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

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Clinical criteria, microsatellite analysis (MSA) and immunohistochemistry (IHC) are important diagnostic tools for identification of hereditary nonpolyposis colorectal cancer (HNPCC) 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 HNPCC criteria is used to allocate individual patients to that alternative pathway that is expected to be least 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 (HNPCC) 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 HNPCC.[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 HNPCC 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.[15,16] 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 HNPCC Consortium was established to provide comprehensive medical care to members of HNPCC 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,

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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 instable. In this case, the result was interpreted as MSI-H if at least 30% of all markers showed instabilities; otherwise it was regarded as MSI-L (low-level MSI). Immunostaining for MLH1 and MSH2 was performed by a reference pathologist at each center following a standardized procedure as previously described.[25]

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

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

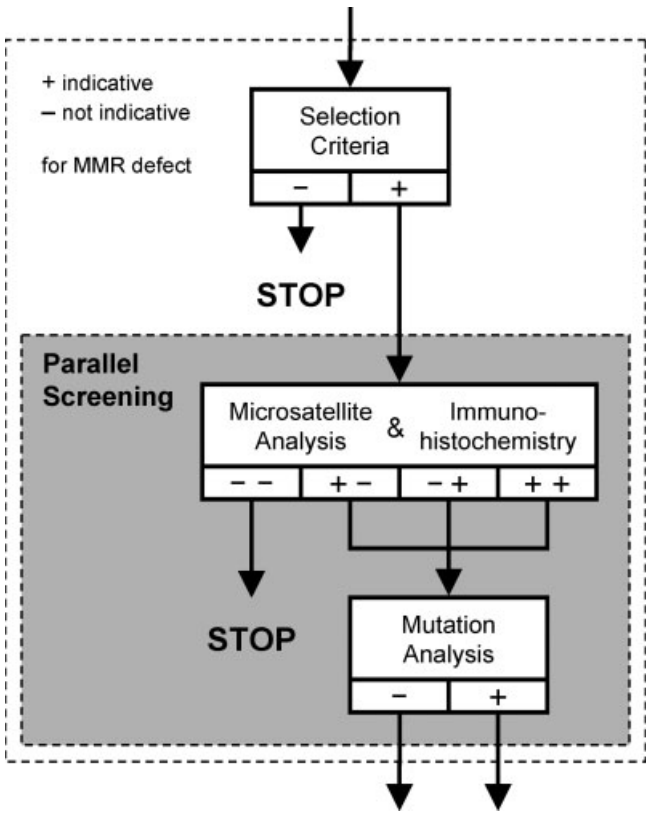

FIGURE 1 – Current diagnostic strategy of the German HNPCC Consortium using MSA and IHC in parallel.

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_{MSA} and C_{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:

$$C^{abs}_{MSA+IHC} = C_{MSA} + C_{IHC} \quad (1a)$$

$$C^{abs}_{IHC\rightarrow MSA} = C_{IHC} + (1 - p_{IHC}) \cdot C_{MSA} \quad (2a)$$

$$C^{abs}_{MSA\rightarrow IHC} = C_{MSA} + p_{MSA} \cdot C_{IHC} \quad (3a)$$

The variables p_{IHC} and p_{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 $R = C_{MSA}/C_{IHC}$ is considered (that is, each equation describes the costs relative to the costs of one IHC):

$$C^{rel}_{MSA+IHC} = 1 + R \quad (1b)$$

$$C^{rel}_{IHC\rightarrow MSA} = 1 + (1 - p_{IHC}) \cdot R \quad (2b)$$

$$C^{rel}_{MSA\rightarrow IHC} = R + p_{MSA} \quad (3b)$$

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:

$$R_{BEP} = \frac{1 - p_{MSA}}{p_{IHC}} \quad (4)$$

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.[30] Multivariate logistic regression analysis was used to estimate the probability of having an abnormal IHC or MSA outcome. The logistic model of the form $\log [p/(1 - p)] = \beta_0 + \beta_1 \cdot x_1 + \beta_2 \cdot x_2$ 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. β_0, β_1 and β_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.

