

A National Cancer Institute Workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition: Development of International Criteria for the Determination of Microsatellite Instability in Colorectal Cancer

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Abstract

In December 1997, the National Cancer Institute sponsored "The International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition," to review and unify the field. The following recommendations were endorsed at the workshop. (a) The form of genomic instability associated with defective DNA mismatch repair in tumors is to be called microsatellite instability (MSI). (b) A panel of five microsatellites has been validated and is recommended as a reference panel for future research in the field. Tumors may be characterized on the basis of: high-frequency MSI (MSI-H), if two or more of the five markers show instability (i.e., have insertion/deletion mutations), and low-frequency MSI (MSI-L), if only one of the five markers shows instability. The distinction between microsatellite stable (MSS) and low frequency MSI (MSI-L) can only be accomplished if a greater number of markers is utilized. (c) A unique clinical and pathological phenotype is identified for the MSI-H tumors, which comprise ~15% of colorectal cancers, whereas MSI-L and MSS tumors appear to be phenotypically similar. MSI-H colorectal tumors are found predominantly in the proximal colon, have unique histopathological features, and are associated with a less aggressive clinical course than are stage-matched MSI-L or MSS tumors. Preclinical models suggest the possibility that these tumors may be resistant to the cytotoxicity induced by certain chemotherapeutic agents. The implications for MSI-L are not yet clear. (d) MSI can be measured in fresh or fixed tumor specimens equally well; microdissection of pathological specimens is recommended to enrich for neoplastic tissue; and normal tissue is required to document the presence of MSI. (e) The "Bethesda guidelines," which were developed in 1996 to assist in the selection of tumors for microsatellite analysis, are endorsed. (f) The spectrum of microsatellite alterations in noncolonic tumors was reviewed, and it was concluded that the above recommendations apply only to colorectal neoplasms. (g) A research agenda was recommended.

Introduction

The discovery of MSI³ in colorectal cancers and its linkage to HNPCC in 1993 opened new chapters in tumor biology and in the

clinical management of patients with heightened cancer susceptibility. Over the past 5 years, the scope of MSI has been expanded to encompass a unique form of genomic instability broadly involved in the genesis of cancer and limited to neither HNPCC nor colorectal cancer. MSI is caused by a failure of the DNA MMR system to repair errors that occur during the replication of DNA and is characterized by the accelerated accumulation of single nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences that occur ubiquitously throughout the genome. MSI is seen in most HNPCC tumors and in a proportion of nonhereditary colorectal tumors. The presence of MSI in tumor tissue is associated with certain unique clinical and pathological characteristics. The literature on MSI is growing rapidly, and the need for development of uniform criteria for its detection and definition is recognized.

A series of prior workshops led to the progressive evolution of criteria and guidelines to deal with the emerging information on the subject. The "Amsterdam Criteria" were developed in 1991 to permit the uniform identification of familial clusters of colorectal cancer so that HNPCC families might be recognized and studied (1). In 1995, the Early Detection Branch of the National Cancer Institute convened a workshop to discuss the role of genetic testing for HNPCC. In 1996, a second NCI sponsored workshop entitled "The Intersection of Pathology and Genetics in the Hereditary Nonpolyposis Colorectal Cancer (HNPCC) Syndrome" was held, which led to the development of the "Bethesda guidelines" for testing of colorectal tumors for MSI (2). To facilitate communication among investigators, the Early Detection Branch sponsored a third workshop entitled the "International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition" on December 8-9, 1997, in Bethesda, MD, to define uniform criteria for MSI; to propose technical guidelines for its detection; to review the literature pertaining to the implications of this phenotype; and to develop a research agenda for future research. In particular, the identification of potential areas of clinical application to cancer detection, prognosis, and therapeutic response was a high priority.

MSI was initially described in independent publications from three groups that appeared nearly simultaneously in 1993, and each used unique descriptors for their findings. Thibodeau *et al.* (3) referred to the process as MSI (which they abbreviated MIN), and recognized that tumors with MSI predominately occurred in the proximal colon, were associated with an enhanced survival, and were notable for the absence of "LOH." Peltomaki *et al.* (4) used the term RER phenotype, and the same international collaborating group, Aaltonen *et al.* (5), linked this to a locus that would later yield the first of several DNA MMR genes responsible for HNPCC. Ionov *et al.* (6) referred to the

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³ The abbreviations used are: MSI, microsatellite instability; HNPCC, hereditary nonpolyposis colorectal cancer; MMR, mismatch repair; NCI, National Cancer Institute; LOH, loss of heterozygosity; RER, replicative error; MSI-H, high-frequency MSI; MSI-L, low-frequency MSI; MSS, microsatellite stable; FAP, familial adenomatous polyposis; SEER, Surveillance, Epidemiology, and End Results; RER, replicative error.

process as ubiquitous somatic mutations at simple repetitive sequences, linked the process to familial cancer, and made other observations on this novel tumor progression pathway (7). Over the past 5 years, a variety of laboratories have developed their own tools for measuring MSI, and the profusion of approaches has made it difficult to compare results from different groups and has confused investigators attempting to enter the field.

At the 1997 Workshop, ~120 investigators and other interested parties from North America, Europe, Asia, and Australia convened to discuss MSI. The NCI intramural and extramural programs, academia, and biotechnology industry were represented. After a general session that reviewed the state of the art, three "breakout" groups were formed. The first of these was charged with developing a consensus definition of MSI, establishing the criteria for the measurement of MSI, and recommending markers that would facilitate uniformity among those doing research in the field. The second group reviewed the existing published and unpublished data on the natural history of colorectal tumors with MSI, discussed animal models relevant to the study of MSI, and explored whether the presence of MSI carries implications for therapy at this time. The third group was asked to address the issue of MSI in noncolonic tumors and in colorectal (and other) tumors outside of the context of HNPCC. After meeting individually, each of the breakout groups presented their findings to the entire workshop for the development of consensus.

