Original articles

Sensitivity and specificity of clinical criteria for hereditary non-polyposis colorectal cancer associated mutations in *MSH2* and *MLH1*

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Abstract

***Background and aims*—There are multiple criteria for the clinical diagnosis of hereditary non-polyposis colorectal cancer (HNPCC). The value of several of the newer proposed diagnostic criteria in identifying subjects with mutations in HNPCC associated mismatch repair genes has not been evaluated, and the performance of the different criteria have not been formally compared with one another.**

***Methods*—We classified 70 families with suspected hereditary colorectal cancer (excluding familial adenomatous polyposis) by several existing clinical criteria for HNPCC, including the Amsterdam criteria, the Modified Amsterdam criteria, the Amsterdam II criteria, and the Bethesda criteria. The results of analysis of the mismatch repair genes *MSH2* and *MLH1* by full gene sequencing were available for a proband with colorectal neoplasia in each family. The sensitivity and specificity of each of the clinical criteria for the presence of *MSH2* and *MLH1* mutations were calculated.**

***Results*—Of the 70 families, 28 families fulfilled the Amsterdam criteria, 39 fulfilled the Modified Amsterdam Criteria, 34 fulfilled the Amsterdam II criteria, and 56 fulfilled at least one of the seven Bethesda Guidelines for the identification of HNPCC patients. The sensitivity and specificity of the Amsterdam criteria were 61% (95% CI 43-79) and 67% (95% CI 50-85). The sensitivity of the Modified Amsterdam and Amsterdam II criteria were 72% (95% CI 58-86) and 78% (95% CI 64-92), respectively. Overall, the most sensitive criteria for identifying families with pathogenic mutations were the Bethesda criteria, with a sensitivity of 94% (95% CI 88-100); the specificity of these criteria was 25% (95% CI 14-36). Use of the first three criteria of the Bethesda guidelines only was associated with a sensitivity of 94% and a specificity of 49% (95% CI 34-64).**

***Conclusions*—The Amsterdam criteria for HNPCC are neither sufficiently sensitive nor specific for use as a sole criterion for determining which families should undergo testing for *MSH2* and *MLH1* mutations. The Modified Amsterdam and the Amsterdam II criteria increase sensitivity, but still miss many families with mutations. The most sensitive clinical criteria for identifying subjects with pathogenic *MSH2* and *MLH1* mutations were the Bethesda Guidelines; a streamlined version of the Bethesda Guidelines may be more specific and easier to use in clinical practice.**

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Hereditary non-polyposis colorectal cancer (HNPCC), the most common hereditary colon cancer syndrome, is a dominantly inherited disorder associated with increased lifetime risks of a range of cancers including colorectal and endometrial as well as extracolonic gastrointestinal, urinary tract, ovarian, and brain cancers.[1] HNPCC has been associated with germline mutations in several DNA mismatch repair genes, including *MSH2*, *MLH1*, *MSH6*, *PMS1*, and *PMS2*.[2–8] Of these, *MSH2* and *MLH1* mutations are the most common and testing is currently commercially available for only these two genes.

Colon cancers from patients with HNPCC are characterised by expansion or contraction of short repeat sequences of DNA (microsatellites) at multiple loci.[9–12] This phenomenon, known as microsatellite instability (MSI), is thought to be responsible for the rapid accumulation of somatic mutations in oncogenes and tumour suppressor genes that have crucial roles in initiation and progression of tumours.[13–16] Because the effort and cost of MSI analysis is less than mutation analysis, MSI screening has been suggested as a cost effective way to identify subjects with a greater likelihood of carrying mutations in mismatch repair genes.[16 17]

