Spectrum and frequencies of mutations in *MSH2* and *MLH1* identified in 1,721 german families suspected of hereditary nonpolyposis colorectal cancer

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Mutations in DNA MMR genes, mainly *MSH2* and *MLH1*, account for the majority of HNPCC, an autosomal dominant predisposition to colorectal cancer and other malignancies. The evaluation of many questions regarding HNPCC requires clinically and genetically well-characterized HNPCC patient cohorts of reasonable size. One main focus of this multicenter study is the evaluation of the mutation spectrum and mutation frequencies in a large HNPCC cohort in Germany; 1,721 unrelated patients, mainly of German descent, who met the Bethesda criteria were included in the study. In tumor samples of 1,377 patients, microsatellite analysis was successfully performed and the results were applied to select patients eligible for mutation analysis. In the patients meeting the strict Amsterdam criteria (AC) for HNPCC, 72% of the tumors exhibited high microsatellite instability (MSI-H) while only 37% of the tumors from patients fulfilling the less stringent criteria showed MSI-H; 454 index patients (406 MSI-H and 48 meeting the AC of whom no tumor samples were available) were screened for small mutations. In 134 index patients, a pathogenic *MSH2* mutation, and in 118 patients, a pathogenic *MLH1* mutation was identified (overall detection rate for pathogenic mutations 56%). One hundred sixty distinct mutations were detected, of which 86 are novel mutations. Noteworthy is that 2 mutations were over-represented in our patient series: *MSH2*,c.942+3A>T and *MLH1*,c.1489_1490insC, which account for 11% and 18% of the *MSH2* and *MLH1* mutations, respectively. A subset of 238 patients was screened for large genomic deletions. In 24 (10%) patients, a deletion was found. In 72 patients, only unspecified variants were found. Our findings demonstrate that preselection by microsatellite analysis substantially raises mutation detection rates in patients not meeting the AC. As a mutation detection strategy for German HNPCC patients, we recommend to start with screening for large genomic deletions and to continue by screening for common mutations in exon 5 of *MSH2* and exon 13 of *MLH1* before searching for small mutations in the remaining exons.
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Hereditary nonpolyposis colorectal cancer (HNPCC, MIM 114500) is an autosomal dominant tumor predisposition with high penetrance. The most common HNPCC tumor manifestation is in the colorectum. With an estimated proportion of 2–5% of all colorectal cancers, HNPCC represents the most frequent monogenic predisposition to colorectal malignancies.[1–3] In addition, the incidence of other malignant tumors, *e.g.*, endometrial cancer, urothelial cancer, small bowel cancer and ovarian cancer is also increased in HNPCC patients.[4,5] Another typical finding in

HNPCC families is a younger age of onset when compared to patients with sporadic tumors. According to the literature, the mean age of onset is approximately 40 years for HNPCC colorectal cancers compared to approximately 60 years in sporadic colorectal malignancies.[6] As recently demonstrated, a better survival rate in HNPCC patients can be achieved by early detection of colorectal tumors *via* frequent colonoscopies.[7] A lifelong cancer surveillance program addressing colorectal cancer and other common HNPCC malignancies is currently regarded to be of benefit for HNPCC families. However, the precise differentiation between HNPCC patients who truly are at increased cancer risk and patients with sporadic colorectal cancers who are not is still a major challenge.

The clinical diagnosis of HNPCC is made when the patient's family meets the Amsterdam criteria (AC).[8,9] However, the diagnosis of HNPCC should also be considered when the patient's individual history and family history do not meet the AC but 1 or more of the less stringent Bethesda guidelines.[10,11]

TABLE I – CLASSIFICATION OF PATIENTS IN DIFFERENT GROUPS ACCORDING TO DIAGNOSTIC CRITERIA

Designation of group	Diagnostic criteria
Amsterdam positive (AC)	Family meets the Amsterdam criteria I or II
Less stringent inclusion criteria	Family meets all of the Amsterdam criteria II except the age criterion (i.e., none of the tumors was diagnosed before age 50) or
	Individuals with 2 HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, or small bowel cancer or transitional cell carcinoma of the renal pelvis or ureter) (Bethesda criterion 2) or
	Individuals with colorectal cancer and a first degree relative with colorectal cancer and/or an HNPCC-related extracolonic cancer and/or a colorectal adenoma; 1 of the cancers diagnosed at age < 45, the adenoma at age < 40 (Bethesda criterion 3) or
	Individuals with colorectal or endometrial cancer diagnosed at age < 45 (Bethesda criterion 4)
Other criteria suspicious of HNPCC	Patient/family meets none of the above criteria but a strong suspicion for HNPCC was raised as the patient/family history is very close to the Bethesda criteria

Another approach to identify HNPCC patients is provided by molecular genetics. A deficient DNA mismatch repair system was found to be the genetic basis for a majority of HNPCC cases. To date, a broad variety of mutations in the DNA MMR genes *MSH2*, *MLH1*, *MSH6* and *PMS2* have been identified in HNPCC families, while the role of *MLH3* and *PMS1* remains unclear.[12] Most of the mutations currently listed in the databases (*e.g.*, in the database of the international collaborative group on HNPCC; http://www.nfdht.nl) were identified in the genes *MSH2* and *MLH1*. Identification of the predisposing germline mutation in a patient confirms the clinical diagnosis of HNPCC. The finding of a definitely pathogenic germline mutation also represents the prerequisite for predictive genetic testing in family members at risk. However, performing mutation analysis in every patient suspected of HNPCC, according to the Bethesda guidelines, has often been regarded as an excessively cost-intensive and time-consuming approach, even when it is restricted to *MSH2* and *MLH1*, where the chance of detecting an underlying germline mutation is far higher than in *MSH6* or *PMS2*.

Various approaches have been proposed to reduce the number of candidates for mutation analysis to only those with a high probability of harboring germline mutations. Clinical preselection, either by Bethesda criteria or other pedigree data, was successfully applied by all studies.

Moreover, tumor tissue analysis of suspected HNPCC patients was found to be useful for this purpose: A characteristic finding in DNA mismatch repair deficient tumors is genomic instability which is reflected in high microsatellite instability (MSI-H).[13–15] The use of microsatellite analysis in combination with clinical criteria as a prescreening method has been advocated by many authors and is currently regarded as the gold-standard of tumor tissue analysis in HNPCC suspects.[16–19] Immunohistochemical staining of DNA mismatch repair proteins in tumor tissue represents another method to preselect candidates for mutation analysis.[20,21] However, according to current literature data, immunohistochemistry has a lower sensitivity than microsatellite analysis. This problem still awaits further evaluation in large series of HNPCC patients.[22,23]

Besides the search for an optimum prescreening approach many more questions, *e.g.*, regarding the pathogenicity of missense variants in DNA MMR genes or the best cancer prevention strategy in HNPCC patients, were raised in the past. Most of these questions urgently need clinically and genetically well-characterized HNPCC patient cohorts of reasonable size in order to achieve reliable answers. At present, the number of such HNPCC cohorts reported in the literature is quite limited.

