Value of microsatellite instability typing in detecting hereditary non-polyposis colorectal cancer

A prospective multicentric study by the Association Aquitaine Gastro

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SUMMARY

Aim of the study — To detect hereditary non-polyposis colorectal cancer (HNPCC) patients with a strategy combining clinical selection (patient age at onset of cancer less than 50 years or family history of HNPCC tumors) and microsatellite instability typing plus immunohistochemistry, leading to mismatch repair (MMR) germline mutation analysis.

Methods — Tumors were screened for microsatellite instability (MSI) and for hmlh1 and hmsh2 immunohistochemical expression. Germ-line mutation analysis was performed to search for MLH1 and MSH2 mutations in patients with MSI-High and MSI-Low tumors.

Results — 197 adenocarcinomas were studied: 164 patients were ≤ 50 years old, 33 were older than 50 years but had a family history of HNPCC tumors. Fifty tumors (25.4%) were MSI-High, 10 were MSI-Low (5.1%), and 130 were MS-Stable (66%). MSI typing was inconclusive in 7 (3.5%). Immunohistochemistry screening was performed on 165 tumors: sensitivity was 63.6%, specificity was 99%. Germline mutation analysis was performed in 33/60 MSI-High or MSI-Low tumors: 23 mutations were noted (70% of the tested patients).

Conclusion — This proposed strategy of determining microsatellite instability in young colorectal cancer patients or in patients with a family history of HNPCC tumors led to an increased frequency in the detection of MMR germline mutations.

RÉSUMÉ

Évaluation diagnostique de l’instabilité de microsatellites dans la détection de cancers colorectaux héréditaires sans polypose. Une étude prospective multicentrique de l’Association Aquitaine Gastro

Yves BÉCOUARN, Anne RULLIER, Philippe GORRY, Denis SMITH, Bruno RICHARD-MOLARD, Emmanuel ECHINARD, Patrick TEXEREAU, Richard BEYSSAC, Jean-Louis LEGOUX, Hervé LAMOULIATTE, Thierry FREBOURG, Sylviane OLSCHWANG, Brigitte GILBERT, Laurence VENAT, Véronique PICOT, François PARAF, Michel LONGY

Objectifs — Identifier des tumeurs survenant dans le cadre d’un syndrome HNPCC, à l’aide d’une démarche stratégique reposant sur : 1) la sélection clinique des malades (survenue d’un cancer avant 50 ans ou antécédents familiaux) ; 2) un typage allélique microsatellitaire couplé à une étude immunohistochimique, permettant de poser l’indication de la recherche de mutation constitutionnelle des gènes MMR.

Méthodes — Les tumeurs ont fait l’objet d’un typage allélique microsatellitaire, d’une analyse immunohistochimique hmsh2 et hmlh1 et pour les malades présentant un profil MSI-High ou Low, d’une recherche de mutation constitutionnelle des gènes MMR.

Résultats — 197 adénocarcinomes ont été sélectionnés ; 164 malades avaient moins de 50 ans, 33 avaient plus de 50 ans, mais des antécédents familiaux ; 50 tumeurs (25,4 %) se sont avérées MSI-High, 130 MSI-Low (66 %), recherche non contributive dans 7 cas (3,5 %). L’immunohistochimie a été réalisée pour 165 tumeurs : la sensibilité de la technique était de 63,6 %, la spécificité était de 99 %. La recherche de mutations constitutionnelles a pu être réalisée pour 33 des 60 MSI-High ou Low. Vingt-trois mutations ont été mises en évidence, soit dans 70 % des cas recherchés.