Table 1 Clinical criteria for HNPCC

Name	*Criteria*
Amsterdam*[18]	Three relatives with colorectal cancer (CRC), one of whom is a first degree relative of the other two; CRC involving at least two generations; one or more CRC cases diagnosed before the age of 50
Modified Amsterdam†[20 24]	(1) Very small families, which cannot be further expanded, can be considered as HNPCC even if only two CRCs in first degree relatives; CRC must involve at least two generations, and one or more CRC cases must be diagnosed under age 55. or (2) In families with two first degree relatives affected by colorectal cancer, the presence of a third relative with an unusual early onset neoplasm or endometrial cancer is sufficient.
Amsterdam II[21]	Three relatives with an HNPCC associated tumour (CRC, endometrial, small bowel, ureter, or renal pelvis), one of whom is a first degree relative of the other two; involving at least two generations; one or more cases diagnosed before the age of 50
Bethesda†[19]	(1) Subjects with cancer in families that fulfil Amsterdam criteria (2) Subjects with two HNPCC related cancers, including synchronous and metachronous CRCs or associated extracolonic cancers (3) Subjects with CRC and a first degree relative with colorectal cancer and/or HNPCC related extracolonic cancer and/or colorectal adenoma; one of the cancers diagnosed at age <45 years and the adenoma diagnosed at age <40 years (4) Subjects with CRC or endometrial cancer diagnosed at age <45 years (5) Subjects with right sided CRC with an undifferentiated pattern (solid/cribiform) on histopathology diagnosed at age <45 years (6) Subjects with signet ring cell type CRC diagnosed at age <45 years (7) Subjects with adenomas diagnosed at age <40 years

*All criteria must be met.
†Meeting all features listed under any of numbered criteria is sufficient.

Several clinical diagnostic criteria have been proposed to define HNPCC. The classical criteria, proposed by the International Collaborative Group and known as the Amsterdam criteria,[18] have been the most widely used in the research setting, but have been criticised as being too stringent for use in clinical practice.[19] Several additional criteria have been proposed for the diagnosis of HNPCC, including the Modified Amsterdam criteria[20] and the Bethesda Guidelines.[19] Most recently, the International Collaborative Group proposed its own modification of the Amsterdam criteria, which they termed Amsterdam II.[21] Each of the existing criteria try to address a variety of issues relevant to the diagnosis of HNPCC; for example, the Modified Amsterdam criteria were designed to take into account small families and a wide variety of HNPCC extracolonic tumours, and the Bethesda Guidelines' goals were to guide who should undergo MSI analysis.

The value of several of the newer proposed diagnostic criteria in identifying subjects with mutations in *MSH2* and *MLH1* has not been evaluated, and the performance of the different criteria have not been formally compared with one another. We compared the sensitivity and specificity of the various existing clinical criteria for HNPCC in identifying and excluding mutations of *MSH2* and *MLH1* in a cohort of 70 families with features of hereditary colorectal cancer.

Materials and methods

SUBJECTS

Families were identified by self or health care provider referral and were enrolled on the basis of multiple cases of CRC, early age of CRC diagnosis, or the familial association of CRC with other HNPCC associated tumours, as previously described.[22] Informed consent was obtained from each participant. 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.

For this analysis, we classified each pedigree by whether they fulfilled the Amsterdam,[18] Modified Amsterdam,[20] and the Amsterdam II criteria (table 1). For each pedigree, the designated proband, who had developed early onset colorectal neoplasia, was evaluated according to the recently proposed Bethesda Guidelines for the identification of HNPCC patients (table 1).[19]

MSH2/*MLH1* SEQUENCING

Genetic analysis was performed on a specimen from the proband in each family. Genomic DNA was isolated from peripheral blood using a Midi kit from Qiagen. Each of the exons plus some flanking intron from the *MSH2* and *MLH1* genes was amplified by the polymerase chain reaction. (Primer sequences and conditions can be provided on request.) Sequencing reactions were assembled on a Packard Multiprobe robot. The resulting fluorescent sequencing products were analysed on a semi-automated sequencing apparatus (Model 377 Perkin-Elmer Applied Biosystems Division, Foster City, CA).

STATISTICAL ANALYSES

Sequencing results were classified into (1) definitive disease causing mutation, (2) mutation of unknown significance, or (3) no mutation.[22] The clinical diagnosis of HNPCC using each of the defined criteria were used as tests in two by two contingency tables to determine the sensitivity (the proportion of patients with a mutation in *MSH2* or *MLH1* who met each of the clinical criteria for HNPCC) and the specificity (the proportion of patients who did not have a mutation in the *MSH2* or *MLH1* who did not fulfil the respective clinical criteria for HNPCC). Associated 95% confidence intervals were calculated for each of the estimates of sensitivity and specificity. In addition, we evaluated the strengths and weaknesses of each of the criteria by analysing the clinical characteristics of the families with *MSH2* and *MLH1* mutations that were identified and missed by each of the respective criteria.

