Novel strategy for optimal sequential application of clinical criteria, immunohistochemistry and microsatellite analysis in the diagnosis of hereditary nonpolyposis colorectal cancer

Christoph Engel14, Jochen Forberg1, Elke Holinski-Feder2, Constanze Pagenstecher3, Jens Plaschke1, Matthias Kloor5, Christopher Poremba6, Christian P. Pox7, Josef Rüssloff8, Gisela Keller9, Wolfgang Dietmaier10, Petra Rümmele10, Nikolaus Friedrichs11, Elisabeth Mangold3, Reinhard Buettner11, Hans K. Schackert4, Peter Kienle12, Susanne Stemmler13, Gabriela Moeslein14, Markus Loeffler1 and the German HNPPC Consortium

1Institute of Medical Informatics, Statistics and Epidemiology, University of Leipzig, Leipzig, Germany
2Department of Medical Genetics, Ludwig Maximilians University, Munich, Germany
3Institute of Human Genetics, University Hospital, Bonn, Germany
4Department of Surgical Research, Dresden University of Technology, Dresden, Germany
5Department of Molecular Pathology, Institute of Pathology, University of Heidelberg, Heidelberg, Germany
6Department of Pathology, Heinrich Heine University, Düsseldorf, Germany
7Medical Department, Knappschaftskrankenhaus, Ruhr University Bochum, Bochum, Germany
8Department of Pathology, Klinikum Kassel, Kassel, Germany
9Institute of Pathology, Klinikum rechts der Isar, Technical University, Munich, Germany
10Institute of Pathology, University Regensburg, Regensburg, Germany
11Institute of Pathology, University of Bonn, Bonn, Germany
12Department of Surgery, University Hospital of Heidelberg, Heidelberg, Germany
13Institute of Human Genetics, Ruhr University Bochum, Bochum, Germany
14Department of Surgery, Heinrich Heine University, Düsseldorf, Germany

Clinical criteria, microsatellite analysis (MSA) and immunohistochemistry (IHC) are important diagnostic tools for identification of hereditary nonpolyposis colorectal cancer (HNPPC) patients who are likely to carry pathogenic germline mutations in mismatch repair genes. Based on MSA and IHC results and subsequent mutation analyses of 1,119 unrelated index patients meeting the Amsterdam II criteria or the classical Bethesda guidelines, we analyzed the value of these tools to predict MLH1 and MSH2 mutations with the aim of establishing optimal strategies for their most efficient sequential use. The overall prevalence of pathogenic germline mutations in our cohort was 20.6% (95% CI = 18.3–23.0%) and 61.8% (95% CI = 56.8–66.6%), respectively, after MSA/IHC-based preselection. IHC was highly predictive (99.1%) and specific (99.6%) with regard to MSA. However, 14 out of 230 mutations (6%) escaped detection by IHC. Thus, IHC cannot be recommended to substitute MSA fully. Nonetheless, IHC is important to indicate the gene that is likely to be affected. To combine both methods efficiently, we propose a novel screening strategy that provides 2 alternative ways of sequential IHC and MSA application, either using IHC or MSA in the first place. A logistic regression model based on the age of the index patient at first tumor diagnosis and the number of fulfilled HNPPC criteria is used to allocate individual patients to that alternative pathway that is expected to be less expensive. A cost analysis reveals that about 25% of the costs can be saved using this strategy.

Key words: hereditary nonpolyposis colorectal cancer; Amsterdam criteria; Bethesda guidelines; immunohistochemistry; microsatellite analysis; mutation analysis; tumor screening; diagnostic strategy; cost analysis

Hereditary nonpolyposis colorectal cancer (HNPPC) is a highly penetrant cancer susceptibility syndrome characterized by the early onset or familial clustering of colorectal tumors and a variety of extracolonic malignancies.1–2 Germline mutations in DNA mismatch repair genes, most commonly in MLH1 and MSH2, have been identified as a cause for HNPPC.3–6 The identification of pathogenic germline mutations in such families is an important issue since it enables predictive testing of family members and targeted surveillance of mutation carriers. However, mutation analysis is time-consuming and expensive and therefore cost-effective screening strategies are needed to preselect those families that are likely to carry a pathogenic mutation. Information on the clinical presentation of the syndrome within the members of HNPPC families can be used in the first selection step. The Amsterdam criteria and the Bethesda guidelines are based on such clinical information and allow an increase in the probability to detect a mutation.7–11

