, and random apoptosis counts were compared across MSI levels using a Kruskal-Wallis non-parametric analysis of variance. Also, K-*ras*, *p53*, and BAX status were compared with CD3 or random apoptosis counts using a Kruskal-Wallis analysis of variance. Differences in the frequency distributions of categorical factors, such as sex, stage, and degree of differentiation, across MSI levels were examined using Pearson's χ^2 statistic. Spearman's non-parametric correlation coefficient (rho) was used to measure the degree of association between CD3 or CD8 count and random apoptosis per cent. In addition, the degree of association between CD3 count or random apoptosis and Bcl-2 or p53 protein expression was measured using Spearman's correlation coefficient. A p value less than 0.05 was considered statistically significant.

Results

CLINICOPATHOLOGICAL FEATURES OF MSI-H, MSI-L, AND MSS COLORECTAL CANCERS

Clinicopathological data from the 102 sporadic colorectal carcinomas surgically resected from 101 patients are shown in table 1. The majority of MSI-H cancers (28/32, 87.5%) occurred in the right colon (proximal to the splenic flexure). In contrast, MSI-L and MSS cancers were found predominantly in the left colon (distal to the splenic flexure) and rectum (table 1). The MSI-H cancers were also characterised by a significantly higher proportion of mucinous carcinomas (composed of >50% mucinous lakes). In addition, more than half (18/32, 56.3%) of the MSI-H cancers were poorly differentiated compared with 4/30 (13.3%) MSI-L and 9/40 (22.5%) MSS tumours. There was a tendency towards more advanced disease in the MSI-L and MSS groups as 12/30 (40.0%) MSI-L and 16/40 (40.0%) MSS cancers had metastasised to the lymph nodes or distal sites (Duke's stages C and D) whereas only 7/31 (22.6%) of MSI-H cancers had progressed to stage C or stage D (table 1). However, this trend did not reach statistical significance (p=0.071).

Table 2 Distribution of CD3⁺ and CD8⁺ tumour infiltrating lymphocytes (TILs) in sporadic colorectal cancer, according to level of DNA microsatellite instability (microsatellite instability-high (MSI-H), microsatellite instability-low (MSI-L), and microsatellite stable (MSS))

Variables	*Total*	*MSI-H (n=32)*	*MSI-L (n=30)*	*MSS (n=40)*	*p Value*
CD3 count*					
Mean (SD)		68.4 (46.9)	36.3 (26.5)	24.6 (24.1)	<0.0001†
Median		64.5	25.0	16.0	
CD8 count*					
Mean (SD)		55.5 (43.4)	29.2 (24.6)	18.8 (20.4)	<0.0001†
Median		42.0	20.5	11.0	
TIL					
Negative	65	9 (28.1%)	21 (70.0%)	35 (87.5%)	<0.0001‡
Positive	37	23 (71.9%)	9 (30.0%)	5 (12.5%)	

*Count/1000 tumour nuclei.
†Kruskal-Wallis test.
‡χ^2 test.

TILs IN SPORADIC COLORECTAL CANCER ARE ASSOCIATED WITH BOTH THE MSI-H AND MSI-L PHENOTYPES

Immunohistochemical analysis revealed an abundance of TILs in MSI-H colorectal cancers with a mean count of 68.4 (SD 46.9) positively stained CD3 lymphocytes per 1000 tumour nuclei (table 2). The MSI-L subgroup had an intermediate mean CD3 count of 36.3 (26.5) and the mean CD3 count for the MSS group was the lowest at 24.6 (24.1). The distribution of $CD3^+$ count is summarised according to MSI in fig 1. Altogether, 37 of 102 tumours were scored as TIL positive (containing >50 $CD3^+$ lymphocytes per 1000 tumour nuclei). Of these, 23/32 (71.9%) were MSI-H, 9/30 (30%) were MSI-L, and only 5/40 (12.5%) were MSS (table 2). The difference in both median $CD3^+$ count and percentage of assigned TIL score between these groups was