Conclusions — Cette stratégie d’étude du typage allélique microsatellitaire évaluée chez des malades jeunes atteints de cancers colorectaux ou ayant un contexte d’antécédents familiaux a permis de mettre en évidence des mutations des gènes MMR avec une fréquence particulièrement élevée.
Introduction

Hereditary non-polyposis colorectal cancer (HNPCC) is the most frequent form of hereditary colorectal cancer, accounting for 2 to 4% of all colorectal cancers [1-4]. Since the identification of the causal genes [5-8] formal diagnosis of HNPCC syndrome can now be established. Specific care for digestive and gynecological disorders can then be proposed to patients and their relatives using well established protocols with demonstrated efficacy [1-9,12]. Unfortunately, screening for HNPCC is still insufficient, leading to delayed diagnosis of colorectal cancer. There are several reasons: i) the clinical phenotype is not specific before the development of cancer [13]; ii) the Amsterdam criteria based on patient history and genealogy [14] recommended by the consensus conference [1] are strict and incomplete, and do not recognize nearly 30% of HNPCC families [3]; iii) diagnosis of HNPCC concerns a wide range of specialties (oncology, gastroenterology, gynecology, surgery, radiotherapy, genetics) which might contribute to insufficient knowledge of the syndrome [15]. Schematically, colorectal cancers can be divided into two groups: a) cancers associated with chromosomal instability which accounts for the majority of patients (about 85%); b) cancers associated with microsatellite instability (MSI) found in about 15% of patients, including those with HNPCC [16,17]. Systematic search for germline mismatch repair (MMR) gene mutations is technically difficult and costly. Search for microsatellite instability (MSI), characteristic of MMR defects, is an interesting alternative. MSI screening was proposed recently to identify patients with HNPCC syndrome [2,18-20]. While relatively easy to perform and more rapid and less costly than the earlier method, MSI typing remains insufficiently specific: among the 15% of patients with colorectal cancers associated with microsatellite instability, only one-quarter have HNPCC, the others presenting sporadic cancers [21] related to acquired loss of MLH1 gene expression due to hypermethylation of its promoter [22,23]. Microsatellite allelic typing to identify HNPCC tumors thus requires a supplementary criterion which could be either early onset of cancer or presence of a familial history of HNPCC tumors. Immunohistochemical detection of MMR proteins in tumor tissue is an attractive diagnostic alternative which remains to be fully assessed. In order to analyze the pertinence of this approach, we conducted a multicentric prospective study of 197 colorectal cancers older but with a familial history of colorectal cancer (familial criterion). Microsatellite instability typing and immunohistochemical study for mlh1 and mlh2 protein expression were performed [20,24,25]. When microsatellite instability was identified, genetic counseling was proposed, as well as possible germline mutation analysis to search for MMR genes.

Patients and methods

Patient selection

The study population included patients with colorectal cancer diagnosed between 1998 and 2001 who presented at least one of the following criteria: 1) age over 18 years and less than 50 years, irrespective of familial history; 2) age over 50 years with a history of colorectal cancer in a first-degree relative. Patients came from the Aquitaine and Limousin regions, recruited by members of the “Association Aquitaine Gastro”. An information chart was given to all patients who provided their informed consent for participation in the study. Data were collected, including the pathology report, and centralized to enable access to tissue samples necessary for the molecular biology and immunohistochemical studies.

Diagnosis of HNPCC

Patients whose tumor displayed microsatellite instability were considered suspect to carry MMR germline mutations. An oncogenetic consultation was proposed for these patients in order to: i) collect genealogic data; ii) obtain pathological confirmation; iii) establish an opinion concerning the genetic nature of the patient’s disease and determine which family members could benefit from genetic screening; iv) provide clear detailed information on molecular diagnosis of predisposition, together with information on the technical and ethical aspects; v) prescribe if necessary after obtaining the patient’s written consent, MMR germline mutation analysis. In agreement with current guidelines for the management of HNPCC [1], patients participating in this special screening program were scheduled for regular gastrointestinal and gynecological surveillance while waiting for the results of the genetic diagnosis.

Microsatellite allelic typing

Paraffin-embedded block of tumor and normal tissue were obtained for each patient. The DNA purification method used for such material has been previously described [26]. Briefly, after deparaffinization and hydration, the material was digested with proteinase K (10 μg/100 μL) followed by a double purification procedure using an affinity column (Wizard DNA clean-up system – Promega) and miniembrane filtration (Ultrafree MC30, Millipore). The DNA obtained was suspended in 50 μL tris-EDTA.

