CLINICAL– ALIMENTARY TRACT

Pathogenicity of *MSH2* Missense Mutations Is Typically Associated With Impaired Repair Capability of the Mutated Protein

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Background & Aims: Inherited deleterious mutations in mismatch repair genes *MLH1*, *MSH2*, and *MSH6* predispose to hereditary nonpolyposis colorectal cancer. A major diagnostic challenge is the difficulty in evaluating the pathogenicity of missense mutations. Previously we showed that most missense variants in *MSH6* do not impair MMR capability and are associated with no or low cancer susceptibility, whereas in *MLH1*, functional studies distinguished nontruncating mutations with severe defects from those not or slightly impaired in protein expression or function. The present study was undertaken to evaluate the pathogenicity of inherited missense mutations in *MSH2*. **Methods:** Fifteen mutated MSH2 proteins including 14 amino acid substitutions and one in-frame deletion were tested for expression/stability, MSH2/MSH6 interaction, and repair efficiency. The genetic and biochemical data were correlated with the clinical data. Comparative sequence analysis was performed to assess the value of sequence homology as a tool for predicting functional results. **Results:** None of the studied *MSH2* mutations destroyed the protein or abolished MSH2/MSH6 interaction, whereas 12 mutations impaired the repair capability of the protein. Comparative sequence analysis correctly predicted functional studies for 13 of 14 amino acid substitutions. **Conclusions:** Interpretation was pathogenic for 12, nonpathogenic for 2, and contradictory for 1 mutation. The pathogenicity could not be distinguished unambiguously by phenotypic characteristics, although correlation between the absence of staining for MSH2 and pathogenicity of the missense mutation was notable. Unlike in *MSH6* and *MLH1*, the pathogenicity of missense mutations in *MSH2* was always associated with impaired repair capability of the mutated protein.

D iscovery of an inherited deleterious mutation in a cancer patient permits predictive gene testing in the family and enables targeted cancer surveillance. Hereditary nonpolyposis colorectal cancer (HNPCC) is a dominantly inherited cancer syndrome characterized by familial accumulation of early onset colorectal, endometrial, and other extracolonic tumors and accounts for around 3%–5% of all colorectal cancer cases.[1,2] HNPCC associates with malfunction of postreplicative mismatch repair (MMR). Nearly 500 different germline mutations that are likely to be pathogenic have been reported for MMR genes, most of which affect *MLH1* (52%), *MSH2* (37%), and *MSH6* (6%) (for more information, see international HNPCC mutation database at www.insight-group.org).

The wide variety of clinical phenotypes complicates HNPCC diagnostics. One reason for varying phenotypes among families, who carry mutations in different MMR genes, may be the different functional roles of the 3 proteins MLH1, MSH2 and MSH6 in the repair reaction. MSH2 and MSH6 form a heterodimer, MutSα, which is involved in the recognition of mispaired DNA and activation of the consequent steps in the repair process, whereas MLH1 most probably is involved in assembly of the repairosome and excision termination past mismatch.[3–5] MSH2 and MLH1 proteins are irreplaceable in MMR, but MSH6 protein has been shown to be functionally redundant with another protein, MSH3, in recognition of small insertions and deletions.[6,7] This probably explains why in contrast to HNPCC families linked to *MSH2* and *MLH1* mutations, families associated with *MSH6* mutations often lack typical clinical and molecular characteristics of the syndrome (eg, early age at onset and high microsatellite instability [MSI] in the tumors).[8,9] Moreover, the conservation status of the mutated sequence and the associated biochemical consequences influence cancer susceptibility and phenotype.[10,11] To date, a major challenge in the clinical management of patients with suspected HNPCC mutations is the frequent occurrence of nontruncating mutations, which cannot be interpreted as either deleterious or clinically innocent a priori.

MLH1 is the most commonly mutated gene in HNPCC and about 30% of its mutations are of the missense type (www.insight-group.org). So far, missense mutations in *MLH1* have been included in several functional analyses both in heterologous systems and in homologous human MMR systems. In our recent study on 34 nontruncating mutations in *MLH1*, pathogenic mutations could be distinguished clearly from nonpathogenic ones and the associated biochemical mechanisms also could be determined.[12] We found that pathogenic nontruncating sequence changes in *MLH1* may interfere with different

biochemical mechanisms such as expression/stability, subcellular localization, or repair efficiency, most often involving a reduced amount of the mutated MLH1 protein.[12,13] Furthermore, impaired repair capability of the mutated MLH1 protein was shown to correlate tightly with mutation location. The mutations whose pathogenicity was associated with reduced protein expression and aberrant nuclear localization were scattered throughout the whole *MLH1* gene, whereas most mutations deficient in MMR function were located at the adenosine triphosphate binding/hydrolysis region in amino-terminus.

Much less is known about the functional significance of missense mutations found in *MSH2* and *MSH6* in suspected HNPCC families. In our own functional analyses of 11 missense mutations in *MSH6*, most showed no impairment of MMR capability.[14,15] The results did not exclude the possibility that some of the studied mutations, which were functional in the in vitro MMR assay, still could affect biochemical events preceding the MMR function in vivo—indeed, some discrepancies in results obtained with human in vitro and yeast in vivo systems suggested that this could be the case.[15,16]

In *MSH2*, 24% of reported pathogenic mutations are non-truncating (www.insight-group.org). The number is notable and seems to be increasing.[17,18] To date, the pathogenicity of *MSH2* missense mutations has been studied mainly in heterologous systems, which require that the affected amino-acid residue is evolutionarily conserved to make the functional tests relevant to human variants.[19–22] Seven mutations were included in a comprehensive biochemical study, which showed that most resulted in reduced MSH2/MSH6 molecular switch functions.[23] Our present study significantly adds to the interpretations of missense mutations in HNPCC by investigating the functionality of 15 mutated MSH2 proteins in a homologous human MMR system, in analogy to our previous studies with mutated MLH1 and MSH6 proteins.

Materials and Methods

MSH2 Mutations and HNPCC Families

The present study comprised 14 *MSH2* missense mutations and 1 in-frame deletion found in 23 HNPCC families that have been subjected to molecular genetic and clinical studies.[24–30] The studied mutations and clinical characteristics of the index patients and their families are listed in Table 1. All human investigations were performed after approval of the institutional review boards of the collaborating universities or local ethical committees. Because the HNPCC families included in the study were found and analyzed by many research groups, somewhat different methods were used for mutation detection (Table 1). All mutations were verified by direct sequencing. Multiplex ligation–dependent probe amplification (MLPA)[31] or multiplex polymerase chain reaction (PCR)[32] was applied to exclude large genomic rearrangements in *MLH1* and *MSH2* in 17 families. In addition, *MSH6* and *PMS2* were studied with MLPA in the Family U01–573 (T33P) and Family 12 (Del745-746). The mutations are dispersed in different domains of the MSH2 polypeptide, but mainly are clustered in the amino-terminal connector domain responsible for interdomain interactions, and in the adenosine triphosphatase domain at the carboxyl-terminus (Figure 1).[33,34] The L187P and A272V mutations were found in 2 and the D603N, A636P, and C697F mutations in 3 separate families. The C333Y, A636P, and

A834T mutations also have been reported in families that were not included in the present investigation (www.insight-group.org). In Family 16, the index patient was shown to carry 2 missense mutations, *MSH2* V923E and *MSH6* S1188N, whereas only *MSH2* V923E was found in another colorectal cancer (CRC) patient in the family. Most (17 of 23) families fulfilled the stringent Amsterdam Criteria I or II[35,36] and showed early age at onset (Table 1).

