INTERPRETATION OF GENETIC TEST RESULTS FOR HEREDITARY NONPOLYPOSIS COLONRECTAL CANCER: IMPLICATIONS FOR CLINICAL PREDISPOSITION TESTING

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Genetic testing for cancer susceptibility is evolving from purely research applications to affecting clinical management. In an effort to guide this transition, the American Society of Clinical Oncology has recommended that clinical predisposition testing be offered when (1) the prior probability of a positive result is high because of a strong family history of cancer or very early age of onset of cancer, (2) the test can be adequately interpreted, and (3) the results will influence medical management of the patient or a family member.1

Hereditary nonpolyposis colorectal cancer (HNPCC), the most common hereditary colon cancer syndrome,2 is a dominantly inherited disease associated with increased lifetime risks of a range of cancers including colorectal and endometrial cancers as well as extracolonic gastrointestinal, genitourinary, and ovarian cancers.3-5 Various clinical criteria have been proposed to define HNPCC, including the Amsterdam criteria6 and the modified Amsterdam criteria. Modifications of these criteria aim to identify additional families who exhibit features consistent with a diagnosis of HNPCC.7

The objective of this study was to determine how often genetic test results for hereditary nonpolyposis colorectal cancer (HNPCC) can be definitively interpreted and used to guide clinical management. A case-series study conducted in 1996 to 1998 was designed to evaluate the clinical utility of sequence analysis of hMSH2 and hMLH1 coding sequence and flanking intronic regions. Mutations were categorized as protein truncating and missense. In the case of missense alterations, additional analyses were performed in an effort to assess pathogenicity.

Setting and Participants Families were identified by self-referral or health care provider referral to a cancer genetics program. Participants and kindreds were classified into 1 of 4 categories: (1) Amsterdam criteria for HNPCC, (2) modified Amsterdam criteria for HNPCC, (3) young age at onset, or (4) HNPCC-variant. In addition, each proband was classified according to the Bethesda guidelines for identification of individuals with HNPCC.

Main Outcome Measure Alterations of hMSH2 and hMLH1 genes.

Results Twenty-seven alterations of hMSH2 and hMLH1 were found in 24 of 70 families (34.3%). Of these, deleterious mutations that could be used with confidence in clinical management were identified in 25.7% (18/70) of families. The rates of definitive results for families fulfilling Amsterdam criteria, modified Amsterdam criteria, young age at onset, HNPCC-variant, and Bethesda guidelines were 27 (39.3%), 13 (18.2%), 12 (16.7%), 11 (15.8%), and 21 (30.4%), respectively. The prevalence of missense mutations, genetic heterogeneity of the syndrome, and limited availability of validated functional assays present a challenge in the interpretation of genetic test results of HNPCC families.

Conclusions The identification of pathogenic mutations in a significant subset of families for whom the results may have marked clinical importance makes genetic testing an important option for HNPCC and HNPCC-like kindreds. However, for the majority of individuals in whom sequence analysis of hMSH2 and hMLH1 does not give a definitive result, intensive follow-up is still warranted.

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Amsterdam criteria. This syndrome has been associated with germline mutations in 5 DNA mismatch repair genes, most commonly hMSH2 and hMLH1. Tumors of hMSH2 and hMLH1 mutation carriers exhibit a characteristic phenotype termed microsatellite instability, characterized by expansion or contraction of short repeat sequences of DNA at multiple loci.

The exact definition of the HNPCC syndrome remains problematic. The variety of clinical phenotypes and the multiple associated genes introduce complexity into the evaluation of patients and families both for the clinician and the researcher dealing with HNPCC. The Amsterdam criteria have been criticized as being too stringent, whereas other clinical criteria are more inclusive but less specific for HNPCC. Because mutations in currently known genes do not account for all kindreds meeting even the most stringent Amsterdam criteria, it is assumed that more associated genes will be discovered. Nevertheless, genetic testing for cancer predisposition is an option that can potentially be of great value for members of high-risk families. The identification of a pathogenic germline mutation associated with increased cancer risk in a proband allows identification of asymptomatic mutation carriers who would benefit from intensified surveillance or cancer risk reduction strategies. A definitive negative test result in a member of a family with a known pathogenic mutation can prevent unnecessary surveillance procedures or prophylactic surgery and alleviate the fear of cancer in individuals found not to carry the family mutation.