Microsatellite instability was performed using the method previously described [27] based on allelic typing of six mono- and dinucleotidic markers using the following markers: 4 polyA sequences: BAT25, BAT26, BAT40 [28], LmycVpA [29], and 2 polyCA sequences: D16S512, D16S515 [30].

5 μL of the DNA suspension were used for polymerase chain reaction (PCR) amplification of each marker, one of the primers being labeled with fluorochromat. The PCR products were separated by electrophoresis using an automatic sequencer (ABI prism 377, Applied Biosystems). The results were analyzed for each tumor using GeneScan 5712 (Applied Biosystems). Comparison of the DNA patterns of the normal and tumor tissues demonstrated the presence of any “neo-allele” in the tumor tissue characteristic of microsatellite instability. The following tumor classification was used:

- Microsatellite Stability (MSS) or Replication ETrors (RER) negative: stable tumor devoid of neo-alleles revealed by the markers;
- high-frequency microsatellite instability (MSI-High) or RER-positive: unstable tumor exhibiting neo-alleles revealed by at least two markers;
- low-frequency microsatellite instability (MSI-Low): intermediate tumor stability with neo-alleles revealed by only one of the markers.
- Non-contributive test: less than two interpretable markers.

This methodology differed slightly from that proposed by the National Cancer Institute (NCI) [31] since we used: a) six macrosatellite markers instead of five; b) some different markers; c) the exact number of unstable markers instead of their percentage.

Immunohistochemistry

Search for MMR proteins mlh1 and hms2h was conducted on fixed tissue embedded in paraffin and double-labeled immunohistochemistry with an autoanalyzer (Ventana ES 320, Ventana, Tuckson). After deparaffinization and rehydration, the recovered slices were submitted to heat treatment in citrate buffer (pH 7) for antigenic restoration. Anti-mlh1 antibodies (G168-15, Pharmigen International) were diluted at 1/30 and incubated for 32 min. Anti-hms2h antibodies (FE11, Oncogene Research) were diluted at 1/20 and incubated for 32 min. The reaction was amplified using the peroxidase-coupled streptavidin labeled avidin-biotin system. Nuclear labeling appeared as a brownish deposit after histoenzymatic reaction with diaminobenzidine. Labeling was interpreted as follows:

- loss of mlh1 or hms2h expression, defined as absence of nuclear labeling in tumor cells, in the presence of positive controls (basal cells from the colonic mucosa crypts and/or lymphocytes); preservation of protein expression, defined as presence of nuclear labeling in tumor cells and internal controls [27,32-34]; non-interpretable labeling, defined as absence of labeling in tumor cells and internal controls or slice detachment.

Search for MMR germline mutations

Search for point mutations of the MSH2 and MLH1 genes was performed in MSI-High and MSI-Low tumor tissues. The search for germline mutations and their characterization was based on denaturing high pressure liquid chromatography (dHPLC) screening and/or direct sequencing using an automatic sequencer (ABI Prism 377, Applied Biosystem) for
exons 1 to 16 of the MSH2 gene and exons 1 to 19 of the MLH1 gene. Each exon was submitted to PCR amplification using intronic primers on leukocyte DNA obtained from blood samples from the corresponding patients [35]. Search for large MSH2 and MLH1 gene rearrangements was performed in certain patients when point mutations were not identified. Germline mutation analysis was performed with PCR multiplex QMPSF [36].

**Statistical analysis**

Immunohistochemical results were compared with microsatellite allelic typing results. The agreement between loss of hmlh1 or hmsh2 expression and MSI-High and MSI-Low tumors was used to define sensitivity. Agreement between preservation of hmlh1 or hmsh2 expression and MSS tumors was used to define specificity.

**Results**

During the three-year period of this study, 197 colorectal adenocarcinomas were examined to search for microsatellite instability. The corresponding clinical data are summarized in table 1.

