Title: **Loss of hMSH3 and Outcome of hMLH1-Deficient Colorectal Cancers**

Running Title: hMSH3 status and Nodal Metastasis in MSI CRC

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ABSTRACT

Background and Aim. CRC with microsatellite-instability (MSI) have a better outcome than their chromosome-unstable counterpart. Given the heterogeneity of MSI CRC, we wanted to see whether any of MSI-associated molecular features is specifically associated with prognosis.

Methods and Findings One hundred and nine MSI CRC were typed for primary mismatch repair (MMR) defect and for secondary loss of MMR proteins. Frameshifts at 7 target genes, mutations in the RAS-pathway, and methylation at hMLH1/p16 promoters were also searched. The interplay of molecular findings with clinico-pathological features and patient survival was analysed.

Results. A higher rate of distant-organ metastasis in patients with hMSH6-deficient cancer (2/3, 66%) versus hMLH1- (7/84, 8.3%) or hMSH2-deficient CRC (1/22, 4.5%) was the only association ($p<0.001$) between primary MMR defect and tumor stage. Of 84 hMLH1-deficient CRC, 31 (36.9%) had hMSH3 and 11 (13.1%) had hMSH6 loss ($p<0.001$), bi-allelic frameshifts at mononucleotide repeats accounting for most (78%) hMSH3 losses. hMSH3 loss was associated with a higher number of mutated target genes (3.94±1.56 vs. 2.79±1.75; $p=0.001$), absence of nodal involvement at pathology (N0) (OR 0.11, 95%CI 0.04-0.43, $p<0.001$), and better disease-free survival at Kaplan-Meier ($p=0.05$). No prognostic value was observed for K-ras status and for hMLH1/p16 promoter methylation. The association between hMSH3 loss and N0 was confirmed in an independent cohort of 71 hMLH1-deficient CRC (OR 0.23, 95%CI 0.06-0.83, $p=0.02$).

Conclusions. hMLH1-deficient CRC not expressing hMSH3 have a more severe MSI, a lower rate of nodal involvement, and a better postsurgical outcome.

Translational Relevance

The microsatellite unstable CRC are known to be less invasive than their chromosomal-unstable counterpart. However, MSI can result from a variety of mismatch repair defects, which determine a wide spectrum of mutations. We wanted to see whether MSI CRC with distinct molecular features have a different outcome. We investigated a large series of patients with MSI CRC. Loss of hMSH3 expression, in hMLH1-deficient CRC, was found to be the only molecular feature independently associated with downstaging at diagnosis and better postsurgical survival. Biologically, the study supports the hypothesis that a severe mutator phenotype might limit the invasiveness of MSI CRC. Clinically, our findings caution against assuming a better outcome for patients with CRC based only on the presence of MSI, as hMLH1-deficient MSI cancers retaining hMSH3 do not behave differently from chromosomal-unstable tumours. As a consequence, hMSH3 expression should be tested in hMLH1-deficient CRC before planning a differentiated postsurgical management.

INTRODUCTION. Several proteins participate in the task of the DNA Mismatch Repair (MMR) system, which is to correct base substitution mismatches and insertion-deletion mispairs generated during DNA replication. These proteins need to dimerize in order to form MutS and MutL functional complexes which bind to mismatches. Heterodimerization of hMSH2 with hMSH6 or hMSH3 forms hMutSα and hMutSβ, whereas heterodimers of hMLH1 with hPMS2, hPMS1 or hMLH3 produce hMutL. MutSα recognizes single base-base and insertion/deletion loops of 1 or 2 nucleotides, MutSβ preferentially recognizes larger mismatches, and MutL acts as a molecular matchmaker (1).

Hereditary nonpolyposis colorectal cancer (HNPCC) and about 10% of sporadic colon cancer are caused by MMR defects (2, 3). MMR dysfunction leads to cancer development through the accumulation of unrepaired frameshift mutations in simple repeat sequences, called microsatellites, of target genes involved in cell growth regulation (4). In fact, widespread microsatellite instability (MSI) is the hallmark of this carcinogenetic pathway also referred to as the "mutator" pathway. MSI can arise from germline mutations in *hMSH2* (5, 6) and *hMLH1* (7, 8), less frequently in *hPMS2* (9) and in *hMSH6* (10) genes, or from epigenetic silencing of *hMLH1,* which accounts for the vast majority of MSI sporadic colorectal cancers (11, 12). In addition to the primary MMR defect, secondary losses of MMR protein can occur as a consequence of *hMSH3* and *hMSH6* frameshift mutations promoted by *hMLH1* inactivation (13-15), or because of hMSH3 and hMSH6 protein degradation in cancers not expressing the heterodimeric partner hMSH2 (16, 17). As a result, single or combined defects of MMR subunits (MutL, MutSα, MutSβ) can variably underlie the genetic instability of MSI colorectal cancers.

