Novel and recurrent germline alterations in the *MLH1* and *MSH2* genes identified in hereditary nonpolyposis colorectal cancer patients in Slovakia*

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Hereditary non-polyposis colorectal cancer (HNPCC) is associated with germline mutations in DNA mismatch repair genes, predominantly *MSH2* and *MLH1*. Mutation carriers develop cancers in the colorectum, endometrium, ovary, stomach, small intestine and the upper urinary tract. We describe here the results of a mutational analysis of 11 unrelated HNPCC patients by direct genomic sequencing of *MLH1* and *MSH2*. The alterations found include 7 novel changes and 4 different pathogenic mutations described previously in Poland, Moldavia, Finland, Germany, France and USA. Four novel pathogenic mutations in the *MLH1* gene include two frameshift mutations (c.1150delG and c.1210_1211delCT), one missense mutation (c.793C>A) and one intron-exon border mutation (c.546-2A>C). The last change resulted in the skipping of exon 7, as shown by sequencing of RT-PCR products. The only novel *MSH2* pathogenic change was a nonsense mutation c.1129C>T. The novel intronic change c.381-41A>G in *MLH1* was found in a patient carrying a previously-described mutation in the *MSH2* gene. Interestingly, two unrelated patients carried also a novel change in the promoter region of *MLH1* in one of the CpG islands (c.-269C>G). However, this alteration does not abrogate transcription, as shown by RT-PCR analysis. In summary, most (~80%) pathogenic germline mutations detected in the studied group of patients by direct genomic sequencing of *MLH1* and *MSH2* were located in the *MLH1* gene. These and previous data indicate that the majority of germline point mutations and small deletions/insertions in HNPCC families in Slovakia affect the *MLH1* locus.

Key words: HNPCC, *germline mutations*, *MSH2*, *MLH1*

During recent years, molecular diagnostic methods for several hereditary cancer syndromes were established in the Slovak Republic, with the ultimate goal of reducing cancer mortality through early identification of at-risk individuals and effective prophylaxis [1–7]. Much of our attention is dedicated to hereditary non-polyposis colorectal cancer (HNPCC), the most common familial colorectal cancer (CRC) syndrome, which accounts for ~4% of all CRC cases [8]. In Slovakia, where about 2600 CRC cases are diagnosed annually with the incidence of 58.7 per 100 000 inhabitants [9], this translates to ~80 HNPCC cases *per annum.*

HNPCC (MIM#114500), also called the Lynch syndrome, is an autosomal dominant disease with high penetrance (85%) and younger age of onset when compared to patients with sporadic tumors. Although Lynch syndrome patients are primarily afflicted with cancers of the colon and endometrium, the tumor spectrum of HNPCC includes also ovarian, gastric and brain tumors, as well as cancer of the small bowel, hepatobiliary tract and upper urologic tract [10].

Lynch syndrome is associated with germline mutations in DNA mismatch repair (*MMR*) genes (see [11] for a recent review). According to InSIGHT (International Society for Gastrointestinal Hereditary Tumors) database, approximately 50 and 40% of the HNPCC-causing mutations are found in the *MLH1* (MIM#120436; GDB: 249617) and *MSH2* (MIM#120435; GDB: 203983) genes, respectively,

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with mutations in *MSH6*, *MSH3*, *PMS1* and *PMS2* comprising the remaining 10%. Accordingly, recent strategies of mutation detection focus primarily on *MLH1* and *MSH2* [12, 13, 14].

Despite substantial advances in our understanding of HNPCC, much remains to be learned about the correlations between the genetic changes and clinical features of the disease. Description of novel or recurrent alterations of *MMR* genes in clinically well defined HNPCC families has the potential to reveal new properties of the disease, mechanisms of pathogenicity and links to new biological pathways, which may turn out to be useful in the design of preventive and therapeutic strategies in the future. The aim of this study is to describe the novel and recurrent mutations of clinically well defined HNPCC families identified during DNA sequencing screening of *MLH1* and *MSH2* genes. The identification of pathogenic germline *MMR* gene mutations is one of the key prerequisites for the identification of at-risk individuals.