Workshop Summary

Definitions and Technologies

Since its initial description, numerous studies have reported MSI in colorectal cancer and other extracolonic malignancies. Despite the large number of studies, there remains some confusion over how to define this phenomenon, specifically, how many markers should be used, which markers should be used, and how many must display instability before a tumor is defined as having a particular tumor phenotype? Because of the absence of uniform criteria defining this phenomenon, there has been considerable variability in the frequency of MSI reported within a given tumor type. Clearly, if we are to determine the underlying molecular basis of microsatellite instability, as well as potential clinical and pathological associations, it is critical that uniform definitions and criteria be established.

The workshop was established in part to define uniform criteria for the identification and characterization of tumors with MSI. The breakout session "Definition, Correlations, and Technologies," chaired by S. N. T. was charged specifically with this task, as well as discussing other methodological and quality control issues. Overall, there were seven major areas of discussion: Nomenclature, Definition, Methodology, Quality Control, Alternative Strategies, Criteria for Colon *versus* other Malignancies, and Indications for Testing. It is important to note that the discussion and recommendations reported here pertain only to colorectal cancer. As will be discussed later, the applicability of the recommendations to other tumor systems and premalignant states remains uncertain.

Nomenclature. Considerable variability has arisen in the literature as to how to best describe alterations in the length of microsatellites discovered in certain tumors when compared to normal tissue. As a result, there are several different acronyms currently in use. Although there are advantages and disadvantages for each of the different terms, there was general agreement that a single term would be preferred. Proposed terms included: MSI, MIN, or MI (for MSI); RERs, USM (for ubiquitous somatic mutations), and MMP (for the microsatellite mutator phenotype). The majority of participants agreed that MSI was the most appropriate term to describe this phenomenon. It is important to recognize that MSI refers solely to novel length alleles and is

distinct from the observation of LOH or allelic imbalance, in which one of the pre-existing alleles has been lost in the tumor.

Definitions. MSI is defined as a change of any length due to either insertion or deletion of repeating units, in a microsatellite within a tumor when compared to normal tissue. It is important to stress that MSI, as defined, does not describe a particular tumor phenotype but refers only to the observation of instability at a given marker. The definitions and criteria used to define a particular tumor phenotype are discussed below.

Several studies (8–12) have now shown that a subset of colorectal cancer demonstrates the phenomenon of MSI and that such tumors can be divided into several groups. The first is characterized by MSI-H (that is, the majority of markers exhibit MSI); a second group with MSI-L (i.e., only a minority of markers exhibit MSI); and a third group lacking apparent instability (none of the markers exhibit MSI; MSS). Overall, ~60–70% of tumors fall into the group lacking MSI, and the remaining tumors are nearly evenly split between the MSI-H and MSI-L groups. The distinction between tumors demonstrating MSI-H and those demonstrating MSI-L was generally accepted. However, there was considerable debate and controversy regarding the separation of tumors with low frequency *versus* those lacking MSI. Arguments in favor of combining the MSI-L and MSS groups included the fact that the baseline mutation rate for microsatellites in apparently stable colorectal cancers is not precisely known. Second, the distinction between these two groups is highly dependent on both the type and the number of microsatellites analyzed. Given the use of enough markers, it may be that all colorectal cancers will exhibit some level of MSI. With the "reference" panel of five microsatellites suggested below, there may be an insufficient number of markers to conclude with certainty whether or not an apparently stable tumor would demonstrate MSI if an expanded panel were analyzed. Finally, as discussed in another section, differences between MSI-H and MSI-L/MSS but not MSI-L and MSS have been noted with many clinical and pathological parameters, such as tumor site, stage, sex, ploidy, histology, and clinical outcome (8–16). Arguments in favor of the distinction between MSI-L and MSS included the likelihood that a baseline of instability would eventually be established for MSS tumors. Furthermore, given that the cohort of human genes responsible for MSI is not yet fully known and that some of these appear to be associated with more attenuated phenotypes (e.g. *hMSH6*), it may be important to separate those lacking apparent instability from MSI-L, at least in the investigational setting, at this time. Those arguing in favor of the distinction between MSI-L and MSS groups recognized that it is conceivable, in the future, the additional clinical correlation may prove the distinction between MSI-L and MSS to be superfluous.

The next discussion addressed the minimum criteria required to define and distinguish the MSI-H and MSI-L tumor groups. How many and which markers should be used, and how many markers need to demonstrate instability to be placed in the MSI-H group? Critical to the discussion was whether or not markers had been tested for their usefulness in identifying the various tumor phenotypes. Ideally, a given microsatellite sequence should demonstrate MSI frequently (e.g. >80% of the time) for a tumor defined as MSI-H but infrequently (e.g. <20% of the time) for a tumor in the MSI-L group. It was clear that some, but not many, studies examining these specific issues have been performed. Although dinucleotide repeats have been used most frequently to study MSI, instability has also been observed with mono-, tri-, and tetranucleotide repeats (11, 17). The likelihood that a microsatellite will be susceptible to instability may relate to the inherent mutation rate at that locus. For example, some markers that demonstrate very high rates of instability may be desirable markers for clonality (18) but might overestimate the presence of MSI resulting from certain causes such as defective MMR. It was generally

agreed, therefore, that the proper selection of markers was critical for the identification of MSI-L and MSI-H tumor phenotypes and that certain markers should not be used. Several mononucleotide repeats, such as the ones defined by *BAT25*, *BAT26*, and *BAT40* (11, 17, 19), have proven to be very useful for the identification of the MSI-H group of tumors. Instability at these loci appears in the majority of tumors with widespread MSI (*i.e.*, MSI-H) but rarely in tumors defined as MSI-L. Another potential advantage for some of these markers (*e.g.*, *BAT26*) is the presence of a quasimonomorphic profile in populations studied to date (17, 19). The separation of shortened unstable alleles can be clearly distinguished from the normal allele in the majority of cases. Although the availability of matching normal DNA is not an absolute requirement when the germ-line allele size is considered constant in the population, it was recommended that all studies assessing MSI using these markers should continue using matched normal/tumor pairs. This concern was based on a lack of information regarding possible wild-type length variability among different racial and ethnic groups.

