

## Microsatellite Instability as a Prognostic Factor in Resected Colorectal Cancer Liver Metastases

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**Background:** Two distinct genetic mutational pathways characterized by either chromosomal instability or high-frequency microsatellite instability (MSI-H) are currently recognized in the pathogenesis of colorectal cancer (CRC). Recently, it has been shown that patients with primary CRC that displays MSI-H have a significant, stage-independent, multivariate survival advantage. Untreated CRC hepatic metastases are incurable and are associated with a median survival of 4 to 12 months. Conversely, surgical resection in selected patients results in a 20% to 50% cure rate. The aim of this study was to investigate the prognostic importance of MSI-H in patients undergoing resection of hepatic CRC metastases.

**Methods:** DNA was extracted from paraffin-embedded, resected metastatic CRC liver lesions and corresponding normal liver parenchyma from 190 patients. MSI-H status was determined by polymerase chain reaction–based evaluation of the noncoding mononucleotide repeats BAT-25 and BAT-26.

**Results:** MSI was detected in tumors from 5 (2.7%) of the 190 CRC patients. All MSI-H tumors were in patients with node-positive CRC primary tumors. The median survival after hepatic resection of MSI-H and non-MSI-H tumors was 67 and 61 months, respectively ( $P = .9$ ).

**Conclusions:** These data suggest that MSI-H is not a common feature in resected CRC liver metastases and do not suggest a role for MSI in stratifying good versus poor prognosis in these patients.

**Key Words:** Microsatellite instability—Colorectal cancer—Hepatic metastases—Molecular markers.

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Sporadic colorectal cancer (CRC) develops through at least two distinct molecular pathways characterized by either chromosomal instability (CIS) or microsatellite

instability (MSI).<sup>1</sup> Most CRC demonstrates CIS and has a molecular profile characterized by allelic losses (loss of heterozygosity) and by chromosomal amplifications and translocations. Many of these changes directly affect genes implicated in the development and progression of colorectal neoplasia, such as the Adenomatous Polyposis coli (APC) and p53 genes. In contrast, approximately 15% of sporadic CRCs feature widespread or high-frequency MSI (MSI-H).<sup>2</sup> These tumors are characterized by subtle DNA sequence changes, with small frameshift mutations throughout the tumor genome that result from mutation of DNA mismatch repair genes (especially hMLH1 and hMSH2).<sup>3</sup> MSI-H CRC tumors have an increased mutation rate of short tandemly repeated DNA sequences known as *microsatellites*, and genes that con-

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tain short repetitive sequences are most vulnerable to mutation through this mutational mechanism. These genes include transforming growth factor- $\beta$ R2, insulin-like growth factor-2R, and BAX. It is interesting to note that primary CRC with MSI-H is associated with distinct clinicopathologic features: it occurs more frequently in the proximal colon, has a higher frequency in females, is often histologically high grade, displays mucinous differentiation, and is associated with peritumoral lymphoid infiltrates.<sup>4</sup> Furthermore, it has been shown that primary CRC that displays MSI-H has a significant, stage-independent, multivariate survival.<sup>2</sup>

The liver is the most common site for CRC metastases, and hepatic metastases develop in up to 40% of CRC patients at some point in the course of their disease. Although liver metastases are usually incurable, a small subset of patients with CRC hepatic metastases has a more favorable natural history that is amenable to potentially curative resection.<sup>5</sup> Long-term survival and cure have been reported in up to 20% to 50% of patients treated in this way.<sup>5</sup> Clinical factors predictive of long-term survival for patients undergoing hepatic resection for CRC have been well studied, but to date, molecular markers relevant to CRC hepatic metastases have been less well characterized.<sup>5-7</sup> Although MSI-H has been shown to be a predictor of improved outcome in primary CRC, its frequency and importance in secondary CRC are less well defined. The aim of this study was to investigate the frequency of MSI-H in CRC liver metastases and to determine whether there is a correlation between this molecular marker and clinical outcome.

## MATERIALS AND METHODS

### Patient Population

A cohort of consecutive patients undergoing hepatic resection for CRC metastases by two University of Toronto surgical oncologists between 1983 and 2000 was identified. Patients for whom tissue was available were analyzed for MSI status. Corresponding clinical data were obtained from a prospective database and retrospective review of patient records. Follow-up survival data were also obtained.