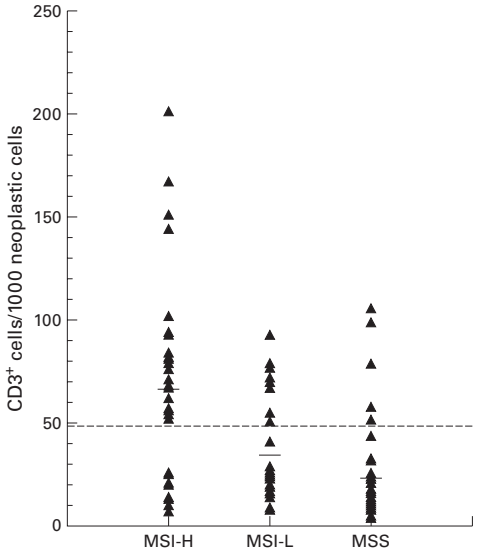

Figure 1 Distribution of $CD3^+$ tumour infiltrating lymphocytes (TILs) in colorectal cancer. All 102 tumour $CD3^+$ counts are represented by a triangle. The score of 50 $CD3^+$ lymphocytes was chosen as the cut off for TIL positive cancers (broken line) and the horizontal bars represent the mean count for each group. MSS, microsatellite stable; MSI-L, microsatellite instability-low; MSI-H, microsatellite instability-high.

highly significant (p<0.001) (table 2), confirming that these TILs were predominantly associated with the MSI and not the MSS phenotype. Further immunohistochemistry and quantitation with anti-CD8 found $CD8^+$ counts similar to $CD3^+$ counts in all 102 cancers, with $CD8^+$ cells comprising 75–80% of the $CD3^+$ population (fig 2, table 2). Therefore, TILs appeared to be predominantly $CD3^+/CD8^+$ T cells. This result was confirmed by the absence of positive intraepithelial anti-CD20 (B cell) staining in serial sections of the same tumours (fig 2D and data not shown). CD4 staining was also attempted but was unsuccessful on this series of tumours (data not shown).

