Accuracy of Revised Bethesda Guidelines, Microsatellite Instability, and Immunohistochemistry for the Identification of Patients With Hereditary Nonpolyposis Colorectal Cancer

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Context The selection of individuals for hereditary nonpolyposis colorectal cancer (HNPCC) genetic testing is challenging. Recently, the National Cancer Institute outlined a new set of recommendations, the revised Bethesda guidelines, for the identification of individuals with HNPCC who should be tested for microsatellite instability.

Objective To establish the most effective and efficient strategy for the detection of MSH2/MLH1 gene carriers.


Interventions Microsatellite instability testing and MSH2/MLH1 immunostaining in all patients regardless of age, personal or family history, and tumor characteristics. Patients whose tumors exhibited microsatellite instability and/or lack of protein expression underwent MSH2/MLH1 germline testing.

Main Outcome Measures Effectiveness and efficiency of both microsatellite instability testing and immunostaining, either directly or previous selection of patients according to the revised Bethesda guidelines, were evaluated with respect to the presence of MSH2/MLH1 germline mutations.

Results Two hundred eighty-seven patients (23.5%) fulfilled the revised Bethesda guidelines. Ninety-one patients (7.4%) had a mismatch repair deficiency, with tumors exhibiting either microsatellite instability (n=83) or loss of protein expression (n=81). Germline testing identified 11 mutations (0.9%) in either MSH2 (7 cases) or MLH1 (4 cases) genes. Strategies based on either microsatellite instability testing or immunostaining were equivalent and highly effective (sensitivity, 81.8% and 81.8%; specificity, 98.0% and 98.2%; positive predictive value, 27.3% and 29.0%, respectively) to identify MSH2/MLH1 gene carriers. Logistic regression analysis confirmed the revised Bethesda guidelines as the most discriminating set of clinical parameters (odds ratio, 33.3; 95% confidence interval, 4.3-250; P=.001).

Conclusion The revised Bethesda guidelines constitute a useful approach to identify patients at risk for HNPCC. In patients fulfilling these criteria, both microsatellite instability testing and immunostaining are equivalent and highly effective strategies to further select those patients who should be tested for MSH2/MLH1 germline mutations.


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Collaborative Group on HNPCC established clinical criteria, known as the Amsterdam criteria, which provided a pivotal definition of this syndrome and were critical in identifying its molecular basis. In response to criticism that the Amsterdam criteria were too stringent, the extended Amsterdam II criteria were developed to include extracolonic HNPCC-associated cancers.

The use of the Amsterdam criteria achieved the original purpose of classifying a family as having HNPCC, but its limited sensitivity hampered decisions about which patients should undergo genetic testing. In 1996, an international workshop on HNPCC hosted by the National Cancer Institute outlined a set of recommendations, known as the Bethesda guidelines, for the identification of individuals with HNPCC who should be tested for microsatellite instability and/or genetic testing. More recently, a second HNPCC workshop revised these criteria and proposed a new set of recommendations, the revised Bethesda guidelines.

As it was previously mentioned, tumor microsatellite instability is a phenotypic indicator of defective DNA mismatch repair. The fact that more than 90% of HNPCC-related cancers exhibit microsatellite instability suggests that screening of tumors for microsatellite instability may be an efficient way of selecting individuals for HNPCC genetic testing. On the other hand, it is well known that most mutations in either MSH2 or MLH1 genes result in abnormal MSH2 or MLH1 protein expression. As a consequence, immunostaining for DNA mismatch repair proteins were performed in all patients regardless of age, personal or family history, and tumor characteristics. To avoid variability in the quality of results, microsatellite instability testing and immunostaining were centralized in 2 single centers, respectively. Researchers scoring immunostaining were blinded to the microsatellite instability results and vice versa.

Tumor Microsatellite Instability Analysis

Tissue samples from tumor and healthy colonic mucosa were obtained from each patient, immediately frozen in liquid nitrogen, and stored at −70°C until use. In those cases in which frozen tissue was not available, archival formalin-fixed, paraffin-embedded samples were used. Genomic DNA was

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isolated using the QiaAmp Tissue Kit (Qiagen, Courtabeuf, France).

