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

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HEREDITARY NONPOLYPOSIS COLORECTAL CANCER (HNPCC), also named Lynch syndrome, is an autosomal dominant disorder that accounts for approximately 1% to 3% of all colorectal cancers. HNPCC is caused by germline mutations in DNA mismatch repair genes, mainly MSH2 and MLH1. Defects on this pathway lead to changes in the length of nucleotide repeat sequences of tumor DNA, termed microsatellite instability.

Although a great advance in the understanding of the molecular basis of HNPCC has taken place in the last decade, optimal selection of individuals for HNPCC genetic testing remains controversial. In 1991, the International Gastrointestinal Association of the Spanish Society of Gastroenterology (EAIG) and the Spanish Association of Cancer Prevention (AEPC) recommended a cascade, optimal selection of individuals for HNPCC has taken place in the last decade. A prospective, multicenter, nationwide study (the EPICOLON study) in 20 hospitals in the general community in Spain of 1222 patients with newly diagnosed colorectal cancer between November 1, 2000, and October 31, 2001.

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). Germ-line testing identified 11 mutations (0.9%) in either microsatellite instability (n=8) or loss of protein expression (n=81). Germ-line 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 previous selection of patients according to the revised Bethesda guidelines were the most 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.
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 the 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 with HNPCC who should be tested for microsatellite instability and/or 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 these 2 proteins is associated with microsatellite instability, but this association is not without exceptions. Indeed, a mutant protein product can be expressed and detected by immunostaining, whereas germline mutations may occur in patients with microsatellite instability-negative tumors. These conflicting results have precluded the establishment of a unique method for primary screening of mismatch repair deficiency. Specific characteristics of performed studies may have contributed to this fact because most of the studies were performed in selected high-risk populations. Different criteria were used to define the microsatellite instability status, immunohistochemical evaluation was not performed systematically, or results were not referred to the presence of germline mutations.

Our prospective, multicenter, nationwide study was aimed at establishing the most effective and efficient strategy for the detection of MSH2 or MLH1 gene carriers in patients with newly diagnosed colorectal cancers. For this purpose, performance characteristics of both microsatellite instability testing and MSH2/MLH1 protein immunostaining, either directly or previous selection of patients according to the revised Bethesda guidelines, were evaluated with respect to the presence of MSH2 or MLH1 germline mutations. A logistic regression analysis was also performed to determine independent predictors of MSH2/MLH1 mutations.

**METHODS**

Between November 1, 2000, and October 31, 2001, all patients with newly diagnosed colorectal cancers in 25 hospitals were included in the EPICOLON study, a clinical epidemiology survey aimed at establishing the incidence of HNPCC in Spain. Twenty of the 25 centers agreed to participate in a nested molecular epidemiology study, which required tissue sample collection. Exclusion criteria were familial adenomatous polyposis, personal history of inflammatory bowel disease, and patient or family refusal to participate in the study. The study was approved by the institutional ethics committee of each participating hospital, and written informed consent was obtained from all patients.

Demographic, clinical, and tumor-related characteristics of probands, as well as a detailed family history were obtained using a preestablished questionnaire. Personal parameters at baseline included date and place of birth, sex, smoking history, personal history of neoplasia (ie, HNPCC-related tumors and colorectal adenomas), date of colorectal cancer diagnosis, presenting symptoms, serum carcinoembryonic antigen concentration, presence of synchronous colorectal neoplasms, and treatment. Tumor-related parameters included location, size, pathology TNM stage, degree of differentiation, presence of lymphocytic infiltration, and mucinous carcinoma type (defined by the presence of >50% mucinous carcinoma cells). Synchronous lesions were assessed through colonoscopy before or immediately after surgery, as well as through systematic review of the resected specimen. Pedigrees were traced backward and laterally as far as possible, or at least up to second-degree relatives, regarding cancer history. Age at cancer diagnosis, type, location, and tumor stage of the neoplasm, and current status were registered for each affected family member. As a check on overreporting of disease, all patients were queried about the occurrence of stroke in their relatives to assess possible recall bias. Furthermore, an effort was made to verify reported neoplasia in relatives by obtaining medical records and pathology confirmation.

Microsatellite instability testing and 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
isolated using the QiaAmp Tissue Kit (Qiagen, Courtaboeuf, France).