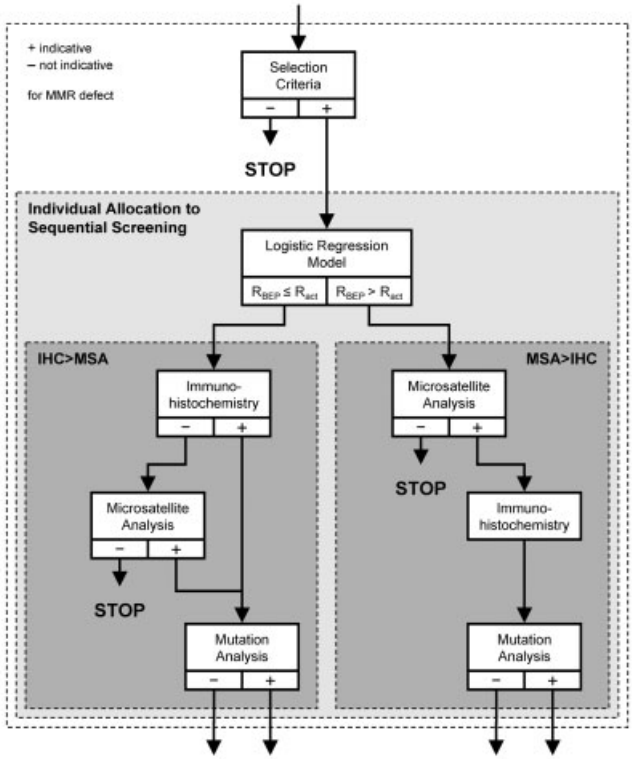

FIGURE 2 – Two-way sequential screening strategy. A logistic regression model is used to allocate patients to that pathway that is expected to be least expensive.

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-

TABLE II – FREQUENCIES OF ABNORMAL TUMOR TISSUE SCREENING AND PATHOGENIC GERMLINE MUTATIONS FOR DIFFERENT SELECTION CRITERIA

	Groups of clinical criteria							Number of families						
								Total		With abnormal IHC and/or MSA		With pathogenic mutation		
	A1	A2	$A2^+$	B2	B3	B4	B7	*n*	%	*n*	% of total (95% CI)	*n*	% of total (95% CI)	% of abnormal IHC/MSA (95% CI)
Total			#	#	#	#	#	1,119	100.0	372	33.2 (30.5–36.1)	230	20.6 (18.3–23.0)	61.8 (56.8–66.6)
A1	+	+	+					222	19.8	139	62.6 (56.1–68.7)	110	49.5 (43.0–66.1)	79.1 (71.6–85.1)
A2 and not A1	−	+	+					29	2.6	20	69.0 (50.8–82.7)	17	58.6 (40.7–74.5)	85.0 (64.0–94.8)
A2		+	+					251	22.4	159	63.3 (57.2–69.1)	127	50.6 (44.5–56.7)	79.9 (73.0–85.4)
$A2^+$ and not A2	−	−	+					70	6.3	10	14.3 (7.9–24.3)	5	7.1 (3.1–15.7)	50.0 (23.7–76.3)
$A2^+$ and not B2/3/4/7			+	−	−	−	−	102	9.1	17	16.7 (10.7–25.1)	8	7.8 (4.0–14.7)	47.1 (26.2–69.0)
$A2^+$ only	−	−	+	−	−	−	−	54	4.8	4	7.4 (2.9–17.6)	1	1.9 (0.3–9.8)	25.0 (4.6–69.9)
B2/3/4/7 only	−	−	−	#	#	#	#	798	71.3	203	25.4 (22.5–28.6)	98	12.3 (10.2–14.7)	48.3 (41.5–55.1)
B2 only	−	−	−	+	−	−	−	140	12.5	27	19.3 (13.6–26.6)	10	7.1 (3.9–12.6)	37.0 (21.5–55.8)
B3 only	−	−	−	−	+	−	−	37	3.3	4	10.8 (4.3–24.7)	3	8.1 (2.8–21.3)	75.0 (30.1–95.4)
B4 only	−	−	−	−	−	+	−	349	31.2	68	19.5 (15.7–24.0)	22	6.3 (4.2–9.4)	32.4 (22.4–44.2)
B7 only	−	−	−	−	−	−	+	37	3.3	2	5.4 (1.5–17.7)	1	2.7 (0.5–13.8)	50.0 (9.5–90.5)
$A2^+$			+					321	28.7	169	52.6 (47.2–58.0)	132	41.1 (35.9–46.6)	78.1 (71.3–83.7)
B2/3/4/7				#	#	#	#	1,017	90.9	355	34.9 (32.0–37.9)	222	21.8 (19.4–24.5)	62.5 (57.4–67.4)
B2				+				339	30.3	167	49.3 (44.0–54.6)	119	35.1 (30.2–40.3)	71.3 (64.0–77.6)
B3					+			390	34.9	215	55.1 (50.2–60.0)	161	41.3 (36.5–46.2)	74.9 (68.7–80.2)
B4						+		718	64.2	279	38.9 (35.4–42.5)	176	24.5 (21.5–27.8)	63.1 (57.3–68.5)
B7							+	84	7.5	26	31.0 (22.1–41.5)	18	21.4 (14.0–31.3)	69.2 (50.0–83.5)