On the basis of those studies performed to date, it was clear that many markers could be used to define and distinguish MSI-H from MSI-L/MSS tumors. For the purposes of providing some uniformity in this area, however, the workshop participants agreed to identify a working "reference" panel of markers with guidelines for defining the MSI-H tumor groups (Table 1). The recommended panel validated by Ruschoff and Fishel (11, 20) is composed of two mononucleotide repeats (*BAT26* and *BAT25*) and three dinucleotide repeats (*D5S346*, *D2S123*, and *D17S250*). Using this reference panel, MSI-H tumors are defined as having instability in two or more markers, whereas MSI-L tumors are defined as having instability in one marker. Tumors showing no apparent instability may either be included in the MSI-L group or the MSS group, with the limitations discussed above. Importantly, historical data suggests that these reference markers will identify those tumors characterized by defective mismatch repair, primarily in the two major mismatch repair genes, *hMSH2* and *hMLH1* (11). If it is important to distinguish MSI-L from MSS tumors, then the evaluation of additional markers may be necessary. If more than five markers are used to identify these particular tumor phenotypes, then the participants recommended that the criteria be modified to assess the percentage of markers demonstrating instability

rather than the absolute number. In this case, the MSI-H group of tumors would be defined as having MSI in ≥ 30 –40% of the markers tested, whereas the MSI-L group would exhibit MSI in < 30 –40% of the markers. Additional microsatellites that have proven to be of use for such studies are shown in Table 1. However, unless problems are encountered or additional information is desired to address specific issues, the use of the "reference marker panel" should be more than adequate to distinguish the MSI-L/MSS group from the MSI-H group of colorectal cancers.

Although these loci are presented as a "working reference panel," the group recognizes that other loci and panels may prove to be of equal utility. Specifically, an argument can be made for choosing markers from the alternate panel, which are in regions displaying high levels of allelic loss (*e.g.*, 17p and 18q), because, if the tumor proves to be MSS, the same assay may provide prognostic information about LOH. This reference panel, therefore is not intended to replace existing markers or panels that have been validated, or to be the only panel available for such studies. Rather, this reference panel provides an opportunity to compare existing markers in the field against which to test the utility of new markers.

As the MSI assay is applied to clinical testing in the future, further analysis of those tumors demonstrating instability in only one or two shifted loci should be considered. This may allow the determination of the validity of studying a limited (*e.g.*, five or fewer) number of markers. Furthermore, such studies may provide more detailed information to determine whether the MSI-L and MSS groups should or should not be separate. Additional data may also help in the identification of the genes responsible for the presence of low-level instability in the MSI-L group.

When results involving MSI are published, the group recommends that as much detail as is feasible should be provided in regard to each tumor and the specific loci shifted. Ideally, it should include a two-dimensional matrix comparing tumors on one axis with the loci tested on the other axis. Such information will be critical for future comparison of new loci and clinical correlation, as well as providing the potential for merging data sets. It may also help eliminate redundant markers and replace them with more informative ones.

Overall, these recommendations are consistent with those being formulated by the International Collaborating Group on HNPCC (ICG-HNPCC), represented by Dr. Hans Vasen, and R. F., and G. N. R. at the workshop. Given the similarities between the two, a single international set of criteria is recommended.

Methodology. In general, a variety of techniques to characterize MSI have been found to be equally effective. A number of studies have shown good correlation of results across multiple sites using different technologies for assessing MSI (20). Instability can be detected in both fresh and paraffin-embedded material, but in either case, it is essential to compare normal to tumor DNA in adjacent lanes.

It is generally recognized that tissue processing should include microdissection to enrich for tumor cell populations. Enriching for tumor cells will enhance the likelihood of detecting novel shifted microsatellites in inherently heterogeneous cell populations. Microdissection should be done in collaboration with an anatomic pathologist for purposes of quality control.

Quality Control. Although they have presented a reference panel here, the group recognizes the need for and encourages the replacement of loci in the current panel with others that have been quality controlled to work on formalin-fixed/paraffin-embedded material and shown to be more sensitive at detecting underlying gene defects resulting in MSI.

When these analyses are performed, a variety of technical and interpretative problems may result at a given locus. One potential

Table 1 International guidelines for evaluation of MSI in colorectal cancer

Reference panel			
Marker	Repeating unit	GenBank accession no.	
<i>BAT25</i>	Mononucleotide	9834508	
<i>BAT26</i>	Mononucleotide	9834505	
<i>D5S346</i>	Dinucleotide	181171	
<i>D2S123</i>	Dinucleotide	187953	
<i>D17S250</i>	Dinucleotide	177030	
Criteria for interpretation			
No. of markers Exhibiting instability Length changes	5 loci analyzed	>5 loci analyzed	Interpretation
	≥ 2	≥ 30 –40%	
	1	< 30 –40%	MSI-L
	0	0	MSS or MSI-L
Alternative loci ^a			
<i>BAT40</i>	<i>D18S55</i>	<i>D3S1029</i>	<i>D5S107</i>
<i>BAT34C4</i>	<i>D18S58</i>	<i>D10S197</i>	<i>D8S87</i>
<i>TGF-β-RII</i>	<i>D18S61</i>	<i>D13S175</i>	<i>D18S69</i>
<i>ACTC (635/636)</i>	<i>D18S64</i>	<i>D17S588</i>	<i>D13S153</i>
			<i>D17S787</i>
			<i>D7S519</i>
			<i>D20S100</i>

^a Marker information was contributed by J. Ruschoff, R. Fishel, S. Hamilton, R. Fodde, J. Gilbert, R. Hamelin, I. Kirsch, S. Markowitz, B. Bapat, and S. N. Thibodeau.

problem is PCR failure, in which a locus simply does not amplify. In situations in which one or more PCR failures are present, the need to reanalyze is dependent upon the information obtained from the remaining markers that successfully amplified. For example, a case having one PCR failure, one marker not demonstrating MSI, and three markers demonstrating MSI could be scored as MSI-H, regardless of whether the PCR failure would have been positive or negative for instability (*i.e.*, three or more of five). In the instance in which there is one PCR failure and only one marker positive for MSI, then reamplification would be required because the results of the one missing marker could alter the final interpretation (*i.e.*, one of five for MSI-L or two of five for MSI-H). Although it is not feasible to outline all of the possible combinations here, the participants agreed that scoring should be done in the context of what would be informative for these five markers.