It has been shown that the presence of high-level microsatellite instabilities is a strong and extremely sensitive predictor for germline mutations in cases with early tumor onset or a positive family history.12–14 This genomic instability is characterized by small deletions or insertions within simple repeat sequences in tumor DNA caused by the inability of the defective mismatch repair system to correct DNA replication errors.15–18 Another predictive but less expensive screening method is the analysis of tumor tissue for reduction or loss of expression of mismatch repair proteins by immunohistochemical staining with monoclonal antibodies.17,18 In contrast to microsatellite analysis, this method provides also information about the mismatch repair gene that is likely to be affected.

To date, microsatellite analysis (MSA) is regarded as the standard screening method prior to mutation analysis. Several investigations have compared the diagnostic performance of MSA and immunohistochemistry (IHC), and screening strategies using both methods sequentially have been proposed.19–21 However, the question of whether either MSA or IHC should be used in the first place, in particular with regard to the total costs of the screening process, has not been addressed in detail so far.

In 1999, the German HNPPC Consortium was established to provide comprehensive medical care to members of HNPPC families. Located at 6 universities, an interdisciplinary team consisting of medical geneticists, pathologists, gastroenterologists and surgeons offer genetic counseling, molecular testing and a standardized surveillance program. Within the framework of a prospective registry, comprehensive information about family pedigrees, Grant sponsor: Deutsche Krebshilfe (German Cancer Aid), Bonn, Germany.

*Correspondence to: Institute for Medical Informatics, Statistics and Epidemiology, Haerlestrasse 16-18, 04107 Leipzig, Germany, Fax: +49-341-9716109, E-mail: engel@imise.uni-leipzig.de

Received 26 January 2005; Accepted after revision 18 April 2005 DOI 10.1002/ijc.21313

Published online 7 July 2005 in Wiley InterScience (www.interscience.wiley.com).
individual tumor histories of family members, molecular and histopathologic tumor analyses and the results of regular surveillance examinations is gathered in a central database of the consortium.

At present, the German HNPCC Consortium has collected data on 1,119 unrelated families that fulfill defined clinical criteria and that have completed a well-defined diagnostic process using microsatellite analysis, immunohistochemistry and mutation analysis.

In this work, we sought to define a screening strategy with optimal sequential application of clinical information, microsatellite analysis and immunohistochemistry. To do so, we characterized the performance of different clinical criteria for family selection, analyzed the predictive values of IHC and MSA and compared different screening strategies with regard to their costs.

Material and methods

Patient recruitment and diagnostic procedures

Families were enrolled into the study if a member fulfilled at least one of the clinical selection criteria shown in Table I. These criteria correspond to the revised Amsterdam (Amsterdam II) criteria and the Bethesda guidelines. However, the 50-year age restriction of the Amsterdam II definition (which requires that at least one case must be diagnosed before the age of 50 years) was not applied. After declaration of written informed consent, one patient from each family (index patient) underwent the following diagnostic procedure: tumor material of the index patient was analyzed both for microsatellite instabilities (MSI) and for a reduction or loss of MLH1 and MSH2 protein expression by immunohistochemical staining. Analysis for MSI was performed as recommended by the international guidelines for the evaluation of MSI in colorectal cancer.22–24 Tumors were classified as MSI-H (high-level MSI) if at least 2 markers of the reference panel (BAT25, BAT26, D5S346, D2S123, D17S250) exhibited instabilities. Tumors showing no instabilities in these markers were classified as microsatellite-stable (MSS). A second panel of 5 alternative markers (BAT40, D10S197, D13S153, MYCL1, D18S58) was applied if only one marker of the first reference panel was unstable. In this case, the result was interpreted as MSI-L if at least 30% of all markers showed instabilities; otherwise it was regarded as MSI-L (low-level MSI). Immunostaining for MLH1 and MSH2 expression was normal. Analysis for pathogenic germline mutations in MLH1 and MSH2 was only performed if microsatellite analysis revealed the MSI-H phenotype and/or immunohistochemistry showed a reduction or loss of protein expression. By protocol, no mutation analysis was planned if the tumor was MSS or MSI-L and protein expression was normal.