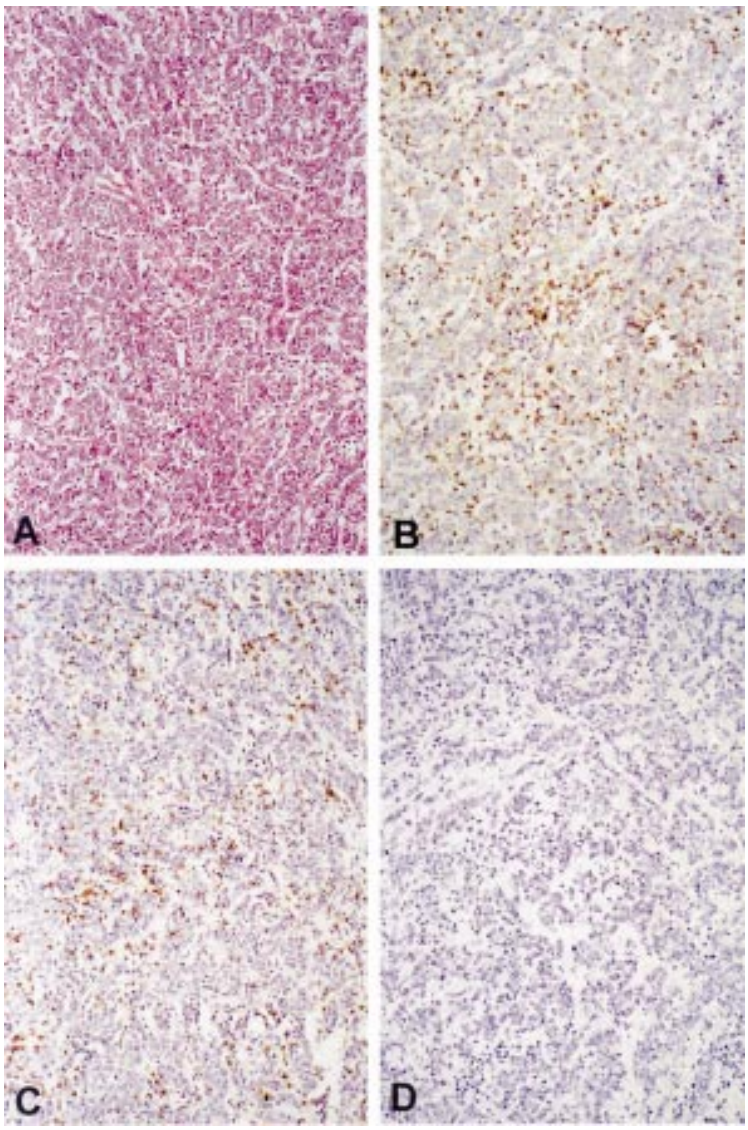

Figure 2 Example of a typical undifferentiated microsatellite instability-high (MSI-H) cancer heavily infiltrated with lymphocytes. (A) Standard haematoxylin and eosin stained section. (B) Serial section of the same tumour stained with anti-CD3, revealing T lymphocytes. (C) Serial section stained with anti-CD8. Note that most $CD3^+$ T cells also show positive staining with anti-CD8. (D) Serial section of the same tumour stained with anti-CD20, a B cell marker. Note that no intraepithelial cells have stained positively in this section (original magnification ×60).

MICROSATELLITE INSTABILITY IS ASSOCIATED WITH ENHANCED APOPTOSIS OF MALIGNANT EPITHELIAL CELLS

To determine if the presence of TILs correlates with an increased level of tumour cell apoptosis, the frequency and distribution pattern of apoptotic cells in the same selected series of 102 cancers were detected by performing immunohistochemistry with the M30 CytoDEATH antibody. The M30 antibody binds to a neoepitope on cytokeratin-18 exposed only after caspase cleavage thus allowing highly specific and early detection of apoptotic epithelial cells.[17] M30 CytoDEATH staining is markedly more sensitive than the morphological identification of apoptosis by haematoxylin and eosin staining (fig 3). Pockets of $M30^+$ malignant epithelial cells were found randomly distributed throughout the 102 examined lesions. Significantly, the MSI-H cancers showed on average a twofold higher rate of apoptosis (3.55 (1.99)%) than the MSS cancers (1.53 (1.47)%) while the MSI-L subgroup had an intermediate level of apoptosis (2.52 (1.92)%) in 10 randomly selected fields from each tumour (table 3). This difference in the level of tumour cell apoptosis was highly significant (p<0.0001). On average, apoptotic counts at the surface and growing edge were also significantly higher in the MSI-H subgroup, intermediate in the MSI-L, and lowest in the MSS cancers (p<0.0001 and p=0.002, respectively) (table 3). In addition, epithelial cell apoptosis in the glandular lumen was most prevalent in MSI-H cancers (table 3) although this difference did not reach statistical significance (p=0.059). M30 negative lumen may contain apoptotic TILs, necrotic debris, neutrophils. and phagocytic cells. Importantly, when serial sections were compared there was a paucity of $M30^+$ staining in areas heavily infiltrated with high $CD8^+$ T cells (fig 4) and similarly, M30 CytoDEATH/CD3 double staining showed little colocalisation between $CD3^+$ TILs and $M30^+$ apoptotic tumour cells (fig 5). Overall, there was a significant but weak-moderate linear correlation between the number of $CD3^+$ TILs and $M30^+$ random apoptosis scores (r=0.347, p<0.001). The linear correlation between $CD8^+$ lymphocytes and $M30^+$ apoptosis was of a similar magnitude (r=0.299, p<0.002). However, these linear correlations were lost when the tumours were subdivided into their three subgroups according to the level of MSI.

We also explored the relationship between apoptotic level and mutations in the *p53*, K-*ras*, and BAX genes, as well as p53 and Bcl-2 protein expression. In the 79 tumours previously analysed for K-*ras* status in this laboratory,[3] there was a trend towards decreased apoptosis (p=0.135), with the 19 tumours harbouring mutant K-*ras* demonstrating a mean apoptosis score of 1.56% compared with 2.51% in the 60 tumours with wild-type K-*ras*. In the 81 tumours from this series previously analysed for *p53* mutation,[6] no significant correlation was found between *p53* status and apoptosis, with 42 wild-type and 39 mutant *p53* cancers having similar mean apoptosis scores (2.39% *v*