LOH or MSI? Another potential scoring problem may arise when one of the normal alleles for a given marker is missing and no other novel fragments are present for that marker. One cannot easily discern whether this represents true LOH or MSI in which the shifted allele has comigrated with the remaining wild-type allele. In this situation, it is recommended that such a finding for that locus be scored as negative for MSI. Although scoring results in this fashion would appear to bias the data in favor of the MSI-L group of tumors, this does not appear to be a serious problem because most of the tumors in the MSI-H group tend to show MSI with the majority of markers. Furthermore, it was felt that the frequency that a shifted allele would comigrate with the remaining wild-type allele in the absence of other novel fragments would be relatively low. This issue, however, may require further clarification.

LOH and MSI? Conversely, when a specific marker shows both allelic imbalance (apparent LOH) and the presence of novel fragments (MSI), it will be difficult to distinguish whether the apparent allelic loss occurred as a result of the MSI or whether it was truly present in addition to the MSI. In this situation, the participants suggested that those markers showing MSI (novel fragments) should be scored as noninformative for LOH in those tumors exhibiting the MSI-L phenotype, whereas all markers should be scored as noninformative for LOH in those tumors exhibiting the MSI-H phenotype. As before, these recommendations may require further study. In general, markers should be scored as having either LOH and MSI, but not both.

As this assay becomes more widely applied clinically, it will be important to establish a bank of paired genomic DNAs (with tumors representing each of the categories) that can be distributed to laboratories for the purposes of quality control. In some cases, additional markers may be required to properly distinguish MSI-L from MSI-H tumors. Those markers shown in Table 1 are alternative markers that can be used for such purposes.

Alternative Strategies. Immunohistochemistry represents a useful alternative strategy for identifying tumors with defective MMR activity. Several studies reported on the close correlation between tumors displaying MSI-H and the absence of protein expression for either hMSH2 or hMLH1 (11, 12, 21). This technique should be evaluated in more detail to explore the potential utility of this approach. Current data suggest that the combined approach of concurrent testing for MSI and hMSH2/hMLH1 immunohistochemistry may provide the highest detection rate for the MSI-H group of tumors. Additionally, immunohistochemistry provides information on the specific defective gene involved and may, therefore, be cost-effective by limiting the numbers of genes to be sequenced to identify the at-risk individuals in a given kindred.

Criteria for Colorectal versus Other Malignancies. The reference panel is recommended for the characterization of MSI in colorectal cancer only. The utility of these markers in other malignancies,

such as endometrial or gastric cancers, has not yet been fully evaluated. The committee encourages evaluation of this panel in other tumors but fully recognizes that the optimal set of loci to diagnose MSI in cancers other than colorectal cancer is likely to be different. Additionally, the role of defective MMR in other malignancies is still not well understood.

Indications for Testing. The group briefly discussed indications for testing and generally felt that the Bethesda guidelines (2) were reasonable. These criteria suggest that tumors should be tested for MSI in the following situations: (a) individuals with cancer in families that meet the Amsterdam Criteria; (b) individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, small bowel adenocarcinoma, or transitional cell carcinoma of the renal pelvis or ureter); (c) individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma, in which one of the cancers was diagnosed at age <45 years, and the adenoma was diagnosed at age <40 years; (d) individuals with colorectal cancer or endometrial cancer diagnosed at age <45 years; (e) individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cirriiform, defined as poorly differentiated or undifferentiated carcinoma composed of irregular, solid sheets of large eosinophilic cells and containing small gland-like spaces; medullary carcinoma) on histopathology diagnosed at age <45 years; (f) individuals with signet ring cell-type colorectal cancer (composed of >50% signet ring cells) diagnosed at age <45 years; and (g) individuals with adenomas diagnosed at age <40 years.

Clinical Associations and Preclinical Models

The second breakout session, chaired by S. R. H., reviewed the current state of knowledge of clinical associations with MSI and preclinical models of MMR. The participants discussed the clinical importance of MSI and tumor pathology, the natural history and prognosis of patients with MSI+ tumors, and the potential impact of MSI on therapeutic decisions. Additional issues mentioned included interactions of environmental factors with deficient MMR, animal models, and *in vitro* systems that may be useful in research. These presentations were made prior to the development of uniform terminology for MSI, and a variety of criteria and definitions were used by individual groups. The data are presented in the terms used by the investigative group.

Pathology and Clinical Correlates of Tumors with MSI. Dr. Jeremy Jass of the University of Queensland presented a study, performed in collaboration with Dr. Barbara Leggett and S. J. M., that examined the clinical tumor traits of 303 sequential frozen colorectal cancers, according to the MSI status of the tumors. MSI status was based on examination of two mononucleotide markers, three dinucleotide markers, and one tetranucleotide microsatellite marker. Eighty % of tumors showed no MSI. Eleven % showed weak positivity, and 9% showed strong positivity. The weak and negative groups were combined for comparison with the strongly reacting group because the clinical features were similar in the first two groups, except for possibly the Crohn's-like/lymphoid reaction in some tumors. The strongly reacting MSI+ group was significantly different from the MSI- group in terms of proximal colonic location, mucinous and undifferentiated histology, and the presence of tumor-infiltrating lymphocytes. There was a trend toward disease stage without liver metastases and expansile growth pattern. Decision trees were developed to use pathological characteristics to predict MSI status. The combined pathological characteristics had a 93% accuracy for predicting

MSI status. These indicative pathological results deserve prospective investigation.