### DNA Extraction From Tissues

Paraffin-embedded tissue blocks were obtained, and DNA was extracted from metastatic liver lesions and corresponding normal liver parenchyma. Histopathologic review was performed to confirm the presence of tumors of high cellularity (>50% neoplastic cells) and normal tissue. For each case, tumor and normal samples were microdissected from two to three 10- $\mu$ m unstained

slides. The tumor and normal sections were microdissected from the slides and incubated in lysis buffer containing 100 mM Tris (pH 8.0), 100 mM ethylenediaminetetraacetic acid (pH 8.0), 1% Tween-20, and proteinase K (200  $\mu$ g/mL) at 60°C overnight.

### MSI Testing

Genomic DNA was amplified by polymerase chain reaction with primers for two mononucleotide microsatellite loci (BAT-25 and BAT-26) that are contained in the recommended National Cancer Institute panel for testing for MSI.<sup>8</sup> MSI at the BAT-26 locus has been shown to be predictive of widespread genomic instability.<sup>9-11</sup> In some cases, tumors showed intermediate BAT-26 allelic size variations, but these were too small to confidently determine the MSI status. These cases were resolved as either MSI-H or microsatellite stable (MSS) by studying the BAT-25 locus.<sup>10</sup>

### Immunohistochemistry

The MSI-H cases were further stained for hMLH1 and hMSH2. Formalin-fixed, paraffin-embedded tissues were sectioned at 4  $\mu$ m. Deparaffinization and rehydration were performed with xylene and alcohol. The slides were submitted to microwave antigen retrieval for pretreatment (10 mM citrate buffer; pH 6.0). Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide, and nonspecific binding was blocked by 20% Protein Blocker (Signet Laboratories, Inc., Dedham, MA) in Tris-buffered saline. Sections were incubated for 1 hour at room temperature with mouse monoclonal antibodies against hMLH1 protein (G168-728; PharMingen, San Diego, CA; prepared with full-length protein) at 1/40 dilution and hMSH2 protein (FE11; Oncogene Research Products, Cambridge, MA; prepared with the carboxy-terminal fragment) at 1/100 dilution. The antibodies were detected with the avidin-biotin complex method by using diaminobenzidine as the chromagen. The slides were counterstained with hematoxylin.<sup>12</sup> Cancers were considered to be deficient in hMSH2 or hMLH1 protein expression when there was complete absence of detectable nuclear staining in neoplastic cells. Intact nuclear staining of adjacent nonneoplastic epithelium, stromal cells, or lymphocytes served as an internal positive control.

### Statistical Analyses

Patient and pathologic characteristics—including age, sex, primary CRC staging, primary CRC, lymph node status, and site of primary CRC—were compared between MSI-H and MSS patients. Continuous data were compared by using the *t*-test, whereas categorical data

was compared by using Fisher's exact test. Overall survival after curative-intent hepatectomy for CRC metastasis was calculated according to the Kaplan-Meier method. The effect of MSI-H or MSS status on survival was examined in a similar fashion and compared by using the log-rank test.

Furthermore, Fong et al.<sup>5</sup> have previously shown that certain clinical factors are predictive of survival after hepatectomy. We evaluated the univariate predictive value of these factors, when available, in our patient population by the Kaplan-Meier method and log-rank tests. In addition, we calculated the clinical risk score (CRS) as described by Fong et al.<sup>5</sup> Adverse predictive factors identified by Fong et al. include (1) node-positive primary tumor, (2) disease-free interval from CRC primary resection to discovery of liver metastases <12 months, (3) number of tumors >1, (4) preoperative carcinoembryonic antigen level >200 ng/mL, and (5) size of largest tumor >5 cm. Each criterion was assigned one point to generate a CRS. Survival analysis was then performed with the population stratified into two groups: the first group was composed of patients with low CRS (0–2), and the second group was composed of patients with high CRS (3–5).

All statistical tests were performed with SPSS version 12 (SPSS Inc., Chicago, IL). Differences were considered significant at  $P < .05$ .

