## CLINICAL VALIDITY

**18. How often is the test positive when the disorder is present?**
**19. How often is the test negative when the disorder is not present?**

Clinical Sensitivity of Microsatellite Instability (MSI) Testing

MSI analysis has an important advantage and some limitations for the recognition of hereditary colorectal cancer. MSI has been found in most cases of HNPCC tumors from patients who fulfill the Amsterdam criteria (clinical sensitivity of about 90 percent – 8 of 9; Hoedema 2003). High clinical sensitivity is also indicated by the finding that 80 to 95 percent of HNPCC tumors have been shown to be MSI positive (Aaltonen *et al*., 1994; Lynch and Smyrk, 1998 Farrington *et al*., 1998; Bapat *et al*., 1999; Calistri *et al*., 2000; Debniak *et al*., 2000; Loukola *et al*., 2001). Analysis by one research group of all published results showed that, among kindreds with suspected HNPCC, germline mutations could be detected in 16 out of 22 colorectal cancer patients with MSI positive tumors, as compared with one out of 37 MSI negative tumors (Liu *et al*., 2000). Thus, clinical sensitivity was 16 of 17 or 94 percent. *Overall, these data are consistent with about a 90 percent clinical sensitivity for MSI testing.* This high clinical sensitivity is an important advantage of MSI testing, because it can exclude the diagnosis of HNPCC from most colorectal cancers without requiring sequencing of mismatch repair genes. To standardize MSI testing, a set of specific markers (i.e. short DNA sequences called BAT-5) has been recommended by the National Cancer Institute (Table 5). If two or more of the five generate multiple bands, then the sample is considered MSI-high, and DNA sequencing of germline DNA is indicated. One limitation is that tumor sample is required; the test cannot be performed on blood or other nontumor tissue.

**Table 5. International guidelines for evaluation of MSI in colorectal cancer**

| **Markers** | **Repeating unit** |
|---|---|
| BAT25 | Mononucleotide |
| BAT26 | Mononucleotide |
| D5S346 | Dinucleotide |
| D2S123 | Dinucleotide |
| D17S250 | Dinucleotide |

| **Interpretation** | **5 Loci** | **> 5 Loci** |
|---|---|---|
| MSI High | ≥ 2 | ≥ 30-40% |
| MSI Low | 1 | < 30-40% |
| MSS or MSI Low | 0 | 0% |

From Giardiello et al., 2001.

Clinical Specificity of Microsatellite Instability (MSI) Testing

Clinical specificity for MSI testing is reported in one study (Aaltonen *et al*., l998) to be 85 percent. Thus, about 15 percent of sporadic colorectal cancers will have MSI-high results. In another study (de la Chapelle 2003), 12 percent of consecutive patients with colorectal cancer, unselected for age or family history, had MSI-high tumors; 25 percent of these had HNPCC mutations. Thus, the false positive rate can be estimated to be about 9 percent. This, too, is an

overestimate, since only about 60 percent of mutations can be identified via sequencing. The best estimate for the false positive from the de la Chapelle study is about 7 percent. *Overall, these data are consistent with about a 10 percent false positive rate for MSI testing, or a 90 percent clinical specificity.*

Clinical sensitivity of mutation testing for detecting HNPCC

To evaluate the clinical sensitivity of a laboratory test, it is important to start with individuals (or families) clinically diagnosed to have the disorder. This means that the laboratory being evaluated cannot first be used to make the original diagnosis. A recently published study (Wagner *et al*. 2003) analyzed 59 clinically well-defined U.S. families with HNPCC. All 59 families had at least four generations of individuals affected with colorectal cancer. Forty-nine fulfilled the stringent Amsterdam criteria. Of the remaining 10 families, nine were characterized by two first-degree relatives with a tumor known to be related to HNPCC (see Table 1), at least one of whom was diagnosed before age 50. The available family member diagnosed at the youngest age was selected for mutation analysis. The reason for choosing this family member is that an HNPCC family may also contain members with sporadic colon cancer. Choosing the member affected at the youngest age minimizes the risk of selecting a sporadic case, thereby leading to a misclassification of the family. Analysis for *MLH1*, *MSH2*, and *MSH6* mutations utilized denaturing gradient gel electrophoresis, Southern analysis, immunohistochemistry, and monoallelic expression analysis. Testing for rearrangements by Southern analysis proved important, because 14 families (24 percent) had rearrangements. Deleterious mutations in these three genes were demonstrated in 52 of the 59 families, giving a clinical sensitivity of 88 percent for this test battery.