+, criterion fulfilled; −, criterion not fulfilled; #, at least one of the criteria is fulfilled.

TABLE III – RESULTS OF IMMUNOHISTOCHEMISTRY, MICROSATELLITE ANALYSIS AND MUTATION ANALYSIS

Result of tumor tissue screening			Families		Result of mutation analysis					
					Pathogenic		Not pathogenic, *n*	Not determined, *n*	Positive predictive value	
IHC MLH1	IHC MSH2	MSA	*n*	% of total	MLH1, *n*	MSH2, *n*			%	95% CI
Normal	Normal	MSS/MSI-L	747	66.8	0	0	49	698	0.0	(0.0–7.3)
Normal	Normal	MSI-H	58	5.2	10	4	44	0	24.1	(15.0–36.5)
Normal	Loss	MSS/MSI-L	1	0.1	0	0	1	0	0.0	(0.0–79.3)
Normal	Loss	MSI-H	165	14.7	0	119	46	0	72.1	(64.8–78.4)
Loss	Normal	MSS/MSI-L	2	0.2	0	0	2	0	0.0	(0.0–65.8)
Loss	Normal	MSI-H	140	12.5	93	0	47	0	66.4	(58.3–73.7)
Loss	Loss	MSS/MSI-L	0	0.0	0	0	0	0	0.0	(0.0–100.0)
Loss	Loss	MSI-H	6	0.5	2	2	2	0	66.7	(30.0–90.3)
Total IHC abnormal			314	28.1	95	121	98	0	68.8	(63.5–73.7)
Total MSA abnormal			369	33.0	105	125	139	0	62.3	(57.3–67.1)
Total IHC and/or MSA abnormal			372	33.2	105	125	142	0	61.8	(56.8–66.6)
Total			1,119	100.0	105	125	191	698	20.6	(18.3–23.0)

dam I). 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 A2'') 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 $A2^+$ only) showed that only one additional mutation could be identified in our study by waiving the age criterion.

Despite the fact that 28.7% of all families met the $A2^+$ definition, only 9.1% were identified exclusively by $A2^+$, that is, they did not fulfill any other of the Bethesda guidelines 2, 3, 4, or 7 (group ''$A2^+$ and not B2/3/4/7''). Surprisingly, the predictive values are low in this group. The largest fraction of families (90.9%) met one or more of the Bethesda guidelines B2 to B7 (group B2/3/4/7). After exclusion of those fulfilling $A2^+$ (group B2/3/4/7 only) as well, a percentage of 71.3% remained. The Bethesda guideline 3 (group B3) was met by 34.9% of all families. However, only 3.3% fulfilled this criterion exclusively (group B3 only), because most patients in this group include B4 cases diagnosed with colorectal cancer before the age of 45 years. Bethesda guideline 4 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.

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

TABLE IV – RESULTS OF LOGISTIC REGRESSION AND ROC ANALYSES

Model	Logistic regression analysis						ROC analysis AUC (CI_{95})
	Constant		Age of diagnosis (x_1)		Clinical criteria (x_2)		
	β_0	p	β_1	p	β_2	p	
p_{IHC}	−1.894	< 0.001	−0.025	0.001	1.176	< 0.001	0.784 (0.752–0.816)
p_{MSA}	−1.366	< 0.001	−0.029	< 0.001	1.141	< 0.001	0.775 (0.744–0.806)