We performed mutation analyses of the hMSH2 and hMLH1 genes in members of 70 kindreds with a personal or family history suggestive of HNPCC. The goal of this study was to evaluate genetic testing for HNPCC from a clinical perspective by assessing the prevalence of mutations in hMSH2 and hMLH1 genes in individuals meeting the several existing clinical criteria for HNPCC and determining how often results of genetic testing for HNPCC can be definitively interpreted and used to guide clinical management decisions.

**METHODS**

**Sample Ascertainment**

Our goal was to assess the utility of genetic predisposition testing for HNPCC within the clinical context in which such testing is currently recommended. Families were identified by self-referral or health care provider referral to our cancer genetics program and were enrolled on the basis of multiple cases of colorectal cancer (CRC), CRC diagnosis at younger than 40 years of age, or familial association of CRC with other HNPCC-associated tumors. Personal and family cancer histories and demographic data were obtained from the proband and participating relatives, and cancer diagnoses and deaths were confirmed by review of medical records, pathology reports, or death certificates. The project was approved by the institutional review board and informed consent was obtained from each participant.

Each pedigree was classified by whether the family history fulfilled Amsterdam, modified Amsterdam, young age at onset, or HNPCC-variant criteria (TABLE 1). For each pedigree, the designated proband, who had developed early-onset colorectal neoplasia, was also evaluated according to the recently proposed Bethesda guidelines for the identification of HNPCC patients.

Genetic analysis was performed on a blood specimen from the proband in each family. Due to difficulty in obtaining the paraffin-embedded tumor samples required for microsatellite instability analysis and the increasing availability of high-throughput technologies for detection of heritable mutations, we opted to use sequencing of DNA isolated from a blood sample rather than beginning with tumor analysis for microsatellite instability.

**hMSH2/hMLH1 Molecular Analysis**

DNA was isolated from peripheral blood using a DNA extraction kit (Qiagen, Valencia, Calif). Each of the exons plus...
some flanking intron from the hMSH2 and hMLH1 genes were amplified by polymerase chain reaction. (Primer sequences and conditions available from the authors.) Sequencing reactions were assembled on a multiprobe robot (104DT; Packard Instrument Company, Meriden, Conn). The resulting fluorescent sequencing products were analyzed on a semiautomated sequencing apparatus (Model 377; Perkin-Elmer Applied Biosystems Division, Foster City, Calif).

Sequencing results for each proband were first analyzed for the presence of a potentially pathogenic mutation. Additional blood samples were analyzed from other family members with cancer or adenomatous polyps when this was indicated and the samples were available. Sequence alterations with an allele frequency of at least 5% were considered normal variants (polymorphisms) and are not reported. For all other alterations, the likelihood of the identified mutation being the cause of cancer predisposition was assessed. Sequence alterations that resulted in the generation of a stop codon, frameshift, or alteration of conserved splicing sequences were classified as protein truncating and therefore deleterious. Determination of the potential pathogenicity of missense alterations leading to amino acid substitutions was done with available data based on the following criteria: (1) evaluation of whether the alteration affected an amino acid conserved evolution...
tionally across species as well as the nature of the amino acid change, (2) estimates of the general population frequency of the alteration, (3) segregation of the alteration with cancer in the kindred (ie, its presence in members of the family with cancer and its absence in those without cancer), and (4) conclusive evidence that the alteration affects gene function.

For each family, genetic results were then assessed for their potential use as a basis for clinical recommendations for the proband and other family members. Results were classified into 2 categories: definitive and inconclusive. Test results were termed definitive if there was a clearly pathogenic mutation in the proband. Results were considered inconclusive if no sequence alteration was found in the proband or if a missense mutation could not be interpreted as pathogenic based on standard criteria for evaluation.