Many of the techniques used in this study (Wagner *et al.*) are not generally available. Therefore, it is necessary to adjust the clinical sensitivity downward in accordance with what is practical in a service setting. Table 6 shows that the 14 mutations involving rearrangements would not have been detected if only standard sequencing of the three genes had been done, yielding a clinical sensitivity of 64 percent, rather than 88 percent. If *MSH6* had not been analyzed, the sensitivity would have been further reduced to 59 percent. If only high MSI individuals had been analyzed, an additional 10 percent of patients with mutations would have been missed (Liu *et al*., 2000, Aaltonen 2003), and the clinical sensitivity would have been further reduced for the most common combination of *MLH1* and *MSH2* to 53 percent.

**Table 6. Clinical Sensitivity of Various DNA Tests Among 59 Families Clinically Diagnosed with Hereditary Non-Polyposis Colorectal Cancer[1]**

| DNA Test | Number Accounted for (%) | Clinical Sensitivity (%) | Adjusted Clinical Sensitivity (%)[2] |
|---|---|---|---|
| *MLH1, MSH2* | 35 ( 59) | 59 | 53 |
| *MSH6* | 3 ( 5) | 64 | 58 |
| Southern Analysis | 14 ( 24) | 88 | 79 |
| No mutation detected | 7 ( 12) | - | - |
| *Total* | 59 (100) | 100 | 90 |

1 Study by Wagner et al., 2003

2 Adjusted downwards by 10 percent to take into account the proportion of mutation positive individuals negative for microsatellite instability

The recommended protocol for identifying HNPCC calls for the family's index case to be the individual with newly diagnosed colorectal cancer, regardless of that person's age. This approach can occasionally lead to a family with HNPCC being initially misclassified. In families with HNPCC mutations, sporadic colorectal cancer can occur among individual family members who do not inherit the mutation. In those individuals, sporadic cancer is more likely when the individual is older. If that person is tested first, it would incorrectly appear as though the family was negative for HNPCC mutations (e.g., a clinical false negative result for the family, but a true negative result for the individual). Subsequent occurrences of colorectal cancer among other family members are likely to offer the opportunity to correctly classify the family as having an HNPCC mutation. This situation is considerably less likely to occur in the study by Wagner et al., because it was possible in that study to preferentially test the individual with the earliest age at diagnosis of colorectal cancer.

Clinical sensitivity of mutation testing in unselected individuals with colorectal cancers

The most useful type of study to answer this question is one in which all colorectal cancer patients in a defined geographic region are investigated. The de la Chapelle study in Ohio meets this criterion. It found that 12 percent of consecutive patients with colorectal cancer unselected for age or family history had MSI-high tumors and that 25 percent of these had mutations in *MLH1*, *MSH2*, or *MSH6*. Although there are many other reports involving HNPCC among patients with colorectal cancer, most of the studies are unsatisfactory for formulating policies. Studies in European countries with founder mutations are not directly applicable to the United States. Some studies in the United States describe patients seen at a single institution or at a specialized clinic, making extrapolation to the nation as a whole problematic. Many other studies report sequencing only on patients meeting the Amsterdam or Bethesda criteria and hence do not permit evaluating these criteria themselves.

de la Chapelle (2003a) calculates that a nationwide testing program of all newly diagnosed colorectal cancer patients could detect 21,000 previously undiagnosed carriers of HNPCC mutations in the first year of testing. The protocol would require performing MSI testing on all colorectal tumors and sequencing DNA from all patients whose tumors were MSI high. This calculation assumes that, for each index case, 10 relatives would be tested for the proband's mutation; this large number might be difficult to achieve in practice, even if counseling and testing were offered free of charge.