Material and methods

Patients and samples. The study involved 11 families (ten of Slovak and one of mixed Slovak/Italian [IT/SK-15] origin). The index patients fulfilled clinical criteria for HNPCC testing, either the strict Amsterdam criteria (AC-I or AC-II), or the less-stringent Bethesda guidelines (BG or RBG). These were recently reviewed by VASEN [15] and are listed in Table 1. The index cases of these 11 families included 6 females and 5 males in the age range of 28–52 years (average age 38 years) at first diagnosis. Samples of peripheral blood leukocytes were obtained from the patients after informed consent during the clinico-genetic consultation. From two patients, additional blood samples were collected using PAXgene Blood RNA Tubes (Qiagen, Hilden, Germany) for preservation of RNA and further use in RT-PCR analyses. The analysis of 100 control chromosomes was performed using the peripheral blood samples of 50 healthy individuals.

DNA sequencing. Genomic DNA for sequencing was isolated using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The *MLH1* gene was divided into 21 fragments covering the promoter and 19 exons with substantial parts of intron regions (exon 12 was subdivided in 2 overlapping fragments). The *MSH2* gene was divided into 17 fragments covering the promoter and 16 exons. The primers (Tab. 2A, B) were designed such that they could be used with identical PCR amplification protocols. This allowed a simultaneous amplification of all PCR fragments and resulted in a significant time-saving. Some of the *MSH2* gene primers in our set were identical to those published previously [16]. The PCR reactions contained 7.5 pmol each primer, 12.5 µl AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, USA) and 50 ng DNA in a final reaction volume of 25 µl. The following cycling conditions were used for all PCRs: initial denaturation at 95 °C for 10 minutes, followed by 8 cycles of denaturation at 98 °C for 10 seconds, annealing step at 60 °C for 30 seconds and extension at 70 °C for 1 minute, followed by additional 37 cycles of denaturation at 96 °C for 10 seconds and annealing-extension step at 68 °C for 2 minutes. After purification by ethanol-sodium acetate method with PelletPaint NF Co-Precipitant (Novagen, Madison, USA) the

Table 1. Clinical criteria of Lynch syndrome (HNPCC)

AC-I	There should be at least 3 relatives with colorectal cancer and all criteria must be satisfied: • One relative should be a first-degree relative of the other two • At least two successive generations should be affected • At least one tumor should be diagnosed before the age of 50 • Familial adenomatous polyposis should be excluded • Tumor should be verified by a histopathological examination
AC-II	There should be at least 3 relatives with colorectal cancer or with HNPCC-associated cancer: cancer of the endometrium, small bowel, ureter or renal pelvis and all Amsterdam criteria I must be satisfied.
BG	1. Individuals with two HNPCC-associated cancers, including synchronous and metachronous colorectal cancers or associated extra-colonic cancers (endometrial, ovarian, gastric, hepatobiliary, small bowel cancer or transitional cell carcinoma of renal pelvis or ureter 2. Individuals with colorectal cancer and a first degree relative with colorectal cancer and/or HNPCC-associated extra-colonic cancer and/or colorectal adenoma; one of the cancers diagnosed at age <45 years, and the adenoma diagnosed at the age <40 3. Individuals with colorectal cancer or endometrial cancer diagnosed at the age <45 4. Individuals with right-sided colorectal cancer with an undifferentiated pattern on histopathology diagnosed at age <45 5. Individuals with signet-ring-cell-type colorectal cancer diagnosed at age <45 6. Individuals with adenomas diagnosed at age <45
RBG	1. Colorectal cancer diagnosed in a patient at age <50 2. Presence of synchronous, metachronous colorectal, or other HNPCC-associated tumors, regardless of age 3. Colorectal cancer with MSI-H phenotype diagnosed in a patient <60 years of age 4. Individual with colorectal cancer and one or more first-degree relatives with an HNPCC-related tumor, with one of the cancers diagnosed under the age of 50 5. Individual with colorectal cancer and two or more first-degree or second-degree relatives with HNPCC-associated tumors, regardless of age