During the discussion, it was noted that the pathological findings of sporadic and HNPCC tumors with MSI appear to be similar, although there may be a difference in the types of alterations that occur in the microsatellite markers, particularly in sporadic tumors showing infrequent MSI. The issue of MSI+ status in appendiceal tumors was also raised, and it was noted that this has not been studied. The significance of the weak MSI+ tumors remains unclear. This pattern is observed in ulcerative colitis and in serrated adenomas and may reflect the early phases of clonal expansion or minor instability in hyperplasia. The implications may thus be different in cancer and precursor lesions. The pathological characteristics of MSI+ extracolonic tumors as well as the immunopathology are not well defined and require further examination.

Several conclusions came from the presentation and discussion. The pathological observations may be used as a surrogate for MSI+ testing with a high degree of accuracy, although the clinical utility of this approach in identifying HNPCC families and managing MSI+ sporadic tumors needs clarification.

Natural History and Prognosis of Tumors with MSI. Dr. Shozo Baba of Hamamatsu University, presented details of the HNPCC registry in Japan. They found 4104 high-risk families, including 394 colorectal cancer cases that met the Amsterdam criteria. In his personal series, Dr. Baba found 137 extracolonic malignancies and 98 colorectal cancers. Among the extracolonic tumors in the series, gastric cancer was most common in men, and gynecological tumors were the most common in women. Other malignancies were similar in occurrence to series in other countries. Only one-third of non-Amsterdam criteria pedigrees were found to have MMR germ-line mutations. One HNPCC family was found to have an hMSH6 mutation. Most families with HNPCC were not identified by Amsterdam Criteria. MSI+ status was found in 95% of HNPCC cancers, 47% of cancers in families with high risk but not meeting Amsterdam criteria, 36% of patients with multiple cancers, and 13% of sporadic tumors. MSI status was positive in 3% of sporadic adenomas and in all HNPCC adenomas. One uterine tumor was MSI+ and exhibited tumor-infiltrating lymphocytes. The crude 5-year survival of patients with colorectal cancer tumors quoted from a study by Jarvinen was 85% in HNPCC, 53% in FAP, and 43% in sporadic cancers. The corresponding survival figures from Japan were 80, 78, and 64%, respectively. There was no clear consensus over whether a positive germ-line mutation in a DNA MMR gene should lead to a recommendation for prophylactic colectomy. Mutations in the transforming growth factor- β type II receptor gene, the *BAX* gene, and the insulin-like growth factor gene were frequent in HNPCC tumors but not in sporadic MSI cases. In contrast, *K-ras*, *p53*, and *APC* gene mutations were unusual in HNPCC but common in sporadic cases.

Dr. Donald Henson from the NCI presented SEER data on colon cancer. Of note, the SEER registry does not include family history data. The purpose of this study was to ascertain if HNPCC could be identified in the SEER database by determining whether features of HNPCC occurred more frequently than would be expected by chance in sporadic tumors. There were 171,229 cases of colorectal cancer from 1973 to 1994, excluding nonadenocarcinoma cases. Thirty-six % of the cases were proximal colonic (cecum and ascending colon only). Age of diagnosis was <50 years in 5.9%. HNPCC-type histology was present in 10.8%. Multiple primary colon cancers were found in 26.4%. The combination of HNPCC features revealed that 89 cases met the criteria, whereas 106 were expected assuming that all HNPCC cancers would have such features. Thus, SEER cases with features of HNPCC were not overrepresented in the sample. The speakers concluded that the occurrence of these tumor features in the SEER

database did not exceed that expected by chance. It was commented from the audience that the frequency of HNPCC may actually be as low as 1% of colorectal cancer cases.

Dr. Anneka Lindblom from the Karolinska Institute briefly presented data on 190 cases from her institution, indicating that MSI status did not correlate with survival even when diagnostic stage was taken into account.

Group participants concluded that population-based studies to determine the frequency and natural history of MSI+ colonic adenomas, cancers, and extracolonic tumors are needed. Similarly, such studies should be done with an emphasis on precursor lesions in persons with known germ-line mutations of MMR genes. The issue of MSI status as an independent predictor of prognosis for any MSI+ lesions is not resolved.

Therapy. Dr. Stephen Howell at the University of California, San Diego, presented data concerning the effects of chemotherapeutic agents in MMR-deficient cell lines. He first discussed cisplatin sensitivity in MMR-deficient cell lines. Tumor cell lines that were deficient in MMR activity exhibited less sensitivity to cisplatin than matched lines made MMR-proficient by stable chromosomal transfer. This difference was observed for cisplatin and carboplatin but not other platinum-containing drugs. These observations suggest that MMR serves to signal the presence of adducts in tumor DNA and is a functional part of the response to chemotherapeutic agents.

Another set of experiments involving a mixture of MSI+ and MSI- tumor cells, both in cell culture and xenograft animal models, demonstrated that treatment with cisplatin enriched the population for MMR-deficient cells. MMR deficiency appears to account for some of the resistance to cisplatin in patients with ovarian cancer, as evidenced by an increased fraction of MMR-deficient cells in tumor biopsies after treatment. These same chemotherapeutic agents also appear to be capable of causing mutations in MMR-deficient cells at a higher rate than in MMR-proficient tumor cells. Loss of MMR also causes resistance to doxorubicin and etoposide but not 5-fluorouracil in certain MMR-proficient and -deficient cell lines.

During the discussion, it was noted that MMR deficiency may be related to a failure to activate proapoptotic signal transduction pathways. There are some data indicating that radiation sensitivity is decreased in MMR-deficient cells, but this is not yet certain. Tumors with defective MMR remain an important target for chemotherapy; it may be possible to identify drugs that selectivity target MMR-deficient cells. The potential of increased toxicity of such agents in patients with germline mutations remains to be clarified. To address these issues, it would be helpful to have truly isogenic MMR proficient and deficient tumor cell lines available.

