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Microsatellite instability in sporadic colon carcinomas has no independent prognostic value in a Belgian study population

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ABSTRACT

Pathological stage is currently the most important determinant of colorectal cancer prognosis. Hence, identification of additional prognostic markers is warranted.

This study aimed to analyse the prognostic relevance of microsatellite instability (MSI) in 241 colon and 90 rectal tumours, using a mononucleotide loci multiplex PCR assay and immunohistochemistry.

Thirty (12.4%) colon tumours and one rectal tumour showed MSI. Although MSI was associated with proximal location and poor differentiation, no survival benefit was observed. The prognostic value of stage and differentiation was confirmed in this study.

Analysis by stage revealed a longer overall (stage II/III) and disease free survival (stage II) for patients with well differentiated tumours. In addition, age and distal localisation were related to longer overall survival in stage II tumours.

In conclusion, our findings show an association of MSI in sporadic colon tumours and certain clinical features; however, they do not suggest a survival benefit for MSI tumours.

1. Introduction

Despite continuing efforts towards early diagnosis and treatment, colorectal cancer (CRC) remains the third most common form of cancer and cancer related deaths in developed countries.[1,2] In Belgium, 6198 new cases of colorectal cancer were diagnosed with an associated 2665 deaths in 2003 (Belgian national cancer registration).

The prognosis of CRC is largely determined by the extent of primary disease at the time of diagnosis. Although some improvements have been achieved with postoperative adjuvant therapy, the prognosis for patients with advanced

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disease remains poor and difficult to predict in individual patients.[3] CRC is not uniformly fatal and there are large differences in survival depending on the stage of the disease. The TNM classification system of the International Union Against Cancer (UICC) is currently the most important determinant of prognosis. However, this staging system does not allow for an accurate prediction of the risk of tumour recurrence after surgery for individual patients. Therefore, identification of additional prognostic markers to supplement standard clinical and pathological staging of the tumour is still warranted.[4]

It is now clear that the genotype of the tumour dictates its behaviour. CRC results from the cumulative effects of sequential genetic alterations in proto-oncogenes, tumour suppressor genes and DNA repair genes through distinct molecular pathways involving chromosomal instability, microsatellite instability (MSI), as well as epigenetic gene silencing through DNA methylation.[5,6]

In sporadic CRC, these alterations are acquired, and are likely to be caused by exogenous and endogenous carcinogens. In contrast, in cancer syndromes like hereditary nonpolyposis colorectal cancer (HNPCC), critical genetic alterations that predispose to malignancy are inherited.[6]

While only 10–15% of sporadic CRC display MSI, predominantly caused by epigenetic hypermethylation of the *MLH1* mismatch repair gene, the majority of HNPCC tumours are characterised by this MSI phenotype. Individuals with a high-frequency MSI (MSI-H) tumour have phenotypically distinct features that are substantially different from those following the chromosomal instability pathway, though not all characteristics have consistently been demonstrated in all studies.[7,8] These distinct features might be useful as diagnostic, predictive or prognostic markers. In addition, MSI-H CRC have different behaviour patterns, response to chemotherapy and possibly a diverse outcome.[9] Therefore, MSI is one of the more promising molecular markers investigated to date.

Although numerous studies have reported MSI in series of colorectal cancer, uncertainty remains as to whether positive MSI status is associated with prolonged survival of the patient. Controversial data have been reported[2,10–12] leaving an area of uncertainty on the usefulness of this molecular marker in clinical practice.

This study aimed to analyse the prognostic relevance of MSI in a cohort of 241 primary colon and 90 primary rectal tumours.

2. Materials and methods

2.1. Patients

Consecutive patients with CRC ($n = 331$), treated at the Antwerp University Hospital in Edegem (between 1994 and 2003), the St. Augustinus Hospital in Wilrijk (between 1998 and 2004), the Ghent University Hospital in Ghent, the H.-Hart General Hospital in Roeselare-Menen and the St.-Jan General Hospital in Bruges (between 1996 and 2000), were included in the study. The only exclusion criterion was stage IV cancer patients because of their worse outcome.

There were 241 patients with colon cancer and 90 with rectal cancer. Due to differences in aetiology, clinical behaviour, applied treatment strategies and survival expectations, colon and rectal cancers were studied separately.