Microsatellite instability status was assessed using BAT-26 mononucleotide marker based on its high sensitivity.30-33 In those cases in which BAT-26 was stable but immunostaining revealed lack of MSH2 or MLH1 protein expression, the 4 additional markers of the Bethesda panel (BAT-25, DSS346, D2S123, and D17S250) were also evaluated. Primers were labeled fluorescently and analyzed on a genetic analyzer using GeneScan Analysis software (ABI 310 Genetic Analyzer; Applied Biosystems, Foster City, Calif).

**Tumor MSH2 and MLH1 Protein Expression**

One block of formalin-fixed, paraffin-embedded tumor tissue was selected per case. Before immunostaining, antigen retrieval was performed by immersing sections in a 10-M concentration of citrate buffer, pH 6.0, and boiling in a pressure cooker for 5 minutes. Sections were then incubated for 20 minutes at room temperature with mouse monoclonal antibodies against MLH1 protein (clone G168-15, dilution 1:40; PharMingen, San Diego, Calif) and MSH2 protein (clone FE11, dilution 1:35; Oncogene Research Products, Boston, Mass). Ultra-Vision streptavidin-biotin peroxidase detection kit (DAKO, Carpinteria, Calif) was used as secondary detection system. The peroxidase reaction was developed using diaminobenzidine tetrachloride as chromogen. Tumor cells were judged to be negative for protein expression only if they lacked staining in a sample in which healthy colonocytes and stroma cells were stained. If no immunostaining of healthy tissue could be demonstrated, the results were considered ambiguous.

**MSH2/MLH1 Germline Mutation Analysis**

Patients found to have tumors with microsatellite instability and/or lack of protein expression of either MSH2 or MLH1 underwent germline genetic testing for MSH2 and MLH1 genes by both multiple ligation probe amplification analysis and sequencing.

Multiple ligation probe amplification was performed using the MLH1/MSH2 exon deletion assay (MRC-Holland, Amsterdam, the Netherlands), which allows the detection of genomic rearrangements in these genes.34 Ligation products were amplified by polymerase chain reaction using a fluorescently labeled primer and analyzed in an ABI 3100 sequencer using GeneScan and Genotyper Analysis software (Applied Biosystems). Peak height of each fragment was compared with those measurements of a control sample and deletions were suspected when peak height was 60% or less of healthy controls. Control DNA samples with known MSH2 or MLH1 genomic rearrangements were included in each batch of experiments. Multiple ligation probe amplification results were confirmed by reverse transcriptase polymerase chain reaction encompassing contiguous exons of the suspected deleted fragment.

Germline mutations in the MSH2 and MLH1 genes were also sought by direct exon-by-exon sequencing. Amplification products were generated with primers located in the flanking introns approximately 50 base pairs from the respective intron/exon borders to detect all possible splice junction mutations. The sequences were determined on the genetic analyzer (ABI 3100, Applied Biosystems) using fluorescently labeled primers and protocols supplied by the manufacturer.

**Statistical Analysis**

Performance characteristics of screening strategies based on microsatellite instability testing and/or protein immunostaining, either directly or through previous selection of patients according to the revised Bethesda guidelines, were calculated with respect to the diagnosis of HNPCC associated with MSH2/MLH1 germline mutations. Mutations were considered deleterious based on sequencing results, published data, and existing mutation databases. Comparison of paired proportions for sensitivity, specificity, and overall accuracy was performed by the McNemar test.

A cost-minimization analysis was also performed to establish the most efficient strategy. For this analysis, costs of microsatellite instability (BAT-26 maker), immunostaining (both MSH2 and MLH1), and genetic testing were established at 100 € (US $130), 200 € (US $260), and 2400 € (US $3120), respectively, or 1200 € per gene (US $1560 per gene), according to hospital clinic current billing.