MSI and Immunohistochemical Analyses

For the MSI analysis, the Bethesda panel (BAT-25, BAT-26, D2S123, D5S346, D17S250, or in some cases D18S69)[37] was applied except in 4 families: HNPCC 183 (D2S123, D2S136, D6S470, D16S663, and HBA1), Family A (BAT-26 and Mdf-15), HNPCC 421 (TP53-Dint, D8S254, NM23, D18S35, D5S346, TP53-Penta, D2S123, D1S2883, D3S1611, and D7S501), and HNPCC 152 (D2S123, D16S663, D5S346, HBA1, and D18S35). Tumors with 2 or more unstable markers were considered to have a high degree of MSI.

The immunohistochemical (IHC) staining was performed with the following primary antibodies: anti-MLH1 (clone G168-15; BD Pharmingen, San Diego, CA), anti-MSH2 (clone FE11; Calbiochem/Oncogene Research Products, San Diego, CA), and anti-MSH6 (clone 44; Transduction Laboratories, Lexington, KY). The percentage of tumor cells staining with the antibodies was recorded for each sample as recommended.[38] Tumor studies were performed on CRCs of the index patients, except in the families UO1-537 and HNPCC B, in which endometrial cancers (ECs) were analyzed, and Family A and HNPCC 62, in which IHC was studied on skin tumors (Table 1).

Functional Assays

Site-directed mutagenesis and production of different expression vectors. The MSH2 variants were created using site-directed mutagenesis as described previously.[14,15,39] All of the primer sequences and PCR parameters are available from the authors on request. Two primer pairs, forward-A with reverse-A and forward-B with reverse-B, were used to create the mutated sites to the wild-type (WT) *MSH2* complementary DNA (cDNA), which was cloned previously into pFastBac1 plasmid between *BamHI* and *XhoI* restriction sites. In the first PCR, fragment A was created using primer pair forward-A and reverse-A and fragment B was created using primers forward-B and reverse-B The nucleotide changes were carried in the primers reverse-A and forward-B. The correct sizes of the fragments were verified on agarose gels. The fragments A and B were used as a template for the second PCR, in which the primers forward-A and reverse-B were used to complete the PCR products. The second PCR products, which contained the mutations, were cloned into the original plasmid between the appropriate restriction sites. Fragments with mutations from amino-terminus until the codon D603 were cloned between *BamH1* and *NdeI* sites and fragments including mutations from the codon A636 until the end of the cDNA between *NdeI* and *XhoI* sites. All constructs were verified by sequencing (ABIPrism 3100 Genetic Analyser; Applied Biosystems, Foster City, CA).

The recombinant baculoviruses for protein production in *Spodoptera frugiperda* 9 (*Sf*9) insect cells were generated using the Bac-to-Bac system according to the manufacturer's instructions (Invitrogen, Groningen, the Netherlands). For protein expression in human cells, the WT and mutated MSH2 cDNAs were

Table 1. Genetic and Clinical Data of HNPCC Families

MSH2 mutation	Family code	Index patient: age at onset, y/ tumor site	All affected patients[a]/ mean age at onset, y	Amsterdam criteria I/II	Method for mutation analysis	MSI status[b]	IHC[b] MSH2	MSH6	MLH1	Reference
T33P	U01-537	45/EC	2/48	−	DS, MLPA	High	+	+	+	This study
V161D	Fam 10	52/CRC	3/53	+	DS, MLPA	High	−	NA	NA	24
G162R	Fam C	56/EC	6/52	+	DS	High	−	+/−	+	59
G164R	HNPCC 420	39/CRC	7/39	+	DS, MLPA	NA	−	NA	+	24
L173P	HNPCC 183	36/CRC	9/45	+	DS, Multiplex PCR	High	−	NA	+	24
L187P	HNPCC 548	41/CRC	5/42	+	DS, MLPA	High	−	NA	+	24
L187P[c]	Family A	42/CRC	11/48	+	DS, MLPA	High	−	−	+	25
A272V	HNPCC 421	41/CRC	3/40	+	DS, MLPA	Low	+	NA	+	This study
A272V	U01-348	41/CRC	1/41	−	DS, MLPA	High	+	+	+	26
C333Y	HNPCC 228	41/CRC	2/41	−	DS, Multiplex PCR	NA	NA	NA	NA	24
D603N	EN 13	50/EC	2/49	−	DS, MLPA	High	−	−	+	27
D603N	EN 26	46/EC	5/55	+	DS	Stable	−	−	+	27
D603N	HNPCC 122	38/CRC	1/38	−	DS	High	NA	NA	NA	28
A636P[d]	HNPCC B	42/CRC, 44/EC	2/44	+	DS	High	−	±	+	29
A636P	U01-051	43/CRC	1/43	−	DS, MLPA	High	−	−	+	26,29
A636P	CG0074	36/EC	5/49	+	DS	High	−	NA	NA	29
C697F[e]	HNPCC 62	27/CRC	5/45	+	DS, Multiplex PCR	High	−	NA	+	24,30
C697F	HNPCC 934	33/CRC	3/38	+	DS, MLPA	High	−	NA	+	24
C697F	1260	40/EC	3/49	+	DS	High	−	+	+	25
Del745-746	Family 12	39/CRC	4/42	+	DS, MLPA	High	−	−	+	This study
E749K	HNPCC 152	29/CRC	7/29	+	DS, MLPA	High	+	NA	±	24
A834T	HNPCC 417	28/CRC	3/39	+	DS, Multiplex PCR	High	−	−	+	24
V923E[f]	Family 16	70/CRC	6/58	+	DS, MLPA	High	±	−	+	This study

EC, endometrial cancer; DS, direct sequencing; NA, not available.
[a]Affected patients with HNPCC tumors.
[b]MSI status and IHC were analyzed on the primary tumor of the index patient. The 3 exceptions are as follows:
[c]IHC not available from index patient; MSH2 and MSH6 loss detected from sebaceous adenoma of a paternal aunt, a verified mutation carrier.
[d]Tumor data are from EC of the index patient.
[e]IHC not available from index patient; MSH2 and MSH6 loss detected from sebaceous carcinoma of sister, a verified mutation carrier.
[f]The index person carries 2 mutations, *MSH2* V923E and *MSH6* S1188N.

cloned from pFastBac1 into pDsRed2-N1 expression vector (BD Biosciences, Palo Alto, CA) between *Sac*I and *Not*I restriction sites, so that the red fluorescent protein was replaced. The WT MSH6 cDNA was cloned from pFastBac1 into pEGFP-N1 expression vector (BD Biosciences) between *Bam*HI and *Not*I restriction sites, replacing the enhanced green fluorescent protein gene. The resulting constructs expressing MSH2 (WT or mutated) and MSH6 (WT) are mentioned here as pMSH2-N1 and pMSH6-N1, respectively.

