Published in final edited form as:

Am J Surg Pathol. 2009 January ; 33(1): 126–133. doi:10.1097/PAS.0b013e31817ec2b1.

Pathologic Predictors of Microsatellite Instability in Colorectal Cancer

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Abstract

Identification of microsatellite unstable (MSI-H) colorectal cancers (CRC) is important not only for the identification of hereditary nonpolyposis colorectal cancer syndrome (HNPCC) but also because MSI-H CRCs have a better prognosis and may respond differently to 5 fluorouracil based chemotherapy. We present two nearly equivalent logistic regression models for clinical use that predict microsatellite instability based on the review of 1649 CRCs from patients of all ages collected in a population-based case control study in northern Israel.

198 of these 1649 tumors demonstrated a high degree of microsatellite instability (12%). Multivariate analysis found that >2 TIL cells per high-power field, the lack of dirty necrosis, the presence of a Crohn's-like reaction, right-sided location, any mucinous differentiation (mucinous or focally mucinous) and well or poor differentiation, and age less than 50 were all independent predictors of MSI-H. We developed two logistic regression models that differ only by the statistical approach used to analyze the number of TIL cells per high-powered field, where the slightly more accurate (and complex) model uses the log of the total number of TIL cells. The simpler clinical model uses a cutoff of 2>TIL cells per high-powered field. The accuracy of both models is high, with an 85.4% vs. 85.0% probability of correctly classifying tumors as MSI-H. By employing the simpler model, pathologists can predict the likelihood of microsatellite instability

by compiling the MSI probability score (see Table 4 and figure 1) from simple histologic and clinical data available during sign-out.

Our model shows that approximately 43% of CRCs have a MSI probability score of 1 or less and hence have little likelihood (<3%) of being MSI-H. While this model is not perfect in predicting microsatellite instability, its use could improve the efficiency of expensive diagnostic testing.

Keywords

Colorectal neoplasia; Microsatellite instability; Pathology; Tumor infiltrating lymphocytes; Mucinous; Differentiation; Dirty necrosis; Logistic regression

Introduction

Molecular genetic studies of colorectal carcinoma (CRC) have found that 10 to 15% of CRCs have high-level microsatellite instability (MSI-H)(10,1,16,37, 25,11). Microsatellite instability most often occurs as a result of sporadic methylation of hMLH1, however, germline mutations in DNA mismatch repair genes also predispose to MSI-H CRCs in patients with Hereditary Non-polyposis Colorectal Cancer Syndrome (HNPCC), also known as Lynch Syndrome (17,32,28,41,31,29, 12).

MSI-H colorectal cancers have been shown to have a better overall prognosis compared to microsatellite stable cancers (MSS) (13, 6,9,8,40,34). There is also evidence that MSI-H CRCs are less responsive to 5 fluorouracil based chemotherapy regimens than MSS tumors (33, 4), although the evidence is conflicting (27,7). Because of these clinical differences and the profound importance in recognizing HNPCC/Lynch Syndrome, there has been increasing pressure on pathologists to identify microsatellite unstable colorectal cancers.

Numerous publications have identified histologic features which are more commonly seen in MSI-H CRCs. Tumors that are poorly differentiated, well-differentiated, mucinous, right-sided, lack dirty necrosis, have increased tumor infiltrating lymphocytes (TIL cells), a circumscribed/expansile growth pattern, histologic heterogeneity and a prominent inflammatory reaction at the advancing edge of the tumor (Crohn's-like reaction) are more likely to be MSI-H (2,5,13, 6,9,8, 18, 21, 22,26, 30,34, 35,39,40, 11, 25, 14). Recently, Jenkins and colleagues published a MsPath score that took many of these histologic factors into consideration to predict the likelihood that an individual tumor is MSI-H (25). Specifically, their model incorporated age at diagnosis, anatomical location, mucinous, signet ring or undifferentiated morphology, poor differentiation and the presence or absence of TIL cells to predict the probability of microsatellite instability (25). Their study focused on identifying HNPCC patients and as such only looked at patients under the age of 60. We present a similar histologic model based on the review of 1649 CRCs from patients of all ages collected in a population-based case control study in northern Israel (83% of whom were over the age of 60). Because of our study design, we were able to assess the likelihood of microsatellite instability in the general population, without a bias towards more HNPCC patients.