To identify parameters associated with MSH2/MLH1 gene mutation carriers, univariate and multivariate analyses were performed. Variables evaluated corresponded to any previously suggested predictors including each individual characteristic of the original and revised Bethesda guidelines, as well as those proposed by Aaltonen et al10 for screening in general population (any first-degree relative with colorectal cancer or endometrial cancer regardless of age, personal history of colorectal cancer or endometrial cancer, and age at diagnosis of colorectal cancer <50 years in the proband) and Wijnen et al14 in familial cancer clinics (age at diagnosis of colorectal cancer <50 years within a family, fulfillment of the Amsterdam criteria, and endometrial cancer in >1 family member). In addition, combined variables corresponding to each of these 4 sets of recommendations were also evaluated. For the univariate analysis, categorical variables were compared by the Fisher exact test. Variables achieving P<.20 in the univariate analysis were subsequently included in a multivariate analysis using a stepwise forward logistic regression procedure. Two different models were evaluated. First, only combined variables (original and revised Bethesda guidelines, and those proposed by Aaltonen et al10 and Wijnen et al14) were included in the model to identify the most discriminating set of parameters. Second, individual characteristics were added to the previously recognized combined variable to identify other independent predictors of MSH2/MLH1 germline mutations that can contribute to refine the model.
Continuous variables were expressed as mean (SD). All P values were 2-sided. \( P < .05 \) was considered statistically significant. All calculations were performed by using SPSS software version 10.0 (SPSS Inc, Chicago, Ill).

RESULTS

During the study period, 1978 patients with newly diagnosed colorectal cancer were included from 25 centers. One hundred six patients were excluded because the tumor developed in the context of a familial adenomatous polyposis (n = 11) or inflammatory bowel disease (n = 14), the patient did not consent to participate in the study (n = 32), or had an incomplete family history (n = 49). Of the remaining 1872 eligible patients, 1222 patients were diagnosed in 20 centers agreeing to participate in the nested molecular epidemiology study and constituted the basis of our analysis. Characteristics of this subset of patients (Table 1) did not differ from the whole group.28

Proband Characteristics

Demographic, clinical, and tumor-related characteristics of patients included in the study are shown in Table 1. According to the extended Amsterdam II criteria, 22 patients (1.8%) belonged to families satisfying the clinical definition of HNPCC. On the other hand, 287 patients (23.5%) fulfilled at least 1 criterion of the revised Bethesda guidelines.

A total of 91 patients (7.4%) were found to have a mismatch repair deficiency. Of these, 83 tumors (6.8%) showed microsatellite instability and 81 (6.6%) loss of protein expression in either MSH2 (21 cases) or MLH1 (60 cases). Loss of MSH2 or MLH1 expression was found in 73 of the microsatellite instability tumors, whereas expression of both proteins was retained in the remaining 10 tumors with microsatellite instability. In addition, loss of MSH2 or MLH1 expression was found in 8 patients whose tumor did not show microsatellite instability according to the BAT-26 marker. Evaluation of the 4 additional markers of the Bethesda panel in these cases confirmed that their tumor was stable. Finally, unequivocal loss of MSH2 or MLH1 was not observed in any of the remaining 1131 tumors not showing microsatellite instability.

Germline testing identified 11 mutations (0.9%) in either MSH2 (7 cases) or MLH1 (4 cases) genes. Characteristics of these patients are shown in Table 2. Three DNA changes corresponded to missense mutations. Although all 3 mutations have been previously reported as deleterious, 1 tumor

<table>
<thead>
<tr>
<th>Case</th>
<th>Amsterdam II Criteria</th>
<th>Revised Bethesda Guidelines</th>
<th>MSI Status</th>
<th>IHC Expression of Protein</th>
<th>Germline Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
<td>Unstable</td>
<td>Present</td>
<td>MLH1 859_860delAA Frameshift at codon 287; stop at codon 305</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>Unstable</td>
<td>Present</td>
<td>MLH1 2136G&gt;A Stop at codon 712</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
<td>Unstable</td>
<td>Absent</td>
<td>MSH2 del exon 9-16 Out-of-frame deletion of exons 9-16</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>Yes</td>
<td>Unstable</td>
<td>Absent</td>
<td>MLH1 1703insA Frameshift at codon 568; stop at codon 571</td>
</tr>
<tr>
<td>5</td>
<td>No</td>
<td>Yes</td>
<td>Absent</td>
<td>Present</td>
<td>MLH1 IVS14-1G&gt;A Out-of-frame deletion of exon 15</td>
</tr>
<tr>
<td>6</td>
<td>No</td>
<td>Yes</td>
<td>Absent</td>
<td>Present</td>
<td>MSH2 IVS15-3TTCC&gt;CTT Splicing defect, out-of-frame deletion</td>
</tr>
<tr>
<td>7</td>
<td>No</td>
<td>No</td>
<td>Unstable</td>
<td>Absent</td>
<td>MSH2 688insA Frameshift at codon 230; stop at codon 232</td>
</tr>
<tr>
<td>8</td>
<td>No</td>
<td>Yes</td>
<td>Unstable</td>
<td>Absent</td>
<td>MSH2 1165C&gt;T Stop at codon 389</td>
</tr>
<tr>
<td>9</td>
<td>No</td>
<td>Yes</td>
<td>Unstable</td>
<td>Absent</td>
<td>MLH1 1852AA&gt;G Stop at codon 618</td>
</tr>
<tr>
<td>10</td>
<td>No</td>
<td>Yes</td>
<td>Stable</td>
<td>Absent</td>
<td>MLH1 965G&gt;A Gly&gt;Gly at codon 322</td>
</tr>
<tr>
<td>11</td>
<td>No</td>
<td>No</td>
<td>Unstable</td>
<td>Absent</td>
<td>MSH2 435T&gt;G Ile&gt;Met at codon 145</td>
</tr>
</tbody>
</table>
did not show microsatellite instability, 2 tumors exhibited contradictory immunostaining results, and 1 patient did not fulfill the revised Bethesda guidelines.