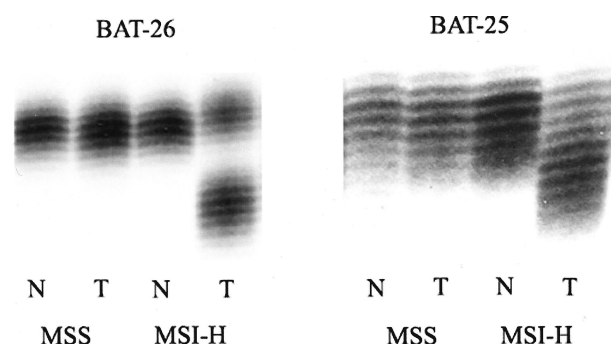
## RESULTS

### Molecular Analysis

Only five cases of resected CRC hepatic metastases (2.7%) were characterized as MSI-H (Fig. 1), and all were subsequently shown to be hMLH1 deficient.

### Clinicopathologic Data

A total of 190 cases were available for study. The clinical characteristics of the overall group, as well as of



**FIG. 1.** Microsatellite instability status as determined by BAT-26 and BAT-25. MSI-H, high-frequency microsatellite instability; MSS, microsatellite stable.

the patients classified by MSI status, are listed in Table 1. Table 2 summarizes the significance of previously recognized prognostic variables with respect to CRC hepatic metastases in the two groups. The median number of liver metastases was 2 (range, 1–8). Forty-six percent of the patients had a solitary liver metastasis, 39% had two or three metastases, and 15% had four or more liver metastases. The median size of liver metastases was 4 cm (range, 1–17 cm). Thirty-two percent of patients had tumors larger than 5 cm.

### Survival and Prognostic Data

Figure 2 shows the Kaplan-Meier survival curve for the entire cohort after liver resection, as well as the survival for MSI-H and MSS patients. Median survival calculated from the time of liver resection was 62 months. Actuarial survival was 91% at 1 year after resection, 52% at 5 years, and 26% at 10 years. The median survival of MSI-H and MSS patients was 67 and 61 months, respectively ( $P = .9$ ). Table 2 summarizes survival analyses based on the clinical criteria derived by Fong et al.,<sup>5</sup> as well as tumor distribution and MSI status in our patient group. The nodal status of the primary tumor and the tumor distribution were the only significant predictors of survival in this cohort. MSI status was not found to add additional prognostic value. However, despite not finding differences in survival at the univariate level for any of the Fong CRS criteria, we found that the CRS stratified by low-risk (CRS 0–2) and high-risk (CRS 3–5) groups was strongly predictive of survival in our cohort of patients (Fig. 3).

## DISCUSSION

There is increasing interest in accurately predicting the natural history of CRC liver metastases. Understanding this has implications for both prognostication and for planning treatment. Large-scale retrospective studies have pointed to a variety of clinical indicators that are useful in this regard.<sup>5–7</sup> The role of molecular markers in further defining prognosis after hepatic resection for CRC is less clear.

Fong et al.<sup>5</sup> have established clinically relevant prognostic factors for patients with metastatic CRC who are being considered for curative-intent surgery. Positive resection margins, extrahepatic disease, node-positive primary CRC, a disease-free interval <12 months, number of hepatic tumors >1, size of the largest tumor >5 cm, and increased carcinoembryonic antigen level were identified as significant adverse predictors of survival. In this study, we applied the five criteria identified by Fong et al. to our cohort of patients. Of these, only the lymph

**TABLE 1.** Clinical characteristics of entire cohort and by MSI status

Clinical factor	All patients (n = 190)	MSI-H patients (n = 5)	MSS patients (n = 185)	P value
Age (y)	61 ± 11	53 ± 23	61 ± 11	.5
Sex (M:F)	1.5:1	3:2	113:72	.7
Stage of primary CRC				
II	49 (25.8%)	1 (20%)	48 (25.9%)	.3
III	69 (36.3%)	2 (40%)	67 (36.2%)	
IV (synchronous)	72 (37.9%)	2 (40%)	70 (37.8%)	
Site of primary tumor				
Right	57 (30%)	4 (80%)	53 (29%)	.06
Left	133 (70%)	1 (20%)	132 (71%)	
Lymph node—primary tumor				
Positive	114 (62%)	5 (100%)	109 (58.9%)	.09
Negative	76 (38%)	0 (0%)	76 (41.1%)	