MSI colorectal cancers have a better prognosis than microsatellite-stable tumors (18). Down-staging at diagnosis, i.e. a lower prevalence of metastatic disease, accounts for the prognostic advantage of these cancers (19). The dense immune infiltrate of MSI CRC, possibly reflecting the enhanced immunogenicity of truncated peptides (20, 21), might contribute to the reduced metastatic potential. However, the molecular basis for the prognostic advantage of MSI cancers has not been clearly

established. In particular, it is not known whether a correlation exists between the metastatic potential of MSI tumors and primary or secondary MMR defects associated with genetic instability. Aim of this paper was to see whether distinct patterns of MMR protein expression in the primary tumor can identify subsets of MSI cancers with different invasiveness and prognosis.

METHODS.

Pathological Assessment of MSI CRC

Screening for MSI was conducted on 1041 CRC consecutively resected from Caucasian patients at the Istituto Clinico Humanitas, between January 1, 1997 to June 18, 2006.

DNA was purified by standard procedures from paraffin sections of formalin-fixed tissue with a neoplastic cell content >50%. *BAT26* and *BAT25* loci were amplified by fluoresceinated primers, and PCR products analyzed by capillary electrophoresis (ABI PRISM 310 DNA Sequence; PE Applied Biosystems, Foster City, California). The MSI phenotype (high-MSI) was defined by the appearance of shorter alleles at *BAT26* and/or at *BAT25* (22).

One hundred and nine patients with MSI CRC were identified. Following approval of the study by the local Ethical Committee, the informed consent of patients to treatment of their personal data was obtained by the referring physician or by other clinicians involved in the study. Demographics and complete clinical data at diagnosis were made available at hospital Intranet resources. An accurate family history, aimed at recognizing the Amsterdam clinical criteria for HNPCC (AC II) (23), was obtained from all patients.

Densities of $CD3^+$ cells ($CD3^+$ TIL_{IM}) were assessed by immunostaining of tissue sections followed by a computer-assisted measurement of the percent areas of CD3+ TIL immunoreactivity (IRA) at the invasive margin of the tumour, as previously described (24).

An average of 25±12 lymph nodes (mean+SD) was made available for assessing nodal status. Tumor clinico-pathologic staging by AJCC (25) was assessed by combining histopathologic findings with surgical records and perioperative imaging (Malesci et al, 2007)

Molecular Subtyping of MSI CRC

a) Primary MMR Defect

Each MSI CRC was tested at immunohistochemistry for nuclear expression of hMLH1 (G-168 monoclonal antibody, PharMingen, San Diego,CA), hMSH2 (clone FE 11, Oncogene Sciences,

Cambridge, MA), hMSH6 (clone 44, Transduction Laboratories, Lexington, KY), and PMS2 (clone A16-4 , PharMingen,)(26, 27)**.**

All patients with hMLH1- or hMSH2-deficient CRC underwent sequencing of the corresponding gene(27). Mutation-negative patients were further tested by multiplex ligation-dependent probe amplification (MLPA kit, MRC-Holland, Amsterdam, the Netherlands), which detected deletions within the *hMSH2* gene in 3 cases.

b) Secondary Loss of hMSH3 and/or hMSH6

The expression of hMSH3 (clone 52/MSH3, monoclonal antibody, Transduction Laboratories) and hMSH6 protein was also evaluated by immunohistochemistry in the entire series of MSI CRC. Staining was visualized by the avidin-biotin method (Vectastain, Vectro Laboratories, Burlingame, CA).