**Performance Characteristics and Efficiency of Screening Strategies**

Performance characteristics of screening strategies for the identification of MSH2/MLH1 gene carriers are shown in Table 3. Calculations were performed considering both germline mutations (n=11) and unambiguous pathogenic variants (n=8). Approaches based on either microsatellite instability analysis or MSH2/MLH1 immunostaining had an almost identical effectiveness. Conversely, specificity, overall accuracy, and positive predictive value of these strategies increased when patients were previously selected according to the revised Bethesda guidelines. Finally, combinations of the results obtained in both microsatellite instability testing and protein immunostaining did not provide any additional advantage with respect to the corresponding individual strategies.

Efficiency of all the above-mentioned strategies for the identification of MSH2/MLH1-associated HNPCC was evaluated in a cost-minimization analysis. Clinical selection of patients according to the revised Bethesda guidelines followed by either microsatellite instability analysis (11 989 € [US $15 586] per detected mutation) or protein immunostaining (10 644 € [US $13 837] per detected mutation) was more cost-effective than any of these approaches performed directly (32 140 € [US $41 782] and 37 956 € [US $49 343] per detected mutation, respectively). Conversely, performance of both microsatellite instability analysis and protein immunostaining increased the cost (14 900 € [US $19 370] per detected mutation in combination with the revised Bethesda guidelines and 49 020 € [US $63 726] per detected mutation without previous selection of patients according to these clinical criteria) with respect to the corresponding individual strategies.

**Predictors of MSH2 or MLH1 Germline Mutations**

To further refine clinical criteria for selecting patients who should be submitted to genetic testing, variables associated with MSH2 or MLH1 germline mutations were identified. Table 4 shows parameters significantly associated in the univariate analysis. When only combined variables corresponding to the original set of recommendations were included in the logistic regression analysis, the revised Bethesda guidelines turned out to be the most discriminating set of parameters (odds ratio [OR], 33.3; 95% confidence interval [CI], 4.3-230; P = .001). In that sense, specific characteristics of the revised Bethesda guidelines, which allowed the identification of 2 additional gene carriers with respect to those identified by the original Bethesda guidelines, were criteria number 5 (colorectal cancer diagnosed in ≥2 first-degree or second-degree relatives with HNPCC-related tumors, regardless of age) in 2 cases and number 3 (colorectal cancer with the microsatellite instability–high histology diagnosed in a patient aged <60 years) in 1 case. Furthermore, when individual characteristics were added to the logistic regression model, family history of colorectal cancer diagnosed in at least 1 first-degree relative (OR, 3.6; 95% CI, 1.05-12.7; P = .04) was selected as an independent predictor of MSH2/MLH1 germline mutations, along with the re-

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**Table 3. Performance Characteristics of Different Strategies for the Identification of MSH2/MLH1 Gene Mutation Carriers**