## RESULTS

### Sequencing Results

A total of 70 families were enrolled, representing 297 CRC and 364 other cancer diagnoses. Based on sequencing results, published data, and existing mutation databases, 18 families were found to have a clearly deleterious mutation (Table 2). Eight of these deleterious mutations have not been previously reported. One novel deleterious mutation was found in 2 families. Further analysis revealed that the probands shared a common haplotype although there was no evidence based on family history that these 2 families were related.

Missense mutations were identified in 8 families (Table 3). Four of these missense mutations had been previously reported and had been interpreted as pathogenic in at least 1 report (Table 3). We performed supplementary analyses in an attempt to determine pathogenicity for previously published and all novel missense mutations. The basis for attributing elevated cancer risk to missense mutations was not always stated in the published reports, and there was considerable variability in the criteria used to define pathogenicity. For example, 2 mutations (hMLH1 codon

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### Table 3. Assessment of Pathogenicity of Missense Alterations

<table>
<thead>
<tr>
<th>Proband No.</th>
<th>Family Type</th>
<th>Exon</th>
<th>Amino Acid Change</th>
<th>Source, y</th>
<th>hMLH1</th>
<th>Nature of Amino Acid Change</th>
<th>General Population Frequency</th>
<th>Segregation Analysis</th>
<th>Yeast Functional Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF5</td>
<td>Amsterdam</td>
<td>3</td>
<td>T82I</td>
<td>Novel</td>
<td>+</td>
<td>Polar to nonpolar</td>
<td>0/166 Alleles</td>
<td>Inconclusive†</td>
<td>Loss of function</td>
</tr>
<tr>
<td>DF232</td>
<td>Amsterdam</td>
<td>19</td>
<td>R755S</td>
<td>Novel</td>
<td>+</td>
<td>Basic positive charge to neutral</td>
<td>0/166 Alleles</td>
<td>Not possible‡</td>
<td>Not tested</td>
</tr>
<tr>
<td>DF260</td>
<td>Amsterdam</td>
<td>13</td>
<td>V506A</td>
<td>Liu et al, 1996</td>
<td>+</td>
<td>Nonpolar to nonpolar</td>
<td>0/184 Alleles</td>
<td>Not possible‡</td>
<td>Loss of function</td>
</tr>
<tr>
<td>DF260</td>
<td>Amsterdam</td>
<td>16</td>
<td>K618A</td>
<td>Maulion et al, 1996; Weber et al, 1997; Wijnen et al, 1997</td>
<td>–</td>
<td>Basic positive charge to nonpolar</td>
<td>1/186 alleles</td>
<td>Not possible‡</td>
<td>Loss of function</td>
</tr>
<tr>
<td>DF1751</td>
<td>Amsterdam</td>
<td>19</td>
<td>V716M</td>
<td>Kowalski et al, 1997</td>
<td>+</td>
<td>Nonpolar to nonpolar</td>
<td>1/180 Alleles</td>
<td>Inconclusive†</td>
<td>Loss of function</td>
</tr>
<tr>
<td>DF597</td>
<td>Amsterdam</td>
<td>5</td>
<td>A272V</td>
<td>Novel</td>
<td>hMSH2</td>
<td>+</td>
<td>0/186 Alleles</td>
<td>Inconclusive†</td>
<td>NA§</td>
</tr>
<tr>
<td>DF3002</td>
<td>HNPCC-variant</td>
<td>6</td>
<td>G315V</td>
<td>Novel</td>
<td>+/-</td>
<td>Nonpolar to nonpolar</td>
<td>0/172 Alleles</td>
<td>Not possible‡</td>
<td>NA§</td>
</tr>
<tr>
<td>DF1448</td>
<td>Amsterdam</td>
<td>6</td>
<td>G322D</td>
<td>Maliaka et al, 1996; Liu et al, 1995; Wu et al, 1997; Herfarth et al, 1997; Froggatt et al, 1996</td>
<td>+</td>
<td>Nonpolar to acidic negative charge</td>
<td>0%-3%¶</td>
<td>Inconclusive</td>
<td>NA§</td>
</tr>
<tr>
<td>DF1370</td>
<td>Young age at onset</td>
<td>6</td>
<td>G322D</td>
<td>Maliaka et al, 1996; Liu et al, 1995; Wu et al, 1997; Herfarth et al, 1997; Froggatt et al, 1996</td>
<td>+</td>
<td>Nonpolar to acidic negative charge</td>
<td>0%-3%¶</td>
<td>Inconclusive</td>
<td>NA§</td>
</tr>
</tbody>
</table>