Since 1999, a registry for HNPCC families has been established by the German HNPCC Consortium. In a multidisciplinary approach, 6 university hospitals collect clinical data of HNPCC families or patients suspected of HNPCC, provide genetic and clinical counseling, tumor tissue analysis, molecular genetic workup, predictive testing and surveillance examinations. Data storage, quality control and biostatistical analyses are performed

centrally. A reference pathology center is in charge of review of histopathology data. The cohort established by the German HNPCC consortium represents an ideal population for future evaluation of diagnostic and therapeutic strategies.

The main focus during the first phase of the project was tumor tissue analysis and collecting clinical and genetic data in patients suspected of HNPCC. Our study presents the spectrum and frequencies of germline mutations detected in the genes *MSH2* and *MLH1* in a large population of mainly German origin. By December 2003, a total of 1,721 unrelated patients or families suspected of HNPCC were registered in the central database. In 1,406 of these index patients, a combination of tumor tissue parameters, patient history and family history was applied in order to preselect patients eligible for mutation analysis in *MSH2* and *MLH1*.

Material and methods

Participating centers

The patients included in our study were recruited from 6 German university hospitals: Bochum (BO), Bonn (BN), Dresden (DD), Düsseldorf (DÜ), Heidelberg (HD) and München/Regensburg (MR). In all centers, human geneticists, molecular biologists, gastroenterologists, pathologists and surgeons were involved in patient ascertainment, analysis and data documentation in accordance with a common study protocol. Patients were referred to the study from other hospitals, institutes of human genetics, private practice physicians, private practice human geneticists or came by self-referral. All centers offered genetic and clinical counseling as well as regular HNPCC surveillance examinations to the participants. The Institute of Medical Informatics at the University of Leipzig is in charge of data storage, data quality management and statistical analyses. Revision of histopathology data was performed at a central reference pathology unit (Department of Pathology, Klinikum Kassel). The study was approved by the ethical committees of all participating clinical centers.

Patients

Modified Bethesda criteria were applied as inclusion criteria (Table I): According to the common study protocol, all patients either meeting the Bethesda criteria as defined by Rodriguez-Bigas and colleagues or whose family meets all of the Amsterdam criteria II as defined by Vasen and colleagues, except the age criterion (*i.e.*, none of the tumors was diagnosed before age 50), were included in the study;[9,10] 1,721 unrelated index patients, suspected of HNPCC due either to their own or possibly their family cancer history, were recruited. Index patients were categorized as either Amsterdam positive patients or as patients meeting the less stringent inclusion criteria (Tables I and II). Four hundred thirty-two patients meet the Amsterdam criteria as previously defined; 1,149 patients were categorized as meeting the less stringent criteria.[9] Another 140 index patients presented in our study did not meet any of the defined inclusion criteria but a suspicion of HNPCC

	All patients	Amsterdam positive	Less stringent inclusion criteria[2]	Other criteria suspicious of HNPCC[2]
Number of patients	1,721	432	1,149	140
Tumor tissue available	1,406	333	961	112
MSA evaluable	1,377	324	943	110
MSS	707	81	549	77
MSI-L	56	9	42	5
MSI-H	614	234	352	28
% MSI-H	44.6	72.2	37.3	25.4

[1]MSA, microsatellite analysis; MSS, microsatellite stability; MSI-L, low microsatellite instability; MSI-H, high microsatellite instability. –[2]For details see Table I.

was raised in these patients because their personal history or family history was very close to the Bethesda criteria (*e.g.*, a patient without HNPCC family history who was diagnosed with colorectal cancer at the age of 46 years) (group designated as ''other criteria suspicious of HNPCC''). All index patients gave written informed consent authorizing data documentation, examination of tumor tissue for HNPCC characteristics and molecular genetic analysis of genes associated with HNPCC. According to our study protocol all patients who meet the Amsterdam criteria and all patients who meet the looser Bethesda criteria and have MSI-H in their tumor were counseled about HNPCC and were advised to undergo regular HNPCC surveillance.

Microsatellite analysis

Analysis for microsatellite instability (MSI) was applied as a prescreening test prior to mutation analysis in the *MSH2* and *MLH1* genes. MSI analysis had been performed on matched pairs of tumor DNA and normal DNA using the National Cancer Institute/International Collaborative Group on HNPCC (NCI/ICG-HNPCC) reference marker panel for the evaluation of MSI in colorectal cancer (BAT25, BAT26, D2S123, D5S346 and D17S250).[24] Tumor DNA was extracted from microdissected tumor tissue; normal DNA was extracted from either normal tissue or peripheral blood leukocytes. Tumors were scored as highly instable (MSI-H) when 2 or more of these 5 markers exhibited additional alleles and as stable (MSS) when none of the 5 markers showed instability. When only 1 marker showed instability, an additional panel of 5 markers (BAT40, D10S197, D13S153, MYCL1 and D18S58) was examined. In these cases, the tumor was classified as MSI-H when 3 or more of the 10 markers showed instability and as low instable (MSI-L) when 1 or 2 of 10 markers were instable.

A subset of tumors had been evaluated for MSI by use of other microsatellite markers as previously described, but were classified as MSI-H, MSI-L or MSS tumors according to the above-mentioned schedule.[17,25]

Search for germline mutations in MSH2 and MLH1

According to the study protocol, a systematic screening for germline mutations was performed in patients with either MSI-H tumors or in patients meeting the AC, of whom no tumor tissue was available for prescreening; 406 patients with MSI-H were screened for small mutations (point mutations or small deletions/insertions) in *MSH2* and *MLH1*. Forty-eight index patients from families meeting the AC, of whom no tumor tissue was available for microsatellite analysis, were also screened for small mutations (Table III). In addition, 11 patients with MSI-L tumors were screened for germline mutations.

Genomic DNA was extracted from EDTA anticoagulated blood samples by standard salting out procedure.[26] Search for germline mutations in the *MSH2* and *MLH1* genes had been performed by a prescreening procedure using denaturing high performance liquid chromatography (DHPLC) in 3 of the centers (BN, DÜ and MR) as described, followed by sequencing of fragments showing aberrant chromatograms.[27] In the other centers (BO, DD and HD),

direct sequencing without prior screening methods was applied. Sequencing was either performed on an ABI 377 or an ABI 3100 DNA Sequencer (Applied Biosystems, Foster City, CA) (BN, BO, DÜ, HD and MR) or on an A.L.F. express sequencer (Amersham Biosciences, Freiburg, Germany) (DD). Five centers (BN, BO, DD, DÜ and HD) stopped DHPLC prescreening or sequence analysis as soon as a pathogenic mutation had been identified. One center (MR) analyzed *MSH2* and *MLH1* by fully prescreening all exons by DHPLC and sequencing all fragments with aberrant chromatograms.