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Any Germline Mutation (n = 11), %</th>
<th>Unambiguous Germline Mutations (n = 8), %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
</tr>
<tr>
<td>Fulfillment of revised Bethesda guidelines</td>
<td>90.9</td>
<td>77.1</td>
</tr>
<tr>
<td>Presence of MSI</td>
<td>90.9</td>
<td>93.9</td>
</tr>
<tr>
<td>Loss of protein expression</td>
<td>81.8</td>
<td>94.2</td>
</tr>
<tr>
<td>Presence of MSI or loss of protein expression</td>
<td>90.9</td>
<td>93.4</td>
</tr>
<tr>
<td>Presence of MSI and loss of protein expression</td>
<td>72.7</td>
<td>94.8</td>
</tr>
</tbody>
</table>

Fulfillment of revised Bethesda guidelines and Presence of MSI | 81.8 | 98.0* | 27.3 | 99.8 | 97.9* | 100 | 97.9* | 24.2 | 100 | 97.9* |
Loss of protein expression | 81.8 | 98.2* | 29.0 | 99.8 | 98.0* | 100 | 98.1* | 25.8 | 100 | 98.1* |
Presence of MSI or loss of protein expression | 81.8 | 97.8* | 25.7 | 99.8 | 97.7* | 100 | 97.8* | 22.9 | 100 | 97.8* |
Presence of MSI and loss of protein expression | 72.7 | 98.3* | 28.6 | 99.7 | 98.1* | 100 | 98.4* | 28.6 | 100 | 98.4* |

Abbreviation: MSI, microsatellite instability.
* P < .001 (McNemar test) with respect to the corresponding paired strategy without previous selection of patients according to the revised Bethesda guidelines.

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vised Bethesda guidelines (OR, 13.7; 95% CI, 1.47-125; \( P = .02 \)).

TABLE 5 summarizes performance characteristics of strategies based on the combination of the 2 independent variables identified in the multivariate analysis. Genetic testing driven by this combination of clinical parameters was not substantially more accurate than when the revised Bethesda guidelines were only used (Table 3).

COMMENT

Our study represents the first reported attempt to our knowledge to determine the effectiveness of different strategies for identifying MSH2/MLH1 gene carriers in the context of the recently proposed revised Bethesda guidelines. The strength of our study relies on the fact that it was performed on a general population basis; it involved the largest number of patients evaluated so far; microsatellite instability analysis and protein immunostaining were performed in a parallel and blinded fashion; results were evaluated according to the presence of germline mutations; and finally, costs were also considered. Results of our study suggest that microsatellite instability testing and protein immunostaining are equivalent strategies in terms of cost-effectiveness, and that when either of these screening methods are performed in patients selected according to the revised Bethesda guidelines, they are highly accurate in identifying patients with MSH2/MLH1-associated HNPCC. However, the relatively low number of identified mutations, as well as the uncertain significance of the 3 missense mutations, may constitute a drawback of the study and, consequently, could have influenced the results. Nonetheless, recalibration of effectiveness and efficiency of the revised Bethesda guidelines, either alone or in combination with microsatellite instability testing or immunostaining, and considering only those unambiguous pathogenic mutations does not modify the conclusions of the study.

Because molecular definition of HNPCC was established,\(^{35-38}\) identifying the use of different sets of clinical criteria and methods for determining mismatch repair deficiency (ie, micro-