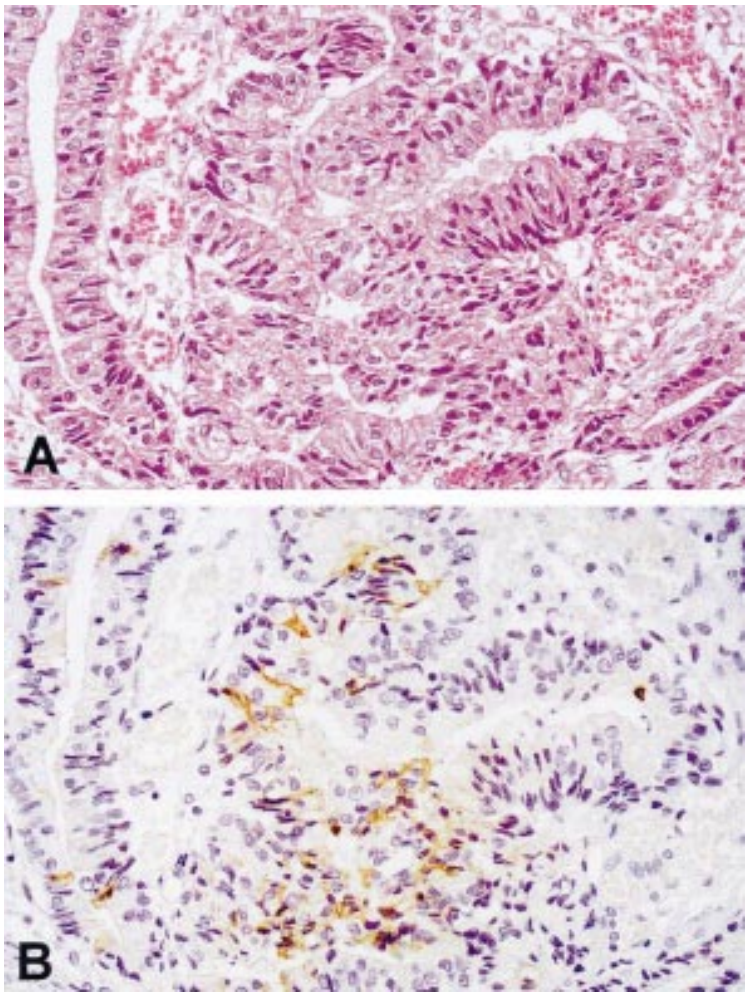

Figure 3 Serial sections of a microsatellite instability-low (MSI-L) cancer (A) stained routinely with haematoxylin and eosin and (B) staining with the M30 cytoDEATH antibody specific for epithelial cell apoptosis. The brown cytoplasmic staining indicates the many apoptotic tumour cells present in this field (original magnification ×120).

2.20%, respectively). However, over expression of mutant p53 protein, as determined previously by immunohistochemistry,[18] was correlated with a low level of apoptosis in 85 tumours from this series (p=0.041). Bcl-2 protein expression was also assessed previously in 65 tumours from the current series.[18] No correlation was found when random apoptosis counts were compared across Bcl-2 expression (p=0.618). Finally, BAX status was assessed in the 32 MSI-H cancers of this series, as described previously.[6] The apoptotic index in the 16 tumours with insertion or deletion in the polyG tract in the BAX gene (3.78%) was similar to that of the 16 tumours without BAX mutation (3.32%) (p=0.361).

Table 3 Distribution of apoptosis in colorectal cancer according to the level of DNA microsatellite instability (microsatellite instability-high (MSI-H), microsatellite instability-low (MSI-L), and microsatellite stable (MSS))

Variables	*Total†*	*MSI-H (n=32)*	*MSI-L (n=30)*	*MSS (n=40)*	*p Value*
Apoptosis					
Random % (mean (SD))		3.55 (1.99)	2.52 (1.92)	1.53 (1.47)	<0.0001‡
Median		3.20	1.95	1.15	
Surface—apoptosis*					
0 (⩽ 1%)	18	1 (3.45%)	3 (11.11%)	14 (41.18%)	<0.0001‡
+ (1–5 %)	35	7 (24.14%)	13 (48.15%)	15 (44.12%)	
++ (5–10 %)	17	9 (31.03%)	5 (18.52%)	3 (8.82%)	
+++ (10–15 %)	11	9 (31.03%)	1 (3.70%)	1 (2.94%)	
++++ (>15%)	9	3 (10.34%)	5 (18.52%)	1 (2.94%)	
Growing edge—apoptosis					
0 (⩽ 1 %)	41	7 (21.21%)	10 (33.33%)	24 (61.54%)	0.002‡
+ (1–5 %)	47	17 (51.52%)	16 (53.33%)	14 (35.90%)	
++ (5–10 %)	14	9 (27.27%)	4 (13.33%)	1 (2.56%)	
Lumen—apoptosis**					
0 (little or none)	53	12 (41.38%)	13 (46.43%)	28 (71.79%)	0.059‡
+ (some)	34	12 (41.38%)	13 (46.43%)	9 (23.08%)	
++ (widespread)	9	5 (17.24%)	2 (7.14%)	2 (5.13%)	