Production of recombinant proteins in insect and human cells. The recombinant proteins were produced in *Sf*9 insect cells using Bac-to-Bac baculovirus expression system (Invitrogen) as previously described.[14,15,39] The bacmid DNAs containing cDNA inserts encoding WT *MSH2* (*MSH2*-WT), mutated *MSH2* (*MSH2*-T33P, *MSH2*-V161D, *MSH2*-G162R, *MSH2*-G164R, *MSH2*-L173P, *MSH2*-L187P, *MSH2*-A272V, *MSH2*-C333Y, *MSH2*-D603N, *MSH2*-A636P, *MSH2*-C697F, *MSH2*-Del745-746, *MSH2*-E749K, *MSH2*-A834T, and *MSH2*-V923E), or
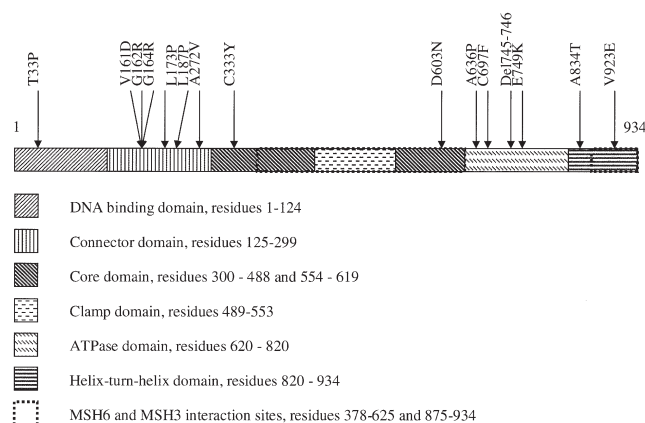
Figure 1. Human MSH2 protein showing the locations of the studied alterations and functional domains. The functional domains are predictions based on crystal structures of prokaryotic MutS proteins[33,34] and the MSH2/MSH6 and MSH2/MSH3 interaction regions.[53]

WT *MSH6* (*MSH6*-WT) were used to transfect *Sf9* cells. For protein production, *Sf9* cells were co-infected with *MSH2* and *MSH6* recombinant baculoviruses because the functional MutSα-complex requires both proteins and MSH6 has been shown to be unstable without its cognate partner MSH2.[40–42] The total protein extracts (TEs) including the heterodimeric MutSα were prepared as in previous reports.[14,15]

Production of recombinant MutSα (heterodimer of MSH2 and MSH6) variants in LoVo human colon adenocarcinoma cell line (MSH2-/-) (American Type Culture Collection, Manassas, VA) was performed as described[43] with minor modifications. A total of 200,000 cells was seeded (in 1 well of a 6-well plate), and transfected after 24 hours with 2 μg of pMSH2-N1 (WT or mutant) and 2 μg pMSH6-N1 vectors using 8 μL of Tfx-20 transfection reagent (Promega, Madison, WI). Forty-eight hours after transfection, the cells were collected by trypsinization and TEs were produced as described previously.[12]

The expression levels and correct sizes of recombinant proteins were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analyses. The protein complexes were run on 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, blotted to nylon membranes, and detected with anti-MSH2 (MSH2 Ab-2, NA27; Calbiochem, EMD Biosciences Inc, Darmstadt, Germany, 0.4 μg/mL in insect cell and 0.1 μg/mL in human cell expression) and anti-MSH6 (MSH6/GTBP, clone 44; BD Transduction Laboratories, Lexington, KY; 0.17 μg/mL in insect cell and 0.5 μg/mL in human cell expression). The naturally expressed β-tubulin protein (clone 5H1, anti-β-tub; [BD Biosciences, San Diego, CA], 0.5 μg/mL) was used as control when the expression levels of MutSα variants produced in LoVo cells were compared.

Combined co-immunoprecipitation and Western blot analysis. The interactions of MSH2 variants with their counterpart MSH6-WT were studied with combined co-immunoprecipitation and Western blot analysis as previously described[14,15] with minor modifications. For immunoprecipitation, *Sf9* TEs were adjusted to contain similar amounts of recombinant proteins (40 μg of MutSα-WT, 40 μg of MutSα-T33P, 120 μg of MutSα-V161D, 120 μg of MutSα-G162R, 120 μg of MutSα-G164R, 120 μg of MutSα-L173P, 120 μg of

MutSα-L187P, 40 μg of MutSα-A272V, 120 μg of MutSα-C333Y, 120 μg of MutSα-D603N, 30 μg of MutSα-A636P, 60 μg of MutSα-C697F, 120 μg of MutSα-Del745-746, 40 μg of MutSα-E749K, 40 μg of MutSα-A834T, and 50 μg of MutSα-V923E). The protein extracts were rotated overnight on a rotating wheel with 1 μg of anti-MSH6 antibody (MSH6/GTBP, clone 44; BD Transduction Laboratories). A total of 30 μL of protein A/G agarose beads (SC2003; Santa Cruz Biotechnology, Santa Cruz, CA) were added and the rotation was continued for 3 hours. The agarose beads, containing the precipitated antibody–protein complexes, were collected by centrifugation and washed 3 times. The precipitated complexes were detected by Western blot (see previously).

In vitro mismatch repair assay. In vitro MMR assay followed the previous protocols.[14,15] The repair reaction included 100 ng of circular heteroduplex DNA and 75 μg of LoVo nuclear extract, which lacks the intrinsic MSH2 protein (MSH2-/-). The functionality of the mutated MSH2 proteins was studied by complementing LoVo nuclear extract with the TE including overexpressed MutSα The total protein amounts were adjusted to contain equal quantities of recombinant MutSα (4 μg of MutSα-WT, 4 μg of MutSα-T33P, 12 μg of MutSα-V161D, 12 μg of MutSα- G162R, 12 μg of MutSα-G164R, 12 μg of MutSα-L173P, 12 μg of MutSα-L187P, 4 μg of MutSα-A272V, 12 μg of MutSα-C333Y, 12 μg of MutSα-D603N, 3 μg of MutSα-A636P, 6 μg of MutSα-C697F, 12 μg of MutSα-Del745-746, 4 μg of MutSα-E749K, 4 μg of MutSα-A834T, and 5 μg of MutSα-V923E). The purified MutSα (1 μg) and the MutSα-WT TE were used as positive controls, and LoVo nuclear extract as a negative control in the assay. The repair reaction was performed for 45 minutes, after which the DNA was extracted, purified, and digested with *Bsa*I and *Bgl*II restriction enzymes. The *Bsa*I enzyme was used to linearize the DNA. The repair reaction converts the G · T heteroduplex, not susceptible to cleavage by the endonuclease *Bgl*II, to the A · T homoduplex, which is cleaved by *Bgl*II. Thus, the repair efficiency can be measured by the cleavage efficiency. The digested DNA was run on 1% agarose gels and the repair efficiencies were quantified by comparing the intensities of the uncorrected and corrected fragments using Image-Pro 4.0 (Media Cybernetics, Silver Spring, MD). The repair percentages were calculated as an average ± SD of 3 independent experiments and the repair percentages of the proficient WT controls were used as a reference level.

Comparative Sequence Analysis

Homologues of human MSH2 were obtained from GenBank by the Entrez search and retrieval system. Full sequences for 240 bacterial and 42 eukaryotic MSH2 homologues were identified. Partial sequences were not included in the alignments. If more than one sequence was returned for a species, the most complete sequence was chosen as a representative for that species. The following 36 eukaryotic species were used in the analyses: human (gi18204306), *Cercopithecus aethiops* (gi52632375), *Canis familiaris* (gi73969550), *Bos taurus* (gi75775312), *Pongo pygmaeus* (gi55733310), *Mus musculus* (gi28436763), *Rattus norbegicus* (gi1103621), *Macaca fascicularis* (gi 67972282), *Xenopus laevis* (gi1079288), *Pan troglodytes* (gi55595712, gi3821282), *Danio rerio* (gi68441231), *Strongylocentrotus purpuratus* (gi72126137), *Cryptococcus neoformans* (gi57223235), *Paramecium tetraurelia* (gi50057558), *Trypanosoma*

brucei (gi71748512), *Plasmodium falciparum* (gi23509476), *Theileria parva* (gi68352933), *Plasmodium chabaudi* (70950501), *Cryptosporidium parvum* (gi66361202), *Anopheles gambiae* (gi55236493), *Ustilago maydis* (gi71017883), *Magnaporthe grisea* (gi39973949), *Neurospora crassa* (gi3914053), *Aspergillus fumigatus* (gi66852271), *Gibberella zeae* (gi42552104), *Saccharomyces cerevisiae* (gi172002), *Drosophila melanogaster* (gi22946491), *Ashbya gossypii* (gi44985261), *Dictyostelium discoideum* (gi66819479), *Schizosaccharomyces pombe* (gi4151103), *Yarrowia lipolytica* (gi50554795), *Kluyveromyces lactis* (gi50310365), *Tetraodon nigroviridis* (gi47210), *Candida albicans* (gi68481255), *Leishmania major* (gi68128853), and *Debaryomyces hansenii* (gi56311471, gi50428111).