AC-I/AC-II – Amsterdam criteria I, II; BG – Bethesda Guidelines; RBG – Revised Bethesda Guidelines

Table 2A. Primers for the amplification of the genomic regions of *MLH1*

Region	Forward 5'-3'*	Reverse 5'-3'*	Product length (bp)
Promoter	M13F-ACCACCAAATAACGCTGGGTC	M13R-AATCACCTCAGTGCCTCGTGC	536
Exon 1	M13F-TGACTGGCATTCAAGCTGTCC	M13R-CTGACTGGCACGTCAGGGAAC	470
Exon 2	M13F-TTGGAGTTTGTTATCATTGCTTGG	M13R-ACTGACACATCCCCTGAACAGTG	398
Exon 3	M13F-CAAAGAGATTTGGAAAAATGAG	M13R-CAAACCTTATTTATCTATGTTGAG	294
Exon 4	M13F-CAGATAACCTTTCCCTTTGGTGAG	M13R-TGGTGTTGAGACAGGATTACTCTGAG	281
Exon 5	M13F-TGATATGATTTTCTCTTTTCCCC	M13R-GTGCAAATTCATTTTATTATTACCC	300
Exon 6	M13F-CTTTTGCCAGGACATCTTGGG	M13R-TTTCACCATCTAGCTCAGCAACTG	302
Exon 7	M13F-TAAAAGGGGGCTCTGACATC	M13R-AAACATCATAACCTTATCTCCACC	245
Exon 8	M13F-ATGTTTCAGTCTCAGCCATG	M13R-CAAGCCTGTGTATTTGACTAAAG	316
Exon 9	M13F-CAAAGTTAGTTTATGGGAAGGAAC	M13R-CATGAGGTTTCTCCATGTTTAATC	372
Exon 10	M13F-TGTCTTTCCTGAGGTGATGTCATG	M13R-CCTTTTGCCAGTGGTGTATGGG	359
Exon 11	M13F-GGTTTTGACCACTGTGTCATCTG	M13R-AATCTGGGCTCTCACGTCTG	382
Exon 12-I	M13F-TACTGCTCCATTTGGGGACCTG	M13R-AGCATCTCCTCATCTTGCTGCC	400
Exon 12-II	M13F-GGTCTATGCCCACCAGATGG	M13R-CATGAAAAGCCAAAGTTAGAAGG	392
Exon 13	M13F-GCTTGCTCCCAAATGCAACC	M13R-GCCCATCAAAACTGTAGTGCCAC	380
Exon 14	M13F-TCTAGTTCTGGTGCCTGGTGCTTTG	M13R-GCCTGTGCTCCCTGGACCATTG	300
Exon 15	M13F-TCTGTCTCATCCATGTTTCAGGG	M13R-CGATCAGTTGAAATTTCAGAAGTG	250
Exon 16	M13F-TCATTTGGATGCTCCGTTAAAG	M13R-AATTTTATTTGGAGAATACAACAGAAG	301
Exon 17	M13F-CAGATAGGAGGCACAAGGCCTG	M13R-TTGCCCTTTCCCTCCAGCAC	293
Exon 18	M13F-CAGTCCCATTTACAGTTTTAACGCC	M13R-TGTCCTAGTCCTGGGGTGCC	323
Exon 19	M13F-AGATAACACCAAGTCTTTCCAGAC	M13R-TCAATCCACTGTGTATAAAGGAATAC	474

*Forward primers have M13F tail attached for sequencing: TGTAAAACGACGGCCAGT; Reverse primers have M13R tail attached for sequencing: CAGGAAACAGCTATGACC

