Microsatellite instability in a population of sporadic colorectal cancers: correlation between genetic and pathological profiles

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Background. *Tumours with high-frequency microsatellite instability exhibit unique genotype and phenotype features, whereas the difference between low-frequency microsatellite instability and apparently stable tumours is far from being clear.*
Aims. *To identify distinctive genetic and pathological characteristics of low-frequency microsatellite instability tumours.*
Methods. *Microsatellite instability status of 57 sporadic colorectal cancers and its correlation with genetic, pathological and clinical features was analysed.*
Results. *High frequency microsatellite instability and low-frequency microsatellite instability and apparently stable cancers were different in terms of tumour localisation (p=0.015), frequency of APC mutations (p=0.012), occurrence of Crohn's-like/lymphoid reaction (p=0.0353) and morphological evidence of origin from an adenoma (p=0.0338). Specifically, in low-frequency microsatellite instability cancers, APC mutations were very frequent (76.9%, 10/13) and a Crohn's-like/lymphoid reaction was common (38.5%, 5/13). High-frequency microsatellite instability tumours were preferentially located in the right colon and exhibited a higher frequency of loss of heterozygosity at the FHIT locus compared with low-frequency microsatellite instability and apparently stable cases (p=0.0243). Dukes' stage (p=0.0021), tumour localisation (p=0.0410) and pattern of cancer growth (p=0.0374), were the only factors affecting patient survival. However, a borderline improvement was noted in overall survival in high-frequency microsatellite instability and low-frequency microsatellite instability cancer patients (p=0.062).*
Conclusions. *These results indicate that low-frequency microsatellite instability tumours have different genetics and histological features and suggest that they are a distinct group of colorectal cancers.*

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Introduction

It is generally accepted that colon cancer results from the progressive accumulation of mutations in genes involved in the control of the cell cycle [1]. It has been suggested that inactivation of the APC gene initiates colorectal neoplasia and during the adenoma-carcinoma sequence additional alterations involve proto-oncogenes and tumour suppressor genes [2,3]. However, the possibility that some colorectal tumours could be initiated by genomic instability was highlighted by the discovery of colon cancers with widespread microsatellite instability (MSI) caused by replication errors, a phenomenon highly suggestive of an underlying genetic instabili-

ty[4]. Almost all patients with hereditary non-polyposis colorectal cancer (HNPCC) have MSI and inherited mutations at one of four DNA mismatch repair (MMR) genes. On the contrary, only some 15% of sporadic colon cancers have MSI and, in these cases, the inactivation of MMR genes is as frequent as in HNPCC[5].

The criteria for classifying MSI in tumours have recently been reviewed[6] and a panel of five microsatellites has been recommended as a reference panel. Accordingly, it has been proposed that tumours may be defined as high-frequency MSI (MSI-H) if two or more of the five markers show instability (i.e., have insertion/deletion mutations), and low-frequency MSI (MSI-L) if only one of the five markers shows instability.

MSI-H colorectal tumours exhibit unique features in genotype and phenotype that distinguish them from tumours without MSI. MSI-H carcinomas are found predominantly in the proximal colon, have mucinous and undifferentiated histology and are generally associated with a less aggressive clinical course[6]. On the contrary, MSI-L cancers appear to have more in common with apparently microsatellite stable (MSS) than MSI-H. On the other hand MSI-L tumours are poorly characterised, being often grouped together with MSS or with MSI-H cases.

In this study, we evaluated the MSI status of 57 human sporadic colorectal carcinomas, previously characterised for FHIT gene alterations[7], and its correlation with mutations in APC, the "gatekeeper" gene in colon carcinogenesis, and in TGF-βRII, one of the important target genes for the high MSI phenotype. Finally, to determine the possible prognostic significance of the different MSI status, we evaluated a series of clinical and pathological variables such as Dukes' stage, grading, invasive margin (expanding or infiltrating), Crohn's-like lymphoid reaction, presence of residual adenomas within cancer, relapse rate, distant metastases and survival.