Environmental and Genetic Interactions with MMR Deficiency. Dr. Russell Jacoby of the University of Wisconsin discussed the factors influencing adenomas in the MIN mouse model of *APC*. He reported that nonsteroidal anti-inflammatory drugs result in a 10-fold decrease in adenomatous polyps in MIN mice and cause a similar regression in FAP patients. He speculated on the role of genetic background (such as *mom1/pla2s* alleles from AKR versus B6 mice), which are associated with a 4-fold difference in polyp number in these rodents. He also reported that a 2-fold difference in adenomas is observed in germ-free animals compared with conventional control animals. The role of these factors in HNPCC and MSI+ tumors has not examined.

The role of MSI in adenomas was discussed as a predictor of the presence of a germ-line MMR mutation. There is evidence that adenomas with MSI progress more quickly to carcinoma and more often contain foci of cancer. The familial standardized incidence ratio, as a way of measuring familiarity, was correlated with the severity of MSI in colonic neoplasms as a predictor of HNPCC. The familial

standardized incidence ratio ranks the family history relative to the general population mean and incorporates information about the number of cancers, age at diagnosis, and degree of relatedness to the individual tested. This correlation showed that when MSI is more common, family history is stronger. The finding of MSI in adenomas is more specific but less sensitive than finding it in cancers for predicting HNPCC, particularly in smaller adenomas.

In the discussion period, the speculation was raised that HNPCC adenomas may start sporadically, with MMR deficiency being activated after an adenoma is formed.

A number of issues were raised for future research: To what degree are environmental factors involved in the tumor spectrum of HNPCC? How is the declining rate of gastric cancer in HNPCC in the United States related to environmental factors? Why do MSI tumors occur predominantly in the proximal colon? Are MMR-deficient cells more sensitive to mutations induced by environmental mutagenic agents?

In Vitro Models. C. R. B. (workshop chairman) addressed the need for different approaches to *in vitro* studies of MSI. Because gene transfer techniques have been unsuccessful in this setting, human chromosomal transfer has been used. Whole chromosome transfer permits the stable introduction of a physiologically regulated copy of the missing DNA MMR genes, but in addition to the gene of interest, numerous other genes are simultaneously transferred. Families of cell lines have been developed and characterized with the reinstatement of *hMLH1* and *hMSH2*. Studies of these cell lines have revealed that MMR-deficient cells do not respond to DNA damage with the expected G₂-M cell cycle checkpoint arrest. Cells that have had DNA MMR activity restored continue to manifest malignant behavior because they can grow in monolayer culture and nude mice, but they respond to certain types of DNA damage with an appropriate G₂-M cell cycle arrest and cell death. DNA MMR-deficient cells are tolerant of 6-thioguanine, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and 5-fluorouracil when compared with their MMR-proficient counterparts.

MSI in Non-HNPCC, Noncolonic Tumors

The third breakout session, chaired by D. S., addressed the phenomenon of MSI in other settings. MSI has been described across a spectrum of microsatellites and at varying frequencies in other tumor types. Some of these tumors with MSI (*e.g.*, endometrial cancer) occur sporadically in non-HNPCC kindreds and other tumors with MSI are not part of the usual HNPCC spectrum. The classification of these tumors is difficult because the absolute background frequency of a given microsatellite alteration in somatic tissue is largely unknown. Background rates of microsatellite alterations in germ-line tissues have been obtained from germ-line linkage studies and analysis of single sperm (22, 23). In these cells, approximately one new allele is generated at a dinucleotide repeat every 1000 divisions (0.1%) and at tetranucleotide repeats every 30–100 divisions (1–3%). Analysis in peripheral lymphocytes suggests some relaxation of microsatellite replication fidelity occurs in somatic compared to the germline tissues. Analysis of these repeats in primary tumors suggests elevated frequencies of these alterations for a given repeat unit (24, 25). As a group, ~1 in 150 dinucleotides and 1 in 20–30 tri- and tetranucleotides demonstrate an alteration across tumor types. However, reliable studies suggest there is a great difference in these observed frequencies from one marker to another (*i.e.* one dinucleotide compared with a similar dinucleotide) and from one type of cancer to another (*e.g.*, bladder cancer *versus* lung cancer). In addition, investigator variability for microsatellite analysis and potential artifacts (*e.g.*, dilution of malignant cell DNA in heterogeneous tissue specimens containing large numbers of normal cells) and other PCR artifacts may contribute to these differences.

There are two general types of noncolonic non-HNPCC tumors that display elevated frequencies of MSI. The first group, commonly found in gastric (26–46) and endometrial (23, 27, 47, 48) neoplasms, has a similar phenotype to MSI in colorectal cancer and displays instability at dinucleotide and mononucleotide markers and, to a lesser degree, at larger repeats. Like colon cancer, these tumors with a high frequency of MSI (similar to MSI-H) are almost always seen in sporadic cases, and the majority of these cases are not from familial clusters or HNPCC kindreds. Like sporadic cases of colon cancer with MSI-H, they have not been commonly found to have mutations in the known MMR genes (49–55). The reference panel of markers described above (or a similar panel) should be established for the identification and characterization of this group of tumors. The presence of and molecular basis for a non-colonic MSI-L and MSI-H group of tumors remains to be established. The mechanism(s) underlying these phenotypes, such as diminished MMR gene expression or more subtle alterations in the MMR or other pathways, thus remain to be elucidated for these cancers (Refs. 56–66; Table 2).

