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Immunohistochemistry Versus Microsatellite Instability Testing in Phenotyping Colorectal Tumors

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Purpose: **To compare microsatellite instability (MSI) testing with immunohistochemical (IHC) detection of *hMLH1* and *hMSH2* in colorectal cancer.**

Patients and Methods: **Colorectal cancers from 1,144 patients were assessed for DNA mismatch repair deficiency by two methods: MSI testing and IHC detection of *hMLH1* and *hMSH2* gene products. High-frequency MSI (MSI-H) was defined as more than 30% instability of at least five markers; low-level MSI (MSI-L) was defined as 1% to 29% of loci unstable.**

Results: **Of 1,144 tumors tested, 818 showed intact expression of *hMLH1* and *hMSH2*. Of these, 680 were microsatellite stable (MSS), 27 were MSI-H, and 111 were MSI-L. In all, 228 tumors showed absence of *hMLH1* expression and 98 showed absence of *hMSH2* expression: all were MSI-H.**

Conclusion: **IHC in colorectal tumors for protein products *hMLH1* and *hMSH2* provides a rapid, cost-effective, sensitive (92.3%), and extremely specific (100%) method for screening for DNA mismatch repair defects. The predictive value of normal IHC for an MSS/MSI-L phenotype was 96.7%, and the predictive value of abnormal IHC was 100% for an MSI-H phenotype. Testing strategies must take into account acceptability of missing some cases of MSI-H tumors if only IHC is performed.**

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IN THE NEAR FUTURE, there are likely to be important clinical indications for determining the molecular type of colorectal cancers (CRC). One parameter by which colorectal cancers can be classified involves alterations in the DNA mismatch repair process. Specifically, about 85% of CRC possess normal DNA mismatch repair function, whereas 15% have defective DNA mismatch repair. The latter category includes mostly sporadic tumors in which *hMLH1* promotor methylation has rendered the DNA mismatch repair complex incompetent. It also includes the cancers associated with hereditary nonpolyposis colon cancer syndrome (HNPCC)/Lynch syndrome that carry a germline mutation in one of the DNA mismatch repair genes, usually *hMLH1* or *MSH2*.

The tumor phenotype associated with either hereditary or acquired loss of DNA mismatch repair competency is called microsatellite instability (MSI). MSI phenotypes have been subdivided into those with high (MSI-H) and low (MSI-L) levels of instability, with MSI-H usually defined as instability at $\geq$ 30 of loci studied, and MSI-L defined as instability at 1% to 29% of loci.[1] All other tumors are referred to as microsatellite stable (MSS). Uncertainty exists about the clinical and biologic significance of the MSI-L phenotype because, in most regards, the behavior of MSI-L tumors is similar to that of MSS tumors.

There is a growing body of evidence that there are clinical and histopathologic differences between MSI-H and MSS/MSI-L colorectal cancers. Tumors with an MSI-H phenotype are more likely to have mutations in genes with short repetitive tracts such as the transforming growth factor beta receptor gene, *BAX* genes, *IGF2R* gene, and others.[2-7] MSI-H tumors are less likely to have loss of *APC*,[8-17] or mutations in *p53*[8,10,14-22] or K-*ras*[8-10,14-16,19,20,23-25] compared with MSS tumors. MSI-H tumors are more likely to be diploid or nearly diploid,[8,26-31] Carcinoembryonic antigen expression is less common in MSI-H tumors.[31] MSI-H tumors more often arise in the right colon[26,27,28,32] and are more likely to occur in individuals with a positive family history of colorectal cancer.[26,27] A female predilection for MSI-H tumors has been noted,[7,30,33] and MSI-H tumors may have a better stage-specific prognosis.[26,27]

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Additional differences are noted in the histopathologic examination. MSI-H tumors are more likely to show cribriform/solid growth pattern and signet ring histology or high-grade medullary histology[28,30,31,34] and to be mucinous[30,35,36] and exophytic.[31] MSI-H tumors may show enhanced immunologic response as determined by marked lymphocytic infiltration of the tumor.[30,36]