MSI cancer cell lines were also characterized for MMR protein expression by Western-blotting. Membranes from SW 48, LS174T, HCT116, and DU145 cancer cells were incubated for 1 hour with TBS 5% skimmed milk, then overnight with primary antibodies directed against MMR protein, finally for 1 hour with anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham. GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), visualized by the Amersham enhanced chemiluminescent detection system. To assess the association with secondary MMR protein loss, the presence of frameshift mutations at the $(A)_8$ repeat of exon 7 of *hMSH3* and at the $(C)_8$ repeat of exon 5 of *hMSH6* was tested in DNA extracted from each cell line and from areas of hMLH1-deficient tumors that had been microdissected under the guidance of immunostaining.

c) Frameshift Mutations at Mutator or Target Genes

Frameshift mutations at coding mononucleotide repeats within *hMSH3, hMSH6, TGFβRII*, *BAX*, *TCF4, CASP-5, MBD4, AXIN2,* and *ACVR2* genes were investigated. PCRs were carried out with fluoresceinated specific primers and products were analyzed for the presence of mutant alleles differing by at least -/+ 1bp, according to previously described methods (28).

d) RAS Pathway Mutations

Mutations at *K-RAS* and *B-RAF* hot spots were detected by PCR–RFLP. *K-RAS* codon 12 and 13 mutations were respectively detected by a modified primer that creates a restriction site for BstNI and XcmI (New England Biolabs Inc., Beverly, MA, USA) restriction enzymes (29). *B-RAF*^{V600E} mutations were analysed by exon 15 amplification followed by digestion with TspRI (New England Biolabs, Beverly, MA, USA) restriction enzyme (30).

e) hMLH1, P16/INK4a Promoter Hypermethylation

The DNA methylation status of *hMLH1* and *P16* promoters was determined by methylation-sensitive PCR based upon DNA treatment with sodium bisulfite and amplification with primers specific for methylated and unmethylated DNA (31).

Follow-up of Patients

The observation period started immediately after surgery (mean±SD, 4.7 ± 2.8 years), and the survival was calculated from diagnosis until tumor recurrence, or until data were censored, as of July 1, 2011**.** Thoraco-abdominal CT (1.2 ± 0.5 per patient per year, mean ± SD), abdominal ultrasonography (1.7 ± 0.5), and chest radiography (1.15 ± 0.4) were performed according to common protocols for surveillance. Chemotherapy was always administered on clinical grounds and not in the context of prospective trials. 5-fluoruracil (5-FU) based adjuvant chemotherapy was administered to a total of 39 patients; 18 (36.0%) of 50 patients with stage II disease, and 21 (67.7%) of 31 patients with stage III disease.

Independent series of patients with hMLH1-deficient CRC.

Seventy-one additional tissue specimens of MSI hMLH1-deficient CRC were retrieved from the pathology archive of the Colorectal Cancer Registry in Modena and from the Ospedale di Circolo di Varese. Specimens were selected from consecutive series (1995 to 2006) of MSI-screened CRC, after confirmation of hMLH1-deficiency at immuhistochemistry. Clinico-pathological information for all patients included age, gender, histological subtype, tumor location, pT stage, pN stage, pM stage, and tumor grade.

Statistical Analysis.

Associations between molecular features of MSI cancers or between molecular and clinico-pathological features were tested by Chi-square test for categorical variables and by unpaired Student's T-test for age. All pathological and molecular factors significantly associated with nodal involvement were entered into a multivariate step-wise logistic regression analysis.

Survival curves were drawn according to the Kaplan-Meyer method, and compared using the log-rank test.

For all statistical tests, two-sided P values ≤ 0.05 were considered statistically significant.

RESULTS

Frameshift Mutations and Variable Expression of hMSH3 and hMSH6 Protein in MSI Cancer Cell-Lines

Figure 1 illustrates the correlation between genotype and protein expression for hMSH3 and for hMSH6 in MSI colorectal cancer cell-lines. As hMLH1-deficient cell lines were tested, the expression of hMSH3 was equivalent to that of MMR-proficient cells (HeLa) in SW48 cells carrying no hMSH3 mutation, greatly reduced in DU145 cells harbouring a mono-allelic *hMSH3* frameshift mutation, and abolished in HCT116 cells having a bi-allelic *hMSH3* truncating mutation. hMSH6 was expressed in all cell lines, although the protein levels were reduced in LS174T, DU145, HCT116, and SW48 cells carrying a heterozygous *hMSH6* frameshift mutation. Both hMSH3 and hMSH6 proteins were undetectable in the hMSH2-deficient LoVo cell line.

Single or Combined MMR Defect(s) in 109 Consecutive MSI Colorectal Cancers

Immunohistochemistry recognized lack of hMLH1 (n= 84; 77.1%), hMSH2 (n=22; 20.2%), or hMSH6 (n=3; 2.7%) as the primary MMR defect. No primary PMS2 defect was detected.

