Prognostic Impact of Microsatellite Instability in Colorectal Cancer Patients Treated with Adjuvant FOLFOX

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Abstract. *Background: Colorectal cancer (CRC) patients whose tumours have microsatellite instability (MSI) do not benefit from adjuvant 5-fluorouracil. However, the predictive value of MSI is not known for FOLFOX, now recommended in adjuvant setting. Patients and Methods: MSI phenotype was assessed by the pentaplex method. Three-year relapse and disease-free survival (DFS) of patients treated for CRC with FOLFOX 4 in an adjuvant setting were compared according to MSI phenotype. Results: A total of 105 patients (19 MSI, 86 microsatellite stable, MSS) were included. Stage II patients more frequently exhibited MSI (58%) than MSS (21%); (p=0.002). Patients with MSI relapsed significantly less than those with MSS (10.5% vs. 35.0%; p=0.04). DFS was similar for MSI and MSS (p=0.1). In univariate analysis, stage (p=0.0006) and MSI status (p=0.017) were significant predictors of DFS. Conclusion: MSI status was associated with significantly fewer relapses and a better prognosis. FOLFOX4 did not alter survival of patients with MSI and can be administered to them.*

Worldwide, 945,000 new cases of colorectal cancer (CRC) are diagnosed every year (1). After ablation of the tumour, localised diseases (stages II and III) represent about half of new patients in an adjuvant situation each year. Stage III and stage II patients with a high relapse rate are normally treated by the combination of 5-fluorouracil (5-FU) and oxaliplatin (FOLFOX), a universal standard therapy (2). Despite an increased knowledge of pathological staging and biological characteristics of CRCs (3), new prognostic factors are emerging but are difficult to include in the present classification of CRCs.

Among CRC biological characteristics, the microsatellite instability (MSI) phenotype has been one of the most studied. It is specific of hereditary forms of CRC (HNPCC syndrome), but is also found in 15-20% of sporadic CRC. MSI is characterized by an alteration of the mismatch repair (MMR) system, the function of which is to repair the nucleotide mismatches occurring during DNA replication. In cases of a dysfunction of these proteins (MLH1, MSH2, MSH6 and PMS2), the persistence of errors in replication can be detected in highly repetitive regions of DNA that are difficult to replicate precisely, and which are called microsatellites. Measurement of the size of microsatellites in tumour DNA is an indirect method of assessment of the function of MMR system (4). Interestingly, the somatic mutation V600E of *BRAF* is associated with epigenetic silencing of the *MLH1* gene by promoter methylation in sporadic CRC but not with the HNPCC syndrome (5, 6). Hence, the characterization of the somatic V600E *BRAF* mutation enables exclusion of a genetic alteration of a MMR gene.

Clinically, MSI CRCs are well characterized. They show female predominance (3 women for 2 men), preferential location in the right colon, mucinous histopathology, and, above all, a better prognosis than microsatellite stable (MSS) tumours (7-9).

Presently, there is no biological parameter that can be used to decide whether or not adjuvant chemotherapy is justified among patients susceptible to relapse after curative surgery. Thus, all stage III CRC patients (node-positive patients) and high-risk stage II patients are usually prescribed the same chemotherapy regimen, namely FOLFOX, during a predetermined fixed time period (6 months), which exposes patients to the risk of side-

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effects, mainly peripheral neuropathy. Concerning more specifically MSI tumours, the benefit obtained from 5-FU is controversial and this chemotherapy may even have a detrimental effect on survival. Two studies demonstrated the absence of survival benefit to CRC patients with MSI compared with those with MSS (10, 11). In a presentation at the 2008 ASCO meeting, Sargent *et al*. even showed that patients with MSI treated with 5-FU alone had a poorer survival than those who did not receive any treatment, suggesting a deleterious effect of 5-FU (12). Consequently, it appears useful to assess the benefits of adjuvant chemotherapy with FOLFOX according to the MSI phenotype.

The main goal of this study was to compare relapse-free survival at three years among patients with MSI and MSS. Ancillary goals were the assessment of overall survival and the assessment of MSI status among other predictive factors (stage, age) by means of multivariate analysis.