Experiments have recently shown in vitro differences in the response of MSI-H cell lines to chemotherapeutic agents. DNA mismatch repair-deficient cells are resistant to the alkylating agents melphalan and busulphan; the methylating agents procarbazine and temozolomide; the platinum-containing agents cisplatin and carboplatin; the antimetabolites 6-thioguanine, fluorouracil, and O^6-methylguanine; and the topoisomerase inhibitors etoposide and doxorubicin (reviewed in[37-39]). The clinical significance of these observations remains unclear; however, one recent publication[40] described striking survival benefits in patients with right-sided colonic tumors who received adjuvant chemotherapy compared with those who did not. Little benefit from adjuvant therapy was noted in patients with left-sided tumors. Right-sided colon tumors are much more frequently MSI. The authors therefore suggest additional prospective studies on the predictive value of MSI regarding benefits from adjuvant chemotherapy. It does seem reasonable to suspect the potential for different susceptibilities to chemotherapeutic agents in MSS versus MSI-H tumors in light of the differences catalogued above.

Presently, determination of DNA mismatch repair competency status from CRC is offered in situations in which the diagnosis of HNPCC/Lynch syndrome is being considered; for example, in a proband with a positive family history of CRC or a very young individual with a diagnosis of CRC. As more is learned about differential responses to therapies between tumors with and without DNA mismatch repair competency, one must anticipate that testing will become even more widely conducted, perhaps encompassing all cases of newly diagnosed CRC in order to tailor therapeutic regimens to the biology of the colorectal tumor.

The current "gold standard" for assessing tumor DNA mismatch pair competency is molecular MSI testing. This is a labor-intensive test that involves extracting DNA from both tumor and normal tissue excised at surgery. The DNA is subjected to polymerase chain reaction (PCR) amplification of five or more different chromosomal loci that compare "microsatellites" (simple sequence repeat such as a CA dinucleotide repeat), running the PCR products through a gel to separate DNA fragments by size, comparing the tumor-normal pairs, and scoring for differences (MSI) between the two. Generally, instability at two or more out of five markers (or $>$ 30% of markers tested) defines a tumor as MSI-H.

The intensive and time-consuming nature of this test is particularly clinically troublesome, because surgeons would frequently like to know preoperatively if a patient is likely to have HNPCC. This information might change the extent of the colectomy that is performed and lead to consideration for simultaneous hysterectomy and oophorectomy as well. The time frame from diagnosis to surgery is generally insufficient to allow tumor MSI results to be available at the time surgical decisions are being made.

It is known that in HNPCC, mutations in two of the DNA mismatch repair genes, known as *hMLH1* and *hMSH2*, account for about two thirds of families meeting Amsterdam criteria. However, in HNPCC patients with tumors with the MSI-H phenotype, nearly all families are thought to carry germline mutations in *hMLH1* or *hMSH2* (mutations in *hMSH6*, *hMSH3*, *hPMS2* are extremely uncommon). Furthermore, in sporadic tumors with MSI-H phenotype, a very high proportion of all tumors have methylation of the *hMLH1* promotor. Therefore, *hMLH1* or *hMSH2* are either mutated or methylated in the vast majority of MSI-H tumors identified to date. Monoclonal antibodies to the protein products of both *hMLH1* and *hMSH2* are now commercially available. This technique is far less labor intensive than traditional MSI testing, and the results can be available to inform clinical decisions within 24 hours.

We sought to determine the correlation between tumor MSI status and tumor IHC for the protein products of *hMLH1* and *hMSH2*, reasoning that if IHC was sufficiently sensitive and specific, tumor IHC might provide a rapid and cost-effective method for categorizing colorectal cancers into mismatch repair competency subgroups.