A systematic search for large genomic deletions was performed in 2 of the participating centers (BN and DÜ). For this purpose, a semiquantitative multiplex PCR method as described or a multiplex ligation-dependent probe amplification (MLPA) method according to the manufacturer's description/protocol (MRC-Holland, Amsterdam, The Netherlands) was applied in Bonn.[28] In Düsseldorf, Southern blot analysis or MLPA were applied in order to detect large genomic deletions.

Results

Preselection by microsatellite analysis

Microsatellite analysis was successfully performed in tumors from 1,377 index patients (Table II); 614 patients exhibited MSI-H and 56 patients exhibited MSI-L in their tumors, 707 tumors were found to be microsatellite stable and in 29 tumors no interpretable results were obtained. As expected, the percentage of MSI-H tumors was highest (234/324; 72%) in the group meeting the Amsterdam criteria; 352/943 (37%) of tumors from patients fulfilling less stringent Bethesda criteria exhibited MSI-H. In the group not meeting the inclusion criteria, the number of MSI-H tumors was lowest (28/110; 25%). Tumor tissue from 56 patients showed low microsatellite instability (MSI-L); 9 of these patients met the AC, 42 met the less stringent inclusion criteria and 5 patients met other criteria suspicious of HNPCC.

In accordance with the study protocol, a total of 406 of index patients who exhibited MSI-H in their tumor tissue and 48 patients from the AC positive group in whom no tumor tissue was available for microsatellite analysis were screened for a germline mutation in *MSH2* and *MLH1*. In addition, mutation analysis was performed in 11 patients with MSI-L tumors.

Classification of mutations

The detected sequence variants were categorized either as definitely pathogenic mutation, unspecified variant or frequent polymorphism. In accordance with the present literature data and mutation database information, we designated the *MSH2* variants c.211+9C>G, c.1077-10T>C, c.1511-9A>T, c.1661+12A>G and c.2006-6T>C and the *MLH1* variants c.655A>G, c.1558+14G>A and c.1668-19A>G as frequent polymorphisms. In 324 patients, sequence alterations, not belonging to the polymorphism category, were identified. These mutations were categorized as pathogenic mutations or variants of unknown relevance (UV). The group of pathogenic mutations included sequence alterations with predicted deleterious effects on the MSH2 or MLH1 protein, such as non-

TABLE III – MSI AND MUTATION STATUS IN DIFFERENT PATIENT GROUPS[1]

	All patients	Amsterdam positive	Less stringent inclusion criteria[2]	Other criteria suspicious of HNPCC[2]
Patients analyzed for mutations	454	220	218	16
MSI-H patients	406 (100%)	172 (100%)	218 (100%)	16
Identified with pathogenic mutations	225 (55.4%)	128 (74.4%)	95 (43.6%)	2
Identified with UVs	88 (21.4%)	30 (17.4%)	54 (24.8%)	4
Patients without tumor tissue analysis	48 (100%)	48 (100%)	n.d.	n.d.
Identified with pathogenic mutations	27 (56.2%)	27 (56.2%)	n.d.	n.d.
Identified with UVs	8 (16.7%)	8 (16.7%)	n.d.	n.d.

[1]MSI-H, high microsatellite instability; UVs, variants with unknown relevance; n.d. according to the study protocol, no germline mutation screening was performed in this group. —[2]For details see Table I.

sense mutations, frameshift mutations, resulting in a premature stop codon, mutations at the highly conserved splice site positions AG/GT, mutations destroying the translation start site and genomic rearrangements (Table IV). Based on published or our own studies that demonstrate exonic deletions at RNA level, 5 mutations that do not fall into 1 of these categories were also classified as pathogenic: The mutation MSH2,c.942+3A>T leads to an in frame deletion of exon 5.[29] MLH1,c.677+3A>G was shown to lead to an out of frame deletion of exon 8.[30] MLH1,c.790+2 790+3insT leads to an in frame deletion of exons 9 and 10 and the apparently silent mutation MLH1,c.1731G>A, p.Ser577 was demonstrated to lead to an out of frame deletion of exon 15.[31] The mutation MLH1,c.2103G>C (p.Gln701His) leads to an in frame deletion of exon 18 (own unpublished data). All remaining mutations were categorized as UVs (Table V).

Detection rates for pathogenic mutations in different patient groups

A total of 252 index patients (225 index patients with MSI-H tumors and 27 AC patients without tumor tissue analysis) were found to have a pathogenic germline mutation, corresponding to an overall detection rate of 56% in the preselected cohort of 454 patients (Table III). The detection rate for pathogenic mutations was highest among patients meeting the Amsterdam criteria with MSI-H tumors (128/172; 74%). A pathogenic mutation was found in 27/48 (56%) of the AC positive patients of whom no tumor samples were available for prescreening. In the index patients with MSI-H tumors fulfilling the less stringent inclusion criteria, the mutation detection rate was 95/218 (44%). In addition, 2 pathogenic mutations were identified in 16 MSI-H index patients meeting other criteria suspicious of HNPCC. In the 11 patients whose tumors exhibited MSI-L, no pathogenic MSH2 or MLH1 mutations were detected.

Spectrum of pathogenic mutations

In 134 patients, we detected pathogenic MSH2 mutations, and in 118 patients, a pathogenic MLH1 mutation was detected (Table IV). Overall, we identified 160 different mutations, 86 of these mutations are novel and not listed in the ICG-HNPCC database (http://www.nfdht.nl). The mutations were distributed over the whole MSH2 and MLH1 genes, respectively. Noteworthy is that most of the mutations were identified in 1 or 2 index patients except for 2 mutations that were significantly over-represented and accounted for 14% of all cases with pathogenic mutations in our series. The mutation MSH2,c.942+3A>T was found in 15 index patients and the mutation MLH1,c.1498_1490insC in 21 patients.

In both genes, the majority of pathogenic mutations were point mutations or small deletions/insertions: 45 of the MSH2 mutation carriers were identified with frameshift mutations due to small insertions/deletions, 43 carried nonsense mutations and 31 alterations at the highly conserved splice site positions AG/GT. Fifteen patients with large genomic MSH2 deletions involving 1 or more exons were detected. Forty-nine of the MLH1 mutation carriers had frameshift mutations, 22 nonsense mutations and 25 had mutations of the highly conserved splice site positions. Eight

patients were found to have missense mutations or silent mutations that lead to a splice defect and 1 patient had a mutation destroying the initiation site of MLH1. In 13 patients, large genomic MLH1 deletions were identified. Noteworthy is that 5 families were found with a deletion encompassing the MLH1 exons 1–10.