Multiple alignments for the sequences were constructed using ClustalW[44] and BioEdit; some poorly conserved regions, which showed large gaps in the automated alignment, were aligned visually. The minimum number of nonsynonymous changes for the alignments was determined using the maximum parsimony method implemented in the program protpars. Three substitutions per codon are required to state that absolute conservation of a given amino acid is statistically significant at a *P* value of less than .05.[45,46] The sequence alignment of 36 species contains more than 17,000 variants. This total is well in excess of the necessary 3 substitutions per site (for MSH2, 3×934 amino acids $= 2802$).

Predictions of tolerability of MSH2 amino-acid substitutions was performed using the program Sorting Intolerant From Tolerant (SIFT) (available at: http://blocks.fhcrc.org/sift/SIFT. html). The assumptions and methodology of SIFT have been described previously.[47] The aligned sequences were entered using default settings. SIFT classifies all potential amino acid variants as tolerated or deleterious, based on an algorithm that calculates normalized probabilities for all possible substitutions from the alignment. Positions with normalized probabilities less than .05 are predicted to be deleterious, those greater than or equal to .05 are predicted to be tolerated.

Results

Tumor Phenotypes

The results of MSI and IHC analyses are shown in Table 1. MSI status was available from 21 mutation carriers from 21 families associated with 13 different mutations. In most of the studied tumors (19 of 21) the degree of MSI was high, in HNPCC 421 (A272V) MSI was low, and in EN26 (D603N) the studied endometrial carcinoma showed stable MSI status. IHC staining of MSH2 was performed on tumors originating from verified mutation carriers in 21 families. MSH2 protein was lost in 17 tumors. In 4 tumors associated with mutations T33P, A272V, and E749K, MSH2 staining was retained, although MSI status was high (T33P, A272V, E749K) or low (A272V). In Family 16 (V923E), CRC from the index patient, who also carried a mutation in *MSH6*, showed reduced expression of MSH2 and loss of MSH6, whereas CRC from another family member carrying only *MSH2* mutation showed loss of MSH2 and heterogeneous MSH6. Overall, the loss or reduced expression of MSH6 was observed frequently (9 of 12) and always together with the loss of the MSH2 protein, whereas MLH1 was reduced in 1 tumor (E749K) and normal in all other (18) analyzed samples.
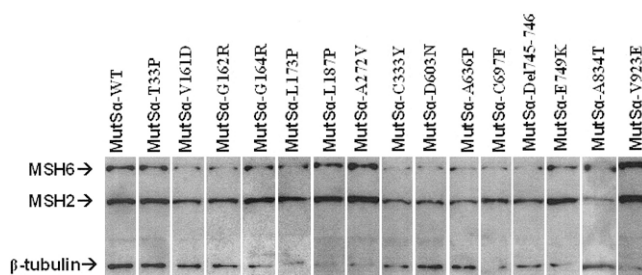

Figure 2. Expression of MutSα variants in LoVo human cells as detected by Western blot analysis. The results were based on at least 3 independent experiments. All 15 MSH2 variants are expressed at similar levels as the WT protein. The naturally expressed β-tubulin is used as an internal loading control.

Expression of MSH2 Protein Variants in Insect and Human Cells

Production of the proteins was successful in both *Sf*9 and LoVo cells. In *Sf*9, production was more efficient than in human cells, although the expression levels in TEs varied (data not shown). Our previous study on stability of MLH1 variants[12] suggested that the expression study in human cells is more sensitive to detect variations in protein quantity than expression in insect cells. Thus, to determine whether the *MSH2* alterations affected the stability of the transcript or the protein, we transiently expressed the MSH2 variants together with WT MSH6 (MutSα) in the LoVo cell line, which has a homozygous deletion in *MSH2* gene and thus does not express MSH2 protein.[48] In human cell expression, all the variants showed similar amounts of MSH2 than WT MSH2 (Figure 2).

Interaction Capability of MSH2 Protein Variants

The combined co-immunoprecipitation and Western blot analysis was performed to study the effect of the mutations on MSH2/MSH6 interaction. The total extract amounts used in the immunoprecipitation assays were adjusted to contain equivalent amounts of MSH2, as estimated from sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis. All the mutant proteins were able to interact with MSH6 and the interaction efficiency was very similar to that of MutSα-WT (MSH2-WT/MSH6-WT) (Table 2).

Repair Efficiency of MutSα Protein Variants

We tested the ability of the recombinant MutSα variants to complement the MMR-defective LoVo extracts in repairing of G · T mispairs in vitro (Figure 3A and B). The LoVo cell line lacks MSH2 protein, and we have previously shown that *Sf*9 TEs containing overexpressed MutSα-WT can be used to restore the MMR proficiency of LoVo cell extracts.[49] Because not all the mutant MutSα protein complexes were expressed in similar quantities, the TEs used in the assays were adjusted to contain equivalent amounts of MutSα. Altogether 11 of the analyzed 15 MutSα variants were completely deficient in the in vitro MMR assay (MutSα-V161D, MutSα-G162R, MutSα-G164R, MutSα-L173P, MutSα-L187P, MutSα-C333Y, MutSα-D603N, MutSα-A636P, MutSα-C697F, MutSα-Del745-746, and MutSα-E749K). They repaired 5%–9% of the added heteroduplex DNA, approximately in the same efficiency as the negative control

Table 2. Functionality of the MSH2 Protein Variants Under Study

MSH2 mutation	Interaction with MSH6	Expression in LoVo cells	In vitro MMR	Prediction based on amino-acid alignment	SIFT score[a]	Assessment of pathogenicity based on functional assays
T33P	Normal	Normal	Decreased	Deleterious	0.02	Pathogenic
V161D	Normal	Normal	Deficient	Deleterious	0.00	Pathogenic
G162R	Normal	Normal	Deficient	Deleterious	0.00	Pathogenic
G164R	Normal	Normal	Deficient	Deleterious	0.02	Pathogenic
L173P	Normal	Normal	Deficient	Deleterious	0.00	Pathogenic
L187P	Normal	Normal	Deficient	Deleterious	0.00	Pathogenic
A272V	Normal	Normal	Normal	Tolerated	0.79	Nonpathogenic
C333Y	Normal	Normal	Deficient	Deleterious	0.00	Pathogenic
D603N	Normal	Normal	Deficient	Deleterious	0.01	Pathogenic
A636P	Normal	Normal	Deficient	Tolerated	0.20	Pathogenic
C697F	Normal	Normal	Deficient	Deleterious	0.00	Pathogenic
Del745746	Normal	Normal	Deficient	NA	NA	Pathogenic
E749K	Normal	Normal	Deficient	Deleterious	0.00	Pathogenic
A834T	Normal	Normal	Normal	Tolerated	0.10	Nonpathogenic
V923E	Normal	Normal	Normal	Tolerated	0.89	Nonpathogenic

NA, not available.