Mutation analysis started with the gene that was likely to be affected according to the preceding immunohistochemistry. The analysis was performed either by prescreening of exons using denaturing high-performance liquid chromatography (DHPLC) and subsequent sequencing of exons showing aberrant chromatograms or by direct sequencing. As a rule, screening or sequencing was continued until a pathogenic mutation was detected or all exons of the 2 genes were completely analyzed. Detected sequence variations were classified as pathogenic mutations, unspecified variants, or frequent polymorphisms. Pathogenic mutations included nonsense mutations, frameshift mutations, mutations at the highly conserved splice site positions AG/GT, mutations that destroy the translation start site and genomic rearrangements. Descriptions of the mutation spectrum found in the families of the German HNPCC Consortium, genotype-phenotype correlations and pathology are reported in separate studies.26–29

The study has been approved by the local ethics committees of the participating centers.

By the end of May 2004, a total of 1,913 index patients from 1,913 unrelated families suspected of having HNPCC were registered in the central database of the consortium. These patients fulfilled at least one of the clinical selection criteria as defined in Table I. In 502 index patients, diagnostic information was not fully complete for historical reasons or because tests were still in progress. The remaining 1,411 index patients underwent tumor

| TABLE I – DEFINITION OF SELECTION CRITERIA OF THE GERMAN HNPCC CONSORTIUM |
| Definition |
| A2 | Modified Amsterdam II (i.e., without 50-year age restriction): family with at least 3 members in at least 2-successive generations who have a histologically verified carcinoma of the colon, rectum, endometrium, small bowel, renal pelvis, or ureter and 1 of the 3 members is a first-degree relative of the other 2 and a familial adenomatous polyposis is excluded |
| B2 | Bethesda guideline 2: individual with 2 HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, small bowel or transitional cell carcinoma of the renal pelvis and ureter) |
| B3 | Bethesda guideline 3: individual with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; one of the cancers was diagnosed at age < 45 years and the adenoma was diagnosed at age < 40 years |
| B4 | Bethesda guideline 4: individual with colorectal or endometrial cancer diagnosed at age < 45 years |
| B7 | Bethesda guideline 7: individual with at least one adenoma diagnosed at age < 40 years |

![Figure 1](Image)

**Figure 1** – Current diagnostic strategy of the German HNPCC Consortium using MSA and IHC in parallel.
tissue analysis as described above, of which 1,338 (95%) had conclusive test results. Of these, 1,119 were included in the present analysis since they were also evaluable regarding mutation analysis. The patients who were not included in the analysis were not significantly different as judged by the frequencies of their clinical selection criteria.

Cost analyses

Cost analyses considered the costs of MSA and IHC for 3 different screening strategies (Figs. 1 and 2). In strategy MSA+IHC, both methods are applied concurrently (this is the current strategy of the German HNPCC consortium). In strategy IHC→MSA, all cases are first subjected to IHC. In case of an abnormal result, gene-specific mutation analysis will be performed without prior conduct of MSA. MSA will be only performed in case of normal IHC. If MSA shows an abnormal outcome, then the mutation analysis will be performed as well. This strategy identifies exactly the same cases for mutation analysis as the concurrent strategy. In strategy MSA→IHC, all cases are first subjected to MSA. IHC is only performed if the preceding MSA is abnormal in order to identify the gene that is likely to be affected and thus to allow gene-specific mutation analysis. This strategy is not identical to the concurrent strategy because cases with normal MSA but abnormal IHC will not be selected for mutation analysis. However, this concerned only 3 out of 1,119 cases of our present cohort and therefore this strategy is considered to be comparably effective.