Frequency of large genomic deletions

In 2 of the participating centers (BN and DÜ), all index patients (with MSI-H tumors or AC patients, of whom no tumor tissue was available) were screened for genomic rearrangements in MSH2 and MLH1. In this subset of 238 patients, 110 small mutations and 24 large deletions were detected, corresponding to a deletion detection rate of 10%. With a total of 134 index patients with a pathogenic mutation, the percentage of large genomic deletions among pathogenic mutations was 18% in this subset.

Unspecified variants

Mutations predicted to result in rare missense, silent and intronic variants or other mutations of unknown pathogenic significance were classified as UVs. Sixty-two distinct UVs (31 in MSH2 and 31 in MLH1) were identified, encompassing 41 missense mutations, 9 silent mutations, 7 intronic variants, 2 variants in the 5′ untranslated region, 1 in frame deletion (MLH1,c.1835 1837delTTG), 1 in frame insertion (MSH2,c.4 21_dup) and 1 out of frame insertion (MLH1,c.2253_2254insAA), and predicted to lead to a prolonged RNA (Table V).

UVs were detected in 97 index patients. Twenty-five of these patients also carried a pathogenic mutation. Among the remaining 72 patients, 62 carried only 1 UV, 9 were identified with 2 UVs and 1 with 3 UVs. From the 72 patients with UVs only, 67 had MSI-H tumors and only 1 patient had an MSI-L tumor. The remaining 4 patients met the AC but no tumor tissue was available for prescreening. In patients from the AC group with MSI-H 30/172 (17%) and in patients meeting the less stringent inclusion criteria, 54/218 (25%) were found to carry 1 or more UVs.

Discussion

Our study presents spectrum, frequencies and distribution of MSH2 and MLH1 germline mutations detected in a large specific cohort of 454 patients that met modified Bethesda criteria after preselection by microsatellite analysis. The patients originated from a cohort of 1,721 patients diagnosed with HNPCC or suspected of HNPCC recruited by 6 German university centers in accordance to a common study protocol. The cohort represents one of the largest specific HNPCC populations reported in the literature to date. The overall mutation detection rate for pathogenic mutations in the preselected series was 56%.

While most of the mutations were detected in 1 or 2 index patients only, 2 mutations were identified significantly more often. MSH2, c.942+3A>T was found in 11% of MSH2 mutation positive patients and MLH1,c.1489_1490insC was detected in 18% of the patients with pathogenic MLH1 mutations. Both mutations are frequent entries in the ICG mutation database. In most other

TABLE IV – PATHOGENIC MUTATIONS DETECTED IN THIS STUDY

Exon/intron	Mutation[1]	Predicted effect	Consequence	Number of alleles
a) Mutations in *MSH2*				
1	c.28C>T[2]	p.Gln10X	Nonsense	1
1	c.29_30insA[2]	p.Gln10fs	Frameshift	1
1	c.82G>T[2]	p.Glu28X	Nonsense	1
1	c.94_103delACCACAGTGC[2]	p.Thr32fs	Frameshift	1
1	c.145_146delGA[2]	p.Asp49fs	Frameshift	1
1	c.166delG[2]	p.Glu56fs	Frameshift	1
1	c.187delG[2]	p.Val63fs	Frameshift	1
2	c.229_230delAG[2]	p.Ser77fs	Frameshift	1
2	c.268_289dup	p.Gln97fs	Frameshift	1
3	c.387_388delTC	p.Ser129fs	Frameshift	1
3	c.388_389delCA	p.Gln130fs	Frameshift	1
3	c.416delA[2]	p.Asn139fs	Frameshift	1
3	c.508C>T[2]	p.Gln170X	Nonsense	1
3	c.518delT	p.Leu173fs	Frameshift	2
3	c.577C>T[2]	p.Gln193X	Nonsense	1
3	c.511_583dup[2]	p.Gly195fs	Frameshift	2
3	c.638_639delTG[2]	p.Leu213fs	Frameshift	1
Intron 3	c.646-3_654delTAGATAATTCAA[2]	r.?[3]	Splice defect	1
4	c.687delA	p.Lys229fs	Frameshift	1
4	c.696_697delTT[2]	p.Ser232fs	Frameshift	1
4	c.704_705delAA[2]	p.Lys235fs	Frameshift	1
4	c.711_714delTTAT[2]	p.Ile237fs	Frameshift	1
4	c.717_721delGGACCinsTTA[2]	p.Gln239fs	Frameshift	1
4	c.754C>T	p.Gln252X	Nonsense	1
4	c.763_766delAGTGinsTT	p.Ser255fs	Frameshift	1
4	c.788_789delAT[2]	p.Asn263fs	Frameshift	1
5	c.795delT[2]	p.Val265fs	Frameshift	1
5	c.810_811delGT	p.Leu270fs	Frameshift	1
5	c.862C>T	p.Gln288X	Nonsense	1
5	c.873_876delGACT	p.Leu291fs	Frameshift	1
5	c.901A>T[2]	p.Lys301X	Nonsense	1
Intron 5	c.942+3A>T	r.[b4]	Splice defect	15
Intron 5	c.943-1G>C[2]	r.?[3]	Splice defect	2
6	c.958_959insA[2]	p.Thr320fs	Frameshift	1
6	c.972_973ins184[2]	p.Gln324fs	Frameshift	1
6	c.973_974insT[2]	p.Ser325fs	Frameshift	1
6	c.1005_1008delCCCC[2]	p.Thr335fs	Frameshift	1
Intron 6	c.1077-2A>G[2]	r.?[3]	Splice defect	2
7	c.1119delG	p.Arg373fs	Frameshift	1
7	c.1120C>T[2]	p.Gln374X	Nonsense	1
7	c.1147C>T	p.Arg383X	Nonsense	4
7	c.1165C>T	p.Arg389X	Nonsense	1
7	c.1183C>T[2]	p.Gln395X	Nonsense	1
7	c.1216C>T	p.Arg406X	Nonsense	2
7	c.1222_1223insT[2]	p.Tyr408fs	Frameshift	2
7	c.1226_1227delAG	p.Gln409fs	Frameshift	5
Intron 7	c.1276+1G>A[2]	r.?[3]	Splice defect	2
Intron 7	c.1277-1G>C[2]	r.?[3]	Splice defect	1
8	c.1285C>T	p.Gln429X	Nonsense	3
8	c.1292T>A[2]	p.Leu431X	Nonsense	1
8	c.1373T>G	P.Leu458X	Nonsense	2
Intron 8	c.1386+1G>A[2]	r.?[3]	Splice defect	2
9	c.1408delG[2]	p.Val470fs	Frameshift	1
9	c.1457delA[2]	p.Asn486fs	Frameshift	1
9	c.1476_1477GC>CT[2]	p.Gln493X	Nonsense	1
9	c.1477C>T	p.Gln493X	Nonsense	4
11	c.1683delA	p.Glu561fs	Frameshift	1
11	c.1705_1706delGA[2]	p.Glu569fs	Frameshift	2
11	c.1720C>T[2]	p.Gln574X	Nonsense	1
11	c.1720delC[2]	p.Gln574fs	Frameshift	1
11	c.1738G>T	p.Glu580X	Nonsense	2
12	c.1764T>G	p.Tyr588X	Nonsense	1
12	c.1779_1782delGACA[2]	p.Gln593fs	Frameshift	1
12	c.1835C>G[2]	p.Ser612X	Nonsense	1
12	c.1857T>G	p.Tyr619X	Nonsense	1
12	c.1968C>G	p.Tyr656X	Nonsense	1
Intron 12	c.2005+1G>T	r.?[3]	Splice defect	1
Intron 12	c.2005+2T>C[2]	r.?[3]	Splice defect	1
Intron 12	c.2006-1G>C[2]	r.?[3]	Splice defect	1
13	c.2038C>T	p.Arg680X	Nonsense	6
13	c.2131C>T	p.Arg711X	Nonsense	1
Intron 14	c.2458+1G>A[2]	r.?[3]	Splice defect	1