2.2. DNA extraction and MSI-analysis

Tumour DNA was obtained from formalin-fixed, paraffin-embedded tissue blocks. After manual microdissection, DNA was isolated as described previously.[13] Quasi-monomorphic mononucleotide markers Bat25, Bat26, SEC63 and CAT25 were used to type all tumours in a multiplex assay as previously described.[14] PCR primers for the amplification of these markers were described elsewhere.[15,16] Fluorescent PCR products were analysed by capillary electrophoresis using an ABI 3100 Genetic Analyzer (Applied Biosystems, Lennik, Belgium) and Genemapper Software 3.7.

Since only four markers were analysed in this multiplex assay, MSI at $\geqslant 2$ loci was defined as MSI-H, instability at a single locus was defined as a low grade of instability (MSI-L) and no instability at any of the loci was defined as microsatellite stability (MSS).

2.3. Immunohistochemistry (IHC)

Four micrometer-thick sections were prepared from formalin-fixed paraffin-embedded tissue blocks for IHC. Sections were deparaffinised, dehydrated and subjected to heat antigen retrieval by Tris EDTA buffer (pH 9) for 10 min at 95 (±1) °C in a heating bath. Sections were subsequently stained using the Dako Autostainer Plus system (DAKO, DakoCytomation, Glostrup, Denmark). Endogenous peroxidase activity was quenched by incubating the slides in peroxidase block EnVision Plus (DAKO EnVision™ kit, DakoCytomation) for 10 min. Incubations with primary monoclonal antibodies were performed as follows: anti-MLH1 (clone G168-15, diluted 1:100, BD Biosciences Pharmingen, San Diego, CA, USA), anti-MSH2 (clone G219-1129, diluted 1:250, BD Biosciences Pharmingen) and anti-MSH6 (clone 44, diluted 1:250, BD Biosciences Pharmingen), all for 30 min at room temperature. Bound antibody was detected with the Envision dual link detection system (DAKO EnVision™ kit, DakoCytomation) using 3,3'-diaminobenzidine (DAB^+) chromogen solution according to the manufacturer's instructions. The sections were then counterstained with haematoxylin. Loss of protein expression was scored as absence of nuclear staining in tumour cells despite nuclear staining in proliferating cells in normal crypts and stroma.

2.4. Clinicopathological data

Clinicopathological factors were retrieved from the hospital records for each patient included in this study. None of the patients with a family history fulfilled the Amsterdam criteria and all colon cancers were therefore believed to be of sporadic origin. The index date for survival time calculation was defined as the date of diagnostic confirmation of colon cancer. The months of observation (=overall survival time) were calculated from the index date to the date of last information/death. For disease free survival time, the months of observation were calculated from the index date to the first date of an unfavourable clinical event or the date of last information.

2.5. Statistical analysis

Prognostic relevance of MSI was assessed by survival analysis. Survival probability was estimated using the Kaplan–Meier method. Differences were tested using the log rank statistic.

Possible associations between MSI-status and clinicopathological parameters of colorectal cancers were investigated using the χ^2-test or Fisher's exact test (when appropriate) for categorical variables and using Student *t*-test or Mann–Whitney *U* test (when appropriate) for continuous variables. In order to assess the independent prognostic contribution of MSI, a multiple Cox regression analysis was conducted. All analyses were conducted using SPSS (version 14.0). Significance for all statistics was two-tailed and recorded if $p < 0.05$.

3. Results

3.1. MSI-analysis

Clinical characteristics of colon and rectum cancer patients included in the study population are summarised in Table 1. All samples could be analysed with the mononucleotide multiplex MSI-analysis system.[14] In all cases showing MSI, at least three markers were affected. Hence, only MSI-H and MSS cases were found. Of the 241 colon tumours, 30 showed MSI-H (12.4%) and the remaining 211 (87.6%) were included in the MSS subgroup. MSI-H tumours were more frequently located proximally in the colon, had a familial history and displayed a poor differentiation. No significant differences were found between patients with MSI-H and MSS tumours for age, gender, tumour stage and lymph node invasion (Table 2).