Table 2B. Primers for the amplification of the genomic regions of *MSH2*

Region	Forward 5'-3'*	Reverse 5'-3'*	Product length (bp)
Promoter	M13F-AAATACTGGGAGGAGGAGGAAGG	M13R-CACCGCCATGTCGAAACCTC	512
Exon 1	M13F-AAACGCAGCCCTGGAAGCTG	M13R-GTGCCTCCGCACTGGAGAGG	494
Exon 2	M13F-GTCCAGCTAATACAGTGCTTG	M13R-CACATTTTTATTTTTCTACTCTTAA	316
Exon 3	M13F-AAAGTATGTTCAAGAGTTTGTTAAATTTTT	M13R-CTTTCCTAGGCCTGGAATCTC	413
Exon 4	M13F-TTTTTGCTTTTCTTATTCCTTTTC	M13R-TGACAGAAATATCCTTCTAA	345
Exon 5	M13F-GTGGTATAGAAATCTTCGATTTTT	M13R-AATCAACATTTTTAACCCTTTT	273
Exon 6	M13F-TTTTCACTAATGAGCTTGCCATTCTT	M13R-GGTAACTGCAGGTTACATAAAACTAACGA	270
Exon 7	M13F-TGATTTAGTTGAGACTTACGTGC	M13R-TTTATGAGGACAGCACATTGC	398
Exon 8	M13F-TTTGTATTCTGTAAAATGAGATCTTT	M13R-CTTTGCTTTTTAAAAATAACTACTG	253
Exon 9	M13F-TAGGATTTTGTCACTTTGTTCTG	M13R-GTGTATAGGACAAAAGAATTATTCC	237
Exon 10	M13F-GGTAGTAGGTATTTATGGAATACTTTTTC	M13R-GCATTTAGGGAATTAATAAAGGG	286
Exon 11	M13F-CATTGCTTCTAGTACACATTT	M13R-CAGGTGACATTCAGAACATTA	231
Exon 12	M13F-TCAGTATTCCTGTGTACATTT	M13R-TTACCCCCACAAAGCCCAA	359
Exon 13	M13F-GTAGCAGAAAGAAGTTTAAAATCTTGC	M13R-GGACAGAGACATACATTTCTATCTTC	341
Exon 14	M13F-CCACATTTTATGTGATGGGAA	M13R-GGTAGTAAGTTTCCCATTAC	385
Exon 15	M13F-TGCTGTCTCTTCTCATGCTG	M13R-AGAAGCTAAGTTAAACTATG	299
Exon 16	M13F-ATTTTAATTACTAATGGGACATTC	M13R-TTAAGTTGATAGCCCATGG	370

*Forward primers have M13F tail attached for sequencing: TGTAAAACGACGGCCAGT; Reverse primers have M13R tail attached for sequencing: CAGGAAACAGCTATGACC

PCR products were sequenced using either the ABI PRISM BigDye v3.1 or, less frequently, the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, USA) kits. The identified DNA sequence alterations were confirmed by sequencing of both DNA strands in two independent PCR products. The mutation nomenclature used complies with the recommendations of DEN DUNNEN and ANTONARAKIS [17, 18], DEN DUNNEN and PAALMAN [19] and the latest nomenclature updates on www.hgvs.org/mutnomen. The sequence variations were described in relation to cDNA reference sequence from a primary sequence database (NCBI GenBank using RefSeq NM_000249.2 and NM_000251.1 for *MLH1* and *MSH2*, respectively). The reference sequences AH003234.1 and AH003235.1 were used for mutation mapping of intronic regions of the *MLH1* and *MSH2* genes, respectively. The amino acid changes were deduced from nucleotide alterations in relation to a protein reference sequence from SWISS-PROT, using ID: P40692 for MLH1 and ID: P43246 for the MSH2 protein.

RT-PCR analysis. Total RNA was extracted from peripheral blood using PAXgene Blood RNA Kit (Qiagen, Hilden, Germany). RT-PCR was performed using OneStep RT-PCR Kit (Qiagen, Hilden, Germany) with *MLH1*-specific primers located in exons 5 and 11, respectively, as published previously [20]. The RT-PCR products were resolved on 1.5 agarose gels. Individual bands were excised, purified by MinElute Extraction Kit (Qiagen, Hilden, Germany), reamplified by a second identical PCR reaction using Pfu DNA polymerase, then cloned into pUC18 and subsequently sequenced.