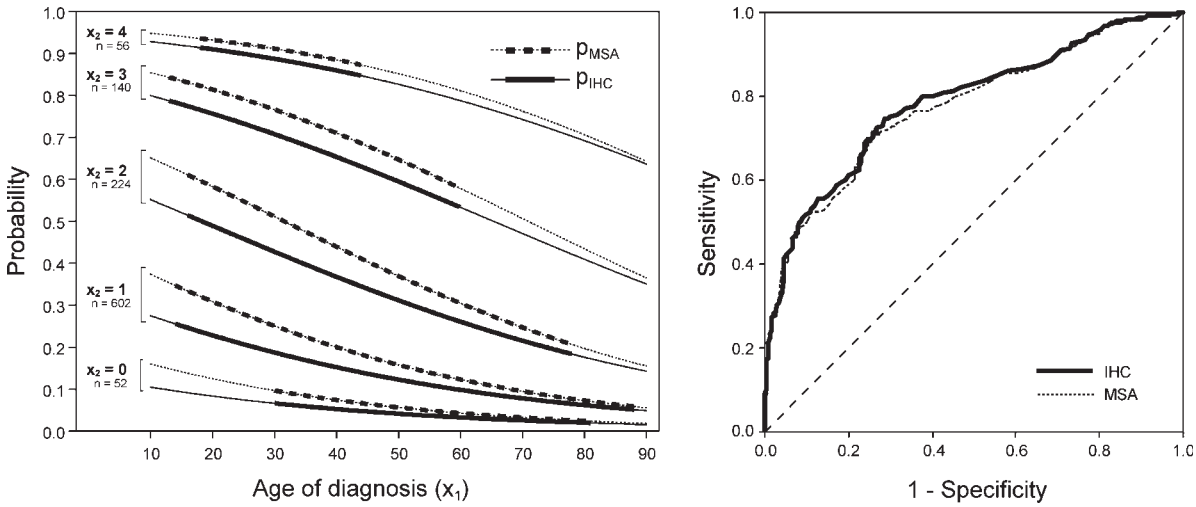

FIGURE 3 – Left: Estimated probability to detect an abnormal outcome in IHC (solid line) or MSA (dashed line) as function of the age of the index patient at first tumor diagnosis (x_1) and the number of fulfilled clinical selection criteria (x_2). Bold parts of the curves indicate ranges in which observations were available in the present data set. Right: ROC analysis comparing predicted with observed IHC (solid line) and MSA (dashed line) outcome.

despite abnormal IHC. There were 6 cases with abnormal MSA and IHC for both *MLH1* and *MSH2*, of which 4 cases had pathogenic mutations (2 in *MLH1* and 2 in *MSH2*). If the result of IHC is compared with the corresponding result of MSA, the positive predictive value and specificity of the IHC were 99.1% and 99.6%, respectively. However, about 6% of the pathogenic mutations (14 out of 230) escaped detection by IHC. The positive predictive value of the combination of both screening methods was 61.8%, which is 3 times higher than the prevalence of pathogenic mutations in the total cohort. This means that preselection by parallel application of IHC and MSA results in a 3-fold enrichment of mutation-positive patients.

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_{BEP} 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_{BEP}. 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 A2, B2, B3 and B4 (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_{BEP} 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}. If R_{BEP} is less than or equal to R_{act}, then the patients should be allocated to the strategy that uses IHC in the first place (IHC→MSA). Conversely, if R_{BEP} is less than or equal to R_{act}, then MSA should be applied first (MSA→IHC). As shown in Figure 5, the costs of this individualized allocation strategy lie between 73% and 75% compared with the parallel strategy, that is, a cost reduction of about 25% can be achieved.

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.[19,20,31–36] 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