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Germline Mutation (n = 11)</th>
<th>No Germline Mutation (n = 1211)</th>
<th>( P ) Value</th>
<th>Positive Predictive Value, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal cancer in ( \geq 1 ) first-degree relative</td>
<td>6 (54.5)</td>
<td>151 (12.5)</td>
<td>.001</td>
<td>3.8</td>
</tr>
<tr>
<td>Colorectal or endometrial cancer in ( \geq 1 ) first-degree relative</td>
<td>6 (54.5)</td>
<td>180 (14.8)</td>
<td>.003</td>
<td>3.2</td>
</tr>
<tr>
<td>Personal history of colorectal or endometrial cancer( ^\dagger )</td>
<td>4 (36.4)</td>
<td>90 (7.4)</td>
<td>.007</td>
<td>4.3</td>
</tr>
<tr>
<td>Colorectal cancer diagnosed at age (&lt; 50 ) y</td>
<td>3 (27.3)</td>
<td>55 (4.5)</td>
<td>.01</td>
<td>5.2</td>
</tr>
<tr>
<td>Colorectal or endometrial cancer in ( \geq 1 ) first-degree relative, personal history of colorectal or endometrial cancer, or age at diagnosis of proband colorectal cancer (&lt; 50 ) y( ^\ddagger )</td>
<td>8 (72.7)</td>
<td>306 (25.3)</td>
<td>.001</td>
<td>2.5</td>
</tr>
<tr>
<td>Fulfillment of Amsterdam criteria( ^\dagger )</td>
<td>4 (36.4)</td>
<td>14 (1.1)</td>
<td>&lt;.001</td>
<td>22.2</td>
</tr>
<tr>
<td>Endometrial cancer in ( \geq 1 ) family member</td>
<td>4 (36.4)</td>
<td>63 (5.2)</td>
<td>.002</td>
<td>5.9</td>
</tr>
<tr>
<td>Colorectal cancer diagnosed at age (&lt; 50 ) y within a family, endometrial cancer in ( \geq 1 ) family member, or fulfillment of Amsterdam criteria( ^\ddagger )</td>
<td>7 (63.6)</td>
<td>156 (12.9)</td>
<td>&lt;.001</td>
<td>4.3</td>
</tr>
<tr>
<td>Synchronous, metachronous colorectal, or other HNPCC-related cancer( ^\dagger )</td>
<td>5 (45.5)</td>
<td>98 (8.1)</td>
<td>.001</td>
<td>4.9</td>
</tr>
<tr>
<td>Colorectal or other HNPCC-related cancer( ^\dagger )</td>
<td>4 (36.4)</td>
<td>61 (5.0)</td>
<td>.002</td>
<td>6.2</td>
</tr>
<tr>
<td>Colorectal or endometrial cancer diagnosed at age (&lt; 50 ) y</td>
<td>3 (27.3)</td>
<td>78 (6.4)</td>
<td>.03</td>
<td>3.7</td>
</tr>
<tr>
<td>Right-sided colorectal cancer with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at age (&lt; 50 ) y</td>
<td>1 (9.1)</td>
<td>5 (0.4)</td>
<td>.05</td>
<td>16.7</td>
</tr>
<tr>
<td>Sigmoid-ring cell-type colorectal cancer diagnosed at age (&lt; 50 ) y</td>
<td>1 (9.1)</td>
<td>14 (1.1)</td>
<td>.13</td>
<td>6.7</td>
</tr>
<tr>
<td>Fulfillment of Bethesda guidelines( ^\ddagger )</td>
<td>8 (72.7)</td>
<td>216 (17.8)</td>
<td>&lt;.001</td>
<td>3.6</td>
</tr>
<tr>
<td>Synchronous, metachronous colorectal, or other HNPCC-related tumors, regardless of age</td>
<td>5 (45.5)</td>
<td>99 (8.2)</td>
<td>.001</td>
<td>4.8</td>
</tr>
<tr>
<td>Colorectal cancer with the MSI-high histology** diagnosed at age (&lt; 60 ) y</td>
<td>3 (27.3)</td>
<td>52 (4.3)</td>
<td>.01</td>
<td>5.4</td>
</tr>
<tr>
<td>Colorectal cancer diagnosed in ( \geq 1 ) first-degree relatives with an HNPCC-related tumor, with ( \geq 1 ) cancer diagnosed at age (&lt; 50 ) y</td>
<td>4 (36.4)</td>
<td>57 (4.7)</td>
<td>.001</td>
<td>6.6</td>
</tr>
<tr>
<td>Colorectal cancer diagnosed in ( \geq 2 ) first- or second-degree relatives with HNPCC-related tumors, regardless of age</td>
<td>5 (45.5)</td>
<td>83 (6.8)</td>
<td>.001</td>
<td>5.7</td>
</tr>
<tr>
<td>Fulfillment of revised Bethesda guidelines( ^\ddagger )</td>
<td>10 (90.9)</td>
<td>277 (22.9)</td>
<td>&lt;.001</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Abbreviations: HNPCC, hereditary nonpolyposis colorectal cancer; MSI, microsatellite instability.

*When not specified, characteristics are referred to probands.

†Patients with endometrial cancer also had colorectal cancer, which qualified inclusion into the study.