Microsatellite instability status was assessed using BAT-26 mononucleotide marker based on its high sensitivity. 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, D5S346, D2S123, and D17S250) were also evaluated. Primers were labeled fluorescently and amplified 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. 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 al 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 al 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 the 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 al and Wijnen et al) 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%) responded to missense mutations. Although all 3 mutations have been previously reported as deleterious, 1 tumor

<table>
<thead>
<tr>
<th>Table 1. Characteristics of Patients With Colorectal Cancer (N=1222)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Characteristic</strong></td>
</tr>
<tr>
<td>Age, mean (SD), y</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Site of tumor relative to splenic flexure</td>
</tr>
<tr>
<td>Degree of differentiation</td>
</tr>
<tr>
<td>Mucinous carcinoma type</td>
</tr>
<tr>
<td>Fulfillment of guidelines</td>
</tr>
</tbody>
</table>

*Stage I indicates tumor 1-2 (T1-2), no regional lymph node metastasis, and no metastasis; stage II, T3-4, no regional lymph node metastasis, and no metastasis; stage III, any tumor, node 1-3, and no metastasis; and stage IV, any tumor, any node, and distant metastasis.
†Well differentiated tumors resemble identifiable tissue types by the expression of cell markers or by extremely focal and subtle histologic and/or cytologic findings.
‡For an explanation of Amsterdam criteria, see Vasen et al; extended Amsterdam II criteria, see Vasen et al; original Bethesda guidelines, see Rodriguez-Bigas et al; and revised Bethesda guidelines, see Umar et al.

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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 $13 387] 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 or second-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 referring Bethesda guidelines.

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>Sensitivity</td>
<td>Specificity</td>
<td>Positive Predictive Value</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>

Abbreviation: MSI, microsatellite instability.

aP < .001 (McNemar test) with respect to the corresponding paired strategy without previous selection of patients according to the revised Bethesda guidelines.
vised Bethesda guidelines (OR, 13.7; 95% CI, 1.47-125; \( P = .02 \)).

**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, \(^{35-38}\) identification of MSH2/MLH1 gene carriers has become a critical and difficult issue. \(^{38}\) Currently, several approaches including the use of different sets of clinical criteria and methods for determining mismatch repair deficiency (ie, micro-

**Table 4. Predictors of MSH2/MLH1 Germline Mutations (Univariate Analysis)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%) of Patients</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) 151 (12.5)</td>
<td>.001 3.8</td>
<td></td>
</tr>
<tr>
<td>Colorectal or endometrial cancer in ( \geq 1 ) first-degree relative</td>
<td>6 (54.5) 180 (14.8)</td>
<td>.003 3.2</td>
<td></td>
</tr>
<tr>
<td>Personal history of colorectal or endometrial cancer†</td>
<td>4 (36.4) 90 (7.4)</td>
<td>.007 4.3</td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer diagnosed at age &lt; 50 y</td>
<td>3 (27.3) 55 (4.5)</td>
<td>.01 5.2</td>
<td></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‡</td>
<td>8 (72.7) 306 (25.3)</td>
<td>.001 2.5</td>
<td></td>
</tr>
<tr>
<td>Fulfillment of Amsterdam criteria †</td>
<td>4 (36.4) 14 (1.1)</td>
<td>&lt; .001 22.2</td>
<td></td>
</tr>
<tr>
<td>Endometrial cancer in ( \geq 1 ) family member</td>
<td>4 (36.4) 63 (5.2)</td>
<td>.002 5.9</td>
<td></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]</td>
<td>7 (63.6) 156 (12.9)</td>
<td>&lt; .001 4.3</td>
<td></td>
</tr>
<tr>
<td>Synchronous, metachronous colorectal, or other HNPCC-related cancer¶</td>
<td>5 (45.5) 98 (8.1)</td>
<td>.001 4.9</td>
<td></td>
</tr>
<tr>
<td>Colorectal or other HNPCC-related cancer¶ and/or a colorectal adenoma in ( \geq 1 ) first-degree relative, 1 of the cancers diagnosed at age &lt; 50 y, and the adenoma diagnosed at age &lt; 40 y</td>
<td>4 (36.4) 61 (5.0)</td>
<td>.002 6.2</td>
<td></td>
</tr>
<tr>
<td>Colorectal or endometrial cancer† diagnosed at age &lt; 50 y</td>
<td>3 (27.3) 78 (6.4)</td>
<td>.03 3.7</td>
<td></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) 5 (0.4)</td>
<td>.05 16.7</td>
<td></td>
</tr>
<tr>
<td>Signet-ring cell-type colorectal cancer diagnosed at age &lt; 50 y</td>
<td>1 (9.1) 14 (1.1)</td>
<td>.13 6.7</td>
<td></td>
</tr>
<tr>
<td>Fulfillment of Bethesda guidelines‡</td>
<td>8 (72.7) 216 (17.8)</td>
<td>&lt; .001 3.6</td>
<td></td>
</tr>
<tr>
<td>Synchronous, metachronous colorectal, or other HNPCC-related tumors,¶ regardless of age</td>
<td>5 (45.5) 99 (8.2)</td>
<td>.001 4.8</td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer with the MSI-high histology** diagnosed at age &lt; 60 y</td>
<td>3 (27.3) 52 (4.3)</td>
<td>.01 5.4</td>
<td></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) 57 (4.7)</td>
<td>.001 6.6</td>
<td></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) 83 (6.8)</td>
<td>.001 5.7</td>
<td></td>
</tr>
<tr>
<td>Fulfillment of revised Bethesda guidelines‡</td>
<td>10 (90.9) 277 (22.9)</td>
<td>&lt; .001 3.5</td>
<td></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.
‡Combination of individual variables according to the original sets of recommendations. \(^{7,9,11,14}\)
§Criteria suggested by Aaltonen et al. \(^{10}\)
¶Including endometrial, ovarian, gastric, hepatobiliary, small-bowel, or urinary tract cancer.
**Criteria suggested by Wijnen et al. \(^{14}\)
††Including endometrial, ovarian, gastric, hepatobiliary, small-bowel, urinary tract, pancreatic, and brain cancer, sebaceous gland adenomas, and keratoacanthomas.
**G**