Results and discussion

Clinical data and family histories of the patients with identified germline mutations are summarized in Table 3. Six patients belonged to families fulfilling the AC-I criteria and one met the AC-II criteria. Three patients complied with BG and one with RBG. All but one index cases, had colon cancer. Only patient IT/SK-15 had a tumor in the rectum. Three patients presented with additional cancers in extracolonic sites; two (SK7, SK12) had endometrial and one (SK10) ovarian cancer. Metachronous or synchronous tumors were present in four (SK7, SK12, SK17, SK19) and two (SK9, SK10) cases, respectively. Right-sided colon tumor localization was described in the medical records of six patients (SK7, SK8, SK9, SK11, SK12, SK19) and mucinous histopathology was confirmed in two tumors (SK7, SK8). Microsatellite instability and immunohistochemical analyses of most of these tumors will be described elsewhere [FRIDRICHOVA et al, manuscript in preparation], as part of a study evaluating the efficacy of pre-screening methods for HNPCC diagnosis in a large cohort of patients.

In the present study, we describe the alterations (Tab. 4) found in 11 HNPCC families by DNA sequencing of the *MLH1* and *MSH2* genes. To our knowledge, seven of these alterations have not been previously reported in the literature and are not included in the database of International Society of Gastrointestinal Hereditary Tumors (http://www.insight-group.org/) or in the Human Gene Mutation Database [21].

Genetic changes in the *MLH1* promoter appear to be rare [22, 23]. We found a novel change, c.-269C>G, in two apparently unrelated patients (SK-7 and SK-9). However, both patients carried also additional mutations in the same gene. The first (SK-7) had an A>C change at position -2 of the splice acceptor site upstream of exon 7 (c.546-2A>C). The mutation would cause skipping of exon 7, resulting in an out-of-frame deletion that would lead to a premature stop codon and truncated protein (p.Arg182SerfsX187). This change, coupled with the promoter mutation, was identified in another affected family member and in 4 asymptomatic relatives, while 5 other asymptomatic relatives carried neither alteration. This indicated that both mutations affected the same allele. RT-PCR of exons 5-11 yielded the expected 536 bp fragment in addition to a shorter product of 493 bp. As expected, the cloning and subsequent DNA sequencing of the

Table 3. Clinical data and cancer family histories of the patients included in this study

ID	Tumor localization (age at onset)	Affected 1st, 2nd and 3rd degree relatives (age at onset)	Criteria fulfilled
SK-7	colon (52), endometrium (55)	1st: C(33, 43, 48); C(45) 2nd: En(41); C(60); C(61); C(74)	AC-I
SK-8	colon (49)	1st: C(55); Br(50)	RBG
SK-9	duplex in colon (34)	2nd: En(42); En(60); C(68)	BG
SK-10	colon + ovary (42)	1st: C(33); C + En(32); C(33) 2nd: C; C(37); C + Ov(29); C(49); En(58); St(37)	AC-I
SK-11	colon (29)	1st: polyp at En(?) 2nd: En(?); M(59) + polyp in the colon (?); En(57)	BG
SK-12	endometrium (52), colon (53)	1st: C(57); En(45) + C(46)	AC-I
SK-13	colon (30)	1st: duplex C(41); C(28) 2nd: C(40); C(?); C(61) 3rd: C(?)	AC-I
IT/SK-15	rectum (28)	1st: Br(37) 2nd: C(38); C(40); C(55); C(29); En(75)	AC-I
SK-17	colon (32, 47, 50)	1st: C(73)	BG
SK-19	colon (32, 39, 54, 62)	1st: C(42) 2nd: En(50) + Br(48); En(50)	AC-II
SK-24	colon (42)	1st: C(32), C(27), C(39)	AC-I