Patients and Methods

Patients and treatments. Specimens from 105 patients with histologically confirmed stage II/III CRC were studied. Patients were consecutively included at Bordeaux Nord Clinic, Avicenne Hospital, Curie Institute and Le Raincy-Montfermeil Hospital, France. All analysed specimens were derived from primary tumours. All patients with stage III CRC received FOLFOX adjuvant chemotherapy. Only high-risk stage II patients (with T4, perineural invasion, capillary invasion, occlusion, perforation and young age) received this treatment. In addition, after neo-adjuvant radiotherapy, patients with rectal cancer also received FOLFOX postoperatively in cases of nodal invasion on the surgical specimen (13). Follow-up was performed by means of clinical examination, determination of carcinoembryonic antigen, pelvic and abdominal ultrasonography and CT scans every 4 months during the first 2 years and then every 6 months until three years of follow-up. Patients also had a chest X-ray or a thoracic CT scan at least yearly.

All patients were treated with FOLFOX regimens in an adjuvant setting. The FOLFOX 4 regimen consisted of a combination of folinic acid, 5-FU bolus and continuous infusion of oxaliplatin. The treatments in the Medical Oncology Departments of Avicenne Hospital, Curie Institute and Raincy-Montfermeil Hospital were based on FOLFOX 4 (2).

Determination of the microsatellite status. Blocks of formaldehyde-treated paraffin-embedded specimens were obtained and fractions selected by pathologists were microdissected and the DNA was extracted using a QIAamp DNA Minikit (Qiagen, Courtaboeuf, France) following the manufacturer's recommendations. Five quasimonomorphic mononucleotide markers (NR21, NR24, NR27, BAT 25, BAT 26) were studied as previously described without the need for matching normal DNA (19). The five loci were amplified by pentaplex PCR. Fluorescent products were separated on an ABI Prism®3100-Avant Genetic Analyzer (Applied Biosystems, Courtaboeuf, France) followed by length determination with the Genotyper software (Applied Biosystems, Courtaboeuf, France). Specimens with a minimum of three unstable markers were scored as highly unstable (MSI-H), whereas specimens with less than three unstable markers were scored as stable (MSS). There were no specimen with only two unstable markers.

Table I. *Characteristics of patients*.

	Tumour status		*p*-Value
	MSI (n=19)	MSS (n=86)	
Median age (years) range	60.4 (34-77)	60.6 (24-85)	NS
Gender no. (%)			
Male	8 (42)	50 (58)	
Female	11 (58)	36 (42)	NS (0.1)
Stage: no. (%)			
I/II High risk	11 (58)	19 (23)	
III	8 (42)	64 (77)	0.003
Tumour site: no. (%)			
Right colon	14 (67)	29 (34)	
Left colon	4 (21)	31 (36)	0.001
Rectum	0 (0)	21 (25)	
Metastasis: no. (%)	2 (10%)	29 (36%)	0.01
Location of metastases: no. (%)			
Liver	0 (0)	11 (38)	
Lung	0 (0)	5 (17)	
Peritoneum	2 (100)	11 (38)	ND

NS: Non-statistically significant; ND Not determined.

Detection of V600E *BRAF* mutation was assessed by allelic discrimination using TaqMan probes on a Taqman 7000 thermocycler (Applied Biosystems, Courtaboeuf, France). Patients were informed of the specific pathological analysis needed for the study. Their written informed consent was obtained for the determination of MSI status.

Statistical analysis. For the analysis of the outcome, patients were classified according to their MSI or MSS status. The independence of qualitative factors was tested by the Fisher's exact test and the means of both groups were compared using the Student's *t*-test. Survival times were analysed as Kaplan-Meier curves and compared using the log-rank test. For multivariate analysis, adjusting for predictive factors, the independent effect of prognostic factors on survival was assessed by the Cox's proportional hazard model. Statistical analyses were carried out using SAS, version 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics. Among the 105 tumour specimens, there were 30 (29%) from patients with stage II (with high risk of relapse) and 72 (69%) with stage III tumours; tumors in 5 patients (5%) could not be staged. Median age was 60.3 years. There were 58 men, and 84 colon and 21 rectal tumours (Table I). The 19 MSI patients included 8 men (42%) and 11 women (58%). There were significantly more MSI tumours in the right colon than in the left colon and rectum (14/43 *vs*. 4/56, respectively; chi-square=10.6; p=0.001). MSI phenotype predominated in high-risk stage II patients (11 stage II MSI/29 *vs*. 8 stage III MSI patients/71; chi-