†Total is the horizontal sum from each of the three MSI subgroups.
‡χ^2 test.
*Some samples were not available for testing.
**Glandular lumen is not applicable to undifferentiated tumours and therefore these were excluded from this analysis.

Discussion

There is now a large body of evidence that demonstrates significant phenotypic and genotypic differences between colorectal cancers with MSI compared with those that are MSS.[2–10] However, the over representation of poorly differentiated tumours in the MSI-H group has been difficult to rationalise with the better prognosis of these tumours reported in a number of series.[5 10 11] One of the major phenotypic features that may provide some explanation for this better prognosis is the presence of a significant lymphocytic infiltrate in MSI cancers. Previous work from this laboratory suggested that 33% of MSI-H cancers had a significant lymphocytic infiltrate.[2] However, the current study clearly shows that immunohistochemical assessment of TILs is markedly more sensitive than previous study methods based on morphological analysis by routine haematoxylin and eosin staining. Anti-CD3 staining identified 2–5-fold higher numbers of TIL positive cancers than were assessed by eye[2] and the presence of a gradient of TIL infiltrate between MSI-H, MSI-L, and MSS cancers. The number of TILs identified in MSI-H cancers (median 6.8%) is similar to the ratio of IEL to epithelial cells in the normal colonic mucosa (5 IEL:100 epithelial cells), and significantly greater than TIL number in the MSI-L (3.6%) and MSS (2.5%) subgroups. We also found that 75–80% of TILs were $CD3^+/CD8^+$ T lymphocytes, consistent with a colonic intraepithelial origin and with the findings of Dolcetti and colleagues.[12] The remaining 20–25% of $CD3^+$ $CD8^-$ TILs are most likely to be $CD4^+$ T cells, giving a similar CD8:CD4 ratio within tumours to that found in the normal human intestinal IEL population.[16 19]

The presence of the lymphocytic infiltrate was investigated further by a detailed analysis of epithelial/tumour cell apoptosis, using the M30 CytoDEATH antibody. Apoptotic counts were significantly higher (p<0.0001) in the MSI-H cancers (3.55 (1.99)%) than in the MSS cancers (1.53 (1.47)%). The MSI-L subgroup had an intermediate level of apoptosis (2.52 (1.92)%). The presence of both higher numbers of TILs and a higher apoptotic index in the MSI-H cancers was also demonstrated by Dolcetti and colleagues.[12] However, their further observations showing close apposition of $CD8^+$ TIL and TUNEL positive epithelial cells were not supported statistically by any linear correlation in that study. In our larger series of sporadic cancers, there was a weak to