Results

A total of 70 families were enrolled, representing 297 CRCs and 364 other cancer diagnoses. Clearly deleterious mutations were found in

Table 2 Sensitivity and specificity of clinical criteria for identifying kindreds with pathogenic hMSH2 or MLH1 mutations

	No of families with mutations in MSH2 or MLH1	*% Families fulfilling criteria (n=70)*	*% Families with mutations missed by criteria (n=18)*	*Sensitivity of criteria (95% CI)*	*Specificity of criteria (95% CI)*
Amsterdam	11	40 (28)	39 (7)	61% (43%, 79%)	67% (50%, 85%)
Modified Amsterdam	13	56 (39)	28 (5)	72% (58%, 86%)	50% (34%, 66%)
Amsterdam II	14	49 (34)	22 (4)	78% (64%, 92%)	61% (45%, 78%)
Bethesda	17	80 (56)	6 (1)	94% (88%, 100%)	25% (14%, 36%)
Bethesda (1–3)	17	63 (44)	6 (1)	94% (88%, 100%)	49% (34%, 64%)

18/70 (25.7%) of families. An additional five families were found to have missense mutations of unclear significance despite supplementary analyses to determine their pathogenicity.[22]

Twenty eight families fulfilled the Amsterdam criteria, 39 fulfilled the Modified Amsterdam Criteria, 34 fulfilled the Amsterdam II criteria, and 56 fulfilled at least one of the seven Bethesda Guidelines for the identification of HNPCC patients (table 2).

The sensitivity and specificity of the Amsterdam, Modified Amsterdam, Amsterdam II, and Bethesda criteria for detecting families with definitive mutations and excluding those without mutations were calculated (table 2). The sensitivity and specificity of the Amsterdam criteria were 61% (95% CI 43-79) and 67% (95% CI 50-85), respectively. The sensitivity of the Modified Amsterdam and Amsterdam II criteria were 72% (95% CI 58-86) and 78% (95% CI 64-92), respectively. Overall, the most sensitive criteria for identifying families with pathogenic mutations were the Bethesda criteria, with a sensitivity of 94% (95% CI 88-100); the specificity of these criteria was 25% (95% CI 14-36).

The results above represent analysis using only definitive pathogenic mutations, as these are the only genetic test results which would be used to make clinical recommendations. Although at the current time we were not able to determine conclusively the pathogenicity of the missense mutations, many or all of them may ultimately prove to be pathogenic. To determine the impact of this scenario, the analysis was repeated including the missense mutations as being pathogenic. There was no significant change in the results of the two analyses; the sensitivity and specificity of each of the criteria varied by less than 5%.

The ideal clinical criteria would identify all families with mutations (have 100% sensitivity) and test the fewest families without mutations (100% specificity). We thus evaluated our cohort to determine which families were found to have mutations but were not identified by one or more clinical criteria to have HNPCC (table 3). As expected, the Amsterdam criteria missed the most families with mutations; 39% of families would not have been identified if genetic testing were limited to Amsterdam families alone. The Amsterdam II criteria identified more families with mutations than the Modified Amsterdam criteria, while being more specific. However, the Amsterdam II criteria still missed 22% of mutations.

Evaluation of table 3 indicates that there was significant overlap in the families not identified by the Amsterdam, Amsterdam II, and Modified Amsterdam criteria primarily because of the requirement of a cluster of three relatives with cancer, where one is a first degree relative

Table 3 Cases of discordance between clinical criteria and the presence of MSH2 or MLH1 mutations