ID – patient/family ID number, C – colon, En – endometrium, St – stomach, Br – breast, Ov – ovary, M – malignant melanoma. Individuals are separated by a semicolon, (?): age at onset not known. AC-I/AC-II – Amsterdam criteria I, II; BG – Bethesda Guidelines; RBG – Revised Bethesda Guidelines.

shorter product showed that exon 6 was spliced directly onto exon 8 (Fig. 1, left panel). This suggests that the skipping of exon 7 represents the pathological mutation, as the promoter mutation did not affect transcription from this allele. In the second patient (SK-9) carrying the *MLH1* promoter change, a frameshift mutation c.1150delG in exon 12 of *MLH1* was detected in the germline. This change should lead to a truncated protein p.Val384PhefsX400 and is very likely the primary pathogenic mutation, even though we could not perform segregation analysis in this family. Thus, although the promoter change c.-269C>G was not present in 100 control chromosomes and does not seem to be a polymorphism, we assume that it is not pathogenic.

Despite the fact that patient SK-8 fulfilled solely the least stringent HNPCC criteria (RBG), she was included in the mutational screening, as the presence of the mucinous right-sided colon tumor was highly indicative of HNPCC. Interestingly, the identified mutation, c.210_213delAGAA, was previously described in a Finnish family meeting the strict AC-I criteria for HNPCC. This deletion, located three nucleotides downstream from the 5' end of *MLH1* exon 3, should lead to a frameshift and result in truncated protein (p.Glu71IlefsX90). However, the authors showed that the consequence at the RNA level is the skipping of exon 3 due to disruption of a 5' splice site [24]. Interestingly, in our family the same mutation was identified also in three of the patient's nephews, suggesting that their mother, the proband's sister, was an obligate carrier. Unfortunately, this could not be confirmed, as she died at a relatively early age of breast cancer, a tumor the inclusion of which in the HNPCC-tumor spectrum is still controversial [25–29].

Patients SK-10 and SK-24 carried identical mutations at the last nucleotide of *MLH1* exon 8 (c.677G>T), which was originally described in a Moldavian family [30]. This transversion could be classified as a missense mutation (p.Arg226Leu), however, its location at the 3' splice site region may suggest skipping of exon 8. The same mutation was previously found by our group in another Slovak family [1]. High frequency of this mutation in our and other populations supports the hypothesis that exonic CpG dinucleotides in *MLH1* and *MSH2* might be hotspots for recurrent HNPCC mutations, which can be either of ancestral origin or derived from *de novo* events.

Patient SK-11 presented with a right-sided colon cancer at 29 years of age. The transversion c.793C>A in exon 10 found in the germline of this patient is a missense mutation, presumably leading to a change from a positively-charged arginine to a polar serine (p.Arg265Ser). A transition at the same position c.793C>T, leading to a change of arginine to cysteine, was found previously in a Czech family [31]. Another alteration affecting the same codon (c.794G>A) and resulting in a change from arginine to histidine was found in an Italian HNPCC family. However, it was classified as a rare polymorphism (not found in 196 control chromosomes), as it co-segregated with an additional mutation in the same gene, which resulted in a frameshift [32]. Recent structural studies showed that Arg265 corresponds to Arg261 of *E. coli* MutL, which destabilizes the P loop if mutated to His. This mutation reduces ATP binding and ATP-dependent activation of MutH endonuclease [33, 34]. We thus assume that the mutation found in patient SK-11 might have pathological consequences. The alteration was not present in 100 control chromosomes, but was detected in two other members of family SK-11 presenting with endometrial and colonic polyps, respectively. In addition, the colon cancer of the proband lacked MLH1 in IHC analysis (unpublished data) and no additional alterations were found in *MLH1* or *MSH2*. Taken together, we suggest that the missense mutation c.793C>A in exon 10 of *MLH1* may predispose to HNPCC.