\( C_{\text{MSA}} \) and \( C_{\text{IHC}} \) describe the absolute costs for MSA (using 5 or 10 markers) and IHC (analyzing MLH1 and MSH2), respectively, spent per index patient. The absolute costs of the 3 strategies are described by the following cost functions:

\[
\begin{align*}
C_{\text{MSA+IHC}} &= C_{\text{MSA}} + C_{\text{IHC}} \\
C_{\text{IHC→MSA}} &= C_{\text{IHC}} + (1 - p_{\text{IHC}}) \cdot C_{\text{MSA}} \\
C_{\text{MSA→IHC}} &= C_{\text{MSA}} + p_{\text{MSA}} \cdot C_{\text{IHC}}
\end{align*}
\]

(1a) The variables \( p_{\text{IHC}} \) and \( p_{\text{MSA}} \) are the probabilities to observe abnormal IHC and MSA outcomes, respectively, in the population of interest. The cost functions can be simplified if only the cost ratio \( R = C_{\text{MSA}} / C_{\text{IHC}} \) is considered (that is, each equation describes the costs relative to the costs of one IHC):

\[
\begin{align*}
C_{\text{MSA+IHC}}^\text{rel} &= 1 + R \\
C_{\text{IHC→MSA}}^\text{rel} &= 1 + (1 - p_{\text{IHC}}) \cdot R \\
C_{\text{MSA→IHC}}^\text{rel} &= R + p_{\text{MSA}}
\end{align*}
\]

(1b) The cost ratio \( R \) at which both sequential strategies will yield identical costs (so-called break-even point; BEP) can be determined by equating Equation 2b with Equation 3b:

\[
R_{\text{BEP}} = \frac{1 - p_{\text{MSA}}}{p_{\text{IHC}}}
\]

(4)

Statistical analysis

Categorial outcome data were reported as absolute or relative frequencies where appropriate. Ninety-five percent confidence intervals for proportions were calculated using Wilson’s score method. Multivariate logistic regression analysis was used to estimate the probability of having an abnormal IHC or MSA outcome. The logistic model of the form \( \log \left[ \frac{p}{1-p} \right] = \beta_0 + \beta_1 \cdot x_1 + \beta_2 \cdot x_2 \) considers the logarithm of the odds of having an abnormal outcome as a linear function of the weighted sum of the predictors

\( x_1 \) and \( x_2 \). The variable \( x_1 \) represents the age of first tumor diagnosis of the index patient and \( x_2 \) represents the number of fulfilled selection criteria A2, B2, B3 and B4 (see Table I for definition). The variable \( x_2 \) was chosen in this particular way for the following reasons. First, all 4 mentioned selection criteria were significantly predictive in a logistic model using separate indicator variables for each criterion. Second, in order to simplify the approach and because the odds ratios did not differ largely between the 4 variables, the summary predictor \( x_2 \) was defined. \( \beta_0, \beta_1 \) and \( \beta_2 \) are regression coefficients that were estimated using the maximum likelihood method. The receiver-operating-characteristic (ROC) method was used to compare the predicted outcome classifications with the observed outcomes. The predictive value of the model was characterized by the area under the ROC curve (AUC), which is the probability to classify correctly a randomly chosen pair of truly positive and negative cases, \( p \)-values below 0.05 were considered significant. SPSS 10.0.7 (SPSS, Chicago, IL) was used for all statistical data analyses.

Results

Diagnostic performance of clinical selection criteria

To characterize the diagnostic value of the different clinical selection criteria, we compared the number and percentage of index patients showing abnormal tumor tissue screening results and pathogenic mutations between groups of patients fulfilling different sets of selection criteria (Table II). The Amsterdam I definition (group A1) was met by 19.8% of all families with considerably higher frequencies of patients with abnormal screening results and pathogenic mutations (62.6% and 49.5%, respectively) compared with the total cohort (33.2% and 20.6%, respectively). The additional benefit of the Amsterdam II over the Amsterdam I definition for patient selection is shown by the group “A2 and not A1” (comprising families that met Amsterdam II but not Amster-
This group made up only 2.6% of all families, however, with similar frequencies of abnormal tumor screening results (69.0%) and pathogenic mutations (58.6%) compared with A1. In our study, the 50-year age restriction within the Amsterdam II definition was not applied in order to increase sensitivity. The positive predictive values in the group identified by this modification (group “A2 and not A1”) were considerably low with 14.3% tumor screening abnormality and 7.1% mutation positivity. Moreover, some cases in this group also fulfilled one or more of the Bethesda guidelines 2, 3, 4 and 7. Exclusion of these cases (group “A2 and not A2”) was met by 34.9% of all families. However, only 71.3% remained. The Bethesda guideline A2 was the most frequent criterion (group B4; 64.2%) and 31.2% of the patients were selected exclusively by this criterion (group B4 only). Bethesda guideline 7 was exclusively fulfilled in 3.3% of the patients; however, only one pathogenic mutation was detected. In general, predictive values were considerably lower in the groups fulfilling only one criterion than in the groups that fulfilled more than one selection criterion.