PATIENTS AND METHODS

Patient/Tumor Ascertainment

Tumors for this study came from a number of different sources. Three centers from the Cooperative Family Registry for Colon Cancer Studies (CFRs) participated in this study (additional information about the CFRs resource can be found at http://www-dccps.ims.nci.nih.gov/cfrccs/q&a.html). Patients from the Mayo CFR site were recruited from three sources: (1) Mayo Clinic Rochester patients; (2) North Central Cancer Treatment Group patients, a consortium of community-based oncology practices throughout the middle United States; and (3) via the Minnesota Cancer Surveillance System, a population-based state cancer registry. Patients from the Australia CFR site were recruited from multiple family cancer clinics throughout Australia. Patients from the Ontario CFR site were recruited from a population-based cancer registry from throughout Ontario. All CFR sites had appropriate institutional review board review of protocols, and participants gave written informed consent for collection of blood and tumor tissue for use in cancer research. Patients completed extensive epidemiology questionnaires, family history was obtained, and additional affected and unaffected relatives were also invited to participate in the CFRs. The tumors from a variety of hospitals throughout the United States, Australia, and Canada were preserved in a variety of ways, and

Table 1. Summary of Patients Included in This Analysis

Ascertainment Site	No. of Cases	Age (years)	
		Mean	Range
Mayo CFR	337	54	28-77
Mayo CRA consecutive case series	255	69	29-91
Australia CFR	284	50	17-80
Australia consecutive case series	136	67	18-96
Ontario CFR	132	NA	
Total	1,144		

tumor blocks varied in age from less than 1 year to more than 15 years since resection. The numbers and ages of patients in this study are listed in Table 1.

In addition, we pooled CFRs data with a series of unselected, consecutive cases of colorectal cancers resected at Mayo Clinic Rochester. These cases (hereafter called the Cancer Risk Assessment [CRA] cases) were obtained from 257 of 514 patients who underwent surgical resection during a 1.5-year period from December 1995 to April 1997 (57.2% of those approached did agree to participate) (Thibodeau et al, manuscript submitted for publication). There is no overlap with the CFRs cases. The male/female ratio was 1.47 (153 men, 104 women). For the nonparticipants (n = 199), the male/female ratio was 1.1, indicating that male subjects were more likely to participate than female subjects. The nonparticipants were also older than the participants (median age, 72 *v* 69 years; $P = .005$). Note that this aggregate data set is collected via oversampling of high-risk colon cancer probands, and thus is not suitable for determining such things as the frequency of MSI phenotypes in the general colon cancer population.

DNA Extraction

DNA was extracted from frozen or paraffin-embedded tissues as described previously.[33] Briefly, DNA from microdissected frozen tissue sections (10 μm) was extracted by a standard phenol/chloroform procedure. For tumor DNA, only those areas containing more than 70% tumor cells were used. For DNA extraction from paraffin-embedded tissues, the Qiamp tissue kit (Qiagen, Inc, Santa Clarita, CA) was used according to the manufacturer's instructions. The corresponding normal control DNA for each patient was derived from peripheral blood. For these specimens, DNA was extracted using the Puregene nucleic acid isolation kit (Gentra Systems, Minneapolis, MN).

MSI

For the CFRs tumors, paired normal and tumor DNA was analyzed for MSI with 10 markers: mononucleotide markers BAT25, BAT26, BAT40, BAT34C4; dinucleotide markers D5S346, D17S250, ACTC, D18S55, and D10S197; and penta-mono-tetra compound marker MYCL.

For the CRA group, paired normal and tumor DNA were analyzed for MSI with six dinucleotide microsatellite markers (D5S346, TP53, D18S34, D18S49, D18S61, and ACTC) and one mononucleotide repeat (BAT 26). PCR and gel electrophoresis were carried out as described by Thibodeau et al.[26] Tumors were classified as MSI-H if $\geq$ 30% markers demonstrated instability, MSI-L if $<$ 30% demonstrated MSI, and MSS if no marker exhibited MSI.[1,33]

Immunohistochemical Analysis

For immunohistochemical (IHC) analysis performed at the Mayo Clinic, tissue sections were cut at 6 μm and mounted on Probe On charged slides (Fisher Scientific, Pittsburgh, PA). After deparaffinization, slides were steam pretreated in EDTA buffer, pH 8.0, in a Black & Decker Handy Steamer Plus (Black & Decker, Shelton, CT) for 30 minutes. After rinsing in cool water, slides were loaded onto the Tech Mate 500 (Ventana Medical Systems, Tuscon, AZ) automated immunohistochemical stainer. The stainer uses capillary gap technology as the primary mode of operation. In order for successful staining performance, a gap measuring between 75 and 200 μm must be formed between two slides where the tissues are face to face.