Patient SK-12 was diagnosed with endometrial cancer

Table 4. Novel and reccurrent *MLH1* and *MLH2* germline alterations detected in the present study

ID	Gene	Region	Nucleotide Change[2]	Consequences[4]	First description
SK-7	*MLH1*	promoter	c.-269C>G[3]	?	novel
		SAS[1] of exon 7	c.546-2A>C	r.546_588del (skipping of exon 7)	novel
SK-8	*MLH1*	exon 3	c.210_213delAGAA	splice defect (skipping of exon 3)	Renkonen et al., 2004
SK-9 *	*MLH1*	exon 12	c.1150delG	p.Val384 PhefsX400	novel
SK-10	*MLH1*	exon 8	c.677G>T	p.Arg226Leu + splice defect	Maliaka et al., 1996
SK-11	*MLH1*	exon 10	c.793C>A[3]	p.Arg265Ser	novel
SK-12	*MLH1*	exon 12	c.1210_1211delCT	p.Leu404ValfsX415	novel
SK-13	*MLH1*	exon 4	c.350C>T	p.Thr117Met	Maliaka et al., 1996
IT/SK-15	*MLH1*	intron 4	c.381-41A>G[3]	?	novel
	MSH2	exon 12	c.1861C>T	p.Arg621X	Maliaka et al., 1996
SK-17	*MLH1*	exon 10	c.883A>C[3]	r.791_884del (skipping of exon 10)	Kurzawski et al., 2006
SK-19	*MSH2*	exon 7	c.1129C>T	p.Gln377X	novel
SK-24	*MLH1*	exon 8	c.677G>T	p.Arg226Leu + splice defect	Maliaka et al., 1996

*The patient carried also the same promoter change as patient SK-7; [1]splice acceptor site; [2]the changes were experimentally determined at DNA level, numbering is based on cDNA sequence, position +1 corresponds to the A of the ATG translation initiation codon in the RefSeq; [3]the change was not found in 100 control chromosomes; [4]the consequences at RNA level (r.) were experimentally determined, the consequences on the protein level (p.) are theoretically predicted.

aged 52 and with right-sided colon cancer a year later. A 2-bp deletion (c.1210_1211delCT) in exon 12 of *MLH1* was detected. This mutation leads to a premature stop codon 33 nucleotides downstream (codon 415 in exon 12). At the protein level it results in a reading frame change starting at Leu404, whereby the -2 open reading frame remains open for 10 amino acids. The truncated protein, if stable, will lack the interaction domain of MLH1 with PMS2 [35].

In the genomic DNA of patient SK-13 we identified a transition mutation (c.350C>T) in exon 4 of *MLH1*, which was previously described in other populations [30, 31, 36, 37, 38]. This mutation presumably leads to a non-conservative amino acid change of a polar threonine to a hydrophobic methionine (p.Thr117Met) in the highly-conserved region of MLH1. The mutation segregated with two other affected family members and IHC showed absence of MLH1 (unpublished data). We therefore assume that the SK-13 mutation is pathogenic.

Patient IT/SK-15 is of Italian origin with offspring living in Slovakia. His family met the AC-I criteria and its germline mutation was apparently linked to the maternal line of the patient. However, the mother died of breast cancer at the age of 37, which is not typically associated with HNPCC, as already discussed. At first, we sequenced the *MLH1* gene and observed a variation c.381-41A>G in intron 4. Although this alteration was not present in 100 control chromosomes and could not be classified as a polymorphism, we proceeded with the sequencing of the *MSH2* gene, where the change c.1861C>T in exon 12 was detected. This latter change has been described for the first time in Moldavia [30]. Since it is a nonsense mutation and was found also in other HNPCC families from Western Europe and USA (unpublished data in the InSIGHT database), its association with the disease in our patient is very likely, while the consequences of the *MLH1* intronic change are not clear at this time. The frequent detection of the recurrent mutations c.350C>T in *MLH1* and c.1861C>T in *MSH2* in different populations supports the hypothesis that exonic CpG dinucleotides represent mutational hotspots, as already mentioned for mutation c.677G>T in *MLH1*.