Methods and Materials

Study Design and Case Selection

This data was collected as part of The Molecular Epidemiology of Colorectal Cancer study, a population-based, case-control study of invasive colorectal cancer in Northern Israel. Eligibility criteria in this study included all cases of invasive colorectal cancer diagnosed in

the Haifa and Northern District of Israel between March 31, 1998, and December 31, 2002. All of the tumors were originally diagnosed by a small group of pathologists representing the study hospitals in Israel. Uniform histopathologic review was then performed on every tumor by one pathologist (JKG). Demographic and tumor staging data were obtained in Israel from original pathology reports.

This study was approved by the internal review boards of The University of Michigan Health System, Ann Arbor, Michigan, USA and The Carmel Medical Center, Haifa, Israel.

Pathologic analysis

All tumors were reviewed blindly by a single gastrointestinal pathologist (JKG). One or two representative blocks of normal and tumor were sent from Israel to The University of Michigan Department of Pathology where one hematoxylin and eosin-stained (H and E) section and ten 5 micron unstained non-heated sections from each block were prepared. The coded H and E stained sections were reviewed and the following histologic criteria were used to evaluate the tumors. In the majority of cases, the tumor block contained the advancing edge of the neoplasm, as the contributing pathologists were instructed to include this area in the blocks they sent for review.

General

Only resection specimens showing invasive adenocarcinoma of the colon and rectum were accepted into this analysis. Adenomas with “intramucosal carcinoma or carcinoma in-situ” were not included.

Tumor Grade

Tumors were given a single grade of differentiation (well, moderate, or poor) based on the criteria of Jass and colleagues with minor modification(20). The worst grade of tumor seen was used for the overall grade, unless the worst area was a small focus (< 10%) at the advancing margin of the tumor (the presence of tumor budding was not counted as poor differentiation and did not impact the overall grade given to any tumor).

Mucinous differentiation

Tumors with greater than 50% area showing extracellular mucin were classified as mucinous (23). Tumors with less than 50% area showing extracellular mucin were classified as having focal mucinous differentiation (Figure 1).

Signet ring cells

Tumor cells with intracytoplasmic mucin vacuoles were designated as signet ring cells. Tumors were classified as signet ring cell carcinomas if greater than 50 % of their area showed signet ring cell differentiation (23). Tumors with signet ring cells in less than 50% of their area were classified as having focal signet ring cell differentiation.

Histologic heterogeneity

Tumors with at least two distinct growth patterns were classified as showing histologic heterogeneity. This did not include mucinous and non-mucinous areas unless there were other differences in pattern such as tumor grade or architecture (otherwise virtually all mucinous tumors would be classified as having histologic heterogeneity).

Growth pattern of tumor at advancing edge

The advancing edge of the tumor was examined at low power to determine whether the tumor grew with a pushing/expansile pattern or an infiltrative pattern (20). If the advancing edge of the tumor was not present, this field was coded as unknown.

Tumor necrosis

Tumors were assessed for the presence or absence of “dirty or garland necrosis”, often considered a characteristic of colorectal carcinomas. If only a rare focus of necrosis was present (< 10%) then the tumor was classified as negative. Large geographic areas of necrosis (infarcted tumor) were not included.

Prominent Crohn’s-like host response

The advancing edge of the tumor was assessed for the presence of a Crohn’s-like inflammatory response (Figure 1). For the reaction to be considered prominent, a minimum of three lymphoid aggregates was required per section. If the advancing edge of the tumor was not present, this field was graded as unknown.