Materials and methods

Patients and specimens

Tissues were obtained from patients undergoing surgical resection for colorectal cancer between 1994 and 1997. Tumour and normal tissue specimens from 57 patients were frozen immediately and stored in liquid nitrogen. Of these patients, 35 (61.4%) were male and 22 (38.6%) female, ages ranging from 23 to 82 years (mean ± SE 63.56±1.42). The clinical-pathological features of each patient were reviewed and recorded.

The same pathologist, without knowledge of mutational analysis and clinical outcome, reviewed all slides. Tumour histotype and grading were defined according to WHO classification[8]. In each case, we evaluated the pattern of cancer growth (expanding or infiltrating), the occurrence of Crohn's-like lymphoid reaction at the periphery of the cancer, and the presence of residual adenomas within cancers. All cases were staged by the modified Dukes' system[9]: A: cancer limited to bowel wall, B: extra mural spread, C: lymph-node metastasis, D: distant metastasis.

Tumours involving the caecum up to the splenic flexure were designated as "right" colon tumours; the splenic flexure, left colon and sigmoid were designated as "left" colon tumours, while sigmoid junction, intra- and extra-peritoneal rectum were indicated as "rectum".

Follow-up information on patient relapse and survival was obtained from clinical records and only patients whose primary cause of death was recurrent colorectal cancers were considered as events in the survival analysis. Of the 57 patients investigated, 77% were still alive at the end of the study with a mean follow-up of 5.1 years (range 4.2 to 7).

MSI analysis

Total DNA was extracted from frozen tumours and normal tissue using QIAamp tissue kit (QIAGEN, Hilden, Germany), according to the manufacturer's specifications.

Microsatellite analysis was performed using a polymerase chain reaction (PCR)-based approach with primers amplifying four (CA)n and three (A)n polymorphic microsatellite loci: D3S1300 (within intron 5 of the FHIT gene locus, close to exon 5), D3S1234 (located in FHIT intron 7), D2S123 (within the hMSH2 gene), D5S346 (close to the APC gene locus), BATRII (containing a 10-base pair adenine sequence in the coding region of TGF-βRII gene), BAT40 and BAT26. One of two primers from each marker was first end-labelled with γ^{33}P-ATP (2000 Ci/mmol; NEN, Germany) and T4 DNA polynucleotide kinase (Pharmacia Biotech Italia, Cologno Monzese, MI, Italy). PCR reactions were carried out in a 15 µl volume containing about 150 ng of genomic DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 mM primers, 0.2 µl of the end-labelled primer and 1.25 units of Taq polymerase (Advanced Biotechnologies Ltd., Epsom, UK). The PCR conditions of the seven markers were the same: 30 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were separated on a 7% polyacrylamide-urea-formamide gel and visualised by autoradiography. Tumours showing band shift at one or more microsatellite markers were termed MSI. MSI status was sub-