[a]Predictions are based on the SIFT program using alignment as described in the text. SIFT scores represent the probability of the new amino acid appearing in the alignment. Scores $\leq$ .05 are generally considered deleterious.

(5.9%), whereas MutSαWT repaired 44.7%. MutSα-T33P showed a reduced MMR efficiency (23.3%), and 3 variants (MutSα-A272V, MutSα-A834T, and MutSα-V923E) showed no reduction in their mismatch repair capability compared with the wild-type MSH2.

Comparative Sequence Analysis

To assess how well comparative sequence analysis predicts the results of functional assays, we constructed multiple sequence alignments of human MSH2 with eukaryotic and prokaryotic homologues and used the SIFT program to predict the outcome of all missense variants (in-frame deletion was excluded). SIFT classifies each amino acid substitution as tolerated or deleterious. Variants were defined as nonpathogenic if functionality was normal in all assays and as pathogenic if there was an abnormality in any assay. The T33P variant was classified as deficient in MMR activity (pathogenic).

By using the alignment of 36 eukaryotic sequences that included animals, yeast, fungi, and parasites, and a cut-off score of .05, SIFT correctly predicted the functional results for 13 of 14 variants, for an overall predictive value of 92.9%. The positive predictive value was 100% (10 of 10 predicted deleterious had reduced function) and the negative predictive value was 75% (3 of 4 predicted tolerated functioned as WT) (Table 2). Adding
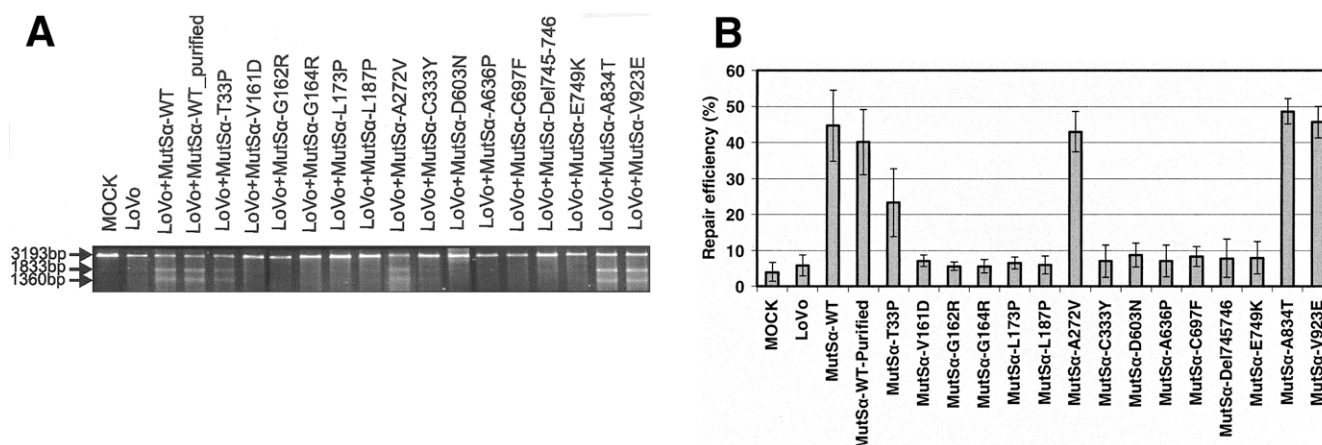

Figure 3. (*A*) In vitro MMR efficiency of LoVo nuclear extract complemented with MutSα protein variants. Mock represents heteroduplex only, with no added nuclear extract or recombinant protein. LoVo nuclear extract lacks intrinsic MSH2 protein (MSH2$^{-/-}$) and is used as a negative control. The upper fragment (3193 bp) represents the unrepaired linearized G ● T heteroduplexes, and the lower bands (1833 and 1360 bp) show the migration of the repaired and double-digested DNA molecules. (*B*) Relative repair percentages of heteroduplex DNA calculated as a ratio of double-digested DNA relative to total DNA added to the reaction. The percentage is an average ± SD of 3 independent experiments. Mock: 4.0% ± 2.6%; LoVo alone: 5.9% ± 2.8%; MutSα-WT: 44.7% ± 9.8%; MutSα-WT-purified: 40.1% ± 9.0%; MutSα-T33P: 23.3% ± 9.4%; MutSα-V161D: 7.2% ± 1.6%; MutSα-G162R: 5.6% ± 1.2%; MutSα-G164R: 5.6% ± 1.9%; MutSα-L173P: 6.5% ± 1.7%; MutSα-L187P: 6.0% ± 2.5%; MutSα-A272V: 42.9% ± 5.6%; MutSα-C333Y: 7.0% ± 4.5%; MutSα-D603N: 8.8% ± 3.4%; MutSα-A636P: 7.1% ± 4.4%; MutSα-C697F: 8.3% ± 2.8%; MutSα-Del745-746: 7.8% ± 5.3%; MutSα-E749K: 8.0% ± 4.5%; MutSα-A834T: 48.6% ± 9.2%; and MutSα-V923E: 45.6% ± 4.3%.

plants to the alignment reduced the overall predictive value to 78.6% (11 of 14), and adding the 240 prokaryotes to the alignment reduced the overall predictive value to 71.4% (10 of 14). By using an alignment of only vertebrates with less evolutionary distance achieved 100% sensitivity, but classified all variants as deleterious (0% specificity in this small data set).

The only incorrect prediction occurred for variant A636P, predicted tolerated but found to be deficient in MMR. Changing the SIFT cut-off score from 0.05 to 0.20 would result in classifying the variant A636P as deleterious but at the same time another tolerated variant A834T (score, 0.10) would be predicted incorrectly as deleterious (Table 2). One variant, V923E, was predicted correctly as tolerated only by the best alignment of animals, yeast, fungi, and parasites. This residue is conserved in mammals. However, it is 10 amino acids from the C terminus of the protein, in a region where most prokaryotic and some eukaryotic sequences already have terminated.

Discussion

Deficiency in MMR predisposes to HNPCC, which is associated primarily with inherited deleterious mutations in the genes *MLH1*, *MSH2*, and *MSH6*.[50] The interpretation of nontruncating alterations in MMR genes is a tremendous challenge to cancer diagnostics and genetic counseling, which rely on the assessment of sequence variants found in cancer patients. The consequence of a nontruncating mutation can vary from none to complete dysfunction of the protein. Without reliable segregation data in a family, functional analyses have been the only way to determine pathogenicity.

In *MSH2*, the second most frequently mutated gene in HNPCC, 24% of reported pathogenic mutations and all variants of unknown significance are nontruncating (www.insight-group.org). Their functional consequences, however, are studied scantily and mainly by yeast-based analyses. Given the known differences in normal functions of MLH1, MSH2, and MSH6 and the different clinical phenotypes associated with their mutations, we wanted to supplement the interpretations of missense mutations in HNPCC by investigating the functionality of mutated MSH2 proteins in the human homologous MMR system, as we had performed previously with MLH1 and MSH6 mutated proteins. We tested the MMR capability of 15 nontruncating *MSH2* mutations found in HNPCC families. Moreover, possible deficiencies associated with quantity of the mutated proteins were studied. Most families fulfilled the international diagnostic criteria for HNPCC, showed early age at cancer onset, and high MSI and loss of MSH2 protein in tumors. The information of cosegregation of mutations with disease in the families usually was missing.