Table III summarizes the results of tumor screening and mutation analysis depending on the outcome of IHC and MSA. About 2/3 (66.8%) of the cases were found to have completely normal screening results. Information about pathogenic mutations is largely not available for this group as mutation analysis was not mandatory according to the study protocol. Nonetheless, 49 out of 732 index patients underwent mutation analysis that, however, revealed no pathogenic mutation in MLH1 or MSH2. Of all index patients, 58 (5.2%) had normal IHC but were MSI-H. Fourteen patients in this group (24.1%) had a pathogenic mutation of MLH1 or MSH2. As expected, the largest numbers of index patients carrying pathogenic mutations were found in the groups that were MSI-H and showed a loss of protein expression (positive predictive values for mutation: 72.1% for MLH1 and 66.4% for MSH2). No pathogenic mutation could be detected in 3 cases with MSI-H.

Predictive value of immunohistochemistry and microsatellite analysis

Table III summarizes the results of tumor screening and mutation analysis depending on the outcome of IHC and MSA. About 2/3 (66.8%) of the cases were found to have completely normal screening results. Information about pathogenic mutations is largely not available for this group as mutation analysis was not mandatory according to the study protocol. Nonetheless, 49 out of 732 index patients underwent mutation analysis that, however, revealed no pathogenic mutation in MLH1 or MSH2. Of all index patients, 58 (5.2%) had normal IHC but were MSI-H. Fourteen patients in this group (24.1%) had a pathogenic mutation of MLH1 or MSH2. As expected, the largest numbers of index patients carrying pathogenic mutations were found in the groups that were MSI-H and showed a loss of protein expression (positive predictive values for mutation: 72.1% for MLH1 and 66.4% for MSH2). No pathogenic mutation could be detected in 3 cases with MSI-H.
from about 3.25 to 5, which is larger than RBEP. This means that approximately 80% of the patients in Germany, CMSA amounts to approximately 260 € and 400 € (5 and 10 markers, respectively) and C_{IHC} amounts to approximately 80 €. Thus, R_{act} (= C_{MSA} divided by C_{IHC}) ranges from about 3.25 to 5, which is larger than R_{BE}. This means that the costs of strategy IHC→MSA are lower (77–79% of the costs of the parallel strategy) than those of strategy MSA→IHC.

The cost reductions demonstrated so far are achieved by subjecting all patients to the same strategy (fixed allocation). However, for further cost reduction, one may wish to allocate patients individually to that sequential screening strategy that is expected to be less costly than the other depending on individually predicted values for p_{IHC} and p_{MSA} (individual allocation). Logistic regression analysis shows that the age of the index patient at first tumor diagnosis (x_1) and the number of fulfilled selection criteria (x_2) are highly significant predictors for both IHC and MSA outcome (Table IV). The predicted probabilities for p_{IHC} and p_{MSA} cover a broad range 0.01 and 0.94 for the cases in our cohort (Fig. 3). ROC analysis revealed predictive values (AUC) of the regression models of 0.78 and 0.76 for IHC and MSA, respectively. Using this predictive model, R_{BE} can be individually determined as a function of the variables x_1 and x_2 as shown in Figure 4. This graph can be used to choose the optimal sequential screening strategy, given the individual clinical information and the actual MSA-to-IHC cost ratio R_{act}. Because the cost functions are crossing, R_{act} determines which of the 2 alternative strategies is less expensive. The break-even point R_{BE} yields 2.4 in our specific cohort (see Equation 4, p_{IHC} = 0.28 and p_{MSA} = 0.33). According to the present official regulation of charges for physicians in Germany, C_{MSA} amounts to approximately 260 € and 400 € (5 and 10 markers, respectively) and C_{IHC} amounts to approximately 80 €. Thus, R_{act} (= C_{MSA} divided by C_{IHC}) ranges from about 3.25 to 5, which is larger than R_{BE}. This means that the costs of strategy IHC→MSA are lower (77–79% of the costs of the parallel strategy) than those of strategy MSA→IHC.