TABLE IV – PATHOGENIC MUTATIONS DETECTED IN THIS STUDY (CONTINUED)

Exon/intron	Mutation[1]	Predicted effect	Consequence	Number of alleles
15	c.2536C>T	p.Gln846X	Nonsense	1
15	c.2575G>T[2]	p.Glu859X	Nonsense	1
15	c.2579C>A	p.Ser860X	Nonsense	1
Intron 15	c.2634+1G>T[2]	r.?[3]	Splice defect	1
Intron 15	c.2635-1G>T[2]	r.?[3]	Splice defect	1
	Deletion Exon 1		Large deletion	1
	Deletion Exon 1–2		Large deletion	2
	Deletion Exon 1–6		Large deletion	2
	Deletion Exon 1–10		Large deletion	1
	Deletion Exon 1–16		Large deletion	1
	Deletion Exon 11–14		Large deletion	2
	Deletion Exon 12–15		Large deletion	1
	Deletion Exon 15–16[2]		Large deletion	1
	Deletion Exon 3		Large deletion	1
	Deletion Exon 3–5[2]		Large deletion	1
	Deletion Exon 7		Large deletion	1
	Deletion Exon 8		Large deletion	1
b) Mutations in *MLHI*				
1	c.2T>A	p.Met1?	Initiation	1
1	c.44_45insT[2]	p.Val15fs	Frameshift	1
1	c.67G>T	p.Glu23X	Nonsense	2
1	c.73delA	p.IIe25fs	Frameshift	1
1	c.76delC[2]	p.Gln26fs	Frameshift	1
1	c.109G>T[2]	p.Glu37X	Nonsense	1
2	c.131_132insAATC[2]	p.Ser44fs	Frameshift	1
2	c.150_151insT	p.Val51fs	Frameshift	1
2	c.184C>T	p.Gln62X	Nonsense	3
2	c.205delA[2]	p.Arg69fs	Frameshift	1
Intron 2	c.207+1G>T[2]	r.?[3]	Splice defect	1
3	c.211G>T[2]	p.Glu71X	Nonsense	1
3	c.298C>T	p.Arg100X	Nonsense	3
Intron 3	c.306+1G>A	r.?[3]	Splice defect	3
4	c.341delC	p.Thr114fs	Frameshift	1
Intron 4	c.380+1G>A[2]	r.?[3]	Splice defect	1
5	c.436C>T	p.Gln146X	Nonsense	1
6	c.503_504insA	p.Asn168fs	Frameshift	1
6	c.513delA[2]	p.Glu171fs	Frameshift	1
Intron 6	c.545+1G>A[2]	r.?[3]	Splice defect	1
7	c.578C>G[2]	p.Ser193X	Nonsense	1
Intron 7	c.588+1delG[2]	r.?[3]	Splice defect	1
8	c.676C>T	p.Arg226X	Nonsense	4
Intron 8	c.677+3A>G	r.[c6]	Splice defect	2
Intron 9	c.790+1delG[2]	r.?[3]	Splice defect	2
Intron 9	c.790+1G>A	r.[d7]	Splice defect	2
Intron 9	c.790+2_790+3insT	r.?[3]	Splice defect	1
10	c.791_794delATCG	p.His264fs	Frameshift	1
10	c.821_824dup	p.Lys274fs	Frameshift	1
10	c.840T>A[2]	p.Tyr280X	Nonsense	1
Intron 10	c.884+2T>G[2]	r.?[3]	Splice defect	2
11	c.954delC	p.His318fs	Frameshift	1
11	c.1023delG	p.Arg341fs	Frameshift	1
Intron 11	c.1039-2A>G[2]	r.?[3]	Splice defect	1
12	c.1068_1075delTGGGGAGA	p.Ser356fs	Frameshift	1
12	c.1310delC	p.Pro437fs	Frameshift	1
13	c.1412_1413insA	p.Lys471fs	Frameshift	1
13	c.1459C>T	p.Arg487X	Nonsense	1
13	c.1463delA[2]	p.Lys488fs	Frameshift	1
13	c.1489_1490insC	p.Arg497fs	Frameshift	21
13	c.1534G>T[2]	p.Glu512X	Nonsense	1
Intron 13	c.1559-1G>A	r.?[3]	Splice defect	1
14	c.1622delC	p.Ala541fs	Frameshift	1
14	c.1640T>A[2]	p.Leu547X	Nonsense	1
15	c.1672G>T[2]	p.Glu558X	Nonsense	1
15	c.1683C>G[2]	p.Tyr561X	Nonsense	1
15	c.1725delG[2]	p.Arg575fs	Frameshift	1
15	c.1731G>A	p.Ser577[8]	Splice defect	3
Intron 15	c.1731+2T>G[2]	r.?[3]	Splice defect	1
Intron 15	c.1732-1G>A[2]	r.?[3]	Splice defect	1
16	c.1749delT[2]	p.Phe583fs	Frameshift	1
16	c.1783_1784delAG	p.Ser595fs	Frameshift	1
Intron 16	c.1896+1G>T	r.?[3]	Splice defect	1
Intron 16	c.1896+2T>C[2]	r.?[3]	Splice defect	1
Intron 17	c.1989+1G>A[2]	r.?[3]	Splice defect	1

TABLE IV – PATHOGENIC MUTATIONS DETECTED IN THIS STUDY (CONTINUED)