Patient SK-17, who presented with three metachronous colon cancers diagnosed at 32, 47 and 50 years of age, carried the transversion c.883A>C in exon 10 of the *MLH1* gene. Since this mutation is located only two nucleotides upstream from the highly-conserved "GT" dinucleotide of a splice donor site, we performed RT-PCR analysis, which detected an additional, shorter fragment suggesting aberrant splicing. Cloning and subsequent sequencing of this fragment confirmed this; the deletion of 94 bp indicated that exon 9 was spliced directly onto exon 11 (Fig. 1, right panel). This indicates that deletion of exon 10 is pathogenic. The patient carried also a common polymorphism (c.655A>G) in exon 8 and the "G" allele co-segregated with the aberrantly-spliced allele. During the preparation of this manuscript, the c.883A>C mutation was found also in a HNPCC family from Poland [39].

Patient SK-19 presented with four metachronous colon tumors at 32, 39, 54 and 62 years of age. DNA sequencing revealed a novel nonsense mutation localized in exon 7 of *MSH2* (c.1129C>T) leading to a change of glutamine at position 377 to a premature stop codon. Interestingly, this was the third family in our study (27%), in which occurrence of breast cancer was reported in first or second degree relatives at early age. This evidence supports attempts to include breast cancer in the HNPCC tumor spectrum [26, 29].

In summary, 9 of 11 (82%) pathogenic germline mutations detected in this study by direct genomic sequencing of the *MLH1* and *MSH2* genes were located in the *MLH1* gene. In our previous mutation study, 4 of 6 (67%) pathogenic germline mutations were also located in the *MLH1* gene [1]. Taken together, this evidence suggests that most (13/17, 76%) point mutations and small deletions/insertions in Slovak HNPCC families occur in the *MLH1* gene. However, germline mutations in the *MSH2* gene are frequently large deletions/insertions, which are not detectable by DNA sequencing [40–43]. In order to eliminate the possibility that direct sequencing failed to identify the latter mutations, we are currently deploying MLPA analysis, which is the preferred method for identification of large gene rearrangements [44].

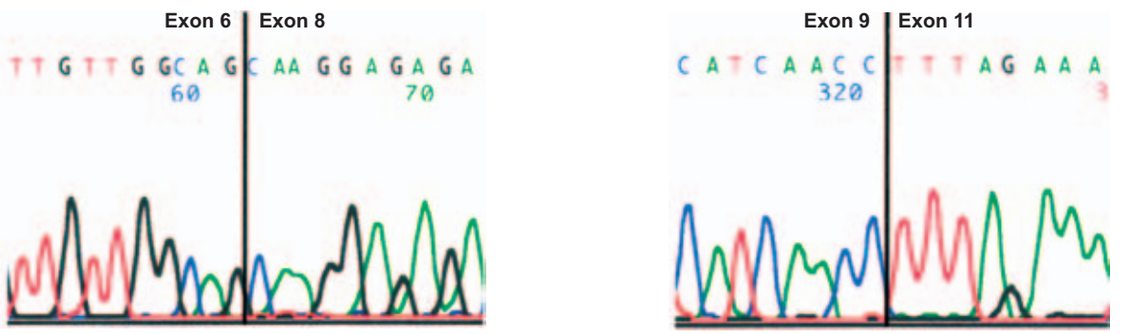

Figure 1. Nucleotide sequence analysis of alternatively-spliced transcripts of the *MLH1* gene. Left panel: splicing of exon 6 directly onto exon 8 in patient SK-7; Right panel: splicing of exon 9 directly onto exon 11 in patient SK-17.

The alterations in the *MMR* genes described in this study were detected as part of a molecular screening program initiated several years ago, the primary objective of which is to identify all HNPCC families in Slovakia [1, 2]. Symptomatic and asymptomatic *MMR* gene mutation carriers who take advantage of this predictive molecular-genetic analysis are followed up with an annual colonoscopy, and, where appropriate, with abdominal and transvaginal sonography. The efficacy of this cancer prevention strategy is currently under investigation.

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