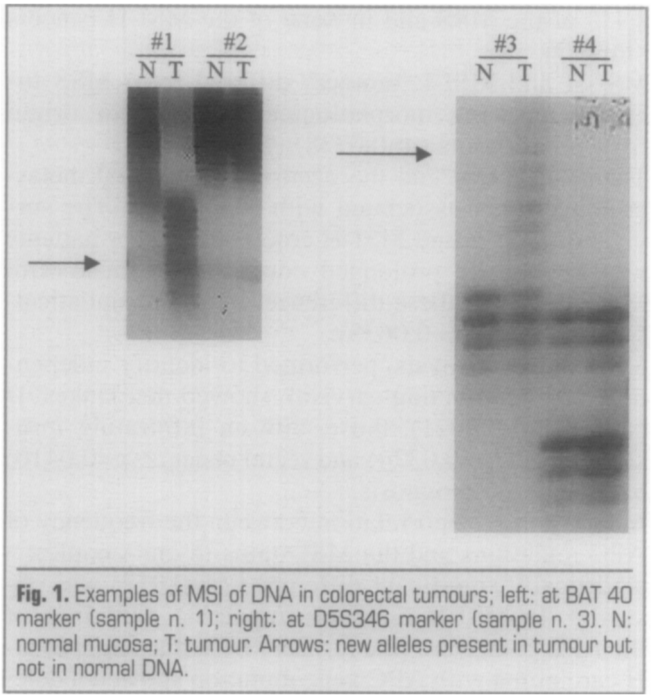

Fig. 1. Examples of MSI of DNA in colorectal tumours; left: at BAT 40 marker (sample n. 1); right: at D5S346 marker (sample n. 3). N: normal mucosa; T: tumour. Arrows: new alleles present in tumour but not in normal DNA.

classified as MSI-H when band shifts were seen at more than 30% of markers and MSI-L when the value was less than 30%. Examples of such analyses are shown in Figure 1.

In vitro synthetised protein (IVSP)

Screening for truncating APC mutations was performed on 51 tumours since, for six tumour specimens, we did not obtain enough tissue to perform the IVSP analyses. The region between nucleotide 2058 to nucleotide 5079 of the APC gene was analysed for APC mutations using the IVSP assay [10]. In brief, two overlapping segments of the APC gene (segment S2 from nt. 2058 to nt. 3651 and segment S3 from nt. 3297 to nt. 5079) were amplified using PCR as follows: 200 ng of genomic DNA; 350 ng each of the appropriate primers:

Segment S2: 5'-GGATCCTAATACGACTCAC-TATAGGGAGACCACCATGGATGCATGTG-GAACTTTG

TGG- 3'and 5'-CTCTTGGCATTAGATGAAGGT-GTGGACG-3',

Segment S3: 5'-GGATCCTAATACGACTCAC-TATAGGGAGACCACCATGGTTTCTCCAT-ACAGGTCA

CGG-3' and 5'-GGAGGATCCTGTAGGAATG-GTATCTCG-3'.

Two units of Taq Polymerase (Advanced Biotechnologies Ltd., Epsom, UK) were used in a 50 µl PCR reaction (10 mM Tris-HCl, 1.5 mM $MgCl_2$). Amplifications were performed in a thermal cycler (Perkin Elmer 9700, Foster City, CA, USA) for 36 cycles of 40' denaturation (95°C), 90" annealing (59°C), 100" extension (72°C). All PCR reactions included a 5-min extension period (72°C) after the thirty-sixth cycle. PCR reaction mixtures (4 µl), purified with chloroform, were used as templates in 25 µl in a linked transcription-translation system (TNT T7 Quick Coupled transcription/translation system, Promega, WI, USA) containing 10 µCi of [^{35}S]methionine (Amersham, Cologno Monzese, MI, Italy) for 90 min (transcription/translation) at 30°C. Samples were diluted in sample buffer, denaturated for 5 min at 95°C and analysed on 12.5% sodium dodecylsulfate-polyacrylamide gel. Proteins were visualised by autoradiography. Tumours with truncated protein were analysed by sequencing to determine the underlying mutation. PCR products for sequencing analysis were purified from agarose gels (QIAquik gel extraction kit, Quiagen, Hilden, Germany). PCR products of the mutated samples were sequenced using internal primers and DNA Sequencing kit (Rodhamine Terminator Cycle Sequencing Ready reaction (ABI PRISM, Perkin Elmer Applied Biosystem, Foster City, CA, USA).

Statistical analysis

Fisher exact test and extension to general RxC tables were performed when appropriate. The level of significance was set at $p<0.05$, two-sided [11]. The correlations between the MSI status and relapse and survival were carried out using univariate and multivariate models by Cox regression analysis in order to identify independent survival factors. Kaplan-Meier curves, used to estimate survival probability as a function of time and patient relapse or survival differences, were analysed by the log-rank test. Statistical analyses were carried out using STATA 6 Statistical Software (StataCorp, College Station, TX, USA).