Tumor infiltrating lymphocytes (TIL)

TILs were identified on H and E stained sections as small blue mononuclear cells that typically had a halo around them (Figure 1). Only cells infiltrating between tumor cells were counted. Care was taken not to count apoptotic cells. The tumor was scanned at low power to look for the area with the most TILs (which was often the more superficial region of a deeply invasive carcinoma). Once this area was identified, 5 consecutive 40x fields of an Olympus BX40 microscope with a UPlanFI objective (Olympus America Inc., Melville, New York) were counted (total area equal to 0.94 mm²). The mean TIL/high-power field for each tumor was then calculated by dividing the total number of TIL by 5.

Molecular analysis

Microsatellite instability assays were performed by microdissecting normal and tumor tissue from unstained, recut slides of paraffin-embedded tumors. Areas for microdissection were selected by one pathologist (JKG) and circled on a hematoxylin and eosin-stained slide for a microdissection template. DNA was extracted by carefully scraping tissue from designated areas of slides with a clean razor blade and transferring the samples to separate non-siliconized tubes. Xylene (350μl) was added to each sample to dissolve the paraffin, and ethanol precipitation was performed by adding 150μl of cold 100% ethanol to each sample. Pellets were lyophilized in a Speed Vac for 10 min on high heat, and the pellets were resuspended in 100 μl of Proteinase K buffer; 200ng/μl proteinase K in 50mM Tris pH 8.3, and incubated overnight at 37°C. Samples were heated at 95°C for 8 min and quickly transferred to ice for 5 min prior to PCR.

Paired normal and tumor samples were evaluated using a consensus panel of 5 microsatellite markers as previously described (3). In brief, forward primers for Bat 25, Bat 26, D2S123, & D5S346 and the reverse primer for D17S250 were labeled with $\gamma^{32}\text{P}$ -ATP. PCR reaction volumes were 20μl/reaction, and the annealing temperatures were: BAT 25 (58 °C), BAT26 (55 °C), D2S123 (60 °C), D5S346 (58 °C), D17S250 (52 °C). PCR products were run on 6% polyacrylamide gels (pre-run for 30 min) using a running buffer of 1X TBE for 3 hours at 65W. Films were exposed at -80 °C, double-scored and double-entered. Markers showing loss of heterozygosity were considered equivocal. Only tumors with complete scoring on all 5 markers were included for statistical analyses. A tumor was considered to be MSI-H if two or more markers showed instability. Tumors with instability in only one marker (MSI-low) were considered together with tumors without instability (MSI-S) for statistical purposes.

Statistical Analysis

Statistical analyses were performed using SAS version 9.1 (Cary, NC), and all p-values reported are two-tailed. Univariate analyses of continuous variables were conducted using an unpaired t-test, and categorical variables were analyzed by contingency table analysis and chi-square tests. A receiver-operator curve was plotted to maximize the sensitivity and specificity for selecting a cutpoint of tumor-infiltrating lymphocytes as a predictor of microsatellite instability. Logistic regression was used to identify independent predictors of microsatellite instability, and variables included in the final model included all variables statistically significant at a p-value <0.05. The area under the receiver-operator curve was calculated for univariate and multivariate models using logistic regression.

Results

A total of 1649 CRCs were included in this study. 198 of these 1649 tumors demonstrated a high degree of microsatellite instability (12%). The age range of our CRC patients was from 21 to 99 with a mean of 70.3. Univariate analysis showed that TIL>2/HPF, well or poor differentiation, a lack of dirty necrosis, mucinous or focal mucinous differentiation, histologic heterogeneity, signet ring or focal signet ring differentiation, right sided location, expansile growth pattern, Crohn's like reaction, age less than 50, low stage (I and II vs III and IV) and female sex were statistically significant predictors of microsatellite instability (Table 1). Relationships between age and gender and microsatellite instability are displayed in table 2.