Clinical specificity of mutation testing in unselected individuals with colorectal cancers

One way to address clinical specificity of DNA sequencing is to evaluate how often a clinical false positive test result occurs. In this report, DNA sequencing for HNPCC mutations is limited to individuals who already have been diagnosed with colorectal cancer. If a known deleterious mutation for HNPCC were to be correctly identified in one of these individuals, that individual would, by definition, have HNPCC. Thus, false positives would occur only if the test 's analytic result was incorrect (Question 10) or if an innocuous missense mutation was interpreted to be pathogenic. Frameshift mutations (resulting from deletion or insertion of a number of nucleotides not divisible by three) or mutations to a stop-codon are predictably deleterious, unless at the extreme 3'-terminus. A missense mutation may be deleterious if it occurs in a region of the gene conserved across species ("conserved" region), if it is not found in normal subjects, and if the mutation co-segregates with cancer in kindreds. The last criterion is the most reliable, but is often not helpful because of the small size of many families or the failure of informative relatives to provide samples. Both of these events are likely to be uncommon, so the clinical specificity is likely to be high, but a specific estimate is not available. For the purposes of analysis, we assume a false positive rate of 1 per 1000 (a clinical specificity of 99.9 percent).

Figures 2a and 2b show the estimated performance of the HNPCC screening protocol evaluated as part of this report. The protocol uses clinical sensitivity and specificity for family history, MSI testing and DNA testing contained in this review and assumes that three first-degree relatives will be offered and will accept DNA testing. All uptake rates are set to 100 percent. This analysis is structurally similar to ones used by de la Chappelle (2003a) and by Ramsey (2001). The analysis begins with a cohort of 147,500 unselected colorectal cancer patients (the approximate number of new cases in the U.S. annually). Among these cases, 5,900 (4 percent) are expected to be HNPCC carriers (Question 1).

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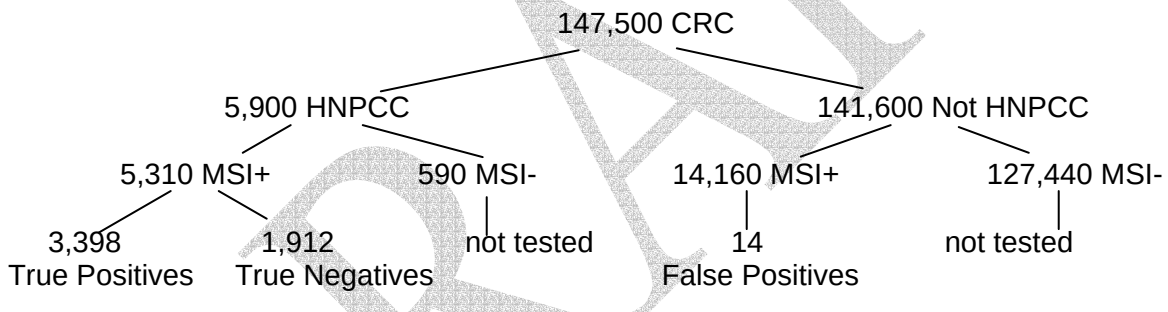
In Figure 2a, all of the colorectal cancer patients receive MSI testing. Among the 5,900 individuals with HNPCC, 5,310 (90 percent) have a positive MSI test result (Question 18/19). Approximately 3,398 (64 percent) of these have a mutation identified via sequencing the *MLH1*, *MSH2* and *MSH6* genes (Question 18/19, Table 6). Among the 141,600 individuals without HNPCC, 14,160 (10 percent) have a positive MSI test (Question 18/19). It is not known how many of these might have a false positive DNA test result, but 1 per 1,000 is used in this analysis. Overall, 147,500 MSI tests are performed, along with 19,470 DNA sequencing tests, to identify 3,398 true positive HNPCC individuals.