**Discussion**

Since the discovery of mismatch repair gene defects as the genetic basis for the cancer disposition syndrome HNPCC, an increasing number of investigations are focusing on the important issue of developing optimal strategies for molecular diagnosis. Clearly, mutation analysis cannot be applied at a large scale and should only be offered to patients with substantial risk of having a deleterious mutation. Also, microsatellite analysis

---

**Cost-saving sequential use of IHC and MSA**

Presently, the German HNPCC Consortium uses both screening methods in parallel (Fig. 1). Alternatively, IHC and MSA can be applied sequentially in 2 different ways as illustrated in Figure 2 (dark shaded areas). These strategies are less costly because the second step depends on the result of the first step. To quantify the resulting cost reduction for the present patient cohort, we calculated the costs of the parallel strategy and both sequential strategies as described above. Figure 5 depicts the costs of the sequential screening strategies IHC→MSA and MSA→IHC relative to the costs of the parallel strategy. The costs are functions of the actual MSA-to-IHC cost ratio R_{act}. Because the cost functions are crossing, R_{act} determines which of the 2 alternative strategies is less expensive. The break-even point R_{BE} yields 2.4 in our specific cohort (see Equation 4, p_{IHC} = 0.28 and p_{MSA} = 0.33). According to the present official regulation of charges for physicians in Germany, C_{MSA} amounts to approximately 260 € and 400 € (5 and 10 markers, respectively) and C_{IHC} amounts to approximately 80 €. Thus, R_{act} (= C_{MSA} divided by C_{IHC}) ranges from about 3.25 to 5, which is larger than R_{BE}. This means that the costs of strategy IHC→MSA are lower (77–79% of the costs of the parallel strategy) than those of strategy MSA→IHC.

**TABLE IV – RESULTS OF LOGISTIC REGRESSION AND ROC ANALYSES**

<table>
<thead>
<tr>
<th>Model</th>
<th>Logistic regression analysis</th>
<th>ROC analysis AUC (CI95)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β0</td>
<td>p</td>
</tr>
<tr>
<td>p_{IHC}</td>
<td>−1.894</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>p_{MSA}</td>
<td>−1.366</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**FIGURE 3** – Left: Estimated probability to detect an abnormal outcome in IHC (solid line) or MSA (dashed line) as function of the age of the index patient at first tumor diagnosis (x_1) and the number of fulfilled clinical selection criteria (x_2). Bold parts of the curves indicate ranges in which observations were available in the present data set. Right: ROC analysis comparing predicted with observed IHC (solid line) and MSA (dashed line) outcome.
and immunohistochemistry, which have been shown to allow effective enrichment of high-risk patients, are presently not recommended for all cases of colorectal tumors. Instead, a sequential approach using preselection by clinical criteria, followed by further selection using MSA and/or IHC and final verification by mutation analysis, is currently applied for diagnosis. However, appropriate definitions of selection criteria and the optimal order of available testing methods are still under debate.

Here we present data from the German HNPCC Consortium on 1,119 unrelated index patients who underwent standardized molecular diagnosis of HNPCC using clinical selection criteria, immunohistochemistry, microsatellite analysis and mutation analysis. Our objective was to analyze and compare the value of these selection and screening methods for the identification of patients with pathogenic germline mutations and to develop optimal screening strategies with regard to costs.

Our analysis was restricted to MLH1 and MSH2 because other genes have not been analyzed to the same systematic extent in our study so far. However, among all genes associated with HNPCC, the majority of mutations is detected in these 2 genes.