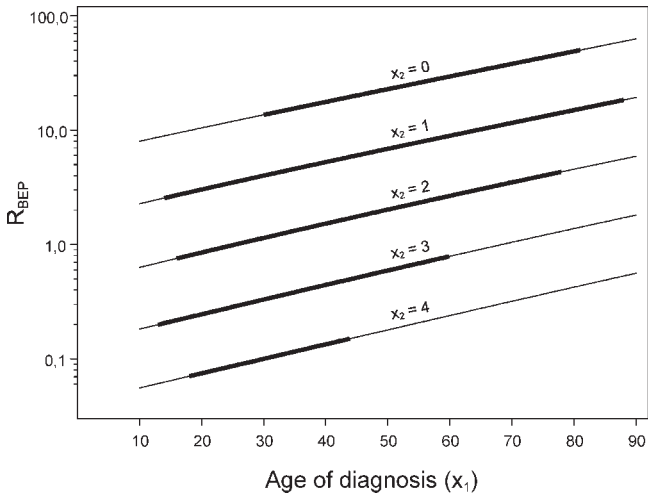

FIGURE 4 – Functional relationship between individual patient characteristics (number of fulfilled clinical selection criteria, age at first tumor diagnosis) and the MSA-to-IHC cost ratio R_{BEP}, at which both sequential screening alternatives yield identical costs. Bold parts of the curves indicate ranges in which observations were available in the present data set.

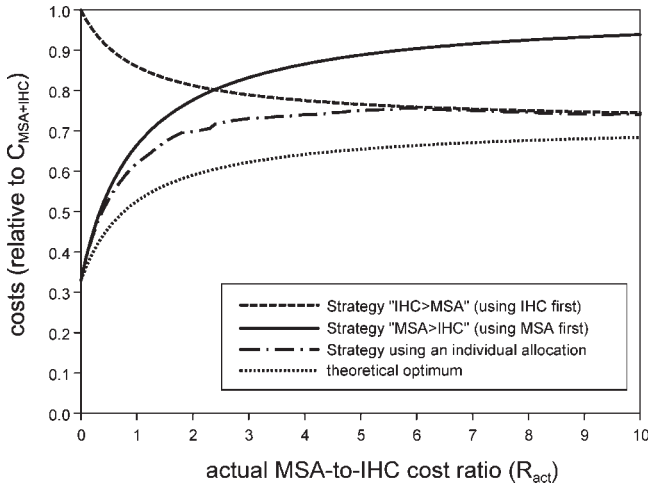

FIGURE 5 – Comparison of the costs of different screening strategies. The gray shaded area indicates the range of the actual MSA-to-IHC cost ratio in Germany.

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.[8] 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*.,[34] 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 connection with the Bethesda guidelines, the Amsterdam criteria 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.[10] 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*.[37] 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.[38,39] 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-

quency), 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 had a pathogenic MLH1 or MSH2 mutation. This group should also include cases with mutations in other MMR genes, such as *MSH6* or *PMS2*, the frequency of which, however, was not comprehensively determined in this study. The extension of immunohistochemical screening to these proteins may lead to a relevant reduction of cases in this group.[38,40]

The positive predictive value of the parallel IHC/MSA test combination to detect pathogenic mutations was 61.8%. Thus, in relation to the overall mutation prevalence of 20.6–25.5%, an enrichment factor of 2.4 to 3 could be achieved by this selection strategy.

Microsatellite analysis is currently regarded as the standard method for selecting patients with a high risk of carrying a pathogenic mutation. However, immunohistochemistry poses a less resource-demanding alternative. Moreover, this method gives information about the presumably mutated gene and thus allows reducing the cost for sequencing. Ideally, the positive predictive value (PPV) and the specificity of IHC with regard to MSA should be 100%. To date, a number of investigations have compared the performance of MSA *versus* IHC, the results of which are in good agreement to those of our present analysis. The largest evaluation has been performed by Lindor *et al*.,[31] who determined a PPV and specificity of 100% for IHC of MLH1 and MSH2 in a series of colorectal cancers from 1,144 patients. Further investigations on smaller series confirm the nearly perfect PPV and specificity.[19,36] The PPV and specificity of IHC in our well-defined cohort were 99.1% and 99.6%, respectively. Three cases with loss of protein expression failed to show the expected microsatellite instability. Fourteen cases (6.1%) with pathogenic mutations escaped detection by 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 IHC is unnecessary because MSA provides no additional information. Therefore, alternative strategies using both methods sequentially should be more cost-effective.