Results

Of the 57 sporadic colorectal cancers analysed, 7 (12.28%) were MSI-H, showing instability at more than 30% of loci. Whilst 13/57 (22.8%) were MSI-L (instability at less than 30% of loci) and 36/57 (63.15%) were MSS.

MSI-H tumours were more common in males (6/7; 85.7%) than in females (1/7; 14.3%) but this difference was not statistically significant (Table I). MSI was not significantly correlated with the age of the patients: the mean age was 62.5±2.0 years in the MSS group, 65.0±2.2 in the MSI-L and 65.3±3.6 in MSI-H.

No differences were seen between the three groups regarding Dukes' stage, histological grade and pattern

Table I. Relationships between MSI colorectal cancers and clinicopathological variables.

	MSS n (%)	MSI-L n (%)	MSI-H n (%)	p
Age (yrs)				ns[c]
≤60	11 (29.7)	4 (30.8)	3 (42.9)	
>60	26 (70.3)	9 (69.2)	4 (57.1)	
Sex				ns
Female	15 (40.5)	6 (46.2)	1 (14.3)	
Male	22 (59.5)	7 (53.8)	6 (85.7)	
Dukes' stage[a]				ns
A	2 (5.4)	2 (15.4)	1 (14.3)	
B	14 (37.8)	4 (30.8)	3 (42.85)	
C	11 (29.7)	6 (46.1)	3 (42.85)	
D	10 (27)	1 (7.7)	0	
Histological grade[d]				ns
Well differentiated	1 (2.8)	0	2 (28.6)	
Moderate differentiated	27 (75)	9 (69.2)	3 (42.8)	
Poorly differentiated	5 (13.9)	1 (7.7)	2 (28.6)	
Mucinous	3 (8.3)	3 (23.1)	0	
Invasive margin[e]				ns
Expanding	10 (28.6)	5 (41.7)	3 (42.8)	
Infiltrating	25 (71.4)	7 (58.3)	4 (57.1)	
Location[b]				0.015
Right colon	7 (18.9)	4 (30.8)	6 (85.7)	
Left colon	13 (35.1)	5 (38.5)	1 (14.3)	
Rectum	17 (45.9)	4 (30.8)	0	
Crohn's-like				0.0353
Yes	3 (8.1)	5 (38.5)	0	
No	34 (91.9)	8 (61.5)	7 (100)	
Ex adenoma				0.0338
Yes	4 (10.8)	5 (38.5)	3 (42.9)	
No	33 (89.2)	8 (61.5)	4 (57.1)	
APC gene[f]				0.012
WT	19 (61.3)	3 (23.1)	6 (85.7)	
Mut	12 (38.7)	10 (76.9)	1 (14.3)	

n: number of cases; p: from Fisher exact test; [a] according to modified Dukes' classification; [b] carcinomas in caecum, ascending and transverse colon are classified as right-sided; carcinomas in splenic flexure, in descending and sigmoid part of colon are classified as left-sided; [c] not significant; [d] data available for 56 patients; [e] data available for 55 patients; [f] data available for 51 patients. Abbreviations: see list.

of cancer growth. No mucinous tumour or Dukes' D tumour was found in the MSI-H group (Table I).

A significant correlation was found between tumour location and MSI status; MSI-H carcinomas were preferentially located in the right colon, compared with MSS and MSI-L tumours (p=0.015) (Table I). MSI-L, MSI-H and MSS tumours were also different regarding Crohn's-like lymphoid reaction (p=0.0353): Crohn's-like lymphocyte infiltration was present in 5 out of 13 (38.5%) MSI-L specimens, in 8.1% of the MSS and in none of the MSI-H cancers (Table I).

MSI-H and MSI-L tumours differed from MSS tumours, showing morphological evidence of origin from an adenoma (p=0.0338) (Table I).

The relapse rate and the occurrence of distal metastases were not associated with MSI status. The survival of MSI-L and MSI-H colorectal cancer patients was seen to be prolonged compared to those with MSS; however, these differences were not statistically significant (p= 0.0635).