Of the 90 rectum tumours, only one showed MSI. This patient was 39 years old and had a stage II cancer. Regrettably, he was lost during follow-up. All other rectum samples showed MSS.

3.2. Immunohistochemical mismatch repair (MMR)-analysis

To verify loss of MMR function, as suggested by MSI, we performed immunohistochemistry in 25/30 (83.3%) MSI-H tumours and in four MSS tumours. Overall, 20 MSI-H tumours (80%) showed loss of staining for either MLH1 (18 tumours) and/or MSH6 (four tumours). No absence of MSH2 was scored. All MSS cases showed intense staining of the MMR proteins examined (Fig. 1). The immunohistochemical analyses showed a significant correlation between MSI and loss of MMR proteins ($p = 0.001$) with a sensitivity of 80% and a specificity of 100%.

Table 1 – Clinical characteristics of the study population of 241 colon and 90 rectum tumours (n = absolute number of patient)

Clinical characteristic		Colon *n* (%)	Rectum *n* (%)
Gender	Female	118 (49)	29 (32.3)
	Male	123 (51)	61 (67.8)
Median age		66 years (range 26–94)	62 years (range 29–88)
Stage	I	26 (11.2)	16 (18.4)
	II	96 (41.4)	34 (39.1)
	III	110 (47.4)	36 (42.5)

Table 2 – Possible associations between MSI-status and clinicopathological parameters of colorectal cancers were investigated using the χ^2-test or Fisher's exact test (when appropriate) for categorical variables and using Student t-test or Mann–Whitney U test (when appropriate) for continuous variables

		MSI	MSS	*p*-Value
Median age		63 ± 15	66 ± 13	0.464
Gender	Female	56.7%	47.9%	0.437
	Male	43.3%	52.1%	
Lymph node invasion	Yes	57.1%	56.6%	1
	No	42.9%	43.4%	
Grade of differentiation	Poor	33.3%	7.9%	<0.0001
	Moderate	30.0%	38.4%	
	Well	36.7%	53.7%	
Localisation	Proximal	83.3%	41.1%	<0.0001
	Distal	16.7%	58.9%	
Stage	0	0.0%	0.5%	0.796
	1	6.7%	11.8%	
	2	46.7%	40.4%	
	3	46.7%	47.1%	
Family history	Yes	20.0%	8.1%	0.049
	No	80.0%	91.9%	
Adjuvant therapy	Yes	40.0%	41.5%	1
	No	60.0%	58.5%	

MSI: microsatellite instability; MSS: microsatellite stability.

3.3. Prognostic relevance in colon cancer

Follow-up for overall survival (OS) and disease free survival (DFS) was available for 228 and 223 colon cancer patients, respectively. At the end of the observation period, 43 (18.9%) patients died while 72 (32.3%) experienced a recurrence of the tumour. Consequently, the median OS and DFS could not be reached in this study population. The 75th percentile survival is shown in Table 3. To quantify the effects of MSI status on survival, a Cox regression analysis was conducted. As expected, tumour stage was the most relevant prognostic factor for OS and DFS. Furthermore, univariate analysis showed that patients with a well differentiated and distal colon tumour had a significantly longer OS and DFS. No statistically significant difference in OS ($p = 0.81$) and DFS ($p = 0.17$) was found between MSI-H and MSS cases (Table 4). In multiple regression analysis, tumour stage and differentiation were significantly correlated with OS and DFS; in contrast, MSI status was not (Table 5).

When patients with a family history of colon cancer were excluded from the study population (23 patients of which six were MSI-H cases), the univariate survival and the multiple regression analysis revealed the same favourable prognostic factors for OS and DFS.

The median and/or 75th percentile OS and DFS, analysed by stage separately, is shown in Table 3. MSI was scored in