*Numbers in parentheses represent Bethesda guideline categories fulfilled by family. Mutations are designated as recommended by the Nomenclature Working Group.‡ Mutations in bold are novel mutations. Plus sign indicates conserved amino acid, minus sign, not conserved. HNPCC, hereditary nonpolyposis colorectal cancer.

†Some affected family members have the same mutation, but 3 or fewer affected members were available per family; therefore, analysis did not yield statistically significant results to enable definitive conclusions about pathogenicity.

‡No functional assay for hMSH2 is available.

§Conserved in mouse, but not in yeast.

¶Based on various reports.
1517T→C, V506A and hMSH2 codon 965G→A, G322D) were both considered likely to be pathogenic on the basis of the location of their missense change in an evolutionarily conserved amino acid.12-43 Consistent with this, the hMLH1 V506A mutation has recently been shown to inactivate the dominant negative phenotype caused by expression of hMLH1 in yeast in a novel functional assay, suggesting that it is pathogenic.44 Conversely, a low frequency of the hMSH2 G322D mutation has been demonstrated in reference populations, which reduces the probability that it is functionally significant.44-47

Three different families were found to carry 2 different potentially pathogenic mutations each. Proband DF260 carries 2 different missense hMLH1 mutations; it is unclear whether either of these is pathogenic. Proband DF1751 similarly carries a protein-truncating hMLH1 mutation and a hMLH1 missense mutation that appeared to be a loss of function mutation in the yeast assay.48 The 2 potentially pathogenic mutations in these families could be in the same copy of the hMLH1 gene or, as noted in a recent article,49 these individuals could be compound heterozygotes. Proband DH11448 has a protein-truncating hMLH1 mutation and a hMLH1 missense mutation that appeared to be a loss of function mutation in the yeast assay.48 The 2 potentially pathogenic mutations in these families could be in the same copy of the hMLH1 gene or, as noted in a recent article,49 these individuals could be compound heterozygotes. Proband DH11448 has a protein-truncating hMLH1 mutation and a hMLH1 missense mutation that appeared to be a loss of function mutation in the yeast assay.48

**Interpretation of Genetic Results for Clinical Counseling**

**Table 4.** Clinical Interpretation of hMSH2 and hMLH1 Genetic Test Results for Hereditary Nonpolyposis Colorectal Cancer (HNPCC)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>No. of Families</th>
<th>Definitive Results, No. (%)</th>
<th>Indeterminate Results, No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cohort</td>
<td>70</td>
<td>18 (25.7)</td>
<td>46 (65.7)</td>
</tr>
<tr>
<td>Amsterdam</td>
<td>28</td>
<td>11 (39.3)</td>
<td>13 (46.4)</td>
</tr>
<tr>
<td>Modified Amsterdam</td>
<td>11</td>
<td>2 (18.2)</td>
<td>9 (81.8)</td>
</tr>
<tr>
<td>HNPCC-variant</td>
<td>19</td>
<td>3 (15.8)</td>
<td>15 (78.9)</td>
</tr>
<tr>
<td>Young age at onset (&lt;40 y)</td>
<td>12</td>
<td>2 (16.7)</td>
<td>9 (75.0)</td>
</tr>
<tr>
<td>Bethesda (B1-7)</td>
<td>56</td>
<td>17 (30.4)</td>
<td>33 (58.9)</td>
</tr>
</tbody>
</table>

Genetic testing for cancer predisposition is now possible for many hereditary syndromes and has the potential to provide valuable information for patients and health care providers. As with any diagnostic test, however, the clinical utility of genetic testing depends on its ability to provide definitive results, whether positive or negative. The onus of obtaining informed consent for testing, providing pretest and posttest counseling, and interpreting genetic test results rests on the health care provider ordering the test.50 Our study highlights some of the advantages and challenges inherent in the optimal implementation of genetic testing for HNPCC in the clinical setting.