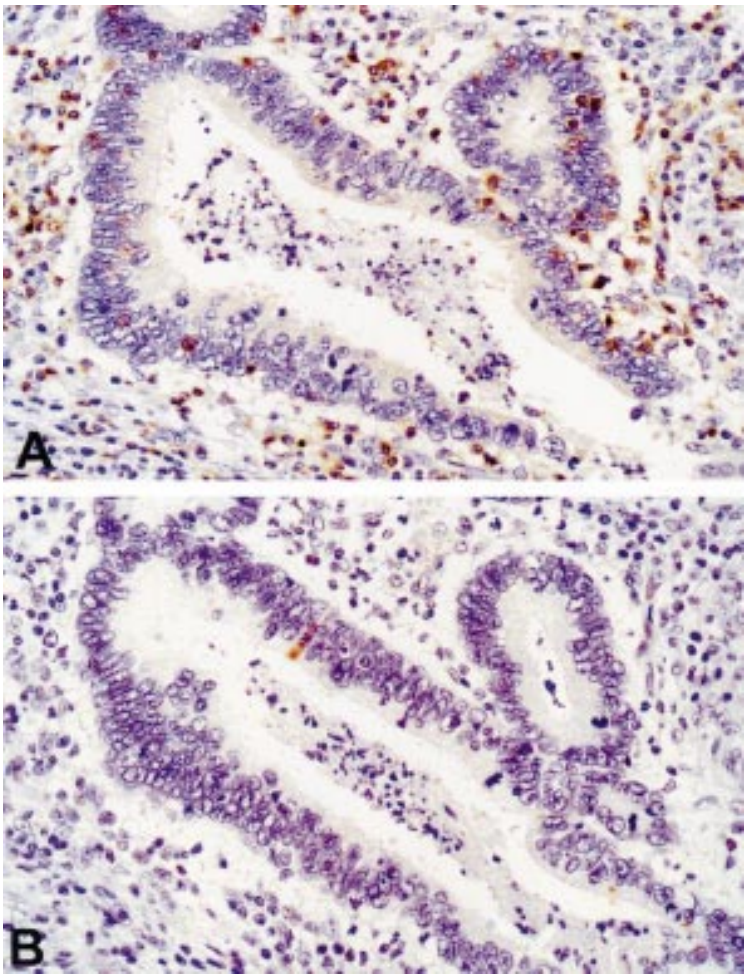

Figure 4 Serial sections of a well differentiated colorectal cancer stained with (A) anti-CD3 and (B) M30 CytoDEATH antibody. Note that only one apoptotic cell is present in this area densely infiltrated with tumour infiltrating lymphocytes (original magnification ×120).

moderate statistically significant correlation between total $CD3^+$ TILs and $M30^+$ random apoptosis scores (r=0.347), with the $CD8^+$:$M30^+$ correlation being of similar magnitude (r=0.299). This significant correlation was lost when the individual groups were analysed stratified by microsatellite status. In addition, no significant difference in apoptosis was found between TIL^+ and TIL^- cancers either overall (3.0 (2.0)% *v* 2.1 (1.8)%, respectively) or within MSI subgroups. In particular, in MSI-H cancers no difference was found between TIL^+ and TIL^- apoptosis with scores of 3.6 (2.0)% and 3.4 (2.1)%, respectively. Furthermore, within individual tumours, areas of TILs and apoptosis did not seem to coincide. Taken together, these results suggest that the enhanced level of apoptosis in MSI-H cancers is spontaneous in nature.

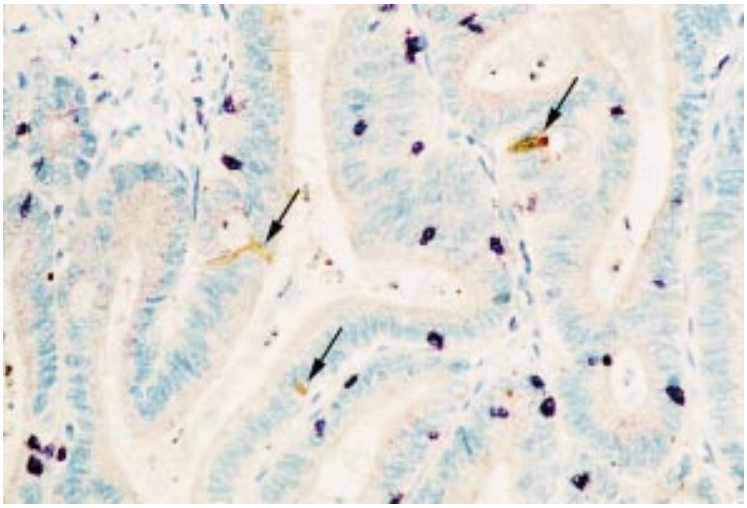

Figure 5 Example of M30 CytoDEATH and CD3 double staining, counterstained with methyl green. The brown DAB staining indicates $M30^+$ apoptotic tumour cells (arrows) and purple Vector VIP staining reveals the $CD3^+$ T cells present in this tumour infiltrating lymphocyte (TIL) positive microsatellite instability-high (MSI-H) cancer. Note that $M30^+$ neoplastic cells and $CD3^+$ TILs do not coincide (original magnification ×120).