Exon/intron	Mutation[1]	Predicted effect	Consequence	Number of alleles
Intron 17	c.1989+1G>T	r.?[3]	Splice defect	1
Intron 17	c.1990-1G>T[2]	r.?[3]	Splice defect	1
18	c.2009delA[2]	p.Lys670fs	Frameshift	1
18	c.2067_2073delGTACATA[2]	p.Gln689fs	Frameshift	1
18	c.2076_2077delTG	p.Ser692fs	Frameshift	1
18	c.2092_2093delTC[2]	p.Ser698fs	Frameshift	2
18	c.2103G>C[2]	p.Gln701His[9]	Splice defect	5
19	c.2198_2199insAACA	p.His733fs	Frameshift	1
19	c.2262delG[2]	p.Glu754fs	Frameshift	1
	Deletion Exon 1–10		Large deletion	5
	Deletion Exon 1–15[2]		Large deletion	1
	Deletion Exon 13[2]		Large deletion	1
	Deletion Exon 13–19[2]		Large deletion	1
	Deletion Exon 2–3[2]		Large deletion	1
	Deletion Exon 3–5		Large deletion	1
	Deletion Exon 4		Large deletion	2
	Deletion Exon 7–10		Large deletion	1

[1]DNA variation numbering based on NCBI RefSeq for *MSH2* mRNA NM_000251 Version NM_000251.1 GI:4557760 with +1 as A of the ATG start codon. –[2]Novel mutation. –[3]r.? effect on RNA-level unknown but expected. –[4]r.[b] leads to an in frame deletion of exon 5[29].–[5]DNA variation numbering based on NCBI RefSeq for *MLH1* mRNA NM_000249 Version NM_000249.2 GI:28559089 with +1 as A of the ATG start codon. –[6]r.[c] leads to an out of frame deletion of exon 8[30]. –[7]r.[d] leads to an in frame deletion of exon 9 and 10.[31] –[8]leads to an out of frame deletion of exon 15.[31] –[9]leads to an in frame deletion of exon 18 (own unpublished observation).

HNPCC cohorts, carriers of *MSH2*, c.942+3A>T are unrelated.[32] Preliminary data show that the high frequency of *MLH1*,c.1489_1490insC in this HNPCC series is due to a founder effect (unpublished observation). Interestingly, to date, *MLH1*, c.1489_1490insC has been reported neither in previously published HNPCC cohorts from the US, the UK, Finland or Australia nor as a mutational hotspot in a specific HNPCC population.[33–36] It is conceivable that the mutation has occurred quite recently and therefore is more frequent in the German HNPCC cohort. Even in the French HNPCC patients who are expected to share many genes with the German HNPCC cohort the mutation *MLH1*,c.1489_1490insC has not been reported.[37,38] Instead, the recent study by Parc *et al.*[38] on 163 French families with a deleterious mutation reports the nonsense mutations *MSH2*,c.2131C>T and *MLH1*,c.676C>T as frequent findings (besides *MSH2*,c.942+3A>T); these nonsense mutations are rare findings in the German cohort.

Another frequent finding among HNPCC patients presented here are large genomic deletions. We detected large deletions in both genes. This observation is in line with previous results on a subgroup of this HNPCC cohort and with HNPCC series from the Netherlands and the CAPP2 chemoprevention study but is in contrast to other studies where the majority of large deletions was described in *MSH2*.[16,33,34,39–41]

Recently, an American founder deletion encompassing *MSH2* exons 1–6 has been reported that could be traced back to a common ancestor of presumably German origin.[33] Noteworthy is that 2 unrelated patients from our study were found to harbor a deletion of exons 1–6 in *MSH2*. However, the deletion in these 2 patients has different breakpoints than the US founder deletion (primers for this examination were kindly provided by R. Fodde).

In the subset of patients in our study who where screened for both small mutations and large genomic rearrangements, 18% of all pathogenic mutations were large deletions. While the proportions of deletions in the North American HNPCC cohort and the CAPP2 series were slightly higher (27% and 24%, respectively), substantially different percentages of 36% and 54.8% were reported from a Dutch and a UK series, respectively.[16,33,34,40] Most likely, the existence of a frequent founder deletion accounts for the higher deletion detection rate in the US cohort.[33] Another fact for consideration is the different methods applied in mutation and deletion detection. However, since the CAPP2 series, the UK HNPCC families and most of our index patients were screened by use of MLPA, these differences might indicate a lower prevalence of genomic deletions in the German HNPCC families.

In our study, more UVs were detected in patients with MSI-H meeting the less stringent inclusion criteria (54/218) than in AC patients with MSI-H (30/172). The most likely reason for this observation is the fact that mutation screening was stopped as soon as a pathogenic mutation had been identified. Since the probability of identifying a pathogenic mutation in patients not meeting the AC is lower than in the AC patients, more patients from the group meeting the less stringent inclusion criteria were screened for mutations in all exons. This raises the probability of identification of polymorphisms and variants.

Owing to the strategy applied in classification of mutations, the group of UVs presented here is a relatively heterogeneous group (Table V). Without taking into consideration the results of functional tests or segregation studies, the criteria used for classifying a mutation as pathogenic were indeed quite strict. For several of the mutations in the UV category, functional tests have shown a pathogenic effect.[42–45] According to extensive segregation analyses and studies in unaffected controls, the variant *MSH2*,c.1906G>C (p.Ala636Pro) is a founder mutation among Ashkenazi HNPCC patients. Additionally, *in silico* tests for a homolog of this mutation strongly suggest that the mutation is disease causing.[46] Nevertheless, we decided to classify only those *MSH2* or *MLH1* mutations as pathogenic that have a predicted deleterious effect on the MSH2 or MLH1 protein and thus provide a reliable result when used for predictive genetic testing. On the other hand, several nondisease causing mutations (*i.e.*, not affecting protein translation or function) are most likely part of the UV group presented here. Most of the intronic variants localized far beyond the highly conserved splice site positions probably fall into this category.

In general, the classification of a large percentage of mutations in *MSH2* and *MLH1* remains problematic. Theoretical calculations (*e.g.*, with splice site prediction programs such as the splice site prediction program of the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seqtools/splice.html) or the program ESE-finder (http://exon.cshl.org/ESE/index.html) for detection of exonic splicing enhancers) are helpful but their results certainly cannot be regarded as a definite proof or disproof of pathogenicity. The evaluation of missense mutations is often hampered by the fact that no reliable functional tests are available. The current database entries are often misleading. In fact, several of the UVs presented here are listed both in the mutation and in the polymorphism category of the ICG-HNPCC mutation database. Support for the assumption that a UV is nonpathogenic might come from