Table II. *Characteristics of relapses in the MSI group.*

	MSI tumour		*p*-Value
	Relapse (n=2)	No relapse (n=17)	
Median age: years (range)	69.5 (69-70)	59 (34.4-77.2)	
Gender (M/F)	0/2	8/9	NS (0.1)
HNPCC: no. (%)	0	6	NS (0.3)
BRAF V600 E: no. (%)			
Mutation	2 (100)	2 (12)	
No mutation	0 (0)	15 (78)	0.048
Stage: no. (%)			
II/II High risk	1 (50)	9 (53)	
III	1 (50)	8 (47)	NS (0.6)
Deaths: no. (%)	1(50)	0	

square=9.51; p=0.002). Among the 19 MSI patients, 6 had HNPCC syndrome. Four patients out of 19 (21%) with MSI tumours displayed the V600E *BRAF* mutation. As expected, none of the patients with confirmed HNPCC syndrome had a V600E *BRAF* mutation.

Relapses. MSI patients had significantly fewer relapses than MSS patients: 2/19 (10.5%) *vs*. 29/83 (35%), respectively (chi-square=4.36; p=0.04). The characteristics of relapse in the MSI group are summarized in Table II. Both MSI relapses were due to peritoneal carcinosis. Of the 31 patients who relapsed, 12 died (60%). One patient who died had MSI status, the other 11 had MSS status, compared with 18 MSI and 72 MSS alive (non-statistically significant difference between MSI and MSS groups). None of the HNPCC patients relapsed during the course of the study. Interestingly, two (50%) of the patients having both MSI tumours and V600E *BRAF* mutation relapsed during the course of the study suggesting a deleterious effect of this mutation (p<0.05). According to a nominal logistic fit, MSI/MSS status did not predict relapse, whereas stage II/III did (p=0.008). There was no statistically significant interaction between those two factors. Disease-free survival (DFS) curves for MSI and MSS patients were not statistically different (log-rank=2.31; p=0.13) (Figure 1). Univariate analysis showed that stage (p=0.0006) and MSI status (p=0.017) were statistically significant predictors of DFS, whereas age was not. Multivariate analysis showed that only stage predicted DFS (p=0.008), with a non significant trend for MSI status (p=0.088).

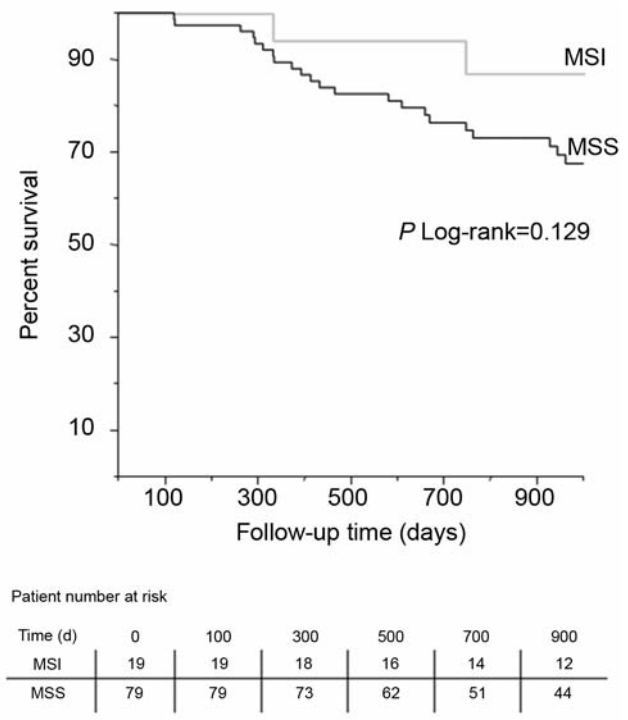

Figure 1. *Kaplan-Meier curves for disease-free survival of 105 colorectal cancer patients treated in an adjuvant setting and stratified by microsatellite status.*

Discussion

To the Authors' best knowledge, this study is the first to find a statistically significant difference in relapse rate in favour of patients with MSI compared with those with MSS, both treated with adjuvant FOLFOX chemotherapy. Multivariate analysis of several predictive factors showed that tumour stage was the only statistically significant predictor of survival, but MSI status showed a non significant trend in favour of a better survival for patients with MSI. This non statistically significant trend was also found by analysing the relapse-free survival curves. Due to the small number of samples analysed, the present study lacked statistical power. It should be noted that the MSI phenotype was more frequent among stage II patients than among stage III, which could be expected from the better prognosis attributed to the MSI tumours. This study has two main limitations: the absence of a comparator arm using 5-FU alone, and its rather small sample size.