**Microsatellite allelic typing**

We observed 50 MSI-High (or RER-positive) tumors (25.4%), 10 MSI-Low tumors (5.1%), 130 MSS (or RER-negative) tumors (66%) and 7 tests which were non-contributive (3.5%).

Most of the MSI-High (RER-positive) tumors (42/50) were found in patients (32 men, 18 women) aged less than 50 years; 33 (66%) of these 42 patients did not have a familial history of colorectal cancer at inclusion. The MSI tumor was located in the rectum (N = 6), the ascending colon (N = 16), the transverse colon (N = 2) and the descending colon (N = 26, 52%).

Nine of the 10 patients (6 men, 4 women) with MSI-Low tumors were aged less than 50 years. One patient aged over 50 years had a familial history of colorectal cancer. The localizations of the MSI-Low tumors were: rectum (N = 3), transverse colon (N = 3) and descending colon (N = 3).

One hundred thirty patients (74 men, 56 women) had MSS (RER-negative) tumors. Most of them (107/130, 82.3%) were aged less than 50 years; 23/130 (17.7%) had a family history of colorectal cancer. These tumors were located in the rectum (N = 30), the ascending colon (N = 55), the transverse colon (N = 8) and the descending colon (N = 37, 28.5%).

There were seven non-contributive tests, including three failures related to insufficient tumor material (tumor remnants of a rectal cancer after radiotherapy-chemotherapy-surgery) and two failures on material fixed in Bouin’s solution. Two failures (2/197, 1%) were unexplained.

**Immunohistochemical findings**

Immunohistochemical analysis was performed on 165 of the 197 tumors (83.75%). Expression of hmlh1 and hmsh2 proteins was preserved in 116/165 tumors (70.3%). Expression of one or both proteins was lost in 30/165 tumors (18.2%): hmlh1 expression was lost in 21 tumors and hmsh2 expression was lost in 9 tumors. Labeling could not be interpreted in 19 tumors (11.5%). These failures resulted from defective tissue fixation or detachment of the histological slice during antigenic revelation.

**Table I.** – Microsatellite instability typing according to clinical characteristics of the tumors.

<table>
<thead>
<tr>
<th>Caractéristiques cliniques et typage microsatellitaire des tumeurs.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Number of patients (%)</th>
<th>MSI-High (%)</th>
<th>MSI-Low (%)</th>
<th>MSS (%)</th>
<th>Non-contributive test (%)</th>
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<tbody>
<tr>
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<td>13</td>
<td>6</td>
<td>1</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>31 - 40</td>
<td>40</td>
<td>11</td>
<td>4</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td>41 - 50</td>
<td>111</td>
<td>25</td>
<td>4</td>
<td>78</td>
<td>4</td>
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<td>23</td>
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</tr>
<tr>
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<td>115</td>
<td>32</td>
<td>6</td>
<td>74</td>
<td>3</td>
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<td></td>
<td>(58.4)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Women</td>
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<td>18</td>
<td>4</td>
<td>56</td>
<td>4</td>
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<tr>
<td></td>
<td>(41.6)</td>
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<td>Cancer location</td>
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<tr>
<td>Rectum</td>
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<td>6</td>
<td>4</td>
<td>30</td>
<td>4</td>
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<td>(20.8)</td>
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<tr>
<td>Left colon</td>
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<td>3</td>
<td>55</td>
<td>4</td>
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<td></td>
<td>(38.1)</td>
<td></td>
<td></td>
<td>(42.3)</td>
<td></td>
</tr>
<tr>
<td>Transverse colon</td>
<td>13</td>
<td>2</td>
<td>3</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(6.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right colon</td>
<td>68</td>
<td>26</td>
<td>3</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(34.5)</td>
<td></td>
<td></td>
<td>(28.5)</td>
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</tr>
<tr>
<td>Total</td>
<td>197</td>
<td>50</td>
<td>10</td>
<td>130</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>(25.4)</td>
<td>(5.1)</td>
<td>(66)</td>
<td>(3.5)</td>
</tr>
</tbody>
</table>
Comparison between immunohistochemistry and microsatellite allelic typing

Immunohistochemical screening was performed in 38 of the 50 MSI-High tumors. Twenty-four (63.15%) lost protein expression of either hmlh1 or hmsh2, in agreement with MSI typing (16 hmlh1 and 8 hmsh2). Protein expression was preserved in 12 tumors (31.6%), in disagreement with MSI typing. Results could not be interpreted for two tumors.