**Figure 2a – MSI testing followed by DNA testing**

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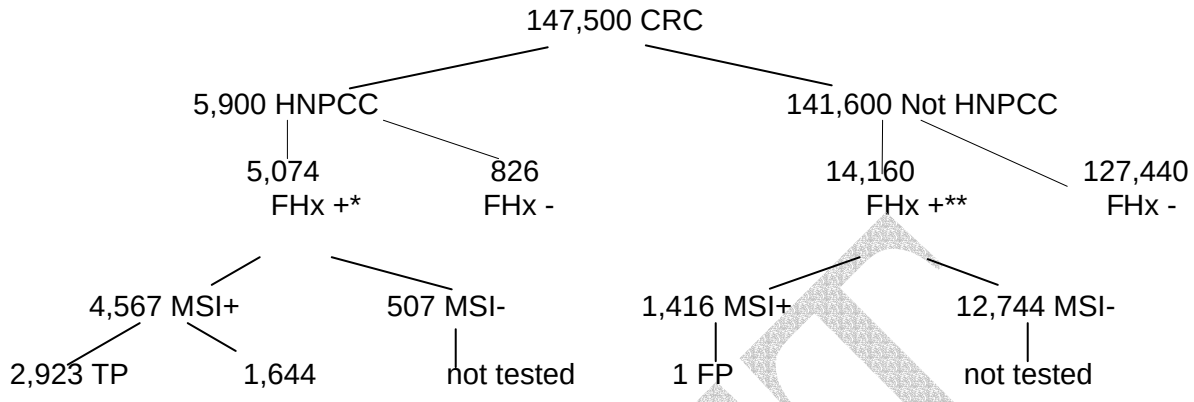
In Figure 2b, all of the colorectal cancer patients are asked about their family history. Among the 5900 patients with HNPCC, 5074 (86 percent) have a positive family history. MSI testing is performed on everyone with a positive family history and is positive in 4567 of the cancer patients in this group (90 percent). DNA testing is then performed on all of the patients whose MSI result is positive, and a mutation associated with HNPCC is identified in 2923 of these individuals (64 percent). Among the 141,600 colorectal cancer patients who do not have an HNPCC mutation, 14,160 (10 percent) have a positive family history. MSI testing is performed on tumor samples from all of these individuals; 1416 of the test results (10 percent) are positive. DNA testing is performed on blood samples from all of these patients, and one false positive result for an HNPCC is reported.

**Figure 2b – Family history, followed by MSI testing and finally, DNA testing**




*86 percent have a family history (Question 18/19)
**10 percent have a family history (Question 18/19)
(give technology – include possibility of variants with unknowns and their interpretation)

An alternative protocol that is aimed at avoiding MSI testing altogether. A formula for calculating the probability of detecting a mismatch repair mutation in a colorectal cancer family based on the family history and age at diagnosis has been proposed (Wijnen *et al*. 1998). It can be useful, not only for the patient with newly diagnosed colorectal cancer considered here, but also for those diagnosed in the past. However, it has several limitations. It relies heavily on the Amsterdam criteria and is derived from Dutch and Norwegian data which may not be applicable to the U.S. population. There is no generally applicable method for estimating the probability of finding a mutation in an HNPCC suspect, unlike the situation for estimating the probability of finding a *BRCA* mutation in an individual with a suspected history of inherited breast / ovarian cancer. This protocol is not addressed anywhere beyond this paragraph.

> **Gap in Knowledge:** This lack of a generally accepted method for estimating the likelihood of detecting a colon cancer-related mutation may be one factor that has impeded HNPCC testing.