‡Combinations of individual variables according to the original sets of recommendations.\( ^{7,8,10,14} \)

§Criteria suggested by Aaltonen et al.\( ^{10} \)

¶Criteria suggested by Wijnen et al.\( ^{14} \)

**Including endometrial, ovarian, gastric, hepatobiliary, small-bowel, or urinary tract cancer.

††Including endometrial, ovarian, gastric, hepatobiliary, small-bowel, urinary tract, pancreatic, and brain cancer, sebaceous gland adenomas, and keratoacanthomas.

**Including presence of tumor-infiltrating lymphocytes, Crohn-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern.
Table 5. Performance Characteristics of Clinical Criteria for the Identification of MSH2/MLH1 Gene Mutation Carriers (Multivariate Analysis)*

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
<th>Overall Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fulfillment of clinical criteria</td>
<td>100</td>
<td>70.3</td>
<td>2.9</td>
<td>100</td>
<td>70.5</td>
</tr>
<tr>
<td>Fulfillment of clinical criteria and presence of MSI</td>
<td>90.9</td>
<td>97.9</td>
<td>28.6</td>
<td>99.9</td>
<td>97.9</td>
</tr>
<tr>
<td>Fulfillment of clinical criteria and loss of protein expression</td>
<td>81.8</td>
<td>98.1</td>
<td>28.1</td>
<td>99.8</td>
<td>97.9</td>
</tr>
<tr>
<td>Fulfillment of clinical criteria and presence of MSI or loss of protein expression</td>
<td>90.9</td>
<td>97.7</td>
<td>26.3</td>
<td>99.9</td>
<td>97.6</td>
</tr>
<tr>
<td>Fulfillment of clinical criteria, presence of MSI, and loss of protein expression</td>
<td>72.7</td>
<td>98.3</td>
<td>28.6</td>
<td>99.7</td>
<td>98.1</td>
</tr>
</tbody>
</table>

*Based on the combination of the revised Bethesda guidelines* and family history of colorectal cancer diagnosed in at least 1 first-degree relative.

A recent study has demonstrated that the use of microsatellite instability testing and immunostaining are being used and there is no unique and universally accepted strategy. Although it can be anticipated that epidemiological diversity among geographical areas and technical disparity among laboratories may preclude the establishment of a definitive, flawless approach, our results suggest that the revised Bethesda guidelines seem to be the most accurate clinical criteria tested so far for the identification of patients at risk for such an inherited disorder. Indeed, its performance characteristics, either alone or in combination with microsatellite instability testing and/or immunostaining, a highly discriminating predictive value in the logistic regression model with respect to other sets of recommendations, and the lack of benefit of adding other independent predictors in terms of both effectiveness and efficiency warrants the use of these criteria in clinical practice. These results are further reinforced by those obtained in a parallel investigation of our group, in which original and revised Bethesda guidelines were compared directly. In that analysis, the latter provided a more accurate and cost-effective approach than the original criteria (unpublished data).

Although current recommendations for HNPCC identification rely on microsatellite instability testing as a screening strategy, immunostaining has been proposed as an alternative approach. Because protein immunostaining is often easier to perform than DNA analysis in a clinical setting, the use of this technique appears to offer a relatively convenient and rapid method for the prescreening of tumors with mismatch repair defects. Equivalence of both strategies has been demonstrated in high-risk colorectal cancer populations, in which detection of microsatellite instability or loss of MSH2/MLH1 expression were both useful criteria for selecting patients who should be submitted to genetic testing. In addition, immunostaining is the method of choice to direct the search for germline mutations, and it may help to solve the status of MSH2 germline variants of uncertain significance because somatic inactivation of MSH2 is a rare event in sporadic microsatellite instability tumors. Finally, results of recent investigations suggest that systematic evaluation of other mismatch repair proteins, such as MSH6 and PMS2, may contribute to increase the effectiveness of immunostaining.

Despite the high correlation between MSH2 or MLH1 germline mutations and the absence of protein expression, some conflicting results were observed in our study. Indeed, 2 patients with missense mutations in either MSH2 or MLH1 exhibited loss of expression of the opposite protein. These contradictory results bring about the issue of determining the pathogenic significance of missense mismatch repair gene mutations. With respect to the MLH1: Lys618Ala variant, although it has been shown to segregate with the HNPCC phenotype, it has also been reported in healthy controls. Regarding the MSH2::Ile145Met variant, it has been reported in other HNPCC families (http://www.insight-group.org), but functional results raised some concerns with respect to its pathogenicity. To overcome this situation, calculations were repeated considering these 3 missense mutations as not proven pathogenic and, although minor variations occurred, the results did not change, thus reinforcing the conclusions of the study.