Immunohistochemical screening was performed in 9 of the 10 MSI-Low tumors. Four displayed preserved protein expression, 4 expressed only one protein (1 loss of hmsh2 and 3 losses of hmlh1). Results could not be interpreted for one tumor.

Immunohistochemical screening was performed in 112 of the 130 MMS tumors. Expression of both proteins was preserved in 97 (86.6%). One tumor had lost expression of hmlh1. Results could not be interpreted for 14 tumors.

Immunohistochemical screening was also performed in 6 of the 7 tumors with non-contributive MSI typing. Protein expression was preserved in three, one tumor had lost protein expression of hmlh1 and results could not be interpreted in two.

After excluding tumors where both techniques failed, our results showed that: 1) the sensibility of immunohistochemistry was 63.6% compared with MSI typing (agreement for 28 positive tumors, disagreement for 16); 2) the specificity of immunohistochemistry was 99% compared with MSI typing (agreement for 97 negative tumors, disagreement for 1); 3) the negative predictive value of immunohistochemistry for preserved protein expression in MSS phenotype was 85.8%; 4) the positive predictive value of immunohistochemistry for loss of protein expression for MSI-High or MSI-Low phenotype was 96.5%.

Germline mutation analysis

Germline mutation analysis was proposed for 50 MSI-High and 10 MSI-Low patients, however this was performed in only 33/60. Twenty-three mutations were identified (70%) (table II), including 19 point mutations and 4 large rearrangements. Germline mutations identified in 21 MSI-High patients (70% of available tumors) were 5 point mutations of MLH1, 12 of MSH2, three large rearrangements for MSH2, 1 large rearrangement for MLH1. Two point mutations, one for MLH1 and one for MSH2, were identified in MSI-Low patients.

Germline mutation analysis was performed in 5 of the 12 MSI-High patients with preserved protein expression recognized by immunohistochemistry. Three MSH2 point mutations were also identified.

The following types of germline mutations were demonstrated: a) 16 MSH2 gene mutations including nonsense mutation (N = 7), insertion/deletion with frameshift (N = 2), splicing mutation (N = 2), missense mutation (N = 2) and large genomic rearrangement (N = 3); b) 7 MLH1 mutations including nonsense mutation (N = 1), deletion with frameshift (N = 1), splicing mutation (N = 3), missense mutation (N = 1) and large genomic rearrangement (N = 1).

Germline mutation analysis could not be performed in 27 tumors due to disease progression or death before undertaking the search (N = 10), patients lost to follow-up (N = 8), or patient refusal (N = 9).

Discussion

Microsatellite instability is observed in about 15% of colorectal cancers and in almost all tumors in the HNPCC disease spectrum [16]. HNPCC tumors account for 2 to 4% of all colorectal cancers [4, 16], representing about one-quarter of unstable tumors. This relatively small proportion limits the pertinence of microsatellite instability analysis as a specific criterion for HNPCC syndrome. The usual clinical criteria for HNPCC syndrome are familial recurrence and young age at onset of tumor development. We aimed to evaluate a screening strategy for patients with colorectal cancer using early age at onset and familial history of colorectal disease as the selection criteria for undertaking microsatellite typing and immunohistochemical detection of MMR proteins. Our goal was to improve the diagnostic yield for HNPCC syndrome. This is the first population-based prospective study testing this strategy. Our discussion will be focused on the results obtained with this strategy, the false positives and false negatives, the patients not selected for screening, and the role of immunohistochemistry. We propose a diagnostic approach applicable in routine clinical practice.