MSI, microsatellite instability; MSI-H, high-frequency MSI; MSS, microsatellite stable; CRC, colorectal cancer.

node status of the primary CRC was significantly predictive of survival in univariate analysis. In addition, the presence of unilobar versus bilobar metastatic disease was significant in this study. It is possible that our smaller series lacked the power to detect differences for the other clinical factors. Nonetheless, other investigators have found the Fong criteria to be imperfect in determining prognosis after hepatic resection.<sup>13</sup> It is interesting to note that when we subgrouped our patients into high- and low-risk groups by using the CRS devised by Fong et al., there was a significant difference in survival between groups (Fig. 3). Nonetheless, it is clear that there are shortcomings in the ability of clinical

determinants to accurately predict the biology of resected hepatic CRC metastases. One of the aims of this study was to evaluate whether MSI analysis could extend the ability to predict the prognosis of resected hepatic metastases.

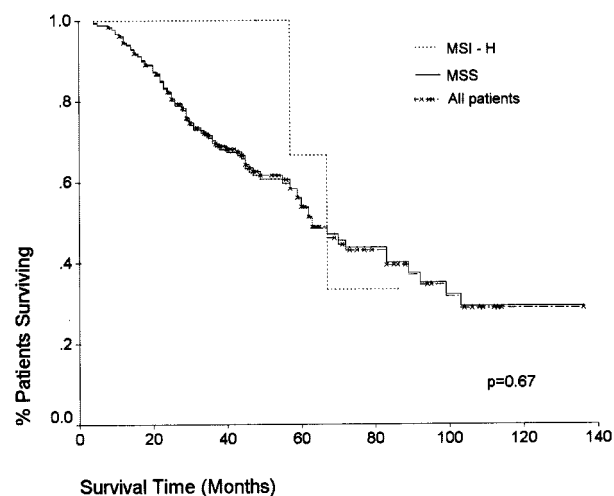
Molecular markers, including MSI, have been shown to be important in understanding the biology of primary CRC. It is increasingly clear that MSI-H denotes primary CRC with clinically significant differences in behavior.<sup>2</sup> Primary CRCs with an MSI-H profile comprise approximately 15% of all primary CRCs and are associated with a better prognosis compared with MSS cancers. Moreover, recent studies have begun to suggest a role for stratifying patients according to the likelihood of response to different chemotherapy regimens.<sup>14,15</sup>

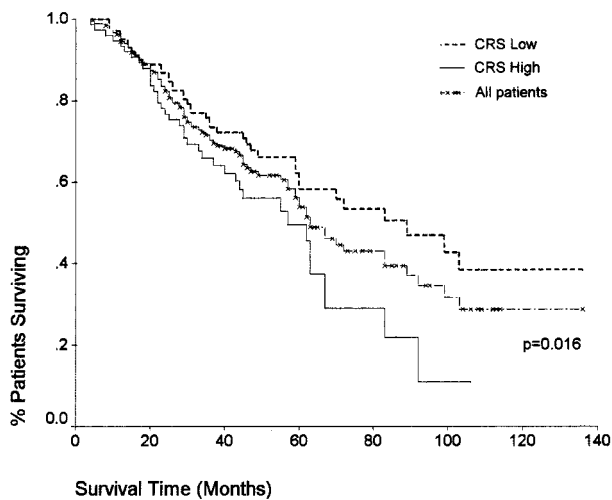
Study of the clinical significance of molecular markers in metastatic CRC is increasingly important as the range

**TABLE 2.** Univariate overall survival analysis of clinical risk factors for the entire cohort

Clinical factor	Median survival (mo)	P value
Primary lymph node		
Positive	60	.03
Negative	89	
Disease-free interval (mo)		
≤12	59	.4
>12	61	
No. hepatic lesions		
1	67	.6
>1	61	
Largest metastasis (cm)		
>5	60	.06
≤5	67	
CEA >200 ng/mL		
Yes	48	.8
No	54	
Tumor distribution		
Unilobar	73	.02
Bilobar	36	
MSI status		
MSI-H	67	.8
MSS	61	

CEA, carcinoembryonic antigen; MSI, microsatellite instability; MSI-H, high-frequency MSI; MSS, microsatellite stable.