Multivariate analysis found that TIL cells per high-power field had the strongest association with unstable tumors. The lack of dirty necrosis, the presence of a Crohn's-like reaction, right-sided location, any mucinous differentiation (mucinous or focally mucinous) and well or poor differentiation were independent predictors of MSI-H (Table 3). Age less than 50 was the only clinical factor that was an independent predictor of MSI-H. The sensitivity, specificity, positive and negative predictive values of these features are listed in Table 4.

Logistic regression analysis found that the likelihood of a tumor being microsatellite unstable could be predicted best with the following formula:

$$\text{Probability of MSI high} = \exp(-4.5797 + \text{PathScore}) / (1 + \exp(-4.5797 + \text{PathScore}))$$

where PathScore = 1.1047[Age under 50] + 0.5355[log(til count + 1)/log(2)] + 1.1039[Well or poor differentiation] + 0.7083[Crohn's like reaction] + 0.7374[right-sided location] + 0.5230[lack of dirty necrosis] + 0.6877[any mucinous differentiation]

A website which allows one to input the various histologic variables of a tumor in order to predict the likelihood of microsatellite instability based on this formula can be accessed here: http://sitemaker.umich.edu/gruber.lab/files/msi_pre.htm A second model that uses a simple break point of TIL/HPF >2 was nearly as accurate (area under the ROC curve 0.85 vs 0.854).

$$\text{Probability of MSI high} = \exp(-4.4712 + \text{PathScore}) / (1 + \exp(-4.4712 + \text{PathScore}))$$

where PathScore = 1.1472 [Age under 50] + 1.3271[Til >=2] + 1.2262[Well or poor differentiation] + 0.8488[Crohn's like reaction] + 0.8052 [right-sided location] + 0.5656[lack of dirty necrosis] + 0.5467[any mucinous differentiation]

Using this second model, a total pathology score (MSI probability score) (Table 5) can be calculated and used to determine risk of MSI-H by using figure 2. A well or poorly differentiated tumor with >2 TIL/HPF would have a MSI probability score of 2.5 that would denote about a 12% risk of being MSI-H. If that same tumor were right-sided and had a Crohn's like reaction, the MSI probability score would be 4.1 and the risk of being MSI-H would be approximately 40%. Note that a tumor with a probability score of 1 has less than a 3% chance of being MSI-H, whereas a tumor with a score of 4.5 has about a 50% chance of being MSI-H. Our data show that approximately 55% of tumors had less than a 5% chance of being MSI-H based upon this model (figure 3). The receiver operator curve for this model is shown in figure 4. The area under the curve is 0.850.

Discussion

Detection of microsatellite instability in colorectal cancer is important for the detection of HNPCC as well as for prognostication and treatment decisions. Currently some centers are testing every colorectal cancer, some centers are testing none and some centers are using an algorithmic approach based on clinical and histologic parameters. A recent editorial in Gastroenterology (the official journal of the American Gastroenterological Association) suggested that every CRC patient be screened for microsatellite instability and/or immunohistochemistry for the detection of deficient mismatch repair gene products (36). While universal testing would certainly allow for the detection of all HNPCC patients, it would come at quite a price. A reasonable compromise may be to use clinical and histologic features as a guideline in deciding which cases should have additional testing, such as those recommended in the revised Bethesda Guidelines for HNPCC (38). These guidelines state that all CRCs occurring in patients under 50 be tested, as well as all CRCs occurring in patients under 60 that have TIL cells, a Crohn's-like inflammatory reaction, mucinous/signet-ring differentiation or a medullary growth pattern. While these guidelines make good sense for identifying HNPCC patients, they do not focus on identifying sporadic MSI-H cases (the majority of which occur over the age of 60).