Altogether, 11 of the 15 variants (V161D, G162R, G164R, L173P, L187P, C333Y, D603N, A636P, C697F, Del745-746, and E749K) showed complete loss of MMR function, T33P showed a decreased repair efficiency, and 3 mutations (A272V, A834T, and V923E) repaired similar to the wild-type MSH2. In accordance, L187P, A636P, and C697F already had been studied previously and interpreted as pathogenic.[25,51,52] In insect cells, the expression levels of the mutated proteins varied and were in some cases lower than the level of expressed MSH2-WT protein but yet sufficient in quantity to conduct in vitro functional analyses. As we have shown previously, production of recombinant proteins in *Sf9* insect cells is effective but not as sensitive as protein expression in human cells to detect decrease in

protein quantity.[12] Because successful production in *Sf9* cells does not necessarily indicate protein stability in human cells, the variants also were expressed transiently in *MSH2*-deficient LoVo cells. Remarkably, all the 15 variants were expressed in similar amounts as wild-type MSH2, suggesting their stability. This is in contrast to *MLH1* missense mutations, whose pathogenicity often was shown to be associated with reduced protein expression.[12] None of the mutations, not even D603N or V923E, which were located in the interaction region, interfered with MSH2/MSH6 interaction in a co-immunoprecipitation assay. MSH2 and MSH6 have 2 distinct interaction regions (codons 378-625 and 875-934 in MSH2) and it has been suggested that the loss of function in one region would not result in the complete loss of heterodimer interaction.[53] Thus, it is possible that some of the mutations interfered with dimerization but because MSH2 and MSH6 were not completely detached they were precipitated together.

Comparative sequence analysis, which was performed to assess the value of sequence homology as a tool for predicting functional effects, predicted deleterious and tolerated mutations with an overall predictive value of 93%. We tested several different sequence alignments, representing different degrees of evolutionary distance. The alignment that gave the best results for MSH2 was the same set of sequences that gave the best results for MLH1 (overall predictive value, 82%) (ie, animals, yeast, and parasites, but excluding plants and bacteria).[12] This degree of evolutionary distance appears to be the most appropriate in the analysis of the MMR system in this kind of small data set because using an alignment of only vertebrates achieved 100% sensitivity but classified all the included variants as deleterious. In the clinical setting, identification of all deleterious variants (100% sensitivity) is the ideal to provide appropriate risk assessment and HNPCC management. A potential strategy might be to use comparative sequence analysis as a screening tool, followed by functional studies to confirm predictions of deleterious variants. It is likely that the optimal strategy for interpreting missense mutations will involve multiple lines of evidence (eg, functional, genetic, and computational).[54] However, we now have shown for 2 MMR genes that comparative sequence analysis using SIFT provides a powerful tool to help interpret pathogenicity of mutations.

For one amino acid substitution, the alignment-based prediction and analysis of repair capability gave conflicting results. By using a SIFT cut-off score of 0.05, the amino acid change, A636P, was predicted to be tolerated but showed a complete loss of function in the MMR assay. A636 is a nonconserved residue adjacent to a conserved region near the important Walker A domain in the adenosine triphosphate–DNA binding region. An inflexible adenine to proline substitution may cause steric hindrance and alter an otherwise conserved region in the protein. A636P has been reported to be a founder mutation in Ashkenazi Jews and is associated with HNPCC in several families.[29,55,56] Its pathogenicity also has been shown in a yeast assay.[51] The published data together with our result of loss of MMR function and with clinical data from the 3 families with A636P (Table 1) suggest the pathogenicity of the mutation.

The variant V923E acted similar to wild-type protein in the functional assay. However, only one comparative sequence alignment correctly predicted (SIFT score, 0.89) the WT in vitro results, whereas other alignments predicted it to be deleterious. Predicting the effects of substitutions in the carboxyl end of the

human MSH2 protein is somewhat difficult because many of the homologous proteins included in the alignment analysis lack those last amino acids. In the bacterial system, 53 last amino acids in the carboxyl end were indeed reported to be unnecessary for MMR function.[34] Moreover, V923 locates in the same domain in human MSH2 as T905R, which was not shown to result in reduced MSH2/MSH6 molecular switch functions in a previous biochemical study.[23] The variant V923E, however, was found in a family fulfilling the diagnostic criteria for HNPCC with 6 affected members and with typical HNPCC tumor characteristics (Table 1), suggesting that an unidentified pathogenic mutation segregates in the family or the limit in sensitivity of in vitro functional tests. The discrepancy between the results of our functional analyses and clinical data and that only one comparative sequence alignment analyses correctly predicted the WT in vitro results unfortunately leaves interpretation contradictory for V923E.

Overall, according to our functional assays, 12 alterations should be classified as pathogenic, and 3 alterations, A272V, A834T, and V923E, as nonpathogenic (Table 2). The variation A272V is located in the conserved connector domain in MSH2, but the codon itself is not absolutely conserved and substitution of alanine to valine in this codon was predicted correctly (SIFT score, 0.79) by comparative sequence analysis to be harmless. In accordance with MMR proficiency, tumors in mutation carriers showed MSH2 expression (Table 1). The MSI-positive phenotypes in the same 2 tumors that express MSH2 are inconsistent with the functional and computational results of A272V. One explanation as to why the MSI status was still high in the 2 tumors would be an unidentified pathogenic mutation. The third mutation A834T, which was proficient in the MMR assay, also was predicted to be tolerated by computational analysis. Moreover, MSH2-A834T previously has been proposed not to be a causative factor for CRC and was reported to be found in a healthy control population.[11] However, the clinical phenotype in the Family HNPCC 417, who showed early age at onset and high MSI and loss of MSH2 in the tumor, was not consistent with results of functional and computational analyses, which led us to find some possible explanations for that discrepancy. With new efforts in mutation screening, another mutation, a deletion of exon 8 in *MSH2*, was found and shown to cosegregate with the disease phenotype in the family (E. Mangold, unpublished data). The typical HNPCC phenotype in the family thus most probably is associated with that mutation.

MMR deficiency was associated mainly with typical HNPCC characteristics such as early age at onset, high MSI, and loss of MSH2 protein in the respective tumors. An endometrial cancer in the family EN 26 with the mutation D603N showed MSI stable status. This was inconsistent with MMR deficiency and loss of MSH2 in that tumor. Moreover, the mutation D603N was associated with 2 other HNPCC families and MSI high phenotype in EC and CRC of their index patients, suggesting its pathogenicity. Only 2 tumors with pathogenic mutations T33P and E749K showed MSH2 expression, which in the T33P tumor may be associated with only reduced MMR efficiency detected in the MMR assay. Unlike T33P, E749K showed complete MMR deficiency and a remarkably young age at onset (age, 29 y) in the mutation carrier, suggesting a strong mutator effect of the mutation. E749K locates in an extremely conserved Walker B domain in the adenosine triphosphatase region. Supporting our data, alterations in the amino acid residues corresponding to E749 in human beings have been reported to show strong mutator phenotypes in bacteria[57] and yeast.[58] Thus, we conclude that although MSH2 deficiency is associated mainly with typical HNPCC characteristics, some pathogenic *MSH2* mutations are not necessarily associated with the loss of the respective protein in a tumor, as assessed by IHC. On the other hand, our study raises a question: why do most tumors show a loss of MSH2 in IHC analyses whereas the respective MSH2 variants seem to be stable in the in vitro expression analyses? An explanation must be associated with differences in protein constructions and abundance in vivo and in vitro. Overall, the correlation between the absence of staining for MSH2 and pathogenicity of the missense mutation was notable and suggest IHC analysis as a sensitive method in HNPCC diagnostics.