**FIG. 2.** Overall survival for the entire cohort (MSI-H and MSS patients). MSI-H, high-frequency microsatellite instability; MSS, microsatellite stable.



**FIG. 3.** The Fong clinical index allowed for prediction of a group of patients with better survival after hepatic colorectal cancer resection. CRS, clinical risk score.

of medical and ablative treatment options for these patients is rapidly increasing. MSI-H is an attractive molecular marker that could be screened for in a relatively straightforward manner. Several publications have suggested that assessment of a single locus, BAT-26, is highly predictive of MSI-H status.<sup>9,10,16</sup> Moreover, it is unlikely that our use of BAT-26 alone to define MSI-H status lacked specificity, because all five MSI-H liver metastases were MLH-I immunodeficient. Although we did not assess the MSI status of the primary CRC, it is generally accepted that molecular progression of a tumor through MSI is an early event during tumorigenesis and that MSI-H primary tumors generate MSI-H metastases and CIS primary tumors generate CIS metastases. As expected, the primary CRC associated with the five MSI-H liver metastases was usually in the right colon (four of five cases; Table 1).

This article presents the largest group of patients with resectable hepatic metastases from CRC for which the MSI status of the hepatic tumors has been studied (Table 3).<sup>17-19</sup> Other reports used less commonly accepted definitions of MSI status and therefore reflect a different subset of patients compared with the specific group de-

finied as MSI-H in this study.<sup>20,21</sup> Similar to results from other investigators, our results demonstrate a very low frequency of this molecular genotype in resected CRC hepatic metastases. Unlike in primary CRC, the rate of MSI-H in resectable CRC hepatic metastases is approximately 2.5%. Two potential explanations explain this discrepancy; either MSI-H primary CRC tumors do not metastasize to the liver as frequently, or these tumors spread to the liver in such a widespread fashion that they are deemed surgically unresectable. The natural downstaging of MSI-H CRC is likely the most important explanation for the discrepancy in frequency of MSI-H primary CRC versus resectable CRC liver metastases.<sup>2,4</sup> However, a completely distinct pattern of metastatic disease associated with MSI-H CRC cannot be ruled out with the current data.

Previous investigators have studied the potential for other biological alterations or genetic changes to predict the outcome of resected hepatic metastases. Such studies have suggested that human telomerase reverse transcriptase, Ki-67, thymidine labeling index, DNA ploidy, transforming growth factor- $\alpha$  expression, p53, epidermal growth factor receptor (EGFr) expression, thymidylate synthase, and DCC (detected in colon cancer) may help discern which patients undergoing hepatic resection are more likely to develop tumor recurrence.<sup>13,22-27</sup> Conversely, investigation by Crowe et al.<sup>27</sup> suggested that a series of other markers do not predict survival. Because of the apparent limitations of clinical predictive scores, it is conceivable that studies evaluating molecular markers will have clinical relevance. This is particularly true given that the range of chemotherapies and molecular therapies available for treating CRC is expanding. Future studies may discover how various prognostic factors correlate with the utility of differing therapies.

## CONCLUSION

Our data suggest that MSI-H is not a common feature in resectable CRC liver metastases. We, like others, found a low frequency of MSI in resected CRC liver metastases. Moreover, this molecular marker does not identify a group of CRC liver metastasis patients with a better or worse prognosis. The low frequency of this marker within this group of patients precludes its clinical utility as an additional selection criterion for hepatic resection of metastatic CRC. It seems that tumors with MSI-H do not develop resectable hepatic metastases as frequently as do primary MSS CRCs. This finding is interesting in that it might be indicative of a different metastatic spread pattern for tumors with MSI. The findings also suggest that the stage IV survival advantage for

**TABLE 3.** Studies of MSI in CRC metastases

Author	Year	No. Liver metastases examined	Frequency of MSI
Kochhar et al. <sup>17</sup>	1997	141	2.5%
Schneider et al. <sup>18</sup>	2000	29	0%
Rosty et al. <sup>19</sup>	2001	56	1.8%
Present study	2004	190	2.7%

MSI, microsatellite instability; CRC, colorectal cancer.

patients with MSI tumors is independent of the surgical resectability of liver metastases.

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