Our findings extend and validate our previous findings (11) and are quite similar to those of Jenkins et al. who found that the presence of mucinous, signet ring cell, poor or undifferentiated histology, right-sided location, a Crohn's-like inflammatory reaction, TIL cells and age under 50 were strong predictors of microsatellite instability (25). While the exact weight of each feature is slightly different in their study compared to ours, this may relate to the different patient populations studied (Jenkins et al had much younger patients which probably enriched the number of HNPCC cases in their study). Over 83% of our CRCs were from patients over the age of 60, including over 78% of our MSI-H CRCs, while all of the patients in the Jenkins study were under 60. One difference between our studies is that we found a lack of dirty necrosis to correlate with MSI-H while they did not evaluate this histologic feature. In addition, we found that any mucinous differentiation, no matter what the overall percentage (rather than having at least 50% mucinous differentiation) correlated with microsatellite instability. While this may be related to our study design, in which only 1 block of tumor was usually evaluated per patient, it may also be explained by having more sporadic MSI-H tumors, which have been shown to have more histologic heterogeneity and more mucinous differentiation than HNPCC tumors (19,24). Nevertheless our results suggest that the current definition of mucinous carcinoma (requiring at least 50 percent of the tumor to be mucinous) may not be biologically relevant in the era of molecular testing (21, 11).

The most accurate model of predicting microsatellite instability employed the log of the number of TIL/HPF. Since this is not readily available during daily surgical pathology sign-out, we present a second model using a binary cut-off of >2 TIL/HPF. Switching to this later

model has no effect on cases with little chance of being MSI-H (MSI probability score less than 1), as all such cases have fewer than 2 TIL/HPF. It only affects cases that have a very large number of TIL/HPF. Since one would opt to test these tumors for microsatellite instability anyway, using this slightly less accurate model has no real clinical relevance. For those interested in looking at the effect of larger number of TIL/HPF the following website can be consulted: http://sitemaker.umich.edu/gruber.lab/files/msi_pre.htm

Whether one wants to employ an algorithmic approach such as ours or simply test all colorectal cancers for microsatellite instability (or none) is currently up to each individual pathology department. As it is not yet standard of care to tailor chemotherapy regimens based on microsatellite instability status, clinical demand for such testing is likely to remain variable both for individual physicians and institutions. However, the clinical necessity to recognize Lynch Syndrome (HNPCC) emphasizes that algorithms like the one that we propose are not a substitute for good clinical judgment. For example, a very young patient with no other pathologic features of MSI-H would have a MSI probability score of 1.1 and MSI probability of 3%, yet a workup for Lynch Syndrome would still be clinically indicated. Even though family history of colorectal cancer in a first-degree relative did not significantly improve the prediction of our model, there are certainly cases where MSI testing may be indicated based entirely on clinical features such as this (which are not routinely available to the pathologist at the time of sign-out).

Our study shows that approximately 50% of a population-based series of representative CRCs have little likelihood of being MSI-H based on clinicopathologic features. While this histologic model is not perfect in predicting microsatellite instability it could cut the number of cases sent for testing in half if tumors with a MSI probability score of less than 1.5 were not tested. Even if a MSI probability score cut-off point of 1 were used, one could eliminate testing on more than 42% of cases unless there were other compelling clinical reasons to pursue testing. In the future, as personalized medicine becomes the norm rather than the exception, we suspect that all CRCs will undergo a battery of molecular testing. Until such time, however, we believe this algorithmic method is a good common sense approach to understanding the probabilities of MSI testing and that it will improve the efficiency of the pathologic evaluation of colorectal cancer.

Acknowledgments

Support: This work was funded by R01 CA81488

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Figure 1.

Histologic features of microsatellite unstable tumors. A. Low-power view of the advancing edge of an MSI-H CRC showing a crohn's-like reaction and focal mucinous differentiation. B. Another example of focal mucinous differentiation in a well-differentiated CRC. C. High-power view of a well-differentiated CRC with large numbers of TIL cells. D. High-power view of a poorly differentiated (medullary type) CRC with large numbers of TIL cells.