In conclusion, the in vitro MMR assay can clearly distinguish completely deficient nontruncating alterations in *MSH2* from those proficient or slightly deficient in repair capability. Remarkably, in contrast to *MSH6* and *MLH1* mutations, a clear majority of *MSH2* missense mutations show a complete elimination of MMR capability. Our study also shows that comparative sequence analysis is a good predictor of functional data and that for interpretation of the pathogenicity, it is important to evaluate the biochemical results together with clinical and genetic data of the families under study.

References

1. Lynch HT, de la Chapelle A. Genetic susceptibility to non-polyposis colorectal cancer. J Med Genet 1999;36:801–818.
2. de la Chapelle A. The incidence of Lynch syndrome. Fam Cancer 2005;4:233–237.
3. Jiricny J, Nyström-Lahti M. Mismatch repair defects in cancer. Curr Opin Gene Dev 2000;10:157–161.
4. Constantin N, Dzantiev L, Kadyrov FA, Modrich P. Human mismatch repair: reconstitution of a nick-directed bidirectional reaction. J Biol Chem 2005;280:39752–39761.
5. Zhang Y, Yuan F, Presnell SR, Tian K, Gao Y, Tomkinson AE, Gu L, Li GM. Reconstitution of 5'-directed human mismatch repair in a purified system. Cell 2005;122:693–705.
6. Acharya S, Wilson T, Gradia S, Kane MF, Guerrette S, Marsischky GT, Kolodner R, Fishel R. hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. Proc Natl Acad Sci U S A 1996;93:13629–13634.
7. Palombo F, Iaccarino I, Nakajima E, Ikejima M, Shimada T, Jiricny J. hMutSbeta, a heterodimer of hMSH2 and hMSH3, binds to insertion/deletion loops in DNA. Curr Biol 1996;6:1181–1184.
8. Wagner A, Hendriks Y, Meijers-Heijboer EJ, de Leeuw WJ, Morreau H, Hofstra R, Tops C, Bik E, Brocker-Vriends AH, van Der Meer C, Lindhout D, Vasen HF, Breuning MH, Cornelisse CJ, van Krimpen C, Niermeijer MF, Zwinderman AH, Wijnen J, Fodde R. Atypical HNPCC owing to MSH6 germline mutations: analysis of a large Dutch pedigree. J Med Genet 2001;38:318–322.
9. 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, Schackert HK. 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;22:4486–4494.
10. Cravo M, Afonso AJ, Lage P, Albuquerque C, Maia L, Lacerda C, Fidalgo P, Chaves P, Cruz C, Nobre-Leitao C. Pathogenicity of missense and splice site mutations in hMSH2 and hMLH1 mismatch repair genes: implications for genetic testing. Gut 2002; 50:405–412.

11. Genuardi M, Carrara S, Anti M, Ponz de Leon M, Viel A. Assessment of pathogenicity criteria for constitutional missense mutations of the hereditary nonpolyposis colorectal cancer genes MLH1 and MSH2. Eur J Hum Genet 1999;7:778–782.

12. Raevaara TE, Korhonen MK, Lohi H, Hampel H, Lynch E, Lonnqvist KE, Holinski-Feder E, Sutter C, McKinnon W, Duraisamy S, Gerdes AM, Peltomäki P, Kohonen-Corish M, Mangold E, Macrae F, Greenblatt M, de la Chapelle A, Nyström M. Functional significance and clinical phenotype of nontruncating mismatch repair variants of MLH1. Gastroenterology 2005;129:537–549.

13. Raevaara TE, Vaccaro C, Abdel-Rahman WM, Mocetti E, Bala S, Lonnqvist KE, Kariola R, Lynch HT, Peltomäki P, Nyström-Lahti M. Pathogenicity of the hereditary colorectal cancer mutation hMLH1 del616 linked to shortage of the functional protein. Gastroenterology 2003;125:501–509.

14. Kariola R, Hampel H, Frankel WL, Raevaara TE, de la Chapelle A, Nyström-Lahti M. MSH6 missense mutations are often associated with no or low cancer susceptibility. Br J Cancer 2004;91:1287–1292.

15. Kariola R, Raevaara TE, Lonnqvist KE, Nyström-Lahti M. Functional analysis of MSH6 mutations linked to kindreds with putative hereditary non-polyposis colorectal cancer syndrome. Hum Mol Genet 2002;11:1303–1310.

16. Kolodner RD, Tytell JD, Schmeits JL, Kane MF, Gupta RD, Weger J, Wahlberg S, Fox EA, Peel D, Ziogas A, Garber JE, Syngal S, Anton-Culver H, Li FP. Germ-line msh6 mutations in colorectal cancer families. Cancer Res 1999;59:5068–5074.

17. Peltomäki P, Vasen HF. Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. Gastroenterology 1997;113:1146–1158.

18. Peltomäki P, Vasen H. Mutations associated with HNPCC predisposition—update of ICG-HNPCC/INSiGHT mutation database. Dis Markers 2004;20:269–276.

19. Drotschmann K, Clark AB, Kunkel TA. Mutator phenotypes of common polymorphisms and missense mutations in MSH2. Curr Biol 1999;9:907–910.

20. Clark AB, Cook ME, Tran HT, Gordenin DA, Resnick MA, Kunkel TA. Functional analysis of human MutSalpha and MutSbeta complexes in yeast. Nucleic Acids Res 1999;27:736–742.

21. Ellison AR, Lofing J, Bitter GA. Functional analysis of human MLH1 and MSH2 missense variants and hybrid human-yeast MLH1 proteins in Saccharomyces cerevisiae. Hum Mol Genet 2001;10:1889–1900.

22. Kijas AW, Studamire B, Alani E. Msh2 separation of function mutations confer defects in the initiation steps of mismatch repair. J Mol Biol 2003;331:123–138.

23. Heinen CD, Wilson T, Mazurek A, Berardini M, Butz C, Fishel R. HNPCC mutations in hMSH2 result in reduced hMSH2-hMSH6 molecular switch functions. Cancer Cell 2002;1:469–478.

24. Mangold E, Pagenstecher C, Friedl W, Mathiak M, Buettner R, Engel C, Loeffler M, Holinski-Feder E, Muller-Koch Y, Keller G, Schackert HK, Kruger S, Goecke T, Moeslein G, Kloor M, Gebert J, Kunstmann E, Schulmann K, Ruschoff J, Propping P. Spectrum and frequencies of mutations in MSH2 and MLH1 identified in 1,721 German families suspected of hereditary nonpolyposis colorectal cancer. Int J Cancer 2005;116:692–702.

25. Ollila S, Fitzpatrick R, Sarantaus L, Kariola R, Ambus I, Velsher L, Hsieh E, Andersen MK, Raevaara TE, Gerdes AM, Mangold E, Peltomäki P, Lynch HT, Nyström M. The importance of functional testing in the genetic assessment of Muir-Torre syndrome, a clinical subphenotype of HNPCC. Int J Oncol 2006;28:149–153.

26. Hampel H, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, Nakagawa H, Sotamaa K, Prior TW, Westman J, Panescu J, Fix D, Lockman J, Comeras I, de la Chapelle A. Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). N Engl J Med 2005;352:1851–1860.

27. Ollikainen M, Abdel-Rahman WM, Moisio AL, Lindroos A, Kariola R, Jarvela I, Poyhonen M, Butzow R, Peltomäki P. Molecular analysis of familial endometrial carcinoma: a manifestation of hereditary nonpolyposis colorectal cancer or a separate syndrome? J Clin Oncol 2005;23:4609–4616.