Staining is performed using an avidin-biotin complex methodology, supplied in kit form from Ventana Medical Systems (Biotek Solutions buffer kit, Biotek Solutions DAB detection kit). This test uses a primary antibody against *hMLH1* (clone G168-728, 1/250; Pharmingen, San Diego, CA) and *hMSH2* (clone FE11, 1/50; Oncogene Research Products, Cambridge, MA) that has been titered on colon cancer sections and also tested on various normal and pathologic tissue specimens.

IHC in Australia used 4-μm sections that were affixed to Superfrost Plus adhesive slides (Fisher Chemical Co, Pittsburgh, PA) and air-dried overnight at 37°C. Antigen retrieval was performed in 0.001 mol/L EDTA, pH 8.0, in an autoclave on "wet" cycle for 30 minutes. The sections were cooled in EDTA buffer for 20 minutes before being transferred to tris-buffered saline (TBS) (pH 7.4). Endogenous peroxidase activity was blocked by immersing the slides in 1.0% H_2O_2, 0.1% NaN_3 in TBS for 10 minutes. Nonspecific antibody binding was inhibited by incubating the sections in 4% commercial nonfat skim milk powder in TBS for 15 minutes; then, after a brief rinse in TBS, the slides were transferred to a humidified chamber and incubated with 10% nonimmune normal goat serum. Excess nonimmune serum was decanted from the slides and sections were incubated with primary antibody overnight at room temperature. The primary antibodies used were *MLH1*, clone G168-15 (BD PharMingen, Franklin Lakes, NJ) 1/75; and *MSH2*, clone G219-1129 (PharMingen), 1/150.

The sections were washed in TBS and then transferred to a Shandon Sequenza staining system (Thermo-Trace, Noble Park, VIC, Australia). To block endogenous biotin-like activity unmasked by the antigen retrieval step, the slides were subjected to biotin blocking using the Dako Biotin Blocking kit (Dako, Carpinteria, CA) according to the manufacturer's instructions. The sections were incubated with biotinylated goat antimouse immunoglobulins (Jackson ImmunoResearch, West Grove, PA), diluted 1/250 in TBS for 45 minutes, then with streptavidin–horseradish peroxidase conjugate (Jackson ImmunoResearch) diluted 1/500 in TBS for 15 minutes. Antigenic sites were identified using 0.05% 3,3'-diaminobenzidine with H_2O_2 as substrate, and were then lightly counterstained with Mayer's hematoxylin before being permanently mounted using DePeX (BDH-Gurr, Poole, United Kingdom).

Statistical Methods

Sensitivity and specificity for IHC classification for MSI-H status was defined using the MSI results as the gold standard. Exact 95% confidence intervals (CIs) were calculated using the binomial distribution. Sensitivity was defined as the absence of *hMLH1* and *hMSH2* expression by IHC in MSI-H tumors. Specificity was defined as intact expression of *hMLH1* and *hMSH2* by IHC in MSS or MSI-L tumors.

RESULTS

Of the 1,144 cases examined, 350 (30.6%) were classified as MSI-H by MSI testing. Of these, 323 showed absence of either *hMLH1* (70.6%) or *hMSH2* (29.4%) expression by

Table 2. Comparison of Colorectal Cancer IHC Results With MSI Results by Center

	No. of Tumors With Intact Expression of *MLH1* and *MSH2* by IHC					No. of Tumors With Absent Expression of *MLH1* by IHC					No. of Tumors With Absent Expression of *MSH2* by IHC				
	A	B	C	D	Σ	A	B	C	D	Σ	A	B	C	D	Σ
Total no. of cases (N = 1,144)	274	204	251	89	818	41	48	116	23	228	22	3	53	20	98
No. of MSS	215	186	199	80		0	0	0	0		0	0	3*	0	
No. of MSI-H	12	0	8	7		41	48	116	23		22	3	50	20	
No. of MSI-L	47	18	44	2		0	0	0	0		0	0	0	0	

NOTE. Patient populations were not comparable across centers. The cases in B were not selected for high-risk findings and the cases in C had the highest risk profiles for hereditary cancers. This study does address incidence of MSI-H phenotype and should not be used for this purpose.