Germ-line predisposing defects were identified in 26 patients (14 with *hMSH2* and 12 with *hMLH1* mutation). Amsterdam II criteria for HNPCC were fulfilled in 24 (22%) patients, 8 of whom had no detectable hMLH1 or hMSH2 germ-line mutation.

All hMSH2-deficient cancers failed to express both hMSH3 and hMSH6 protein. Of 84 hMLH1-deficient cancers (examples given in Figure 2), 31 (36.9%) did not express hMSH3 whereas only 11 (13.1%) failed to express hMSH6 ($p<0.001$). Focal areas of hMSH3 loss were observed in 9 (10.7%) additional hMLH1-deficient cancers. The rate of secondary MMR protein loss in hMLH1 cancers was not significantly different ($p>0.5$) in hereditary (hMSH3 6/14, 42.9%; hMSH6 2/14, 14.2%) and sporadic (hMSH3 25/70, 35.7%; hMSH6 97/70, 12.8%) subsets.

DNA analysis of microdissected areas of hMLH1 cancers confirmed a strong correlation between *hMSH3* genotype and protein expression (Supplemental material 1). Of 40 neoplastic areas not

expressing hMSH3 (31 from cancers with homogeneous protein loss and 9 from tumors with only focal hMSH3 loss), 31 (78%) harboured two $(A)_7$ mutated alleles, 9 (22%) had a heterozygous frameshift, and none had both wild-type alleles. About 30% of cancers still expressing the hMSH3 protein harboured a heterozygous frameshift mutation within the $(A)_8$ repeat. Homozygous mutations of *hMSH6* were also limited to cancers not expressing the protein, but bi-allelic frameshifts accounted for loss of hMSH6 only in 2 of 11 (18%) cancers. The overall frequency of hMSH6 hetero- or homo-zygous frameshift in hMLH1-deficient CRC (17 of 84, 20.2%) was significantly lower ($p<0.001$) than that of hMSH3 mutations (46 of 84, 54.8%).

Mutations in Target Genes

Figure 3 shows the frequency of mutations at 7 well recognized "targets" of DNA-repeat instability (cell growth/survival genes), in MSI CRC subsets defined by MMR protein defect. The primary MMR defect was first considered (Panels A). Two of three hMSH6-deficient CRC had no mutation in the investigated genes and the third one exhibited only a frameshift in *MBD4*. The mutational rate in hMSH6-deficient CRC was significantly lower ($p<0.01$) than that observed both in hMLH1- and hMSH2-deficient tumors. The number (mean$\pm$SD) of mutated targets was higher, although not significantly ($p=0.11$), in hMSH2-deficient CRC (3.95 ± 1.84) than in hMLH1-deficient tumors (3.30 ± 1.65), frameshift mutations of TCF4 being much more frequent in hMSH2-deficient cancers (17/22, 77.3% vs. 23/84, 27.4%; $p<0.001$). The mutational rate was then analyzed in hMLH1-deficient CRC with or without secondary loss of hMSH6 or hMSH3 (Panels B). hMLH1-deficient CRC with additional loss of hMSH3 protein had a mean number of mutated genes significantly higher than hMLH1-deficient CRC with no additional secondary loss ($3.90+1.47$ vs. 2.95 ± 1.77; $p=0.017$), 5 of 7 individual targets having a higher rate of mutation. In particular, the frequency of mutated *caspase-5* was significantly different in the two subgroups (24/31, 77.4% vs. 17/42, 40.5%; $p=0.002$). Conversely, hMLH1-deficient cancers with coexistent loss of hMSH6 had a number of mutations in target genes not different from that of hMLH1-only-deficient tumors (2.78 ± 1.30 vs. 2.95 ± 1.77; $p=0.78$).

The number of target genes with frameshift mutation was higher in hMSH2-deficient cancers (3.95±1.8) than in hMLH1-deficient tumors with no secondary loss (2.95 ±1.8; p=0.04) or with hMSH6 loss (2.78±1.69; p=0.09), but almost identical to that observed in hMLH1-deficient tumors with secondary hMSH3 loss (3.94±1.9; p=0.96).