In our study, microsatellite instability testing was systematically performed by analyzing the BAT-26 marker alone instead of using the 5-marker panel proposed by the National Cancer Institute. The use of this single marker, in a similar manner as it has been performed in previous investigations, was justified by its high sensitivity, which ranged between 93% and 97%. Indeed, in the great majority of tumors, analysis of mononucleotide repeats BAT-25 and BAT-26 is sufficient to establish the microsatellite instability status without reference to the germline DNA, because these markers are quasi-monomorphic in white populations. In addition, Loukola et al demonstrated that the use of BAT-26 alone was feasible in screening for individuals with HNPCC because this marker was able to identify all gene mutation carriers. Nevertheless, there is still some controversy with respect to this issue because some studies have suggested that BAT-26 can miss some cases with mismatch repair defects (ie, those related to the MSH2 gene). With this limitation, we designed our investigation with the hypothesis that simultaneous protein immunostaining would contribute to identify germline mutations in patients with BAT-26 stable tumors. However, our results demonstrated that combination of both
microsatellite instability testing and protein immunostaining did not provide any advantage with respect to each of these approaches individually. More important, performance of the 4 remaining markers of the Bethesda panel did not contribute to identify any additional gene mutation carrier because all tumors stable for the BAT-26 marker and showing loss of protein expression were also stable for the whole panel. Finally, the frequency of MSH2/MLH1 germline mutations observed in our study (0.9%) is very similar to that reported in other investigations using different screening strategies,\textsuperscript{13,16,47} therefore, arguing against the possibility of underestimating the incidence of HNPCC as a result of the screening method used. However, some geographical differences may exist, as noted by the relatively low frequency of MSH2/MLH1 gene carriers among patients fulfilling the Amsterdam criteria in our study, similar to the previously reported studies in Spain.\textsuperscript{48,49} Taking into account all these considerations, evaluation of the BAT-26 marker alone seems to be a simple, rapid, and reliable method to screen for microsatellite instability when the final goal is to select patients who should be tested for MSH2/MLH1 germline defects.

In the last few years, it has been demonstrated that colorectal cancer surveillance of HNPCC gene carriers is effective and considerably less costly than no colorectal cancer surveillance.\textsuperscript{50} In addition, screening patients with newly diagnosed colorectal cancer using the original Bethesda guidelines and microsatellite instability testing to drive subsequent genetic testing for HNPCC is cost-effective, especially if the benefits to the patients’ immediate relatives are considered.\textsuperscript{51} However, there is very little information with respect to other strategies.\textsuperscript{52} In that sense, no study has evaluated the efficiency of the revised Bethesda guidelines or compared microsatellite instability testing and protein immunostaining in terms of cost-effectiveness for the identification of MSH2/MLH1 germline mutations. Our results suggest that clinical selection of patients according to the revised Bethesda guidelines along with either microsatellite instability testing or immunostaining was more efficient than any of these approaches separately. However, because of the noteworthy variations in costs of medical procedures among countries and health systems as well as the minimal difference observed, the superiority of immunostaining vs microsatellite instability testing cannot be clearly established.

In conclusion, our results demonstrate that the revised Bethesda guidelines constitute a very useful approach to select patients at risk for HNPCC. In patients fulfilling these criteria, both microsatellite instability testing and protein immunostaining are equivalent and highly cost-effective strategies to further select those patients who should be tested for MSH2/MLH1 germline mutations. Considering this equivalence and that immunostaining is more available than DNA analysis in a clinical setting, the use of immunohistochemistry may help identify a larger proportion of patients with HNPCC.

**Author Contributions:** Dr Castells had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

- **Study concept and design:** Piñol, Castells, Andreu, Llor, Payá.
- **Acquisition of data:** Piñol, Castells, Andreu, Llor, Xicola, Rodríguez-Moranta, Jover, Bessa.
- **Analysis and interpretation of data:** Piñol, Castells, Andreu, Castellvi-Bel, Alenda, Rodríguez-Moranta, Jover, Bessa.
- **Drafting of the manuscript:** Piñol, Castells, Andreu, Xicola, Rodríguez-Moranta, Jover, Bessa.

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