Proband No	*Clinical description*	*Criteria that did not identify family*	*Reason*
DF2579	Proband with colon (37) and stomach (49); mother with ovarian (48); maternal 1st cousin with colon (40); first cousin's children with pancreas (20s) and colon (30s)	Ams	3 colon cancers not in first degree relatives; extracolonic tumours not counted
DF1851	Proband with 2 breast primaries (37,44); proband's mother with ovarian, colon, breast (42,49,64); maternal aunt with breast (60s); maternal uncle with colon (42); maternal grandmother with endometrial 46	Ams Beth	Absence of 3 colon cancers (Ams); proband does not have colorectal neoplasia (Beth)
DF268	Proband with adenomas (36); brother with colon (28); paternal aunt with colon (51); paternal grandmother with ovarian (40)	Ams Mod Ams Ams II	Absence of 3 colon cancers (Ams); 3 relatives with cancer not connected by 1st degree (Mod Ams, Ams II)
DF1754	Proband with colon (44); mother with TCC of renal pelvis (69) and ureter and stomach (70); maternal aunt with ovarian (60), cholangiocarcinoma (60); maternal grandfather with stomach (41)	Ams Mod Ams Ams II	Lack of 3 colon cancers (Ams); lack of 2 colon cancers (Mod Ams); extracolonic tumours different from those included in criteria (Ams II)
DF951	Proband with 2 colon primaries (38,51) and endometrial (40); 2 sisters and mother with endometrial	Ams Mod Ams	Lack of 3 colon cancers (Ams); lack of 2 colon cancers (Mod Ams)
DF1251	Proband with 5 colon primaries (26); mother with duodenal and jejunal primaries (46); sister with adenomas (36,38)	Ams Mod Ams Ams II	Lack of 3 subjects with colon cancer (Ams); adenomas not counted (Ams, Mod Ams, Ams II)
DF357	Proband with 2 colon primaries (33,34)	Ams Mod Ams Amst II	Requirement for multiple affected subjects, synchronous tumours not counted (Ams, Mod Ams, and Ams II)

Ams = Amsterdam; Mod Ams = Modified Amsterdam; Ams II = Amsterdam II; Beth = Bethesda.

of the other two. The Amsterdam criteria missed seven families; none of these seven families had three family members with colon cancer, where one was a first degree relative of the other two. The Modified Amsterdam criteria missed five families, four families because of the requirement for at least two colon cancers and one because the three relatives with HNPCC associated tumours were not connected by a first degree relative. The Amsterdam II criteria missed four families, two families because of the absence of three subjects with cancer, one because the three subjects were not connected by a first degree relative, and the fourth because the extracolonic tumours in the family (which included ovarian, cholangiocarcinoma, and gastric) were not included as HNPCC tumours in the Amsterdam II criteria.

Only one family was missed by the Bethesda criteria. In this family, the proband (DF1851) had two breast cancers at ages 37 and 44, a tumour not classically a component of HNPCC. Other members of the family, however, did have features suggestive of HNPCC, including early onset colon, endometrial, and ovarian cancers.

As the Bethesda Guidelines were also the least specific criteria and would require the most families to undergo genetic analysis, we then examined the families identified by the Bethesda Guidelines that were found not to have mutations. Results of this analysis showed that there were no mutations in subjects fulfilling only items 4-7 of the Bethesda Guidelines (table 1). That is, no mutations were found in 12 subjects with isolated young onset colorectal cancer, adenomas, or endometrial cancer without other features, such as synchronous or metachronous tumours, or at least one other affected first degree relative with colorectal cancer or adenoma. Not including the pedigrees with isolated colorectal or endometrial neoplasia in the analysis maintained the sensitivity of the Bethesda criteria at 94%, but improved the specificity to 49%. The prevalence of mutations if testing for *MSH2* and *MLH1* were limited to Bethesda Guidelines 1-3 was 39% (17/44), similar to that of the Amsterdam criteria.

Discussion

The definition of HNPCC has been debated for some time owing to the variety of clinical phenotypes associated with the syndrome. The elucidation of the mismatch repair genes that are the genetic basis for many HNPCC families has added another level of complexity for the clinician. Now, one must decide not only how to make the diagnosis of HNPCC, but also which patients should undergo genetic testing. Despite the numerous available clinical criteria proposed for HNPCC, none has been systematically analysed for their performance characteristics as would be expected for most diagnostic tests. Our study was instructive in several respects regarding the use of published criteria in the clinical setting.

First, our analysis confirms what has been suggested by others about the Amsterdam criteria. Although the Amsterdam criteria have been extremely successful in achieving their original purpose of providing a common nomenclature for the HNPCC syndrome for research purposes, using these criteria in the clinical realm must be done with extreme caution. Their limited sensitivity for identifying families with *MSH2* and *MLH1* mutations make it inappropriate to use the Amsterdam criteria as the sole criteria in choosing which patients should undergo genetic testing. Clearly, additional clinical cues must be used both for decisions regarding genetic testing and for making the clinical diagnosis of HNPCC. In addition, the limited specificity of even these most restrictive criteria highlight the importance of the role of additional genes causing HNPCC. Owing to the fact that 40% of families fulfilling the Amsterdam criteria did not have *MSH2* or *MLH1* mutations, and that mutations in other known mismatch repair genes *hPMS1, hPMS2*, and *hMSH6* have been rare, it is most likely that additional genes causing a substantial portion of HNPCC remain to be discovered.