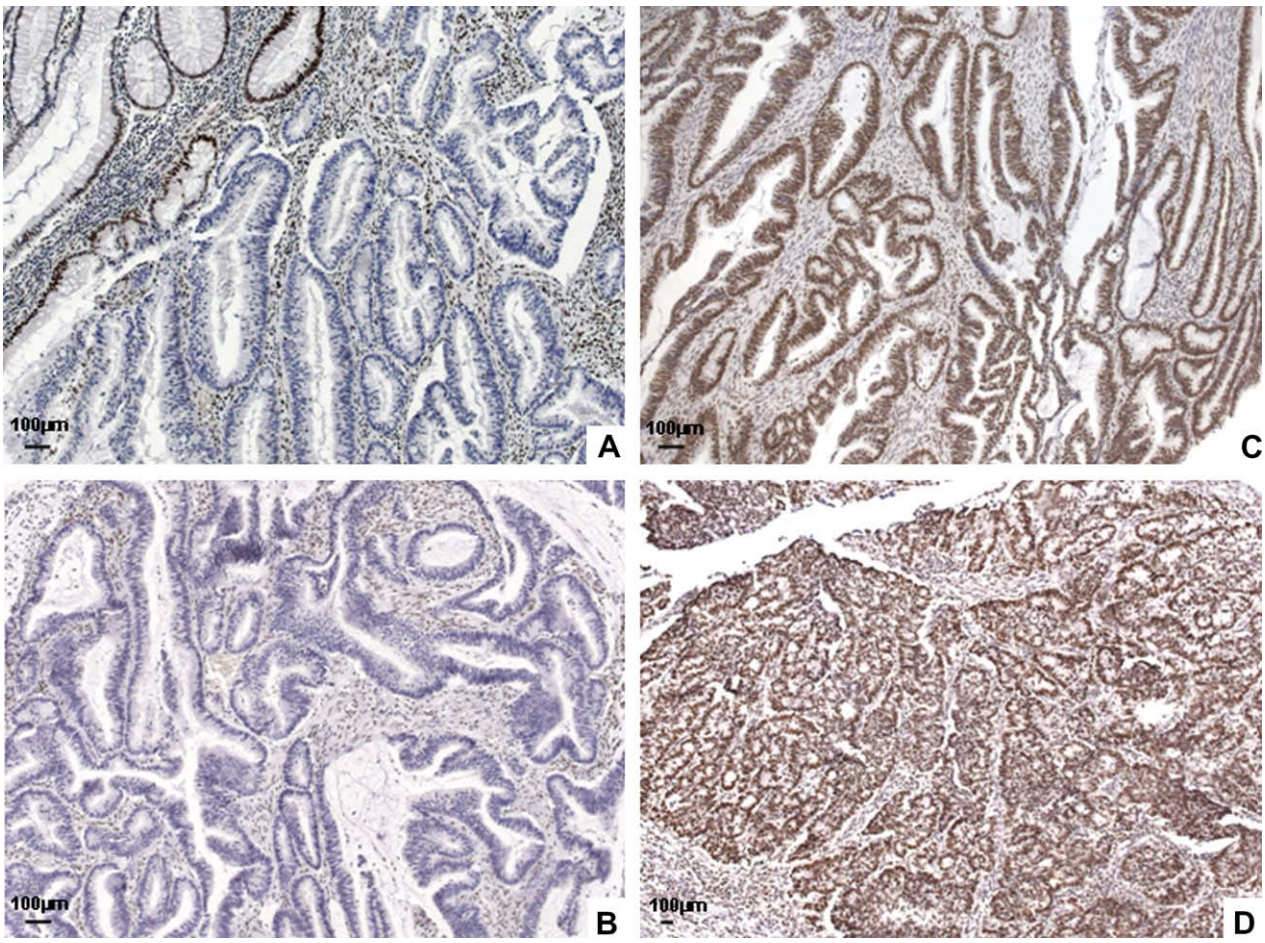

Fig. 1 – Immunohistochemical staining of mismatch repair proteins MLH1 (A–B) and MSH2 (C–D). In (A) nuclear MLH1 protein staining is very abundant in normal crypts and stromal cells (also seen in B), while nuclear staining was absent in tumour cells. C and D show very intense staining of MSH2 protein in stromal as well as tumour cells.

Table 3 – Median and/or 75th percentile OS (overall survival) and DFS (disease free survival) for the overall study population and stage specific survival analysis

		Median survival		75th percentile survival	
		OS (months + 95CI)	DFS (months + 95CI)	OS (months + SD)	DFS (months + SD)
Overall population		NA	NA	67.5 ± 13.7	21.7 ± 5.9
Stage	I	NA	NA	NA	NA
	II	NA	NA	106.7 ± 4.5	56.5 ± 18.9
	III	88.1 (48.8–127.4)	38.4 (20.9–55.9)	33.2 ± 5.6	14.1 ± 2.3

The median OS and DFS could only be reached in stage III tumors during stage specific survival analysis (95CI: 95% confidence interval; SD: standard deviation).