The clinical selection criteria of the present study were identical to the Bethesda guidelines proposed in 1996 with the exception that the 50-year age restriction within the Amsterdam criteria was not applied. However, our data clearly show that waiving the age criterion was not effective as only 1 patient was identified to carry a pathogenic mutation out of 54 patients who were additionally selected through this modification. The Amsterdam criteria were applied using the revised definition of 1999 (Amsterdam II), which considers defined malignancies in addition to colorectal carcinomas. Although the number of Amsterdam families additionally identified by the broader tumor spectrum was comparably low (29 out of 222 families), the predictive values for IHC/MSA and mutation positivity were similar to those observed in the group fulfilling only Amsterdam I. This result clearly confirms a previous analysis of 56 kindreds by Stormorken et al., who found that the revision of the Amsterdam I criteria was of great importance to identify additionally mutation-positive families that would have escaped detection by the original criteria. However, despite the predictive importance of the Amsterdam criteria, about 91% of the index patients of our cohort fulfilled at least one of the Bethesda guidelines 2, 3, 4, or 7. This group comprised 222 out of 230 (96.5%) families with pathogenic mutations, that is, only 3.5% would not have been ascertained if the Amsterdam II criteria would not have been considered for selection. Thus, in contrast to the Amsterdam criteria, the Bethesda guidelines are only of little additional value for the identification of families. Moreover, in contrast to the Amsterdam criteria, which require deeper information about the tumor history within a family, the Bethesda guidelines 2, 3, 4, and 7 require only information about the tumor history of the index patient and his first-degree relatives.

Recently, revised Bethesda guidelines for testing colorectal tumors for microsatellite instability have been proposed. In these new guidelines, the former guideline 7, which recommended analyzing patients with colorectal adenomas diagnosed before the age of 40 years, is no longer proposed. Our present data show that this former guideline is in fact largely ineffective for patient selection (only 2 patients out of 37 had abnormal results of IHC or MSA, 1 had a pathogenic mutation). However, a recent study of de Jong et al. showed that adenomas in young mutation carriers were significantly larger and that a higher proportion had villous components and/or high-grade dysplasia compared with adenomas of noncarriers, suggesting that adenomas with such features should be analyzed by immunostaining to identify patients with suspected HNPCC.

The new Bethesda guideline 5 (hereafter “nB5”), which recommends MSI testing in colorectal cancer patients having 2 or more first- or second-degree relatives with HNPCC-related tumors regardless of age, is similar to A2. In our cohort, 421 (37.6%) patients fulfill nB5, of whom 206 (48.9%) had abnormal IHC and/or MSA and 154 (36.6%) had a pathogenic mutation. Interestingly, 115 (10.3%) patients fulfill nB5 but not A2, of whom 42 (36.5%) patients showed abnormal IHC and/or MSA and 26 (22.6%) had a pathogenic mutation. Thus, the new Bethesda guideline 5 seems to be useful to identify additionally a considerable number of patients with mutations that do not fulfill the Amsterdam II criteria without the 50-year age restriction.

The prevalence of patients with MSI-H was 33.0%, which agrees to findings obtained by others in cohorts complying with the Bethesda guidelines. The overall prevalence of patients with pathogenic mutations was 20.6% in our cohort, which, however, might be subject to verification bias since mutation analysis has largely not been performed according to the study protocol if no abnormal result in IHC and MSA was present. Thus, although the analysis of 49 patients in this group did not reveal any pathogenic mutation, the existence of such cannot be excluded. If one assumes a mutation frequency of 7.3% in this group (upper limit of the 95% confidence interval for the expected mutation fre-

\[
R_{\text{MSA-IHC}} = \frac{1}{1 + \frac{C_{\text{MSA-IHC}}^*}{R_{\text{MSA-IHC}}^*}}
\]

\[
R_{\text{MSA-IHC}}^* = \frac{C_{\text{MSA-IHC}}}{C_{\text{MSA-IHC}} + R_{\text{MSA-IHC}}^*}
\]

\[
R_{\text{MSA-IHC}}^* = \frac{1}{1 + \frac{C_{\text{MSA-IHC}}}{R_{\text{MSA-IHC}}^*}}
\]

\[
R_{\text{MSA-IHC}} = \frac{1}{1 + \frac{C_{\text{MSA-IHC}}}{R_{\text{MSA-IHC}}^*}}
\]