In MSI patients, 5-FU does not seem to be a very effective chemotherapy (10). There is no general agreement on a better treatment such as CPT 11, since the literature on this issue is controversial (14, 15). In daily practice, the only standard therapy in the adjuvant setting is FOLFOX. It is of paramount importance to determine whether or not micrometastases, when they exist, are sensitive to FOLFOX. Preclinical data are available for cisplatin. Nucleotidic alteration induced by cisplatin may activate the MMR system (16). This may result in apoptosis *via* p53 activation (17). Moreover, alterations of MMR genes have been associated

with an increased resistance to cisplatin in many cell lines (18). This does not seem to be true for oxaliplatin. Thus, identification of oxaliplatin adducts in DNA and the existence of colon cancer cell lines resistant to high doses of oxaliplatin appear to be independent of MSI status (19).

Two studies assessing FOLFOX in the adjuvant setting have been published (20, 21). The interpretation of their results was limited by the low numbers of MSI patients treated with FOLFOX, namely 12 patients in each study. The absence of a statistically significant difference in survival between patients with MSI and those with MSS may have been related to a lack of statistical power. In the study by Zaanan *et al*. (20), however, there was no relapse with FOLFOX compared with 8/20 relapses with 5-FU alone (p=0.01), which probably meant that patients receiving oxaliplatin combined with 5-FU had a better response than patients receiving 5-FU alone. Only the MOSAIC and C-07 studies (2), which randomised 5-FU with or without oxaliplatin, included sufficiently high numbers of patients to allow precise conclusions about the beneficial effect of oxaliplatin compared with 5-FU alone.

The present study included both stage II and stage III patients, at variance with the study by Zaanan *et al*. (20), which included only stage III patients. In the present study, patients with MSI comprised the majority of stage II patients. It is well recognised that patients with MSI have fewer metastases than those with MSS, which probably implies that those with MSI have a less severe disease (22). Taken separately, stage was a statistically significant predictive factor, whereas MSI status only tended to influence prognosis. Adjusting for stage did not show a statistically significant prognostic effect of MSI status.

Sargent *et al*. (12) hypothesised a detrimental effect of chemotherapy in stage II patients with MSI. In their study of stage II CRCs, prognosis of patients with MSI treated by 5-FU alone was as poor as that of treated patients with MSS (about 30% relapsed), whereas prognosis of non-treated patients with MSI was excellent (only 15% relapsed).

The response to FOLFOX in the metastatic setting was previously studied according to MSI status. Patients with MSI seem to have a poorer response to FOLFOX therapy (23). Interestingly, in a previous study in the metastatic setting, we found that patients with MSI who responded had received FOLFOX 6, which includes higher doses of 5-FU and oxaliplatin than FOLFOX 4. These results suggested a better response to higher doses of oxaliplatin as in FOLFOX 6 compared with the lower dose as in standard FOLFOX 4. The present study included very few relapses (only 2); it is therefore impossible to assess precisely the chemosensitivity of metastatic cancer. The main limitation of this type of studies is the low frequency of patients with MSI and metastasis, making results difficult to interpret. For example, this is the case for the study by Muller *et al*. (23).

The presence of the V600E *BRAF* mutation in CRC has been associated with poor prognosis (24). The present data, although preliminary, confirm this deleterious impact since 50% of patients with tumours with both MSI and *BRAF* mutation relapsed during the course of this study.

This study raises the issue of whether treatment and follow-up of CRC patients should be adapted to the MSI or MSS phenotype. However, the best treatment for patients with MSS, who represent more than 80% of CRC patients and form a heterogeneous group, could not be determined. A more aggressive treatment may be advocated for these patients, for example by associating targeted therapies to chemotherapy. It seems useful to identify patients with MSI because of their better prognosis that may alleviate follow-up. Further specific studies of MSI patients are necessary.

In summary, FOLFOX is the standard treatment for CRC in adjuvant setting and it has a beneficial effect among patients with MSI. Stage remains a major prognostic factor but it seems useful to determine the MSI phenotype early in the evolution of each tumour in order to distinguish groups of patients with different prognoses and to improve the management of CRC.

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