Mutations in Genes of the KRAS-Pathway

KRAS $^{cod12\text{-}13}$ mutations more frequently occurred in cancers whose primary MMR defect was hMSH2 (10/22, 45.5%) than in those with hMLH1- (15/84, 17.9%; p=0.007) or hMSH6- (0/3; p= 0.06) deficiency. Among hMLH1-deficient cancers, *KRAS* $^{cod12\text{-}13}$ mutations were found in 7 of 44 (15.9%) tumors with no secondary MMR loss, in 6 of 31 (19.4%) cancers with additional hMSH3 loss, and in 2 of 9 (22.2%) CRC with hMSH6-only secondary deficiency. *BRAF* ^{V600E} mutation were detected in 40 of 84 (47.6%) cancers with hMLH1 primary defect but in no hMSH2- (0/22; p<0.001) or hMSH6- (0/3; p=0.05) deficient tumor. No significant difference in the frequency of *BRAF* ^{V600E} mutation was observed between subgroups of hMLH1-deficient tumors (21/44, 47.7%, in cancers with no secondary loss; 17/31, 54.8%, in tumors with secondary hMSH3 loss; 2/9, 22.2%, in cancers with secondary hMSH6 loss).

Methylation of *hMLH1* and $p16_{INK4}$ promoter

hMLH1 promoter methylation was significantly more frequent in hMLH1-deficient CRC (66/84, 78.6%) than in hMSH2- (6/22, 27.3%; p<0.001) and hMSH6- (0/3; p=0.002) deficient tumors. The rate of *P16* methylation was higher, although not significantly, in hMLH1-deficient CRC (47/84, 56.0%) than in hMSH2- (8/22, 36.4%; p=0.10) and in hMSH6-deficient tumors (1/3, 33%).

Twenty-three of 31 (74.2%) hMLH1-deficient tumors with secondary hMSH3 loss, 7 of 9 (77.8%) cancers with secondary hMSH6 loss, and 36 of 44 (81.8%) tumors with no secondary MMR protein loss exhibited *hMLH1* methylation. The methylation rate of *P16* in hMLH1-deficient CRC was also unaffected by secondary protein loss (hMSH3 loss: 18/31, 58.1%; hMSH6 loss: 6/9, 66.7%; no additional loss: 23/44, 52.3%).

Clinico-Pathological Features at Time of Diagnosis

Table I details patient characteristics and tumor pathological features according to tumor MMR defects. As to the primary MMR defect, hMSH2-deficient tumors were diagnosed at younger age than hMLH1-deficient CRC (p=0.001), had a higher likelihood to be HNPCC (p=0.001), included a larger subset of left-sided cancers (p=0.03), and had a lower degree of local invasion (p=0.03). Two of 3 hMSH6-deficient cancers had evidence of distant-organ metastasis at diagnosis (p<0.001 vs. hMSH2- and hMLH1-deficient tumors). hMLH1-deficient cancers were then classified by the occurrence of secondary MMR protein deficiency. To this aim, tumors with only focal areas of hMSH3 deficiency (n=9) were considered as hMSH3 still-expressing tumors. Cancers with additional loss of hMSH3 had a rate of nodal involvement significantly lower than that of CRC with no secondary loss (p<0.001) or with additional loss of hMSH6 (p=0.02), and were diagnosed at an earlier stage than the remaining hMLH1-deficient tumors (p=0.01). hMLH1-deficient cancers with no loss (p=0.05), but not those with additional hMSH3 loss (p=0.19), had a frequency of nodal involvement higher than hMSH2 deficient cancers. Consistently with protein-based results, the rate of nodal involvement of hMLH1-deficient CRC with bi-allelic mutation at $(A)_8$ tract in exon 7 of *hMSH3* (3/25, 12%) was much lower (p=0.001) than that of corresponding tumors with no mutation or with heterozygous *hMSH3* frameshift (29/59, 49.2%).

Low densities of $CD3^+$ TILs were strongly associated with distant-organ metastasis at diagnosis (1^{st}quartile vs. 2^{nd} –to- 4^{th} quartile; OR, 8.67; 95%; CI, 2.06-36.4; p=0.003), but the density of $CD3^+$ lymphocytes at the invasive margin of the tumor was not statistically different in MSI cancers with different primary MMR defect, nor in hMLH1-deficient tumors with or without secondary MMR protein loss. The density of $CD3^+$ peritumoral TILs was also similar in tumours harbouring <3 (median 5.8%, 25^{th}-75^{th} percentile 2.5%-11.1%) or ≥3 (median 4.9%, 25^{th}-75^{th} percentile 2.7%-8.6%; p=0.40) frameshifted target genes.