First, genetic testing has marked clinical value for members of HNPCC families for which sequence analysis yields a definitive result. In such families, when a pathogenic mutation is identified, intensive surveillance and consideration of prophylactic surgery can be limited to those individuals showing the mutation. Family members without the mutation can undergo the same form of surveillance recommended for the general population. In series of HNPCC and HNPCC-like families, including that of this study, the frequencies of pathogenic germline mutations in the hMSH2 and hMLH1 genes of affected members range between 15% and...
and 60%, with higher prevalence in kindreds meeting more stringent criteria for HNPCC.

Second, at present, sequence analysis will not give a definitive result for many families. Failure to identify a clearly pathogenic mutation in the sequence of hMSH2 or hMLH1 in an individual or family meeting a set of clinical criteria for HNPCC should not result in a change in clinical management decisions for at-risk family members. Some mutations in known genes may escape detection by the testing methods used, or an alteration in a different gene may later be found in the kindred. Incorrect interpretation of genetic results potentially has enormous impact because false-negative results may lead to inadequate medical follow-up for individuals at remarkably high risk of cancer. Difficulties in interpretation of genetic test results were illustrated in a recent study of genetic testing in patients with familial adenomatous polyposis where physicians misinterpreted inconclusive genetic test results as being negative in 31.6% of cases, even in patients with the clinical syndrome.

Third, not all detected sequence alterations of associated genes can be presumed to be the cause of the cancer predisposition in a family. A mutation can be considered to underlie cancer predisposition only in the presence of sufficient evidence that the sequence change results in altered gene function. The fact that different investigators have determined the same mutation to be either pathogenic or insignificant highlights that interpretation of sequence results is not always straightforward. The process of determining whether a sequence alteration is meaningful often requires repeated contact, consent, and participation of family members; multiple steps of laboratory analysis; and assessment of the strength of previously published interpretations of pathogenicity.

What options are available for families with inconclusive hMSH2 and hMLH1 results? The most established approach would be to perform tumor microsatellite instability analysis in families in whom no mutation or an alteration of unclear significance has been found. Demonstrating loss of the second allele of the gene containing the sequence change in tumors would provide further evidence that the missense mutation is pathogenic. For those with missense mutations of unclear significance and evidence of microsatellite instability, further analysis to determine the functional significance of the mutations can be pursued. For those with microsatellite instability but no alteration of hMSH2 or hMLH1, analysis of other genes associated with HNPCC can be performed. The identification of additional genes associated with hereditary colon cancer also eventually will lead to more patients receiving informative results regarding the genetic basis of cancer predisposition in their family.

In summary, the prevalence of missense mutations, genetic heterogeneity of the syndrome, and the current lack of validated functional assays present challenges for the interpretation of genetic test results and counseling of HNPCC families. When considering cancer predisposition testing for HNPCC, medical providers and patients should appreciate that genetic analysis and interpretation of these results are likely to be more complex than most traditional diagnostic testing. Such tests may involve multiple family members and still yield inconclusive results despite expense, time, and multiple steps of analysis. However, the identification of pathogenic mutations in a significant subset of families for whom the results may have marked clinical importance makes genetic testing an important option for HNPCC and HNPCC-like kindreds.

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REFERENCES

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Most of us walk unseeing through the world, unaware alike of its beauties, its wonders, and the strange and sometimes terrible intensity of the lives that are being lived about us.
—Rachel Carson (1907-1964)

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