Abbreviations: A, Mayo CFR cases; B, Mayo CRA cases; C, Australian cases; D, Ontario cases; Σ, total for each category; MSS, all tested microsatellites were stable; MSI-H, ≥ 30% or more of microsatellites tested were unstable; MSI-L, 1-29% of tested microsatellites were unstable.

*These three cases would *not* have been reported clinically as MSS because there was insufficient tumor burden in the postirradiated specimens to perform MSI testing reliably, and are included here only to highlight this technical issue in MSI testing. Germline mutations in *hMSH2* were present in the families of these three cases.

IHC, for a sensitivity of 92.3% (95% CI, 88.9% to 94.9%). Of the 794 cases found to be MSS or MSI-L by MSI testing, 794 (100%) showed normal IHC expression of both proteins, for a specificity of 100% (95% CI, 99.5% to 100%). The observed predictive value of absence of expression of either *hMLH1* or *hMSH2* (no cases showed absence of both) for predicting MSI-H status was 100%. The predictive value of normal expression of both of these proteins for predicting MSS/MSI-L status was 96.7%. Results of testing of 1,144 colorectal cancers for MSI testing and IHC for *hMLH1/hMSH2* are listed in Table 2.

DISCUSSION

This comparative study provided an opportunity to assess the strengths and weaknesses of tumor MSI testing versus tumor IHC for determining the competence of the mismatch repair mechanism of tumors. Our interest was to determine the correlation between IHC and MSI, *not* in how to best diagnose HNPCC, nor were we trying to determine frequency of MSI in CRCs. Overall, this study showed that absence of expression of *hMLH1* or *hMSH2* had a 100% specificity for predicting a tumor with MSI-H phenotype (302 of 302). On the other hand, an MSI-H phenotype was present in 3.3% of tumors with normal expression of both *hMLH1* and *hMSH2* (27 of 818 tumors). The sensitivity of IHC for detecting MSI-H tumors was 92.3%. That is, 326 of 353 tumors with MSI-H phenotype had absence of expression of either *hMLH1* or *hMSH2*. Thus, in this mixed patient population that is oversampled for high-risk factors (young age, positive family history), an abnormal IHC test has a 100% predictive value for an MSI-H tumor phenotype, and a normal IHC test for these two proteins has a 96.7% predictive value for an MSS/MSI-L phenotype.

Others have looked at the issue of IHC versus MSI in smaller series in different populations for different reasons. Two groups have found 100% correspondence between tumor MSI results and tumor IHC. Dieumegard et al[41] reported that 15 MSI-H tumors they studied had lack of expression of either *hMSH2* or *hMLH1* in each case, whereas normal expression was found in 17 MSS tumors. Cawkwell et al[42] studied 502 colorectal cancers. Sixty-six showed an MSI-H phenotype and all (100%) were associated with complete lack of expression of either *hMSH2* or *hMLH1*.

On the other hand, other groups have found less than 100% correspondence between these technologies. Debniak et al[43] studied 168 patients with CRC including 25 with suspected or known HNPCC. In this study, IHC was normal in 9% of cases (four of 43) in which an MSI-H tumor phenotype was found. Marcus et al[44] studied the expression of *hMLH1* and *hMSH2* in 72 formalin-fixed, paraffin-embedded tumors. MSI-H phenotype was predicted correctly in 37 of 38 tumors (97%). IHC expression was normal in all tumors without instability (34 of 34). Terdiman et al[45] used IHC on 38 MSI-H tumors, and four tumors (10.5%) had normal IHC (five were equivocal). Chaves et al[46] studied 76 cases of sporadic CRC and found MSI-H phenotype in nine cases; IHC detected only 75% of these. Ward et al[47] studied 308 colon tumors and found that 27 of 33 (82%) of MSI-H tumors had loss of *hMLH1* or *hMSH2*.