A second group of noncolonic non-HNPCC tumors displays elevated frequencies of instability only at highly selected tri- and tetranucleotide repeats. These markers have been empirically tested in certain primary tumors to identify shifts that may be useful as clonal markers for cancer detection (25). In certain tumors, larger repeats are more commonly altered than smaller repeats; this finding stands in stark contrast to what is found in HNPCC and most sporadic gastric and endometrial tumors, which have alterations in mono- and dinucleotide repeats at higher frequency. One group of tetranucleotide alterations, (AAAG)_n, seems to be particularly susceptible to these alterations in non-HNPCC tumors of different types, including lung, bladder, and head and neck cancer.⁴ However, the frequency of alterations at any given locus varies substantially from one tumor type to another. Evidence also suggests a binomial distribution of instability at tri- and tetranucleotide markers in primary lung cancer in which a small peak of tumors demonstrates an overall shift rate of >10% at the most susceptible repeats, and there is a large peak at 0–2% of susceptible repeats for most lung cancers. This observation is reminiscent of the binomial distribution for mono- and dinucleotide alterations reported for MSI-H and MSI-L tumors above. This different type of MSI has been termed elevated microsatellite alterations at selected tetranucleotide repeats, to achieve uniformity of classification in future research studies. The mechanism underlying these phenotypes also remains to be elucidated, but the relative absence of overlap between traditional MSI+ and EMAST+ tumors already suggests that a non-MMR pathway may be involved.

In addition to underlying deficiencies in repair mechanisms, it is possible that environmental factors may play a role in generating some microsatellite alterations. The issue of microenvironment has been raised, with a special focus on DNA damage from oxygen radicals or lipid adducts leading to MSI at a microsatellite locus. General environmental exposures, including smoking and diet, were also raised as potential generators of increased microsatellite alterations in tumors, regardless of the endogenous proficiency of repair pathways. These exposures could act alone or in concert with DNA repair pathways to generate the specific phenotypes described in the different tumor types.

Tumors with MSI have been found to potentially inactivate certain target genes by permitting an increased frequency of mutations in short repeat tracts in the DNA encoding the expressed portions of

⁴ D. Sidransky and J. Jen, unpublished observations.

Table 2 Frequency of MSI in noncolonic, non-HNPCC tumors

Tumor	Loci	MSI ^a	Frequency	Ref
Endometrial	Dinucleotide	≥2/8 loci	10/109 (9%)	67
Endometrial	<i>D1S126</i> <i>D2S393</i> <i>D3S1067</i> <i>D5S644</i> <i>TP53</i>	≥2/5 loci	18/77 (22%)	68
Ovarian		≥2/5 loci	2/68 (3%)	52
Endometrial	Dinucleotide	≥2/8 loci	12/68 (18.5%)	
Gastric	<i>D2S123</i> <i>D2S136</i> <i>D3S1067</i> <i>D11S922</i> <i>TP53</i>	≥2/5 loci	15/24 (62.5%)	69
Gastric	<i>AP(delta)3 (A)₁₈</i> <i>hMSH2 (A)₂₆</i> <i>D1S158</i> <i>D5S421</i> <i>D8S199</i> <i>BAX (G)₈</i> <i>hMSH3 (A)₈</i> <i>hMSH6 (G)₈</i>	≥2/5 loci	25/167 (15%)	70
Gastric	Dinucleotide	≥2/6 loci	16/25 ^a (64%)	
Cervix	Dinucleotide	≥2/30 loci	16/25 ^a (64%)	32
	Tnnucleotide		13/25 ^a (52%)	
	Tetranucleotide		5/98 (2%)	71
Breast	Dinucleotide	≥2/12 loci	3/89 (3.3%)	
Skin cancers	47 loci ^b	≥2 of loci tested	2/100 (2%)	72
Basal cell			1/47 (2%)	
Squamous cell			1/49 (2%)	
Melanoma			0/41 (0%)	
Lymphoma				
MALT ^c	<i>D3S1262</i> <i>D3S1265</i> <i>D3S11</i> <i>D3S1261</i> <i>D6S262</i>	≥2/5 loci	21/40 (52.5%)	74
HIV-positive	Nine loci	≥4/6 (66%)		75
Lung	Dinucleotide	≥2/8 loci	12/35 (34%)	76
Gliomas	<i>D2S123</i> <i>D3S1067</i>	≥1/2 loci		77
Glioblastomas			5/24 (21%)	
Astrocytomas			2/16 (12.5%)	
	<i>TGFβRII(A)₁₀</i>			
Glioblastomas			4/24 (17%)	
Astrocytomas			2/16 (12.5%)	
	<i>TGFβRII(GT)₃</i>			
Glioblastomas			0	
Astrocytomas			0	
Prostate	23 loci	>2/23 loci	4/47 (8.5%)	78

^a These 25 tumors had MSI in ≥2/5 loci.

^b Not all tumors investigated at all loci.

^c MALT, mucosa-associated lymphoid tissue.

these genes. Most of these microsatellite alterations result in frame-shifts that truncate proteins, presumably leading to inactivation of the affected allele. Because MSI affecting repeat tracts within some, but not all, coding regions may represent chance events rather than specific gene targeting, certain criteria are hereby set forth by which to gauge whether an affected gene is a true target of inactivation involved in tumorigenesis. These include: (a) a high frequency of inactivation; (b) biallelic inactivation by simultaneous alteration of the other allele's repeat tract, point mutation, or loss; (c) involvement of the candidate MSI target gene in a bona fide growth suppressor

pathway; (d) inactivation of the same growth suppression pathway in MSI-negative tumors through inactivation of the same gene, or of another gene within the same pathway; and (e) functional suppressor studies in *in vitro* or *in vivo* models, such as cell lines or animals. Several potential target genes have already been identified as listed in Table 3.

Explorations of the genome for additional targets of MSI was thought to hold great potential for elucidating the MMR pathway in human tumorigenesis. Furthermore, the discovery of unstable sequences in the coding regions could help to identify new tumor suppressor genes, as well as to suggest new areas of research into targets of molecular-based anticancer therapy or cancer prevention.