Christensen *et al*.[19] and also Debniak *et al*.[20] 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*.[21] 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 tumor 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 definitive selection criterion for *MSH6* mutation analysis, as some mutations may not be detected by either method.[29,41,42] 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

1. Aarnio M, Mecklin JP, Aaltonen LA, Nystrom-Lahti M, Jarvinen HJ. Life-time risk of different cancers in hereditary non-polyposis colorectal cancer (HNPCC) syndrome. Int J Cancer 1995;64:430–3.
2. Lynch HT, de la Chapelle A. Genetic susceptibility to non-polyposis colorectal cancer. J Med Genet 1999;36:801–18.
3. Bronner CE, Baker SM, Morrison PT, Warren G, Smith LG, Lescoe MK, Kane M, Earabino C, Lipford J, Lindblom A, Tannergord P, Bollag RJ. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. Nature 1994;368:258–61.
4. Fishel R, Lescoe MK, Rao MR, Copeland NG, Jenkins NA, Garber J, Kane M, Kolodner R. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell 1993;75:1027–38.
5. Leach FS, Nicolaides NC, Papadopoulos N, Liu B, Jen J, Parsons R, Peltomaki P, Sistonen P, Aaltonen LA, Nystrom-Lahti M, Guan XY, Zhang J. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. Cell 1993;75:1215–25.
6. Papadopoulos N, Nicolaides NC, Wei YF, Ruben SM, Carter KC, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, Venter JC, Dunlop MG. Mutation of a mutL homolog in hereditary colon cancer. Science 1994;263:1625–9.
7. Vasen HF, Mecklin JP, Khan PM, Lynch HT. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). Dis Colon Rectum 1991;34:424–5.
8. Vasen HF, Watson P, Mecklin JP, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology 1999;116:1453–6.
9. Rodriguez-Bigas MA, Boland CR, Hamilton SR, Henson DE, Jass JR, Khan PM, Lynch H, Perucho M, Smyrk T, Sobin L, Srivastava S. A National Cancer Institute workshop on hereditary nonpolyposis colorectal cancer syndrome: meeting highlights and Bethesda guidelines. J Natl Cancer Inst 1997;89:1758–62.
10. Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Ruschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, *et al*. Revised Bethesda guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 2004;96:261–8.
11. Wijnen J, Khan PM, Vasen H, van der Klift H, Mulder A, van Leeuwen-Cornelisse I, Bakker B, Losekoot M, Moller P, Fodde R. Hereditary nonpolyposis colorectal cancer families not complying with the Amsterdam criteria show extremely low frequency of mismatch-repair-gene mutations. Am J Hum Genet 1997;61:329–35.
12. Aaltonen LA, Salovaara R, Kristo P, Canzian F, Hemminki A, Peltomaki P, Chadwick RB, Kaariainen H, Eskelinen M, Jarvinen H, Mecklin JP, de la Chapelle A. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med 1998;338:1481–7.
13. Liu T, Wahlberg S, Burek E, Lindblom P, Rubio C, Lindblom A. Microsatellite instability as a predictor of a mutation in a DNA mismatch repair gene in familial colorectal cancer. Genes Chromosomes Cancer 2000;27:17–25.