Figure 2.

This map shows the MSI probability score (sum of coefficients from table 4) along the top and the percent likelihood of microsatellite instability on the bottom. Hence a tumor with a MSI probability score of 0 would have a 1% chance of being MSI-H while a tumor with a MSI probability score of 4.5 would have a 50% chance of being MSI-H.

Figure 3.

This histogram shows the number of colorectal cancers for each MSI probability score. Cases that are MSI-H are depicted in purple, while cases that are MSS are light blue. Note that more than 42% of cases have a score of 1 or less and that very few of these cases are MSI-H.

Figure 4.

This receiver operator characteristic curve shows an area under the curve of 0.850. The sensitivity and specificity for a given MSI probability score is listed. Note that for a MSI probability score of 1, the sensitivity is 92% and the specificity is 46%.

Table 1

Univariate analysis of histologic and clinical features associated with microsatellite instability

Feature	Odds Ratio	95% Lower Confidence Limit	95% Upper Confidence Limit
>2 TIL/HPF	7.4423	5.363	10.3278
Well or Poor Differentiation	7.3651	5.3604	10.1195
Lack of Dirty Necrosis	5.3872	3.9406	7.3647
Any Mucin	4.6179	3.3895	6.2916
Histologic Heterogeneity	4.3678	2.9933	6.3735
Any Signet Ring	4.3374	2.1599	8.7103
Right Sided	4.0586	2.8966	5.6867
Expansile Growth	3.467	2.2982	5.2301
Crohn's Like Reaction	2.4685	1.786	3.4119
Age < 50	2.2274	1.3065	3.7975
Low-Stage (I & II vs III & IV)	1.6554	1.1789	2.3245
Female Sex	1.3748	1.0199	1.8533

Table 2

Relationship of Age and Gender to microsatellite instability

Feature	MSI-H (%)	MSS (%)
Female	110 (13.73)	691 (86.27)
Male	88 (10.38)	760 (89.62)
Age < 60	43 (15.41)	236 (84.59)
Age 60+	155 (11.31)	1215 (88.69)

Table 3

Multivariate analysis of histologic predictors of microsatellite instability

Feature	Odds Ratio	95% Lower Confidence Limit	95% Upper Confidence Limit
>2 TIL/HPF	3.77	2.52	5.641
Well or Poor Differentiation	3.408	2.226	5.219
Age < 50	3.149	1.52	6.528
Crohn's Like Reaction	2.337	1.566	3.486
Right-sided	2.237	1.5	3.336
Lack of Dirty Necrosis	1.76	1.097	2.825
Any Mucin	1.728	1.12	2.665

Table 4

Sensitivity, specificity, positive and negative predictive values of histologic features in predicting microsatellite instability

Variable	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
Lack of dirty necrosis	0.594872	0.785815	0.275534	0.934057
Any mucin	0.530612	0.803347	0.26943	0.926045
Well or poor differentiation	0.636364	0.808011	0.311881	0.942029
Right-sided Location	0.704918	0.629485	0.213576	0.937282
Crohn's-like Reaction	0.675532	0.542474	0.17139	0.922687
TIL>2/HPF	0.70202	0.759555	0.286598	0.948785

Table 5**MSI Probability Scoring System**

Pathologic Feature	Coefficient	Score
>2 TIL/HPF	1.3	
2 or less TIL/HPF	0	
Well or Poorly Differentiated	1.2	
Moderately Differentiated	0	
Age < 50	1.1	
Age 50 or greater	0	
Crohn's-like reaction present	0.8	
Crohn's like reaction absent	0	
Right-sided location (cecum, ascending or transverse)	0.8	
Left-sided location (descending, sigmoid or rectum)	0	
Lack of Dirty Necrosis	0.6	
Dirty Necrosis present	0	
Any mucinous differentiation	0.5	
No mucinous differentiation	0	
MSI Probability Score	Total:	