The Amsterdam II and Modified Amsterdam criteria both attempt to resolve some of the deficiencies of the Amsterdam criteria. These two criteria differ in relatively minor ways: the Amsterdam II criteria require a cluster of three neoplasms where one subject must be a first degree relative of the other two, but the three neoplasms can be any of colorectal, endometrial, small bowel, or renal pelvis tumours. The Modified Amsterdam criteria are less restrictive regarding the type of extracolonic tumours (which are only defined as "endometrial or unusual early onset neoplam"), but require a minimum of two colorectal cancers in a family. The Modified criteria also increased the age limit of CRC to 55. We found that although both these criteria did pick up some additional families with mutations, their sensitivities were still limited to less than 80%.

Our analysis indicated that the most sensitive clinical criteria for identifying subjects with pathogenic mutations of the mismatch repair genes *MSH2* and *MLH1* were the Bethesda Guidelines. The Bethesda Guidelines were developed for the identification of tumours that should be tested for microsatellite instability, to aid in detecting families who are not identified by the Amsterdam criteria.[19] We found several main differences between the Bethesda Guidelines and the Modified Amsterdam and Amsterdam II criteria. While all three of these criteria are inclusive of Amsterdam criteria families, the Bethesda criteria made three major modifications that led to a sensitivity of 94%: (1) the allowance of two HNPCC synchronous or metachronous tumours as a sole criterion for diagnosis; (2) the inclusion of early onset (age <40) adenomas as one of the HNPCC tumours; and (3) the need for only two first degree relatives with CRC or adenomas, as long as one is early onset.

As expected, greater sensitivity of the Bethesda guidelines was achieved at the expense of decreased specificity (25%, the low-

est of all criteria evaluated). However, in a secondary analysis, we determined that there were no *MSH2* or *MLH1* mutations in those subjects with isolated sporadic colorectal tumours without other features of HNPCC, such as synchronous or metachronous tumours or other affected first degree relatives with HNPCC cancers. Not including these families in the analysis maintained the sensitivity of the Bethesda Guidelines to 94%, but increased the specificity from 25% to 49%.

Based on the results of our study, we propose that the Bethesda Guidelines be used in clinical practice as the most inclusive clinical criterion for the diagnosis of HNPCC and consideration of genetic analysis. The Bethesda Guidelines were proposed to target who should undergo tumour MSI analysis. However, the use of MSI testing in clinical practice has some major practical obstacles: MSI testing as a routine commercial clinical laboratory test is not widely available and tumour blocks are often difficult to obtain/analyse owing to logistical and technical difficulties.[23] The prevalence of *MSH2* and *MLH1* mutations if only criteria 1-3 of the Guidelines were used was 39%. Therefore, it would be reasonable for subjects whose histories fulfil Bethesda Guidelines 1-3 to proceed directly to sequencing of *MSH2* and *MLH1* and use MSI as a screening tool for those much less likely to carry mutations, such as the isolated young onset sporadic tumours.

It is important to consider several limitations of our study. First, our results pertain only to *MSH2* and *MLH1* mutations. We chose to limit our analysis to these two genes as they are by far the most frequently associated with HNPCC, and are the only ones currently commercially available to clinicians. As more associated genes are identified and become available, similar analyses to ours should be performed to identify optimally the families who should undergo genetic analysis. Second, our results apply only to cohorts ascertained from populations similar to ours. Our study sample is typical of the patient population presenting to cancer genetics programmes and represents a group selected to have a relatively high prior probability of carrying a predisposing mutation. Finally, we did not consider other screening techniques, such as microsatellite instability analysis or immunohistochemistry, as potential methods of screening families before full gene sequence analysis. Further studies formally evaluating the role of such techniques in the diagnostic algorithm of HNPCC families are needed to refine further the most optimal, feasible, and cost effective diagnostic approach to families suspected of having HNPCC.

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