MSI at any locus represents a potential clonal marker for the detection of cancer (25). Although these alterations at anonymous repeat tracts may not provide any growth advantage to neoplastic cells, they are still carried and propagated as genetic material is passed on to daughter cells during clonal propagation. Recent pilot studies in bladder cancer indicate the potential for use of these alterations in cancer detection and monitoring (16, 116). In combination with LOH studies, the majority of bladder cancers were identified by microsatellite analysis of the urine. In three cases, microscopic tumors were identified before direct visualization by cystoscopy (16). These markers may also be useful in the detection of other tumor types by analysis of bodily fluids and can also be found at lower frequency in the serum or plasma of cancer patients (117, 118). These markers may also provide useful prognostic information in addition to establishing the diagnosis in tested patients. Further characterization of these markers in preneoplastic lesions would help establish their role in detection of preclinical lesions for preventive approaches.

Future studies should focus on better cataloging and characterization of microsatellite alterations (including length and type of alteration) in normal tissues and cancers. Precise documentation will help define accurate phenotypes that may eventually lead to novel biological and mechanistic insights. Correlation of these phenotypes in the various MSI groups with emerging molecular events and clinical-pathological data needs to be carried out. To ensure optimal use of this knowledge, a database will need to be set up to collect, store, and disseminate information about the rate and frequencies of MSI in different tumor types. The database will be limited if we do not develop optimal high-throughput assays for running and interpreting microsatellite assays.

To better decipher complex phenotypes and establish parameters for testing different repair pathways, we need to develop new eukaryotic models for MSI and genomic instability. In addition, we need to pursue the establishment of mammalian cell lines and transgenic animals that demonstrate microsatellite or genomic instability. These systems are mandatory as resources for elucidating the underlying mechanisms of various types of instability. In addition, they will be valuable resources for testing novel therapeutic approaches in various tumor types with distinct instability phenotypes.

It may be appropriate to initiate a massive screen of known cDNAs

Table 3 Mutated genes in tumors with MSI

Putative gene target	Frequency	Biallelic inactivation	Suppressor pathway	Inactivated in MSS	Functional data	Refs.
<i>TGF-β</i> type II receptor	High (colon)	Yes	Yes	Yes	Yes	28, 29, 77, 79–95
<i>IGF-IIR</i> ^a	Moderate	Yes	Yes	Yes	Yes	96–106
<i>Bax</i>	High	No	Unclear	No	No	70–107
<i>MSH3/MSH6</i>	Moderate	Yes	Yes	No	No	70, 108–110
<i>PTEN (MMAC1)</i>	High	Yes	Yes	Yes	Yes	111, 112
<i>APC</i>	Moderate	Yes	Yes	Yes	Yes	113, 114
<i>β2-microglobulin</i>	Low	No	No	No	No	115

^a IGF-IIR, insulin-like growth factor type II receptor.

with repetitive DNA tracts in multiple tumor types to identify potential target genes involved in the pathogenesis of MSI tumors. This may be viewed as a gene discovery tool to identify important pathways relevant to neoplasms with instability and many other tumor types. In addition to the above criteria, more definitive assays to confirm the involvement of these new gene targets in tumorigenesis would be useful.

Recommendations for Future Research Initiatives

The identification of colorectal cancer patients with MSI-H tumors is important because: (a) some have germline mutations in a mismatch repair gene which has implications for patient management and family members; and (b) the biology of MSI-H tumors differs from that of MSI-L tumors and those without MSI. At the present time, the workshop members are of the opinion that MSI testing has one clinical purpose: to identify patients with HNPCC. The Bethesda guidelines specify which tumors should be tested for this purpose, including criteria based on age, pathological characteristics, family history and the presence of extracolonic tumors.

For research purposes, studies of MSI should address several areas, as follows: (a) Prognosis. The use MSI-H tumor status as an independent prognostic factor in tumors of the colorectum and extracolonic sites in both the sporadic and HNPCC settings requires further investigation. Several lines of evidence relevant to the area of prognosis were discussed. The natural history and response to therapy of MSI-H tumors appear to be different than the other tumors. *In vitro* studies have shown chemoresistance to some agents (e.g., cisplatin) and decreased radiosensitivity, whereas anecdotal clinical reports have suggested improved response to therapy in some settings (e.g., radiotherapy for glioblastoma multiforme in Turcot syndrome; Ref. 119). Defective MMR also represents a potential for novel therapeutic agents. Finally, the pathology of MSI-H tumors characterized by tumor-infiltrating lymphocytes and a Crohn's-like lymphoid response raised the possibility of evaluating immunomodulating agents, such as levamisole. Further examination of prognosis in patients with MSI-H tumors and design of clinical trials is needed. (b) Prevention. Identification of environmental factors which interact with defective MMR in producing MSI-H tumors and studies of chemopreventive agents are needed. (c) Biology of tumors. Further characterization of the biology of both colonic and extracolonic neoplasms is needed, including information on growth rates, progression to malignancy in pre-malignant lesions, and metastasis. (d) Testing strategies. The utility of immunohistochemistry for defining the expression of *hMSH2* and *hMLH1* gene products needs confirmation. It was suggested that this may be used to supplement MSI testing. Algorithms for efficient characterization of MSI-H in sporadic and HNPCC tumors should be developed and validated in prospective clinical studies. Strategies for distinguishing HNPCC from other inherited predispositions, including attenuated FAP and *APC11307K* pre-mutation in Ashkenazi Jews are needed. (e) High-risk clinics and registries. The continued development of high-risk clinics and registries is encouraged. These can provide optimal identification, education, genetic testing, and management of those with HNPCC. They also serve as a vital resource for basic and clinical investigation of this condition. (f) Preclinical models. Further development and characterization of animal models and *in vitro* systems, including yeast and cell lines, would be of great help in addressing the above issues. In addition, the preclinical models can direct translational application of functional tests for defective MMR. (g) Nomenclature. It is suggested that the name HNPCC be changed to a more appropriate name in view of present genetic knowledge. One suggestion was the "hereditary MMR deficiency syndrome;" however, no consensus was reached on this issue.

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