Multivariate analysis, performed to identify independent factors affecting survival, showed that Dukes' D cancers (p=0.0021), those with an infiltrating invasive margin (p=0.0374) and rectal cancers (p=0.0410) had the worst prognosis.

In analysing the correlation between the frequency of APC mutations and the MSI status in our samples, a statistically significant difference (p=0.012) was observed. In MSI-H tumours, truncating mutations of the APC gene were rare (14.3%, 1/7); the single MSI-H carcinoma with APC gene mutation showed a deletion GAAAAG → GAAAG located in the nucleotide 3752. On the contrary, in MSI-L tumours, APC mutations were very frequent (76.9%, 10/13) compared with MSS (12/31; 38.7%) and MSI-H (14.3%, 1/7) tumours (Table II). Moreover, 30% of MSI-L tumours showing an APC truncated protein, carried two mutations in this gene.

Only two mutations were found in the A_{10} coding sequence of the TGF-βII gene, both in MSI-H tumours; in one case this sequence was reduced to A_8 and in the second it increased to A_{11} (Table II).

Loss of heterozygosity (LOH) at the FHIT locus (D3S1234 and D3S1300) were more frequent in MSI-H tumours compared with MSS (60% versus 9.37% in MSI-H and MSS tumours, respectively; p=0.0242) (Table II).

Table II. Summary of somatic changes in MSI-H, MSI-L and MSS sporadic colorectal carcinoma.

	N. tumours with somatic changes		
	Mutations/total, n(%)		LOH/Informative tumours, n(%)
	APC	TGFβRII (BATRII)	FHT (D3S1234/D3S1300)
MSI-H	1/7 (14.2)[b]	2/7 (28.5)[a]	3/5 (60)[a]
MSI-L	10/13 (76.9)[a]	0/13	1/9 (11.1)
MSS	12/31 (38.7)	0/37	3/31 (9.67)

[a] p<0.05 vs MSS; [b] p<0.05 vs MSI-L. Abbreviations: see list.