In the selected population, 50 of the 197 tumors analyzed (25.4%) were MSI-High, 42 in patients aged less than 50 years (84%) and 8 in older patients (16%). Adding the cases of MSI-Low tumors (N = 10), the frequency of microsatellite instability reached 30.5%. These figures are in agreement with data in the literature obtained with selected patients. The frequency of microsatellite instability ranged from 16 to 26% for tumors in patients with a familial history of colorectal cancer [37, 38] and 17% for tumors in patients aged less than 50 years without a familial history of colorectal cancer [39]. It reached 43% using the Amsterdam or Bethesda criteria for patient selection [19].

Conversely, the frequency of microsatellite instability drops off in unselected populations, ranging from 8 to 15% in several studies [2, 18, 40-42]. This difference in frequency probably represents a selection bias, which favors inclusion of more patients with HNPCC tumors, emphasizing the importance of pertinent clinical selection criteria.

Proof of the underlying mutation causing HNPCC syndrome was obtained in 23/33 patients, and corresponded to 70% of the mutations identified. In the literature, the rate of detection of germline mutations for unstable tumors in populations not selected for age or genealogical criteria has varied from 3.5% [41] to 21.7% [18]. This difference results from the fact that the majority of unstable tumors are not part of the HNPCC spectrum, occurring sporadically, generally in relation with acquired loss of MLH1 gene function by hypermethylation of its promoter [22, 23, 41]. In patients with a family history of colorectal cancer, this mutation rate reaches 26% when done without MSI analysis [43]. Using the Amsterdam criteria to select patients among a population with an unstable tumor, the mutation rate reaches 66% [19, 34], a figure close to ours. Thus it appears that our selection criteria, early onset of colorectal cancer (before the age of 50 years) and/or a familial history of colorectal cancer (even when limited) are discriminating, favoring selection of patients with microsatellite instability and MMR germline mutation in HNPCC tumors.

We identified two mutations in patients with an MSI-Low tumor. This probably occurred because of our definition of MSI-Low which was closer to MSI-High than MSS since a small number of markers were used for allelic typing. This emphasizes the importance of considering MSI-Low tumors when defining selection criteria for detection of MMR gene mutation. This attitude was also recommended after mutations of MSH6 gene were observed in MSI-Low tumors [44]. We did not search for MSH6 gene mutations in our series.

The different types of mutations observed were considered to have a deleterious effect, leading to HNPCC syndrome. However, for the three missense mutations (two in patients with MSI-High tumors and one in a patient with an MSI-Low tumor), the deleterious nature of the mutations is not obvious since the observed alterations could correspond to genetic variants devoid of functional consequences.
Table II. – Tumor microsatellite instability, immunohistochemistry, family history and germine mutations.