14. Loukola A, Eklin K, Laiho P, Salovaara R, Kristo P, Jarvinen H, Mecklin JP, Launonen V, Aaltonen LA. Microsatellite marker analysis in screening for hereditary nonpolyposis colorectal cancer (HNPCC). Cancer Res 2001;61:4545–9.
15. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 1993;363:558–61.
16. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. Science 1993;260:816–9.
17. Hendriks Y, Franken P, Dierssen JW, De Leeuw W, Wijnen J, Dreef E, Tops C, Breuning M, Brocker-Vriends A, Vasen H, Fodde R, Morreau H. Conventional and tissue microarray immunohistochemical expression analysis of mismatch repair in hereditary colorectal tumors. Am J Pathol 2003;162:469–77.
18. Leach FS, Polyak K, Burrell M, Johnson KA, Hill D, Dunlop MG, Wyllie AH, Peltomaki P, de la Chapelle A, Hamilton SR, Kinzler KW, Vogelstein B. Expression of the human mismatch repair gene hMSH2 in normal and neoplastic tissues. Cancer Res 1996;56: 235–40.
19. Christensen M, Katballe N, Wikman F, Primdahl H, Sorensen FB, Laurberg S, Orntoft TF. Antibody-based screening for hereditary nonpolyposis colorectal carcinoma compared with microsatellite analysis and sequencing. Cancer 2002;95:2422–30.
20. Debniak T, Kurzawski G, Gorski B, Kladny J, Domagala W, Lubinski J. Value of pedigree/clinical data, immunohistochemistry and microsatellite instability analyses in reducing the cost of determining hMLH1 and hMSH2 gene mutations in patients with colorectal cancer. Eur J Cancer 2000;36:49–54.
21. Ponz de Leon M, Benatti P, Di Gregorio C, Pedroni M, Losi L, Genuardi M, Viel A, Fornasarig M, Lucci-Cordisco E, Anti M, Ponti G, Borghi F, *et al*. Genetic testing among high-risk individuals in families with hereditary nonpolyposis colorectal cancer. Br J Cancer 2004;90:882–7.
22. Bocker T, Diermann J, Friedl W, Gebert J, Holinski-Feder E, Karner-Hanusch J, von Knebel-Doeberitz M, Koelble K, Moeslein G, Schackert HK, Wirtz HC, Fishel R, *et al*. Microsatellite instability analysis: a multicenter study for reliability and quality control. Cancer Res 1997;57:4739–43.
23. Dietmaier W, Wallinger S, Bocker T, Kullmann F, Fishel R, Ruschoff J. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. Cancer Res 1997;57:4749–56.
24. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S. A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998;58:5248–57.
25. Muller A, Giuffre G, Edmonston TB, Mathiak M, Roggendorf B, Heinmoller E, Brodegger T, Tuccari G, Mangold E, Buettner R, Ruschoff J. Challenges and pitfalls in HNPCC screening by microsatellite analysis and immunohistochemistry. J Mol Diagn 2004;6: 308–15.
26. Mangold E, Pagenstecher C, Friedl W, Mathiak M, Buettner R, Engel C, Loeffler M, Holinski-Feder E, Müller-Koch Y, Keller G, Schackert HK, Krüger S, *et al*. Spectrum and frequencies of mutations in MSH2 and MLH1 identified in 1721 German families suspected of hereditary nonpolyposis colorectal cancer. Int J Cancer 2005;116:692–702.
27. Ruschoff J, Roggendorf B, Brasch F, Mathiak M, Aust DE, Plaschke J, Mueller W, Poremba C, Kloor M, Keller G, Muders M, Blasenbreu-Vogt S, *et al*. Molecular pathology in hereditary colorectal cancer: recommendations of the Collaborative German Study Group on hereditary colorectal cancer funded by the German Cancer Aid (Deutsche Krebshilfe). Pathologe 2004;25:178–92.
28. Plaschke J, Kruger S, Dietmaier W, Gebert J, Sutter C, Mangold E, Pagenstecher C, Holinski-Feder E, Schulmann K, Moslein G, Ruschoff J, Engel C, *et al*. Eight novel MSH6 germline mutations in patients with familial and nonfamilial colorectal cancer selected by loss of protein expression in tumor tissue. Hum Mutat 2004;23:285.
29. Plaschke J, Engel C, Kruger S, Holinski-Feder E, Pagenstecher C, Mangold E, Moeslein G, Schulmann K, Gebert J, von Knebel Doeberitz M, Ruschoff J, Loeffler M, *et al*. Lower incidence of colorectal cancer and later age of disease onset in 27 families with pathogenic MSH6 germline mutations compared with families with MLH1 or MSH2 mutations: the German Hereditary Nonpolyposis Colorectal Cancer Consortium. J Clin Oncol 2004;22:4486–94.
30. Newcombe RG. Two-sided confidence intervals for the single proportion: comparison of seven methods. Stat Med 1998;17:857–72.
31. Lindor NM, Burgart LJ, Leontovich O, Goldberg RM, Cunningham JM, Sargent DJ, Walsh-Vockley C, Petersen GM, Walsh MD, Leggett BA, Young JP, Barker MA, *et al*. Immunohistochemistry *versus* microsatellite instability testing in phenotyping colorectal tumors. J Clin Oncol 2002;20:1043–8.
32. Muller W, Burgart LJ, Krause-Paulus R, Thibodeau SN, Almeida M, Edmonston TB, Boland CR, Sutter C, Jass JR, Lindblom A, Lubinski J, MacDermot K, *et al*. The reliability of immunohistochemistry as a prescreening method for the diagnosis of hereditary nonpolyposis colorectal cancer (HNPCC): results of an international collaborative study. Fam Cancer 2001;1:87–92.
33. Salahshor S, Koelble K, Rubio C, Lindblom A. Microsatellite Instability and hMLH1 and hMSH2 expression analysis in familial and sporadic colorectal cancer. Lab Invest 2001;81:535–41.
34. Stormorken AT, Muller W, Lemkemeyer B, Apold J, Wijnen JT, Fodde R, Moslein G, Moller P. Prediction of the outcome of genetic testing in HNPCC kindreds using the revised Amsterdam criteria and immunohistochemistry. Fam Cancer 2001;1:169–73.
35. Syngal S, Fox EA, Eng C, Kolodner RD, Garber JE. Sensitivity and specificity of clinical criteria for hereditary non-polyposis colorectal cancer associated mutations in MSH2 and MLH1. J Med Genet 2000;37:641–5.
36. Wahlberg SS, Schmeits J, Thomas G, Loda M, Garber J, Syngal S, Kolodner RD, Fox E. Evaluation of microsatellite instability and immunohistochemistry for the prediction of germ-line MSH2 and MLH1 mutations in hereditary nonpolyposis colon cancer families. Cancer Res 2002;62:3485–92.
37. De Jong AE, Morreau H, Van Puijenbroek M, Eilers PH, Wijnen J, Nagengast FM, Griffioen G, Cats A, Menko FH, Kleibeuker JH, Vasen HF. The role of mismatch repair gene defects in the development of adenomas in patients with HNPCC. Gastroenterology 2004; 126:42–8.
38. de Jong AE, van Puijenbroek M, Hendriks Y, Tops C, Wijnen J, Ausems MG, Meijers-Heijboer H, Wagner A, van Os TA, Brocker-Vriends AH, Vasen HF, Morreau H. Microsatellite instability, immunohistochemistry, and additional PMS2 staining in suspected hereditary nonpolyposis colorectal cancer. Clin Cancer Res 2004;10:972–80.
39. Furukawa T, Konishi F, Shitoh K, Kojima M, Nagai H, Tsukamoto T. Evaluation of screening strategy for detecting hereditary nonpolyposis colorectal carcinoma. Cancer 2002;94:911–20.
40. Plaschke J, Kruger S, Pistorius S, Theissig F, Saeger HD, Schackert HK. Involvement of hMSH6 in the development of hereditary and sporadic colorectal cancer revealed by immunostaining is based on germline mutations, but rarely on somatic inactivation. Int J Cancer 2002;97:643–8.
41. Berends MJ, Wu Y, Sijmons RH, Mensink RG, van der Sluis T, Hordijk-Hos JM, de Vries EG, Hollema H, Karrenbeld A, Buys CH, van der Zee AG, Hofstra RM, *et al*. Molecular and clinical characteristics of MSH6 variants: an analysis of 25 index carriers of a germline variant. Am J Hum Genet 2002;70:26–37.
42. Wu Y, Berends MJ, Mensink RG, Kempinga C, Sijmons RH, van Der Zee AG, Hollema H, Kleibeuker JH, Buys CH, Hofstra RM. Association of hereditary nonpolyposis colorectal cancer-related tumors displaying low microsatellite instability with MSH6 germline mutations. Am J Hum Genet 1999;65:1291–8.