7.7% of stage I, 14.6% of stage II and 12.7% of stage III colon tumours.

Univariate survival analysis revealed a significant longer OS (stage II and III) and DFS (stage II only) for patients with a well differentiated tumour. In addition, a longer OS was found in those with distally localised stage II tumours. In the multiple regression analysis in patients with stage II tumours, it was shown that a younger age of onset and distal localisation of the tumour were significantly correlated with longer OS, while better differentiation showed a trend towards a longer OS and was significantly correlated with a longer DFS. For stage III tumours, only well differentiated tumours showed a longer OS (Table 6).

3.4. *Survival in rectal cancer*

Follow-up for OS and DFS was available for 81 and 78 rectal cancer patients respectively. During follow-up, 17 (21.0%) patients died while 34 (43.6%) experienced a recurrence of the tumour. Consequently, the median OS and DFS could not be

Table 4 – Univariate survival analysis of colon cancer patients using a Cox regression model (HR: hazard ratio; 95% CI: 95% confidence interval)

	Overall survival			Disease free survival		
	Univariate survival analysis			Univariate survival analysis		
	HR	*p*-Value	95% CI	HR	*p*-Value	95% CI
MSI	0.89	0.81	0.35–2.27	1.51	0.17	0.85–2.72
Age	1.00	0.87	0.98–1.03	0.98	0.09	0.97–1.00
Gender	0.93	0.82	0.52–1.71	0.88	0.60	0.56–1.41
Grade of differentiation	0.68	0.00	0.53–0.86	0.79	0.01	0.66–0.96
Localisation	0.54	0.05	0.29–1.01	0.62	0.05	0.39–1.01
Stage	3.43	<0.0001	1.86–6.38	2.70	<0.0001	1.76–4.13
Family history	1.11	0.83	0.56–3.17	1.38	0.30	0.75–2.84

Table 5 – Survival analysis of colon cancer patients using a multiple Cox regression model (HR: hazard ratio; 95% CI: 95% confidence interval)

	Overall survival			Disease free survival		
	HR	*p*-Value	95% CI	HR	*p*-Value	95% CI
MSI	0.53	0.22	0.19–1.47	1.06	0.86	0.55–2.07
Age	1.01	0.69	0.98–1.03	0.99	0.30	0.97–1.01
Gender	1.18	0.61	0.62–2.26	0.91	0.74	0.56–1.50
Grade of differentiation	0.61	0.00	0.46–0.81	0.80	0.03	0.65–0.98
Localisation	0.64	0.18	0.33–1.22	0.80	0.39	0.48–1.33
Stage	3.65	0.00	1.92–6.95	2.65	<0.0001	1.70–4.12
Family history	1.19	0.74	0.43–3.28	1.28	0.51	0.62–2.64

Table 6 – Univariate (A) and multiple Cox regression (B) survival analysis for stage II and III colorectal tumours separately (HR: hazard ratio)

	Stage 2				Stage 3			
	Overall survival		Disease free survival		Overall survival		Disease free survival	
	HR	*p*-Value	HR	*p*-Value	HR	*p*-Value	HR	*p*-Value
A								
MSI	2.06	0.39	1.57	0.43	0.71	0.57	1.32	0.45
Age	1.07	0.11	0.97	0.14	1.00	0.83	1.00	0.86
Gender	1.19	0.82	1.12	0.81	1.12	0.75	0.82	0.49
Grade of differentiation	0.57	0.05	0.67	0.03	0.70	0.02	0.87	0.25
Localisation	0.09	0.03	0.57	0.27	0.88	0.73	0.82	0.48
Family history	3.41	0.15	2.19	0.22	0.81	0.73	1.20	0.68
Adjuvant therapy	0.47	0.49	1.75	0.30	0.66	0.26	0.98	0.84
B								
MSI	0.36	0.38	1.10	0.90	0.49	0.28	1.10	0.82
Age	1.14	0.03	0.98	0.29	1.00	0.86	1.00	0.93
Gender	6.45	0.13	1.74	0.35	1.31	0.50	0.84	0.55
Grade of differentiation	0.40	0.06	0.64	0.04	0.64	0.02	0.89	0.36
Localisation	0.03	0.03	0.74	0.59	0.86	0.69	0.84	0.57
Family history	10.40	0.10	1.33	0.71	0.95	0.93	1.16	0.75