\[
R_{\text{MSA-IHC}}^* = \frac{1}{1 + \frac{C_{\text{MSA-IHC}}}{R_{\text{MSA-IHC}}^*}}
\]

\[
R_{\text{MSA-IHC}} = \frac{1}{1 + \frac{C_{\text{MSA-IHC}}}{R_{\text{MSA-IHC}}^*}}
\]

\[
R_{\text{MSA-IHC}}^* = \frac{1}{1 + \frac{C_{\text{MSA-IHC}}}{R_{\text{MSA-IHC}}^*}}
\]

\[
R_{\text{MSA-IHC}} = \frac{1}{1 + \frac{C_{\text{MSA-IHC}}}{R_{\text{MSA-IHC}}^*}}
\]
frequency), about 55 positive cases would be expected and the overall mutation prevalence would increase to 25.5%. There were 58 cases (5.2%) with normal IHC and abnormal MSA, of which 14 cases (2.5%) with normal IHC. In contrast, Ponz de Leon et al. who determined a PPV and specificity of 100% for IHC of MLH1 and MSH2 in a series of screening method, then MSA is applied with the intention to combination to detect pathogenic mutations was 61.8%. Thus, in relation to the corresponding IHC but were detected by MSA. This finding confirms that IHC is currently not able to replace MSA. On the other hand, application of MSA in case of an abnormal finding confirms that IHC is currently not able to replace MSA. Therefore, alternative strategies using both methods sequentially should be more cost-effective.

Christensen et al. and also Debniak et al. proposed a stepwise strategy using IHC in the first place, followed by MSA in case of normal IHC. In contrast, Ponz de Leon et al. suggested a strategy using MSA first, followed by IHC in case of abnormal MSA. We here propose a combination of these 2 alternative ways of sequential IHC and MSA application. If IHC is used as first screening method, then MSA is applied with the intention to compensate for the limited sensitivity of IHC. Conversely, if MSA is used first, then IHC is applied to determine the gene that is likely to be affected. However, this latter alternative does not select the rare cases that have stable microsatellites but abnormal immunohistochemistry (3 out of 1,119 cases in our cohort). In this work, we compared the costs of both sequential alternatives with those of the parallel strategy. Importantly, the cost functions of the sequential strategies intersect. Since the costs depend on the MSA-to-IHC cost ratio and the expected probabilities to obtain an abnormal MSA and IHC outcome, the least expensive alternative is determined by the actual values of these parameters, which can vary between different countries and patient populations. Moreover, our logistic regression analysis shows that the probabilities for abnormal IHC or MSA results are significantly dependent from individual clinical factors (age of the index patient at first tumor diagnosis and the number of fulfilled selection criteria). Therefore, a second important result is that an individualized allocation to one of the sequential screening alternatives is possible instead of using one fixed alternative for all patients. Using the individualized allocation strategy as proposed here, about 25% of the costs (85–120 € per patient in our study, depending on the number of microsatellite markers) can be saved compared with the strategy using IHC and MSA in parallel. However, this does not represent the maximum possible cost saving (as indicated in Fig. 5 by the dashed line), because the logistic model does not perfectly predict the screening outcome as indicated in Fig. 3 by the ROC curve. We are currently investigating whether the model can be improved using further information, e.g., from histopathologic examinations.

It has to be emphasized that the proposed strategy does not allow selection for MSH6 mutations. Several investigations suggest that neither MSA nor IHC should be a defining selection criterion for MSH6 mutation analysis, as some mutations may not be detected by either method. Thus, more sophisticated strategies may be needed if also cost-effective detection of MSH6 mutations is required.

In summary, we conclude that the Bethesda guidelines 2, 3 and 4 are the most important criteria for the selection of patients with high risk of having a pathogenic mutation in MLH1 or MSH2. In order to save costs of tumor screening prior to mutation analysis, we recommend a sequential strategy using either IHC or MSA in the first place. A logistic regression model based on the age of the index patient at first tumor diagnosis and the number of fulfilled selection criteria can be used to determine individually which of the 2 sequential alternatives is expected to be least expensive.

References


Appendix