Appendix

The German HNPCC Consortium, funded by the Deutsche Krebshilfe, comprises centers at the following locations (coworkers are listed in addition to authors): clinical centers: Bochum (F.E. Brasch, J.T. Epplen, S.A. Hahn, K.M. Mueller, E. Kunstmann, W. Schmiegel, K. Schulmann, J. Willert), Bonn (H.P. Fischer, W. Friedl, J. Girmscheid, A. Hirner, C. Lamberti, H. Lauschke, M. Mathiak, P. Propping, T. Sauerbruch, N. Wernert), Düsseldorf (T. Goecke, D. Goedde, A. Hansmann, K.L. Schaefer, T. Vogel, C. Wieland), Dresden (D. Aust, F. Balck, R. Höhl, F. Kreuz, S. Pistorius, S. Krüger), Heidelberg (F. Cremer, J. Gebert, M. Keller, H.P. Knaebel, M. von Knebel Doeberitz, U. Mazitschek, M. Tariverdian), Munich/Regensburg (I. Becker, R. Kopp, Y. Müller-Koch, M. Sarbia, U. Schiemann, M. Scholz, H. Vogelsang); center for reference pathology: Kassel (A. Mueller, T. Brodegger); center for documentation and biometry: Leipzig (M. Herold, J. Schaefer, R. Speer).