reached in this study population. The 75th percentile survival was 59.3 ± 20.3 months for OS and 21.8 ± 3.4 months for DFS. Since only one rectal cancer patient showed MSI in this study cohort, the effect of MSI status on survival in rectal cancer patients could not be analysed.

Nevertheless, univariate survival analysis once again revealed stage as the most relevant prognostic factor for OS and DFS. In addition, younger age of onset was correlated with a longer OS (Table 7).

4. Discussion

Currently, TNM staging is the most commonly used predictor of prognosis for CRC patients.

Table 7 – Univariate and multiple Cox regression survival analysis for rectal cancer (HR: hazard ratio)

	Overall survival		Disease free survival		Overall survival		Disease free survival	
	Univariate survival analysis		Univariate survival analysis		Multiple Cox regression		Multiple Cox regression	
	HR	*p*-Value	HR	*p*-Value	HR	*p*-Value	HR	*p*-Value
Age	1.05	0.04	1.02	0.24	1.07	0.11	1.06	0.23
Gender	0.72	0.51	1.40	0.37	1.14	0.85	1.25	0.76
Grade of differentiation	0.84	0.69	0.74	0.43	1.87	0.23	1.89	0.23
Stage	2.36	0.04	2.07	0.01	NA	NA	1.36	0.49
Family history	2.54	0.17	1.07	0.92	0.99	0.99	0.80	0.83

However, within stages, differences in survival can occur, especially in stage II and III CRC.[17] Identification of additional prognostic markers that reliably discriminate between patients with a favourable or unfavourable outcome in this prognostically diverse group is warranted.[17]

MSI has been proposed as one of the most promising molecular markers investigated to date.[2] Despite numerous investigations, uncertainty on the usefulness of MSI as a molecular prognostic marker remains.[2,10–12,18]

To our knowledge, this is one of the few studies, since the revised Bethesda guidelines for MSI,[19] using exclusively mononucleotide loci in a multiplex assay to investigate the prognostic relevance of MSI in CRC.[14] In accordance to Murphy et al.[20] MSI-L is no longer recognised as a separate category in this assay. Because MSI-L phenomenon is still poorly defined and its clinical significance is not well understood, it has been suggested previously that MSI-L cancer should be grouped with MSS cancers for clinical purposes.[19,20]

PCR-based screening with this panel revealed the presence of MSI-H in 12.4% of the CRC cases investigated, which is in agreement with numerous other studies.[4,12,21–23] The relationship of MSI-H to proximal location, poor differentiation and a family history has also been seen by most studies.[4,12,21–23]

In line with a minority of studies dealing with the prognostic value of MSI (as reviewed in Refs. [2,24]) no significant difference in DFS and OS could be found between patients with stable and unstable tumours in this study. However, our study is limited in power by a relatively small number of patients and fairly low prevalence of MSI-H, which might have influenced the level of significance of our results, especially for the multivariate Cox regression of OS (Table 5) showing a HR of 0.53 and $p = 0.22$. Nevertheless, a significant influence of MSI was found in other groups, analysing comparable population sizes and similar MSI-H prevalences.[17,21,25]

The reasons for the lack of association between MSI and survival in our study remain unclear. The conflicting results might be explained by several important factors.

Generally, the retrospective nature and the heterogeneity of the studies might have biased the results. Retrospective studies are more difficult to control for bias and confounding factors and important information is not always accessible. Nevertheless, in a prospective study design, Lamberti and colleagues were also unable to find a significant survival benefit of MSI-H tumours.[23]

Most studies included patients with colon as well as rectal cancers of any tumour stage while others were site and stage specific.[2,23] However, differences in aetiology, clinical behaviour and histopathology between colon and rectal cancer suggest different preferred tumourigenic pathways.[26,27] In addition, as observed in this study and in accordance with others, MSI-H is a relative rare event in rectal tumours,[26,28,29] and if present, strongly suggests a genetic predisposition.[26] The single MSI-H rectal cancer patient in our study population did not fulfil the Amsterdam criteria but had a family history of a first degree relative with CRC and an early age of onset. Regrettably, this patient was lost during follow-up. In this study, colon and rectal tumours were studied separately and stage IV tumours were excluded because of their poor prognosis.