TABLE V – UNSPECIFIED VARIANTS IDENTIFIED IN THIS STUDY

Exon/intron	Variant[1]	Predicted effect	Consequence	Number of alleles
a) Variants in *MSH2*				
1	c.4G>A[3]	p.Ala2Thr	Missense	6
1	c.4_21dup	?	In-frame Insertion	1
1	c.23C>T[2,3]	p.Thr8Met	Missense	1
1	c.114C>G[2,3]	p.Asp38Glu	Missense	1
3	c.380A>G[3]	p.Asn127Ser	Missense	2
3	c.399C>T[3]	p.Asp133	Silent	1
3	c.482T>A	p.Val161Asp	Missense	1
3	c.490G>A[2]	p.Gly164Arg	Missense	2
3	c.518T>C[2]	p.Leu173Pro	Missense	1
3	c.560T>C[2]	p.Leu187Pro	Missense	1
3	c.569_570TC>CT[2]	p.Leu190Pro	Missense	1
5	c.814_815GC>AT[2,3]	p.Ala272Met	Missense	1
5	c.942G>A[2]	p.Gln314	Silent	1
6	c.965G>A[3,4]	p.Gly322Asp	Missense	10
6	c.998G>A	p.Cys333Tyr	Missense	1
7	c.1275A>G[2]	p.Glu425	Silent	1
10	c.1571G>C	p.Arg524Pro	Missense	1
11	c.1666T>C[3]	p.Leu563	Silent	2
11	c.1737A>G	p.Lys579	Silent	1
12	c.1787A>G	p.Asn596Ser	Missense	1
12	c.1826C>T[2]	p.Ala609Val	Missense	1
12	c.1906G>C[5]	p.Ala636Pro	Missense	1
12	c.1935A>G[2]	p.Gln645	Silent	1
13	c.2045C>T[2,3]	p.Thr682Ile	Missense	1
13	c.2075G>T[2]	p.Gly692Val	Missense	1
13	c.2090G>T	p.Cys697Phe	Missense	2
13	c.2154A>G[3]	p.Gln718	Silent	1
14	c.2245G>A[2]	p.Glu749Lys	Missense	1
14	c.2315C>G[2,3]	p.Thr772Arg	Missense	1
Intron 14	c.2459-12A>G[2]	?	Unknown	1
15	c.2500G>A	p.Ala834Thr	Missense	1
b) Variants in *MLH1*				
Promotor	c.1-42C>T	?	Unknown	1
Promotor	c.1-28A>T	?	Unknown	1
2	c.122A>G	Asp41Gly	Missense	1
2	c.189C>A[2]	Asp63Glu	Missense	1
2	c.199G>A[7]	Gly67Arg	Missense	3
3	c.230G>A[8]	Cys77Tyr	Missense	1
Intron 3	c.306+2_306+3insT[2]	?	Splice defect?	1
6	c.464T>G[2]	Leu155Arg	Missense	1
Intron 6	c.545+3A>G	?	Unknown	1
Intron 8	c.677+51delT[2]	?	Unknown	1
Intron 8	c.677+68A>G[2,3]	?	Unknown	1
9	c.739T>C[2]	p.Ser247Pro	Missense	1
10	c.793C>T[4]	p.Arg265Cys	Missense	3
10	c.843A>T[2,3]	p.Ala281	Silent	1
11	c.986A>C	p.His329Pro	Missense	1
11	c.1038G>C[2]	p.Gln346His	Missense	1
Intron 11	c.1039-8T>A	?	Unknown	2
12	c.1321G>A[3]	p.Ala441Thr	Missense	2
16	c.1745T>C[2]	p.Leu582Pro	Missense	1
16	c.1766C>A[2]	p.Ala589Asp	Missense	1
16	c.1835_1837delTTG[2]	?	In-frame Deletion	1
16	c.1852_1853AA>GC[3,9]	p.Lys618Ala	Missense	4
16	c.1853A>G[2,3,9]	p.Lys618Arg	Missense	2
17	c.1919C>T[2]	p.Pro640Leu	Missense	1
17	c.1959G>T[3]	p.Leu653	Silent	7
17	c.1961C>T	p.Pro654Leu	Missense	3
18	c.2027T>C[2]	p.Leu676Pro	Missense	1
18	c.2041G>A	p.Ala681Thr	Missense	1
19	c.2210A>T[2,3]	p.Asp737Val	Missense	1
19	c.2253_2254insAA[2]	?	Unknown	1
3[1]UTR	c.2268+33_2268+35delTTC	?	Unknown	1

[1]DNA variation numbering based on NCBI RefSeq for *MSH2* mRNA NM_000251 Version NM_000251.1 GI:4557760 with +1 as A of the ATG start codon. –[2]Novel variant. –[3]In one or more carriers of this variant a pathogenic mutation was identified as well.–[4]Functional analysis in a quantitative *in vivo* yeast DNA mismatch repair assay in *Saccharomyces cerevisiae* demonstrates intermediate functional significance for this variant.[42]–[5]Previously reported findings suggest strongly that this variant is disease causing.[46]–[6]DNA variation numbering based on NCBI RefSeq for *MLH1* mRNA NM_000249 Version NM_000249.2 GI:28559089 with +1 as A of the ATG start codon. –[7]Functional analysis in 4 different *Saccharomyces cerevisiae* model systems demonstrates pathogenic significance for this variant.[42–45] –[8]Functional analysis in 2 *Saccharomyces cerevisiae* model systems demonstrates pathogenic significance for this variant.[43,45] –[9]Functional analysis in a yeast 2-hybrid assay demonstrates functional significance for this variant.[45]

the additional finding of a pathogenic mutation in the same index patient, as was the case in 25 of the UV carriers presented here, but certainly this is not final proof of it being absolutely harmless. The possibility of double mutations in a patient also has to be considered. The missense variant MSH2,c.4G>A (p.Ala2Thr) for example, was found in 6 families presented here. At present, there is 1 entry for this variant in the ICG-mutation database in the mutation category. In 1 of the families (BN-2128-X), immunohistochemical analysis in tumors from mother and son (both carriers of the variant) revealed a loss of MSH2 protein expression. This finding further suggests MSH2,c.4G>A (p.Ala2Thr) to be disease causing. The positive segregation of MSH2,c.4G>A with HNPCC tumors in family BN-2128-X in combination with immunohistochemistry data (or a haplotype analysis) supporting its pathogenicity might allow its use as an intragenic marker for predictive testing of other family members. However, such findings cannot be regarded as proof of pathogenicity as another (not detected) mutation in the same allele might be disease causing. Indeed, in family HD-1722-8, the deleterious mutation MSH2,c.1835C>G (p.Ser612X) was detected in addition to MSH2,c.4G>A.