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satellite instability testing and immunostaining) are being used and there is no unique and universally accepted strategy.\(^4\) Although it can be anticipated that epidemiological diversity among geographical areas and technical disparity among laboratories\(^33\) 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.\(^40\) 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 screening strategy,\(^3,41\) immunostaining has been proposed as an alternative approach.\(^2,17-23\) 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.\(^21\) 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.\(^2\) In addition, immunostaining is the method of choice to direct the search for germline mutations,\(^3\) 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.\(^2,13\) Finally, results of recent investigations suggest that systematic evaluation of other mismatch repair proteins, such as MSH6\(^22,26\) and PMS2,\(^26\) 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,\(^23,42-44\) it has also been reported in healthy controls.\(^13\) 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.\(^19\) 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.\(^9\) The use of this single marker, in a similar manner as it has been performed in previous investigations,\(^10,11,13\) was justified by its high sensitivity, which ranged between 93% and 97%.\(^30,33\) 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.\(^10,33\) In addition, Loukola et al\(^11\) 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).\(^23\) 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

<p>| Table 5. Performance Characteristics of Clinical Criteria for the Identification of MSH2/MLH1 Gene Mutation Carriers (Multivariate Analysis)* |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Strategy</th>
<th>Performance Characteristic, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fulfillment of clinical criteria</td>
<td>Sensitivity 100</td>
</tr>
<tr>
<td>Fulfillment of clinical criteria and presence of MSI</td>
<td>Specificity 70.3</td>
</tr>
<tr>
<td>Fulfillment of clinical criteria and loss of protein expression</td>
<td>Positive Predictive Value 2.9</td>
</tr>
<tr>
<td>Fulfillment of clinical criteria and presence of MSI or loss of protein expression</td>
<td>Negative Predictive Value 100</td>
</tr>
<tr>
<td>Fulfillment of clinical criteria, presence of MSI, and loss of protein expression</td>
<td>Accuracy 70.5</td>
</tr>
</tbody>
</table>

Abbreviation: MSI, microsatellite instability.\(^*\) Based on the combination of the revised Bethesda guidelines and family history of colorectal cancer diagnosed in at least 1 first-degree relative.
microsatellite instability testing and protein immunostaining did not provide any advantage with respect to each of these approaches individually. More importantly, 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{13,56,67} 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 found in our study, similar to the previously reported studies in Spain.\textsuperscript{48,49}

In that sense, no study has considered.\textsuperscript{51} However, there is very little cost-effective, especially if the benefits to 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-Morant, Jover, Bessa. Analysis and interpretation of data: Piñol, Castells, Andreu, Castellvi-Bel, Alenda, Rodríguez-Moranta, Jover, Bessa. Drafting of the manuscript: Piñol, Castells, Andreu, Llor, Payá. Critical revision of the manuscript for important intellectual content: Piñol, Castells, Andreu, Castellvi-Bel, Alenda, Llor, Xicola, Rodríguez-Moranta, Payá, Jover, Bessa. Statistical analysis: Castells. Obtained funding: Castells, Llor. Administrative, technical, or material support: Andreu, Llor, Bessa. Study supervision: Piñol, Castells, Andreu, Castellvi-Bel, Alenda, Llor, Xicola, Rodríguez-Moranta, Jover, Bessa.

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