Associations between nodal involvement and pathological or molecular features of 84 hMLH1-deficient CRC are shown in Table II. At univariate analysis, a positive association was seen for

local invasiveness (pT3-pT4 vs. pT1-pT2, p<0.007), grade (G3 vs. G1-G2, p<0.001), and histotype (mucinous/medullary vs. adenocarcinoma, p<0.001), whereas a negative correlation was observed for hMSH3 loss (p<0.001), frameshift mutations at *hMSH3* (p=0.02 vs. wild-type), and ≥ 3 mutated MSI target genes (p=0.01). At multivariate, however, only hMSH3 loss (p<0.001) and grading (p=0.001) were independently associated with nodal status.

Nodal status resulted to be independently associated with hMSH3 loss (OR 0.19, 95% C.I. 0.07-0.48; p<0.001), and with grading (OR 6.13, 95%CI 2.42-15.5, p<0.001) also if the multivariate analysis was applied to the entire series of 109 MSI CRC including hMSH2-deficient cancers (Supplemental material 2).

Patient Survival

In our series of MSI CRC (hMLH1- or hMSH2 deficient cancers), none of the 10 patients with stage I CRC but 10 of 67 (14.9%) patients with stage II/III cancer were diagnosed with postsurgical local recurrence or metachronous metastasis (mean postsurgical follow-up, 5.65±2.8 years). Figure 4 shows the disease-free survival curves from patients undergoing colonic resection for stage II/III MSI CRC, classified by primary MMR defect and by hMSH3 expression. As a reference, the survival of patients with stage II/III pT3/pT4 MSS CRC is also shown.

Among patients with hMLH1-deficient cancers, those with hMSH3-negative tumors had a better disease-free survival (p=0.05), and their survival curve was almost similar to that of patients with primary hMSH2 defect. Grouping patients with tumor loss of hMSH3 irrespectively of the primary defect (hMLH1 or hMSH2), disease-specific survival was significantly better (p=0.01) than that of patients with hMSH3-positive cancer. As the survival of patients with hMLH1-deficient tumor was compared to that of patients with stage II/III MSS CRC, a significant difference was observed for hMSH3-negative cancers (p=0.03), but not for hMSH3-negatives ones. (p=0.76).

Association Between hMSH3 Loss and Nodal Status in an Independent Series.

Of 71 additional hMLH1-deficient cancers resected at other institutions, 20 (28.2%) did not express hMSH3, and 11 (15.5%) showed heterogeneous expression of hMSH3. The fraction of cancers

lacking hMSH3 protein was not significantly different from that of our institutional series (20/71 *vs* 31/84, p=0.25), neither was different the rate of hMSH3 loss in hereditary (8/25, 32%) or sporadic cancers (12/45, 26.7%; p=0.78). The analysis of the $(A)_8$ mononucleotide repeat in the exon 7 of *hMSH3* confirmed the strong correlation between the presence of frameshift mutation and the loss of hMSH3 protein. Of 20 cancers not expressing hMSH3, 15 (75.0%) harboured *hMSH3* homozygous frameshift mutation $(A)_7$, 5 (25.0%) showed heterozygous frameshift mutation, and none had both wild-type alleles. Of 51 cancers expressing hMSH3, 14 (27.5%) harboured a heterozygous frameshift mutation within the repeat and the remaining 37 (72.5%) cancers showed both wild-type alleles. Also in this series, tumors with additional loss of hMSH3 had a rate of nodal involvement (4/20, 20.0%) significantly lower than that of cancers with no secondary loss (25/51, 49.0%; p=0.02). The association of nodal involvement with tumor features is detailed in Supplemental material 3. Again, at multivariate analysis, hMSH3 loss was negatively associated with nodal involvement (OR 0.23, 95% C.I. 0.06-0.81; p=0.02) (Supplemental material 3).

DISCUSSION

The prognostic advantage of MSI CRC over microsatellite-stable cancers essentially reflects the earlier stages of these tumors, i.e., the lower rate of lymph-node and distant-organ metastasis at time of diagnosis (19). This concept is strongly supported by the fact that MSI tumors represent more than 1/3 of all stage I/II CRC, about 15% of stage III, and only 5-10% of stage IV tumors (32, 33). The present study is the first to demonstrate that the outcome of MSI cancers varies according to different patterns of MMR defect, and, in particular, that the secondary loss of hMSH3 protein identifies h-MLH1-deficient tumors at very low risk of nodal involvement and postsurgical recurrence. Given the overall better prognosis of MSI CRC, the identification of a subclass with better outcome is particularly significant, as it might have been easily overlooked. It is also noteworthy that the association between tumor downstaging and hMSH3 loss was confirmed in an independent validation series of MSI CRC.