**20. Are there methods to resolve clinical false positive results in a timely manner?**

Laboratories offering testing are acutely aware of the difficulty of interpreting the clinical significance of missense mutations; some provide a paragraph of interpretation with the test result that evaluates the evidence for pathogenicity. The analytic false positive rate for DNA sequencing of the HNPCC genes is not known, but indirect evidence suggests that it is relatively low (Question 10). In this test setting (individuals with colon cancer, a positive family history and an MSI-high test result) HNPCC is relatively common and, therefore, analytic true positive results will be very common compared to analytic false positive results. Thus, routine re-sampling and testing of those with positive DNA sequencing results is unlikely to be of much use.

**21. What is the prevalence of the disorder in this setting?**

Based on his studies in Ohio, de la Chapelle (2003a,b) estimates that 3 percent of all colorectal cancer patients have a germ-line mutation in *MLH1*, *MSH2*, or *MSH6*. This calculation is based on 12 percent of consecutive colorectal cancers having a high MSI and on 25 percent of the high MSI tumors having a detectable deleterious germline mutation. This estimate rests on the assumption that the MSI test has a negligible false negative rate in cases of HNPCC and ne-

glects the contribution of genes other than *MLH1*, *MSH2*, and *MSH6*. Thus, the true prevalence is likely to be higher, perhaps 4 percent.

**22. Has the test been adequately validated on all populations to which it may be offered?**
The initial clinical studies of testing for HNPCC have been performed in Finland and Sweden, and extensive testing occurs there. Surveys have been conducted in the United Kingdom and Germany. Limited studies have also been conducted in Uruguay and Argentina (Lynch and Lynch, 2000). In the United States, clinical validity has been reported for Caucasians (de la Chapelle, 2003c) but such data are not available for African Americans, Asian Americans and Hispanic Caucasians.

> **Gap in Knowledge:** Information about HNPCC, including performance, is not available for racial/ethnic groups other than Caucasians.

**23. What are the positive and negative predictive values?**
For HNPCC, MSI is the only preliminary test for which adequate data exist. Table 7 relates high MSI to detection of a mutation in the *MLH1*, *MSH2*, or *MSH6* genes. The estimates are derived from a study of consecutive colorectal cancer patients in Ohio (de la Chapelle, 2003a), extrapolated to 10,000 subjects. That study performed MSI testing in all colorectal cancer patients without regard to family history. Twelve percent of the subjects had tumors that were MSI-high, and 25 percent of those with MSI-high tumors had a mutation detected by standard DNA sequencing. Once the table has been constructed, the positive predictive value of MSI for detecting mutations in these three genes can be calculated -- 300/1200 = 25 percent. Only the mutations that can be detected by clinical laboratory methods are taken into account in this calculation.

**Table 7. *MLH1, MSH2,* and *MSH6* Mutations Detectable by MSI Testing Followed by DNA Analysis**

| | Mutations Detectable | | Total |
|---|---|---|---|
| | Yes | No | |
| High MSI Yes | 300 | 900* | 1,200 |
| High MSI No | 33 | 8767** | 8,800 |
| Total | 333 | 9667 | 10,000 |

*150 HNPCC mutations with positive MSI not detectable in clinical laboratories
**20 HNPCC mutations not detectable by either MSI or routine DNA testing

According to Wagner *et al*. (2003) for families which have HNPCC by indisputable clinical criteria, standard DNA analysis reveals only 73 percent of the mutations that are detectable by special techniques such as deletion analysis (includes southern blotting). In clinical practice, this figure is even smaller (64 percent). Therefore, in addition to the 300 mutations that were detected, an estimated 170 were missed. Thus, as shown below, the negative predictive value is D/(C+D) = 8767/8800 = 99.6 percent.

The positive predictive value is the proportion of individuals (unaffected family members) with positive tests who develop the disorder. If DNA sequencing is the test in question and colorec-

tal cancer is the disease, about 80 percent of men and 40 percent of women with a deleterious HNPCC mutation in a mismatch repair gene develop colorectal cancer, but women also have a 39–60 percent risk of endometrial cancer (Cruz-Correa and Giardiello, 2002).