In addition, although clinical data are conflicting, *in vitro* data suggest a strongly reduced sensitivity to 5FU chemotherapy in MSI-H tumours.[9,30] In this retrospective study population, 40% of the MSI-H CRC patients received adjuvant therapy possibly contributing to the lack of association between MSI-H and prognosis.

Furthermore, MSI tumours with a different molecular background may have different biological behaviour and prognosis.[22] Although MSI occurs in both sporadic CRC and in tumours arising in patients with germline MMR gene mutations, cancer survival should not be considered to be equivalent for these two groups, simply because both exhibit similarities in molecular phenotype.[31] Therefore, survival rates might be different between familial and sporadic MSI-H tumours in some studies.

In this study, MSI-H tumours were sporadic of origin and in accordance with the literature, immunohistochemical verification of the MSI status showed a higher incidence of lost MLH1 expression compared to MSH2.[32,33] In addition, in our study population, 23 patients showed a family history of colorectal cancer of whom six had an MSI-H tumour. However, none of these patients fulfilled the Amsterdam criteria, which suggests a somatic nature of origin. When these 23 patients were excluded from the study population, there was still no recorded significant difference in survival according to the MSI status. In addition, within this group of 23 patients with a familial history, MSI-H patients did not show a significantly better OS and DFS.

A recently published systematic review of MSI and CRC prognosis, pooling data from 32 studies and a total of 7642 patients, demonstrated a significant survival advantage for MSI tumours compared to MSS CRC.[2] According to Lamberti and colleagues,[23] in- or exclusion of stage I tumours among the

eligible studies might have influenced the results. However, exclusion of stage I tumours from our study population did not alter the non-significant results. Furthermore, the relative low number of patients in the different stage subgroups could also contribute to the non-significance of the results of our study.

Several population-based studies, although recognising the lower staging of MSI cancers, have proposed MSI as an independent prognostic factor. Nevertheless, a correlation between tumour stage and MSI status could influence significance as seen in several studies.[10,22,28] Given the fact that patients with an MSI tumour have a decreased likelihood of metastasis at diagnosis, these findings indicate that the reduced metastatic potential of the primary tumour may be the key mechanism for the survival advantage of patients with unstable tumours.[22]

Another theory behind a better prognosis in MSI CRC patients is an increased lymphatic infiltration that enhances the host immune response to the tumour.[12] MSI might lead to the production of abnormal proteins, that by acting as neo-antigens, could induce a cellular immune response against the tumour.[34] The lymphatic infiltration in tumours included in other studies, demonstrating no association between MSI and survival, is unknown. A subset (98 samples) of our study population has been scored for the presence of tumour infiltrating lymphocytes (TILs) (data not shown). Although we observed a correlation between MSI-H colorectal tumours and a higher number of TILs, the infiltration of CD3+ (for OS and DFS) and CD8+ (for DFS only) in the invasive margin of the tumour was correlated with a better survival in stage II tumours only, irrespective of the MSI status. Therefore, it was considered that tumour infiltration could reflect a general principle of antitumour immunity, irrespective of MSI, as suggested by Prall and colleagues.[35]

In conclusion, in a series of 241 patients, we detected MSI in 12.4% of colon cancer specimens, using an exclusively mononucleotide loci multiplex assay. In contrast, in a series of 90 rectal cancer patients, only one patient showed MSI.

Although MSI was associated with proximal location and poor differentiation, no survival benefit could be observed compared to patients with MSS tumours. In the current study, the prognostic value of stage and differentiation was confirmed.

Conflicting results of the available data and our incomplete understanding of the process of MSI argues against using MSI alone as a predictor of survival in colorectal cancer. Large prospective population-based studies need to be performed to elucidate the role of MSI in colorectal tumours.

Conflict of interest statement

None declared.

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