Tumor *hMLH1/hMSH2* IHC has many clear advantages over tumor MSI testing. If test costs are set according to actual workload, IHC will be much less expensive than MSI testing, and IHC can be performed more rapidly. Debniak et al[43] estimate that IHC costs only 14% to 28% of what MSI testing costs. On the basis of our experience, using workload recordings, we would agree with this estimate. Another substantial advantage of tumor IHC over MSI testing is that IHC outcome will guide clinicians to the correct gene for genetic testing in individuals/families in which the issue of HNPCC is under investigation. That is, absence of expression of either *hMLH1* or *hMSH2* indicates which gene is likely involved in an HNPCC family. Additionally, tumor IHC can be conducted on tiny tumor fragments such as those typically obtained from a

needle or colonoscopic biopsy. This type of fragment would frequently yield insufficient DNA to conduct MSI testing.

Are there differences in the likelihood of achieving a technically satisfactory test result between IHC or MSI testing? We did not systematically collect all the data required to answer this question, but made some observations as the data were collected. First, there may be a higher success rate for all testing using fresh tissue compared with archived tissues. With the CRA study, which used fresh frozen tissues, there were no technical failures for either MSI or IHC. With the Mayo CFR archival collection, we observed seven tumors in which MSI testing was technically unsuccessful (amplified in only one or two of the 10 attempted markers), but tumor IHC was successful in six of these cases. This included two cases with absence of *hMLH1* expression and two cases with absence of *hMSH2* expression. Alternatively, there were also two cases in which IHC failed but MSI was successful. Additional prospective studies are needed to look systematically at success rates between these two tests.

If the MSI status was determined solely on the basis of the IHC surrogate, what would IHC of *hMHL1/hMSH2* be expected to miss? Tumor MSI is a reflection of DNA mismatch repair function. Two hits to any of the other components of the DNA mismatch repair system (eg, *hMSH6*, *hPMS2*) can cause MSI-H tumor phenotype that would not be predicted by looking only at *hMLH1/hMSH2* IHC. In addition, it is possible that missense mutations in *hMLH1/hMSH2* may exist that transcribe and translate a stable but nonfunctional immunoreactive protein. This would give an apparently normal IHC. Among the discordant cases at Mayo and in Australia, preliminary work indicates that at least some of these cases are because of inactivation of *hMSH6* and *hPMS2*, although some cases remain unexplained.

Note also that in the three Australian cases with absent expression of *hMSH2* but no MSI, all three were rectal cancer cases that had been irradiated before resection. The very few tumor cells that were present in the specimen (estimated as $<$ 3%) were not enough to yield an MSI-H phenotype, but were sufficient to be detected as islands of *hMSH2* deficient tumor cells by IHC. This MSI result would not have been reported clinically because it was apparent that the specimen was not suitable for reliable MSI testing. For the CFRs, sections with more than 70% tumor are generally used. It is clear that discrepant IHC/MSI results should prompt a search for a biologic/clinical/technical explanation for this finding and not just assume one test is "wrong."

What threshold for test sensitivity is acceptable for categorizing colorectal tumors as DNA mismatch repair proficient or not? The answer to that question surely depends on why the test is being ordered. If the goal is to identify HNPCC kindreds for making a rapid surgical decision or in offering genetic mutation analysis, that is different from categorizing for purposes of tailoring potential chemotherapy. IHC appears to offer a faster and less expensive alternative to MSI testing for classifying colorectal cancers by mismatch repair competency with essentially 100% specificity and greater than 92% sensitivity. Clinicians must decide on a case-by-case basis if they are comfortable with the predictive value of the IHC testing for a particular patient. This weighting may shift if the utility of colorectal tumor phenotyping becomes more important in informing treatment decisions.

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