Discussion

Alterations in the length of simple repetitive genomic sequences, manifesting MSI, may characterise a distinct mechanism of colorectal carcinogenesis, differing from the classical suppressor pathway [12].
MSI tumours show, in fact, a unique spectrum of genetic alterations. The frequency of mutations in some oncogenes (K-ras) and tumour suppressor genes (APC, p53) in MSI tumours are reported to be lower than in MSS [13-15] whereas mutations in short repetitive tracts within the coding regions of genes known to be involved in carcinogenesis, like TGF-βRII, IGF2R and BAX, are common [16-18].
Accordingly, we found mutations in the A_{10} coding sequence of the TGF-βRII only in MSI-H tumours. We also noted a higher frequency of LOH at the FHIT gene locus in MSI-H compared with MSS carcinomas (p=0.0243). FHIT is a candidate human tumour suppressor gene located at chromosome 3p14.2, a location that encompasses the fragile FRA3B chromosomal site [19]. Aberrant transcripts have been detected in a variety of primary tumours and homozygous deletions in the FHIT locus have been observed in different tumour cell lines. Recently, Hilgers et al. reported that human pancreatic cancer cell lines with MSI-H phenotype, showed homozygous deletions within the FHIT gene [20]. These results suggest that also FHIT could be a target gene for MSI.
We analysed a segment of the APC containing a region with the highest frequency of mutations. Although our analysis might under-estimate the amount of APC mutations, it shows that, at least in the analysed region, APC gene mutations are very frequent in MSI-L carcinomas compared with MSS and MSI-H (76.9%, 38.7% and 14.3%, respectively) and 30% of these tumours carried two mutations. Konishi et al. [13] also found APC mutations in 65% of the MSI-L, in 38% of the MSS and in none of the MSI-H sporadic colorectal carcinomas.
APC gene and β-catenin mutations have been shown to activate the same signalling pathway and seem to be mutually exclusive [21]. In sporadic colorectal carcinomas, β-catenin mutations are infrequent [22]; on the other hand, it has recently been reported that in hereditary non-polyposis colorectal cancer (HNPCC) [23] and in sporadic MSI-H colorectal carcinomas [24] β-catenin mutations are common. Altogether these data suggest that these mutations are relatively specific to the MMR-deficient genomic instability pathway whereas loss of APC is a sporadic event.
On the contrary, our data demonstrate that in MSI-L carcinomas, APC inactivation occurs preferentially. Accordingly, an abnormal β-catenin expression has recently been reported in MSI-L tumours [25]. The inactivation of APC gene in human colorectal cancers leads to uncontrolled β-catenin accumulation [26].
Our study reveals that MSI-H, MSI-L and MSS tumours have distinct biological characteristics and these differences result in different pathological features. Among the histological and clinical features analysed, we found that MSI-H, MSI-L and MSS cases differ in terms of tumour localisation (p=0.015), Crohn's-like lymphoid reaction (p=0.0353) and morphological evidence of origin from an adenoma (p=0.0338). Almost all MSI-H cases were located in the right colon and 42.9% of the MSI-H and 38.5% of the MSI-L cancers revealed the presence of adenomatous components, suggesting that they are relatively early colorectal carcinomas and that they are the result of adenoma-carcinoma progression.
While a marked host-lymphocytic response is an important histologic feature of MSI-H, it is only infrequently observed in MSS tumours [27 28]. It has been speculated that the peculiar genomic instability of the MSI-H tumours may lead to production of abnormal proteins that can act as neoantigens. In our study, we found that a Crohn's-like/lymphoid reaction was common (38.5%) in MSI-L tumours compared with both MSI-H and MSS (0 and 8.3%, respectively). A Crohn's-like lymphoid reaction seems to be the only different clinical feature between MSI-L and MSS tumours [6].
MSI-L has been found, not only in tumours, but also in chronically inflamed non-neoplastic colonic tissue [29]. Oxidative stress may allow frameshift mutations to accumulate in human colon cancer cells [30] and the exposure of *Escherichia coli* to low levels of H_2O_2 increases the frequency of expansions and deletions within dinucleotide repetitive sequences [31]. These data may explain the mechanism by which MSI-L occurs in inflamed tissue. It is possible that in tumours with weak microsatellite instability, the presence of Crohn's-like lymphoid aggregates could be a cause more than a consequence.
On the other hand, it is also possible that there is a relationship between this marked host-lymphocytic response and the relatively high frequency of APC mutations found in this group of tumours. The activity of the proinflammatory protein cyclo-oxygenase-2 (COX-2) increases dramatically following mutation of the APC gene [32].
The presence of a cytotoxic immune response may have a favourable impact on the clinical outcome of patients and, in fact, a Crohn's-like lymphoid reaction around colorectal carcinomas has been related to improved patient survival [33 34]. However, in our study, multivariate analysis indicated that the only factors affecting prognosis were tumour localisation, Dukes' stage and presence of an invasive margin in the carci-

nomas. We found only borderline (p=0.062) improvement in overall survival in MSI-H and MSI-L cancer patients.

In conclusion, results of the present study provide some evidence that MSI-L colorectal cancers are distinct from both MSI-H and MSS and that this subset of tumours may be more dependent upon APC gene alterations. Further analysis and a longer follow-up are required to provide evidence that the classification of colorectal carcinomas into MSI-H, MSI-L and MSS may be of prognostic value.

List of abbreviations

COX-2: cyclooxygenase-2; HNPCC: hereditary non-polyposis colorectal cancer; IVSP: in vitro synthetised protein; LOH: loss of heterozygosity; MMR: mismatch repair; MSI-H: high-frequency microsatellite instability; MSI-L: low-frequency microsatellite instability; MSI: microsatellite instability; MSS: apparently microsatellite stable; PCR: polymerase chain reaction; TGFβ: transforming growth factor beta.

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