28. Salovaara R, Loukola A, Kristo P, Kaariainen H, Ahtola H, Eskelinen M, Harkonen N, Julkunen R, Kangas E, Ojala S, Tulikoura J, Valkamo E, Jarvinen H, Mecklin JP, Aaltonen LA, de la Chapelle A. Population-based molecular detection of hereditary nonpolyposis colorectal cancer. J Clin Oncol 2000;18:2193–2200.

29. Foulkes WD, Thiffault I, Gruber SB, Horwitz M, Hamel N, Lee C, Shia J, Markowitz A, Figer A, Friedman E, Farber D, Greenwood CM, Bonner JD, Nafa K, Walsh T, Marcus V, Tomsho L, Gebert J, Macrae FA, Gaff CL, Paillerets BB, Gregersen PK, Weitzel JN, Gordon PH, MacNamara E, King MC, Hampel H, De La Chapelle A, Boyd J, Offit K, Rennert G, Chong G, Ellis NA. The founder mutation MSH2*1906G→C is an important cause of hereditary nonpolyposis colorectal cancer in the Ashkenazi Jewish population. Am J Hum Genet 2002;71:1395–1412.

30. Wehner M, Buschhausen L, Lamberti C, Kruse R, Caspari R, Propping P, Friedl W. Hereditary nonpolyposis colorectal cancer (HNPCC): eight novel germline mutations in hMSH2 or hMLH1 genes. Hum Mutat 1997;10:241–244.

31. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 2002;30:e57.

32. Wang Y, Friedl W, Sengteller M, Jungck M, Filges I, Propping P, Mangold E. A modified multiplex PCR assay for detection of large deletions in MSH2 and MLH1. Hum Mutat 2002;19:279–286.

33. Obmolova G, Ban C, Hsieh P, Yang W. Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. Nature 2000;407:703–710.

34. Lamers MH, Perrakis A, Enzlin JH, Winterwerp HH, de Wind N, Sixma TK. The crystal structure of DNA mismatch repair protein MutS binding to a G x T mismatch. Nature 2000;407:711–717.

35. Vasen HF, Mecklin JP, Khan PM, Lynch HT. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). Dis Colon Rectum 1991;34:424–425.

36. 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;116:1453–1456.

37. 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;58:5248–5257.

38. 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, Sanders DS, Morreau H, Muller A, Oliani C, Orntoft T, Ponz De Leon M, Rosty C, Rodriguez-Bigas M, Ruschoff J, Ruszkiewicz A, Sabourin J, Salovaara R, Moslein G, ICG-HNPCC (International Collaborative Group). 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.

39. Nyström-Lahti M, Perrera C, Raschle M, Panyushkina-Seiler E, Marra G, Curci A, Quaresima B, Costanzo F, D'Urso M, Venuta S, Jiricny J. Functional analysis of MLH1 mutations linked to hereditary nonpolyposis colon cancer. Genes Chromosomes Cancer 2002;33:160–167.

40. de Wind N, Dekker M, van Rossum A, van der Valk M, te Riele H. Mouse models for hereditary nonpolyposis colorectal cancer. Cancer Res 1998;58:248–255.

41. Marra G, Iaccarino I, Lettieri T, Roscilli G, Delmastro P, Jiricny J. Mismatch repair deficiency associated with overexpression of the MSH3 gene. Proc Natl Acad Sci U S A 1998;95:8568–8573.

42. Chang DK, Ricciardiello L, Goel A, Chang CL, Boland CR. Steady-state regulation of the human DNA mismatch repair system. J Biol Chem 2000;275:29178.

43. Brieger A, Trojan J, Raedle J, Plotz G, Zeuzem S. Transient mismatch repair gene transfection for functional analysis of genetic hMLH1 and hMSH2 variants. Gut 2002;51:677–684.

44. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–4680.

45. Cooper GM, Brudno M, Green ED, Batzoglou S, Sidow A, NISC Comparative Sequencing Program. Quantitative estimates of sequence divergence for comparative analyses of mammalian genomes. Genome Res 2003;13:813–820.

46. Greenblatt MS, Beaudet JG, Gump JR, Godin KS, Trombley L, Koh J, Bond JP. Detailed computational study of p53 and p16: using evolutionary sequence analysis and disease-associated mutations to predict the functional consequences of allelic variants. Oncogene 2003;22:1150–1163.

47. Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. Genome Res 2001;11:863–874.

48. Umar A, Boyer JC, Thomas DC, Nguyen DC, Risinger JI, Boyd J, Ionov Y, Perucho M, Kunkel TA. Defective mismatch repair in extracts of colorectal and endometrial cancer cell lines exhibiting microsatellite instability. J Biol Chem 1994;269:14367–14370.

49. Kariola R, Otway R, Lonnqvist KE, Raevaara TE, Macrae F, Vos YJ, Kohonen-Corish M, Hofstra RM, Nyström-Lahti M. Two mismatch repair gene mutations found in a colon cancer patient—which one is pathogenic? Hum Genet 2003;112:105–109.

50. Peltomäki P. Lynch syndrome genes. Fam Cancer 2005;4:227–232.

51. Yuan ZQ, Wong N, Foulkes WD, Alpert L, Manganaro F, Andreutti-Zaugg C, Iggo R, Anthony K, Hsieh E, Redston M, Pinsky L, Trifiro M, Gordon PH, Lasko D. A missense mutation in both hMSH2 and APC in an Ashkenazi Jewish HNPCC kindred: implications for clinical screening. J Med Genet 1999;36:790–793.

52. Drotschmann K, Clark AB, Tran HT, Resnick MA, Gordenin DA, Kunkel TA. Mutator phenotypes of yeast strains heterozygous for mutations in the MSH2 gene. Proc Natl Acad Sci U S A 1999;96:2970–2975.

53. Guerrette S, Wilson T, Gradia S, Fishel R. Interactions of human hMSH2 with hMSH3 and hMSH2 with hMSH6: examination of mutations found in hereditary nonpolyposis colorectal cancer. Mol Cell Biol 1998;18:6616–6623.

54. Goldgar DE, Easton DF, Deffenbaugh AM, Monteiro AN, Tavtigian SV, Couch FJ, Breast Cancer Information Core (BIC) Steering Committee. Integrated evaluation of DNA sequence variants of unknown clinical significance: application to BRCA1 and BRCA2. Am J Hum Genet 2004;75:535–544.

55. Guillem JG, Rapaport BS, Kirchhoff T, Kolachana P, Nafa K, Glogowski E, Finch R, Huang H, Foulkes WD, Markowitz A, Ellis NA, Offit K. A636P is associated with early-onset colon cancer in Ashkenazi Jews. J Am Coll Surg 2003;196:222–225.

56. Guillem JG, Moore HG, Palmer C, Glogowski E, Finch R, Nafa K, Markowitz AJ, Offit K, Ellis NA. A636P testing in Ashkenazi Jews. Fam Cancer 2004;3:223–227.

57. Lamers MH, Winterwerp HH, Sixma TK. The alternating ATPase domains of MutS control DNA mismatch repair. EMBO J 2003; 22:746–756.

58. Studamire B, Price G, Sugawara N, Haber JE, Alani E. Separation-of-function mutations in Saccharomyces cerevisiae MSH2 that confer mismatch repair defects but do not affect nonhomologous-tail removal during recombination. Mol Cell Biol 1999;19: 7558–7567.

59. Otway R, Tetlow N, Hornby J, Doe WF, Kohonen-Corish MR. Gene symbol: MSH2. Disease: hereditary nonpolyposis colorectal cancer. Hum Genet 2005;116:539.

CLINICAL–ALIMENTARY TRACT