MSI CRC can undergo secondary loss of MMR protein through different mechanisms. Primary hMSH2-deficiency invariably determines proteolytic degradation of both hMSH3 and hMSH6 (34), whereas hMLH1-deficient cancers may become unable to express hMSH3 and hMSH6 in the course of tumor progression. Loss of hMSH3 follows bi-allelic frameshift mutation at the *A(8)* coding mononucletide repeat in exon 7 of *hMSH3*, as observed in HCT116 cells (15, 35) (Figure 1). Of other molecular events possibly underlying hMSH3 loss in hMLH1-deficient CRC, LOH typically occurs only in microsatellite-stable tumors (36, 37), while gene-promoter methylation has not been investigated. Having detected *hMSH3* bi-allelic frameshifts in about 80% of hMLH1-deficient cancers with hMSH3 loss, we can assume homozygous mutation as the key mechanism for hMSH3 loss in such tumors (35) and even speculate that the detection of heterozygous-only frameshifts in a few hMSH3-negative CRC might simply reflect an imperfect microdissection of cancer cells. Differently from hMSH3 loss, bi-allelic frameshifts at the mononucleotide repeat did not account for the secondary loss of hMSH6 occasionally detected in hMLH1-deficient CRC and,

in this case, a substantial role for somatic missense mutations (15) and single-base substitutions (14) has to be postulated.

hMSH3 expression resulted to be negatively associated with nodal involvement when the analysis was limited to hMLH1-deficient cancers, but also when all MSI CRC, there including hMSH2-deficient tumors, were considered. The association might be mediated by a lower degree of tumor local invasion (pT) in hMSH2-deficient cancers, but not in hMLH1-deficient tumors whose pT was not significantly influenced by the hMSH3-status. Thus, loss of hMSH3 in hMLH1-deficient tumors, although delayed since cancer initiation, has to occur early enough to preceed the spread of the tumor to regional lymphnodes. This is in accordance with the model of progression originally proposed for MSI CRC by Duval et al. who found that hMSH3 frameshifts were second only to TGFβ mutations as early genetic events able to increase the global instability phenotype (38). Our results are also in agreement with the inverse relationship between clinico-pathological staging and accumulation of frameshift mutations observed in a large series of sporadic MSI cancers (39). In contrast, others (40) found an association between hMSH3 loss and advanced tumor stage in a small group of hMLH1-deficient CRC, but the high rate of LOH suggests that that series was somehow enriched with tumors with overlapping patterns of microsatellite and chromosomal instability.

Different hypotheses have been made to explain the more indolent behaviour of MSI CRC. As compared to microsatellite-stable cancers, MSI tumors might undergo a stronger antitumoral immune response induced by highly immunogenic truncated peptides (20, 21, 41). Being lymphocyte infiltrate established as a favourable prognostic biomarker in CRC (24, 42), we found it particularly interesting that hMSH3 frameshifts have been recently demonstrated to act as antigenic epitopes of cytotoxic T cell (20). However, the prognostic value of hMSH3 loss in our series was independent of peritumoral densities of $CD3^+$ cells, nor there was any association between hMSH3 frameshift mutations and lymphocytic infiltrate. Therefore, our result cannot support a role for immune response in the downstaging of hMSH3-deficient tumors. Conversely, nodal involvement was negatively associated with the number of mutated *target* genes, supporting the alternative

concept that genetic instability itself might in fact trigger a "self-limiting" program for the tumor by accumulating mutations even in genes necessary to cancer cell survival and progression (43-45).

Regardless of any possible clinical application for hMSH3 loss as a prognostic biomarker, the analysis of disease-free survival strongly suggests a role for hMSH3 in modulating the progression and the invasiveness of MSI CRC. MutSβ, resulting from heterodimerization of hMSH2 with hMSH3, and MutSα complex, formed by coupling of hMSH2 with hMSH6, are known to be partially redundant in the recognition of DNA mismatches. MutSα plays a predominant role for the recognition of base/base mismatches. Both complexes can detect small insertion/deletion loops up to10 unpaired nucleotides (17, 46-48), but MutSβ exceeds affinity and efficiency of MutSα for loops made by two or more unpaired nucleotides (17, 46, 49). We found that CRC combining MutL and MutSβ deficiency had a higher number of frameshift mutations at mononucleotide repeats of target genes than those carrying a simple loss of MutL. The correlation between the number of frameshifts at target genes and hMSH3-negative tumors might simply reflect the greater likelihood for MSI CRC with a higher mutational rate in target genes to acquire bi-allelic mutations at *hMSH3*, and not viceversa. Therefore, caution must be exerted in interpreting the lower staging and the better outcome of cancers with hMSH3 loss as the result of a more severe mutator phenotype, as protein loss or gene inactivation might directly affect tumor progression in the absence of any effect on MMR efficiency. Along this line, hMSH3 deficiency has been recently demonstrated to sensitize colorectal CRC cells to platinum drugs independently of any influence on the canonical MMR system (50).