The negative predictive value is the proportion of individuals (unaffected family members) with a negative test who will not develop the disorder. Since the lifetime risk of developing colon cancer in the general population is 5.8 percent (Jemal *et al*., 2004), the negative predictive value in families with a known deleterious mutation is 94.2 percent. Very few will develop colorectal cancer due to HNPCCas a result of a false negative DNA test.

**24. What are the genotype/phenotype relationships?**

In HNPCC, *MSH2* mutations are considered to have more frequent extracolonic manifestations than *MLH1* mutations (Eng *et al*., 2001b). For a listing of extracolonic manifestations, please see Table 1. *MSH6* mutations are associated with manifestations starting at a later age (Jacob and Praz, 2002), and they are also associated with a greater likelihood of endometrial cancer. Homozygosity for an inactivating mutation of *MLH1* or *MSH2* apparently produces a phenotype similar to neurofibromatosis-1 and predisposes to leukemia or lymphoma (Whiteside *et al*., 2002). Bisgaard *et al*. (2002) have compared clinically suspect HNPCC families with or without a mutation detected in *MLH1* or *MSH2*. Those without a detected mutation had colorectal cancer diagnosed at a later age, a lower frequency of multiple colorectal cancers, a high percentage involving the rectum, and a lower frequency of cancers in HNPCC-associated organs. Also mentioned in Table 1 is Turcot's syndrome. This is characterized by development of colorectal carcinomas and adenomas, and primary central nervous system tumors (Hamilton *et al.,* 1995).

Missense mutations, if not previously reported as associated with cancer in families, pose the difficulty of determining whether they are pathogenic or innocuous. Proposed criteria for designation of a missense mutation as pathogenic are one or more of the following: (a) rarity of the variant in the general population, (b) segregation of the mutation with the disease phenotype in families, (c) lack of expression of the affected protein, and (4) involvement of amino acids conserved in other species (Pistorius *et al*., 2000). However, a functional test is needed for a conclusive demonstration. Some progress has been made in studying the physical interaction of mutant and normal mismatch repair gene products (Guerrette *et al*., 1999). Functional tests have been proposed for *MSH2* mutations in HNPCC in yeast (Andreutti-Zaugg *et al*., 1997).

> **Gap in Knowledge: The need for a yeast assay to check functionality.** An assay to assess the functional impact of novel variants (not previously reported in mutation databases) identified by sequence analysis of *MLH1, MSH2*, and *MSH6* in colorectal cancer patients would be useful for clinical laboratories in interpretation of their findings. Such an assay is not currently available in any clinical molecular laboratory performing HNPCC testing. Research and development of a functional assay and placement in a clinical laboratory setting could be addressed by a funding initiative.

**25. What are the genetic, environmental, or other modifiers?**

Patients with MSI positive tumors are more likely to be smokers (Slattery *et al*., 2000), suggesting that smoking may increase MSI. Increased dietary heterocyclic aromatic amines are associated with an increased likelihood of MSI positivity in tumors (Slattery *et al*., 2001). Estrogen use is associated with a greater likelihood of an MSI negative tumor, suggesting that estrogen may reduce MSI (Slattery *et al*., 2001a). In culture, treatment with nonsteroidal anti-inflammatory drugs reduces the number of cells exhibiting MSI (Ruschoff *et al*., 1998; Yamamoto *et al*., 1999).

Regarding genetic modifiers of colorectal cancer in HNPCC, the increased penetrance in males (80 percent) and much lower penetrance in females (40 percent) (Mitchell *et al*., 2002) has been mentioned in Question 2. An earlier age at diagnosis has been associated with a specific N-acetyltransferase I allele in Finnish kindreds with mutations in *MLH1* (Mitchell *et al*., 2002) and with a cyclin D1 allele (Kong *et al*., 2000).