<table>
<thead>
<tr>
<th>Patient n°</th>
<th>Microsatellite typing</th>
<th>Immuno-histochemistry</th>
<th>Genealogic data</th>
<th>Mutations</th>
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<tr>
<td></td>
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<td>Mutated gene</td>
</tr>
<tr>
<td>37</td>
<td>MSI-H</td>
<td>MSH2</td>
<td>sporadic</td>
<td>MSH2</td>
</tr>
<tr>
<td>25</td>
<td>MSI-H</td>
<td>not performed</td>
<td>Amsterdam</td>
<td>MSH2</td>
</tr>
<tr>
<td>148</td>
<td>MSI-H</td>
<td>MSH2</td>
<td>family history</td>
<td>MSH2</td>
</tr>
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<td>39</td>
<td>MSI-H</td>
<td>MSH2</td>
<td>Amsterdam</td>
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<td>59</td>
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<td>MSH2</td>
<td>Amsterdam</td>
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</tr>
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<td>sporadic</td>
<td>MSH2</td>
</tr>
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<td>10</td>
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<td>family history</td>
<td>MSH2</td>
</tr>
<tr>
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<td>MSI-H</td>
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<td>family history</td>
<td>MSH2</td>
</tr>
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<td>MSI-H</td>
<td>not performed</td>
<td>Amsterdam</td>
<td>MSH2</td>
</tr>
<tr>
<td>226</td>
<td>MSI-H</td>
<td>normal</td>
<td>Amsterdam</td>
<td>MSH2</td>
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<td>2</td>
<td>MSI-H</td>
<td>not performed</td>
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<td>73</td>
<td>MSI-H</td>
<td>normal</td>
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<tr>
<td>92</td>
<td>MSI-H</td>
<td>MLH1</td>
<td>sporadic</td>
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<td>MLH1</td>
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<td>192</td>
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<td>MLH1</td>
<td>family history</td>
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<td>Amsterdam</td>
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<th>Immuno-histochemistry</th>
<th>Genealogic data</th>
<th>Mutations</th>
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We identified 23 germline mutations involving the two main MMR genes (MLH1 and MSH2) among the 60 MSI-High and MSI-Low tumors. There were consequently a large number of false positives. This should be considered in light of the limitations of our study and data available in the literature since a) we searched for germline mutations in only 33 patients; germline mutation analysis was not performed in 27/60 patients (nearly 40%) because of an unfavorable disease course or patient refusal; b) point germline mutations of MLH1 and MSH2 account for approximately 50% of HNPPC syndromes [43] and large MSH2 rearrangements for about 20% [45], but we did not conduct an exhaustive analysis of gene mutations (we did not search for MSH6 mutations in less than 10% of families fulfilling the Amsterdam criteria [46-48] but apparently more frequent in HNPPC syndromes involving adenocarcinoma of the endometrium [46], MSH3 mutations appear to be very rare [47, 49] to the order of 1 to 2%, the role of PMS1 and PMS2 mutations in HNPPC syndrome does not appear to be confirmed [50]).

Ten tumors exhibited microsatellite instability without point mutations of either MLH1 or MSH2 genes nor large rearrangements (N = 5). The genealogical context was known for 9 of these tumors (table II): 5 were sporadic tumors (50%), 3 occurred in patients with a familial history of colorectal cancer, and 1 in a patient fulfilling the Amsterdam criteria (10%). The pattern of genealogical criteria contrasts with that observed in the patients with an identified mutation: sporadic tumors in 20% (N = 4), family history in 30% (N = 7), and Amsterdam criteria in 50% (N = 12). The familial nature of the disease appears to be much less marked in the group of unstable tumors without an identified mutation, demonstrating the presence of unstable sporadic tumors developing at an early age. We also noted that for these 10 tumors, the immunohistochemical findings were in agreement with the microsatellite typing in 5 (2 with loss of MSH2 expression and 3 with loss of MLH1 expression). Loss of MLH1 expression is well known in sporadic unstable tumors, but loss of MSH2 expression appears to be much rarer, indicating that germline mutations of this gene have not been fully characterized with currently available techniques. In any case, and even though a more meaningful result was not performed in 27/60 patients (nearly 40%) because of an unfavorable disease course or patient refusal; b) point germline mutations of MLH1 and MSH2 account for approximately 50% of HNPPC syndromes [43] and large MSH2 rearrangements for about 20% [45], but we did not conduct an exhaustive analysis of gene mutations (we did not search for MSH6 mutations in less than 10% of families fulfilling the Amsterdam criteria [46-48] but apparently more frequent in HNPPC syndromes involving adenocarcinoma of the endometrium [46], MSH3 mutations appear to be very rare [47, 49] to the order of 1 to 2%, the role of PMS1 and PMS2 mutations in HNPPC syndrome does not appear to be confirmed [50]).

We did not examine the question of false negatives, corresponding to stable tumors with germline MMR gene mutations, in our study, since we did not perform germline mutation analysis in patients with an MSS tumor. However, reports of false negatives seem to be exceptional in the literature [52].