Results published previously from this laboratory on the relationship between microsatellite status and frequency of *p53*, K-*ras*, and BAX mutations, using a similar series of colorectal cancers, indicated that *p53* and K-*ras* mutations are typically found in MSS and MSI-L cancers but very rarely in MSI-H cancers.[3 6] Although these results may seem a reasonable explanation for the differences in apoptotic rates between the MSI-H, MSI-L, and MSS subgroups, in this study no significant correlation was found between either TIL status or apoptotic count and the presence or absence of a K-*ras* mutation. As expected, a trend approaching significance was found between TIL^+ cancers and wild-type *p53* (p=0.143), as both of these are features of MSI-H cancers. However, no significant correlation was found between the presence of *p53* mutations and tumour cell apoptosis, although as might be expected, increased expression of p53, indicative of the stabilisation of mutant p53 protein, was negatively correlated with apoptosis scores (p=0.041).

In contrast with *p53* and K-*ras*, BAX mutations are found in 50% of MSI-H but in only 2% of MSS cancers.[6] Given that BAX frameshift mutations usually result in inactivation of the proapoptotic protein,[7] one might expect a reduced apoptotic rate in these tumours. Surprisingly, we found no significant correlation between the apoptotic index and BAX status of the tumours used in this study. Therefore, our data indicate that apoptosis in MSI-H cancers appears to be independent of BAX expression. Furthermore, no significant correlation was found between expression of Bcl-2 and apoptotic index, even though we have previously shown that colorectal cancers expressing the antiapoptotic Bcl-2 protein are more likely to be MSS.[18] The lack of correlation with BAX and Bcl-2 status may be further indirect evidence that the higher apoptotic rate seen in MSI-H cancers is partially related to the high number of TILs.

Previous studies of the apoptotic index (expressed as a percentage of total epithelial cells counted, as in this study) have used either standard haematoxylin and eosin staining or TUNEL. The former method may give rise to inappropriately low rates due to a lack of sensitivity, as demonstrated in the study by Sinicrope *et al* who found a median apoptotic index of 1.2%.[20] This study did not control for microsatellite status. The higher apoptotic

counts found with TUNEL may be related to the lack of specificity of this method (staining mitotic and necrotic cells), while other potential drawbacks include the need for double staining and lack of sensitivity in picking up the earlier stages of apoptosis compared with the M30 CytoDEATH antibody.[12 17 21 22] In those studies using TUNEL, and not assessing MSI, the overall apoptotic index ranged between 0.95% and 3.1%[23 24] while in the only other study that controlled for microsatellite status, Dolcetti *et al* found an apoptotic index of 4.1% in the MSI-H group and 1.53% in the MSS group.[12] In the same study, MSI-L was not assessed and the authors admitted to some "normal" looking nuclei being stained with TUNEL. This was not observed with the M30 antibody.

Interestingly, in the normal colon the apoptotic rate in the right colon is less than that in the left colon (1.2% *v* 2.2%) using the TUNEL technique.[25] Our study suggests that the majority of MSI-H cancers undergo apoptosis at a rate approximately three times that of the normal epithelium, while the majority of MSS cancers show a reduction in apoptosis by a factor of 0.7. We analysed this further by examining apoptosis in all cancers based on location (right *v* left). This showed a trend towards significance (p=0.089) but was heavily influenced by the number of MSI-H cancers on the right side (28/32). A further comparison of apoptotic index in all MSI-L and MSS cancers (21 right sided, 48 left sided) showed no difference (p=0.620) based on location. Consistent with the suggestion of other studies,[12 25] our study excludes the possibility that the higher rate of apoptosis in the MSI-H cancers is due to their proximal location, rather, this increased apoptotic index appears to be associated with the high level of DNA microsatellite instability in MSI-H cancers.

Importantly, our findings are the first to demonstrate that, at best, the increased frequency of TILs associated with MSI-H cancers may only partially explain the higher apoptotic counts seen within these tumours. The apoptotic counts and the TIL^+ phenotype are closely related to MSI-H status and are not explained by location of the tumour. The better prognosis fits in well with the above findings. However, it does not explain why 30% of MSI-L cancers are characterised by the presence of TILs and an intermediate apoptotic rate, yet no survival advantage has been documented for this subgroup. Correlations between apoptotic rate, TIL count, and K-*ras* status within these subgroups may be useful but were limited by numbers in this study.

In conclusion, this study indicates that TILs are a useful biomarker of MSI-H cancers but TILs and tumour apoptosis are only weak-moderately correlated. While TILs might be expected to explain the increased apoptotic rate and improved prognosis of MSI-H cancers, it is likely that TILs and apoptosis are independent characteristics of MSI-H cancers.