In summary, MSI CRC lacking hMSH3 expression have a lower rate of nodal involvement at diagnosis and a better outcome than other MSI tumors. This applies both to hMSH2-deficient tumors, whose hMSH3 loss is caused by protein degradation, and to hMLH1-deficient cancers in which bi-allelic frameshift mutations at *hMSH3* can determine the protein loss. As a consequence, no prognostic advantage over MSS cancers should be taken as granted for hMLH1-deficient tumors retaining hMSH3 protein expression.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

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Figure 1. hMSH3 and hMSH6 expression in cancer cells with different MMR primary defect, and frameshift mutations at coding repeats of corresponding genes.
MMR-proficient (HeLa), hMSH2-deficient (LoVo), and hMLH1-deficient cancer cell lines (SW48, LS174T, DU145, HCT116) were tested. MMR protein expression was assessed by Western-blot of nuclear extracts from each cell-line. Allelotyping was conducted by running electropherograms of exon7 for *hMSH3* and of exon5 for *hMSH6*, containing $(A)_8$ and $(C)_8$ repeats, respectively. Although not carrying any frameshift mutation, LoVo cells did not express hMSH3 and hMSH6 due to protein degradation in the absence of hMSH2. Of hMLH1-deficient lines, SW48 cells and LS174T cells (wild-type for *hMSH3*, heterozygous mutation for *hMSH6*) exhibited a reduced expression of hMSH6 only, DU145 (heterozygous frameshifts for *hMSH3* and *hMSH6*) showed lower levels of expression for both proteins, and HCT116 (-1bp bi-allelic *hMSH3* mutation and monoallelic *hMSH6* mutation) failed to express hMSH3.

Figure 2. Patterns of hMSH3 expression in hMLH1-deficient colorectal cancers.
Panel A, homogeneous nuclear expression (brown). Panel B, heterogeneous expression with retained nuclear staining on the left side and focal areas (arrows) of protein loss (blue-stained nuclei). Panel C, homogeneous loss of nuclear staining for hMSH3. (20X magnification)

Figure 3. Frameshift mutations at 7 cell growth/survival genes in MSI CRC by their primary MMR defect (A), and in the hMLH1-deficient subgroup by secondaryMMR-protein loss (B).
▨ **,** cumulative number of target genes with a frameshift (out of 7; *mean*± *sd*).
⬠*,TGFβRII*; △,*CASP5;* ○*,BAX;* ⬠*,MBD4;* □*,TCF4;* ✣*, AXIN;* ▯, *ACVR2.*
Panels A. Upper panel. The three hMSH6-deficient cancers had an overall number of mutated target genes significantly lower than that of hMSH2- and hMLH1-deficient tumors. Lower Panel Only a frameshift at *TGFβRII* was detected in three hMSH6-deficient cancers. *TCF4* frameshifts were more frequent in hMSH2-deficient cancers than in hMLH1-deficient tumors ($p<0.001$, bold).
Panels B. Upper Panel. hMLH1-deficient cancers with secondary hMSH3 loss had a number of mutated genes significantly higher than tumors with no additional MMR loss or with secondary loss of hMSH6. The number of target genes with frameshift mutation in hMSH2-deficient cancers was also significantly higher than that observed in with no secondary loss ($p=0.04$) but not different from that seen in hMLH1-deficient tumors with hMSH3 loss. Lower panel. *CASP5* frameshifts were more frequent in hMLH1&hMSH3-deficient cancers than in hMLH1-only -deficient tumors ($p=0.001$, bold).

Figure 4. Disease-free survival after resection of stage II/III CRC. Kaplan-Meier curves from patients with hMLH1-deficient MSI CRC expressing (blue line, n=43; stage II, n=20; stage III, n=23) or not (red line, n=24; stage II, n=21; stage III, n=3) hMSH3 protein, with hMSH2-deficient MSI CRC (black line, n=13; stage II, n=8; stage III n=5), and with MSS CRC (gray line, n=360; stage II, n=176; stage III, n=184). P value at Log-rank test.