One tumor in our series does however raise indirectly the question of false negatives. This tumor did not exhibit microsatellite instability but presented loss of MLH1 expression by immunohistochemistry. This tumor diagnosed in a 45-year-old patient was apparently sporadic. The patient died before germline mutation analysis could be undertaken. This case recalls the report by Liu et al. [53] who observed a missense E578G variant in exon 16 of the MLH1 gene in members of three families with colorectal cancer but not found in 260 healthy controls and not associated with microsatellite instability of tumor tissue. Nevertheless, such observations remain very exceptional and the rate of false negatives is probably negligible [54].

The clinical criteria used to select patients for microsatellite typing might be insufficient. Our age criterion led us to omit patients with a later disease onset. HNPPC syndrome is also observed in some patients whose tumors develop after the age of 50 years. The family history criterion enabled us to avoid this problem, at least partially, but also excluded from our selection patients with a sporadic neo-mutation or weak penetrance. Moreover, genealogical data are often incomplete and must be considered with caution. The difference in the detection rate for mutations in non-selected unstable tumors and our observed rate emphasizes the pertinence of our selection criteria.

We also observed disagreement between microsatellite typing results and the immunohistochemical search for MMR gene protein expression. Considering the microsatellite typing as the gold standard, the sensitivity of immunohistochemistry was 63.6% (28 cases of lost expression among 44 MSI-High or MSI-Low tumors), but the specificity was high (99%, 97 cases of preserved expression among 98 MSS tumors). In the literature other studies have compared the contribution of immunohistochemistry compared with microsatellite typing either for sporadic tumors or for genetically favored tumors. As in our series, specificity has been very high, reaching 100% in several very large series [20, 24, 33]. Two other studies also found specificity close to 100% [25, 42]. The larger series have not reported cases similar to the one tumor in our series which lost MLH1 expression without microsatellite instability.

Sensitivity of immunohistochemistry varies according to different studies. Certain authors have reported very high sensitivity ranging from 92% [20] to 97% [24] or even 100% [33, 39], sometimes with small sample sizes. Other authors have reported sensitivities similar to ours, ranging from 75% to 81% [19, 25, 42]. Our immunohistochemical technique exhibited poor sensitivity in four patients with HNPPC syndrome (patients no 73, 87, 223, 226) in whom a causal mutation was detected. Two of these patients had missense mutations (T557P in patient no 87 and I884S in patient no 73). These variants probably alter protein function but not its translation and consequently would not be detected by immunohistochemistry which evaluates protein expression. The other two patients had a germline splicing mutation (patient no 226) or large rearrangement (patient no 233), and could lead to either i) preserved reading frame with persistent expression by immunohistochemistry because of the c-terminal localization of the epitope used; or ii) total loss of expression of the mutated allele. Most likely, the genetic event involving the wild allele of the tumor cells produced a protein with deficient function but not total loss of expression (for example, missense mutations). The limited sensitivity of the immunohistochemistry technique used in our study could also be explained by two other problems: i) tissue fixation, a key step which controls the quality of the immunolabeling (due to the lack of a regional consensus, each pathology laboratory used its own fixation protocol, leading to considerable variability), ii) search was limited, mainly concerning the two principal MMR proteins, hmlh1 and hmsh2, while mutations of other proteins (particularly hmsh6) could be involved. Nevertheless, immunohistochemistry is a simple low cost technique which can be rapidly applied in routine practice (time to results: 24 hr) and which provides a precise identification of the protein involved (hmsh1 or hmsh2) useful for targeting search for the germline mutation on the corresponding gene. Immunohistochemistry can also avoid the problem of low cellularity which can lead to failure of molecular biology techniques (4 cases in our study).

In order to improve the diagnosis of HNPPC, we now propose in the Aquitaine region of France a diagnostic strategy using a decisional algorithm presented in figure 1 illustrating the use of these different tools.

Immunohistochemistry is a rapid technique with excellent specificity (99%) which can be used in routine practice. We thus use this method as the first intention test for young patients (<50 years) with colorectal cancer or for patients with a familial history of HNPPC spectrum tumor. If the immunohistochemistry demonstrates loss of expression of one of the MMR gene proteins, we propose germline mutation analysis targeting the corresponding gene. However, due to the moderate sensitivity of...


References


References