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1 Thibodeau SN, French AJ, Cunningham JM, *et al*. Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. *Cancer Res* 1998;**58**:1713–8.
2 Jass JR, Do K-A, Simms LA, *et al.* Morphology of sporadic colorectal cancer with DNA replication errors. *Gut* 1998;**42**:673–9.
3 Jass JR, Biden KG, Cummings MC, *et al*. Characterisation of a subtype of colorectal cancer combining features of the suppressor and mild mutator pathways. *J Clin Pathol* 1999; **52**:455–60.
4 Kim H, Jen J, Volgestein B, *et al*. Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences. *Am J Pathol* 1994;**145**:148–56.
5 Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993;**260**:816–19.
6 Simms LA, Radford-Smith G, Biden KG, *et al*. Reciprocal relationship between the tumour suppressors *p53* and BAX in primary colorectal cancers. *Oncogene* 1998;**17**:2003–8.
7 Rampino N, Yamamoto H, Ionov Y, *et al*. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997;**275**: 967–9.
8 Markowitz S, Wang J, Myeroff L, *et al*. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* 1995;**268**:1336–8.
9 Souza RF, Appel R, Yin J, *et al*. Microsatellite instability in the insulin-like growth factor II receptor gene in gastrointestinal tumours. *Nat Genet* 1996;**14**:255–7.
10 Lothe RA, Peltomaki P, Meling GI, *et al*. Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history. *Cancer Res* 1993;**53**:5849–52.
11 Wright CM, Dent OF, Barker M, *et al*. The prognostic significance of extensive microsatellite instability in sporadic clinicopathological stage C colorectal cancer. *Br J Surgery* 2000;**87**:1197–1202.
12 Dolcetti R, Viel A, Doglioni C, *et al.* High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol* 1999;**154**:1805–13.
13 Ropponen KM, Eskelinen MJ, Lipponen PK, *et al*. Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. *J Pathol* 1997;**182**:318–24.
14 Naito Y, Saito K, Shiiba K, *et al.* $CD8^+$ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 1998;**58**:3491–4.
15 Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin embedded tissues: an enhancement method for immunohistochemical staining based upon microwave oven heating of tissue sections. *J Histochem Cytochem* 1991;**39**:741–8.
16 Hirata I, Berrebi G, Austin LL, *et al*. Immunohistological characterization of intraepithelial and lamina propria lymphocytes in control ileum and colon and in inflammatory bowel disease. *Dig Dis Sci* 1986;**31**:593–63.
17 Leers MPG, Kolgen W, Bjorklund V, *et al.* Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. *J Pathol* 1998; **187**:567–72.
18 Biden, KG, Simms LA, Cummings M, *et al.* Expression of Bcl-2 protein is decreased in colorectal adenocarcinomas with microsatellite instability. *Oncogene* 1999;**18**:1245–9.
19 Cerf-Bensussan N, Guy-Grand D, Griscelli C. Intraepithelial lymphocytes of human gut: isolation, characterization and study of natural killer activity. *Gut* 1985;**26**:81–8.
20 Sinicrope FA, Hart J, Hsu H-A, *et al.* Apoptotic and mitotic indices predict survival rates in lymph-node negative colon carcinomas. *Clin Cancer Res* 1999;**5**:1793–804.
21 Cohen GM, Sun X-M, Snowden RT, *et al.* Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem J* 1992;**286**: 331–4.
22 Enright H, Hebbel RP, Nath KA. Internucleosomal cleavage of DNA as the sole criterion for apoptosis may be artifactual. *J Lab Clin Med* 1994;**124**:63–8.
23 Koike M. Significance of spontaneous apoptosis during colorectal tumorigenesis. *J Surg Oncol* 1996;**62**:97–108.
24 Kikuchi Y, Dinjens WNM, Bosman FT. Proliferation and apoptosis in proliferative lesions of the colon and rectum. *Virchows Arch* 1997;**431**:111–17.
25 Liu LU, Holt PR, Krivosheyev V, *et al*. Human right and left colon differ in epithelial cell apoptosis and in expression of Bak, a pro-apoptotic Bcl-2 homologue. *Gut* 1999;**45**:45–50.