In the HNPCC series presented here, microsatellite analysis was applied as a prescreening step to identify patients eligible for mutation analysis (in a subgroup of patients immunohistochemical analysis of DNA MMR protein expression was also performed; data not shown). This preselection approach is justified by the observation that no pathogenic MSH2 or MLH1 germline mutations were identified in a subset of 56 colorectal cancer patients that met the inclusion criteria who were found to have MSS tumors (data not shown) and literature data.[18] In the index patients of the study presented here, microsatellite analysis allowed a considerable reduction of mutation analysis candidates from 961 down to 352 (37%) in the category meeting the looser inclusion criteria. 95/218 (44%) of the patients from this preselected cohort were subsequently found to harbor a pathogenic mutation, corresponding to a calculated overall mutation detection rate of 16% (37% × 44%) in patients that met the less stringent inclusion criteria. In AC positive patients, 72% (234/324) exhibited MSI-H in their tumors and 128 (74%) carriers of pathogenic mutations were identified in 172 preselected patients of this category, corresponding to a calculated mutation detection rate of 53% (72% × 74%) in AC patients without preselection (as expected, this percentage is close to the 56% mutation detection rate among AC positive index patients without tumor tissue analysis in our study). These results demonstrate the value of microsatellite analysis as a prescreening tool in patients fulfilling less-stringent HNPCC criteria. In contrast, in the AC positive group, a high mutation detection rate can be reached also without prior tumor tissue analysis. Nevertheless, our results have proven microsatellite analysis to be useful in the AC group as well.

Two patients identified with pathogenic mutations do not meet any of the inclusion criteria of our study but were found to have other criteria suspicious of HNPCC. The index patient of family HD-1515-5 was diagnosed with colorectal cancer at 47 years; his father probably suffered from colorectal cancer at around 53 years as well as from a brain tumor. The sister of the father was found to have a malignant tumor involving the lymph nodes and a paternal great aunt was diagnosed with uterus carcinoma in her 6th decade. Family BN-2206-6 was initially categorized as Amsterdam positive by the gastroenterology department who had ascertained the index patient. The pathologist found the tumor tissue to exhibit MSI-H morphology. However, during the course of our study, the detailed family history became available and it turned out that in the index patient from family BN-2206-6, a coecum carcinoma was diagnosed 2 months after his 60th birthday. His brother was diagnosed with colorectal cancer at the age of 53 (tumor tissue was no longer available) and an aunt developed laryngeal cancer at 73 years. Since in both cases the index patients were diagnosed beyond the critical age of 45 years, the current Bethesda definition does not raise suspicion of HNPCC in these 2 families.[10] However, recently, the Bethesda guidelines were revised.[11] One of the

modifications is an extension of the age criterion to a critical age of below 50 years (revised Bethesda criterion 1). Thus, with the revised Bethesda guidelines applied, HD-1515-5 would have been correctly categorized as HNPCC suspect, while BN-2206-6 still remains beyond the age limit.

Our study presented has consequences for mutation detection strategies in HNPCC populations at least from Germany. The results of our study underscore the high value of microsatellite analysis as a prescreening method especially in patients not meeting the AC. Given the high a priori chance for mutation detection in AC patients, we recommend including also those AC patients in mutation screening from whom no tumor tissue is available. Tissue examination for MSH2 and MLH1 expression by immunohistochemistry may also indicate the involved mismatch repair gene, but according to the present literature this cannot be regarded as a substitute for microsatellite analysis.[22,47] Since a large proportion of HNPCC mutations are genomic deletions and since MLPA, a fast, efficient and cost-effective method for rearrangement detection is now available, we advocate starting mutation analysis with screening for large deletions. A search for small point mutations or deletions/insertions should be started in MSH2, exon 5 and MLH1, exon 13, since these exons harbor 2 recurrent mutations.

To date, many studies have reported mutation detection in HNPCC suspects but only a few analyzed larger series and the mutation detection rates vary significantly from study to study, which is most likely due to the nature and size of the analyzed populations, various preselection or mutation screening strategies, or different modes of interpreting sequence variants. Liu et al.,[48] who screened MSH2, MLH1, MSH6, PMS2 and PMS1 in 48 AC positive with MSI-H tumors from North America, New Zealand and Europe, reported mutations in 70%. In 35 Finnish HNPCC kindreds, Nyström-Lahti et al.[36] found 30 mutation positive patients, with 2 MLH1 founder mutations, accounting for more than 80% of the HNPCC cases. In 59 clinically well-selected North American HNPCC families who were examined for mutations in MSH2, MLH1 and MSH6, 52 mutations were identified.[33] Lower detection rates of 34/95 and 26/75 mutation positive patients have been reported in Australian and French HNPCC kindreds.[35,37] In comparison to these publications, the overall mutation detection rate of 56% reported here can be designated as intermediate. The most likely reason for the lower mutation detection rate compared to Liu et al.[33] and Wagner et al.[48] is the large number of patients meeting weaker Bethesda criteria in this HNPCC series. On the other hand, the preselection and deletion screening applied in our study contribute to a higher detection rate than those reported in the French or the Australian kindreds where neither microsatellite analysis nor deletion screening were applied.

The most well-selected patients in our study are patients meeting the AC with MSI-H in their tumors. In this category, 74% were identified with a pathogenic mutation, a proportion that is considerably lower than the 92% reported recently in AC positive patients from the North American series.[33] The fact that no routine screening for MSH6 mutations was performed in our study is one factor responsible for this discrepancy. Indeed, in a subset of patients presented here, MSH6 mutations have been identified and will be reported elsewhere.[49] In addition, Wagner and colleagues classified mutations as pathogenic that would not have been designated as pathogenic in the study presented here. Most likely, some of the mutations that were classified as UVs in our study are in fact pathogenic. There may also be mutations located in other genes (e.g., PMS2) or in parts of the gene that are not analyzed, or mutations that were not unveiled by the methods used for screening (e.g., translocations, inversions). A search for large genomic deletions was not included in the mutation detection protocol of all participating centers. Furthermore, not all small mutations could be identified by direct examination of genomic DNA by DHPLC or sequencing that was limited to the exonic sequences and the flanking exon/intron boundaries. Instead, other techniques

might have to be applied in order to identify them. Monoallelic expression analysis (MAMA), for example, has been shown to detect mutations that cannot be uncovered by the methods routinely applied. However, it is unsuitable for routine mutation screening.[50] Another explanation for the existence of so many mutation-negative patients with MSI-H tumors is hypermethylation of the promoter region of *MLH1* in part of the tumors leading to an epigenetic defect of mismatch repair genes reflected by the finding of MSI-H tumors.[2,51] This phenomenon is rare in HNPCC tumors but is a frequent observation in sporadic colorectal cancer.[52] Thus, some of the MSI-H index patients in this series might represent HNPCC phenocopies.

In summary, we detected pathogenic mutations in 56% of patients suspected of HNPCC according to modified Bethesda criteria that had been preselected on the basis of MSI-H. The observations of 2 common mutations and a high percentage of genomic deletions in our cohort are of major importance for mutation detection strategies in central Europe. Recognition of pathogenic mutations will have a great impact on genetic diagnosis and cancer screening and hence prevention of cancer in many HNPCC families. Furthermore, the large number of mutation carriers detected by our study represents an ideal basis for detection of genetic modifiers or exogenous factors influencing the HNPCC phenotype and evaluation of the current surveillance recommendations.

Acknowledgements

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