

ORIGINAL ARTICLE

Nine novel pathogenic germline mutations in *MLH1*, *MSH2*, *MSH6* and *PMS2* in families with Lynch syndrome

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Abstract

Many germline mutations in the DNA mismatch repair genes have been described so far leading to the clinical phenotype of Lynch syndrome (hereditary nonpolyposis colorectal cancer, HNPCC). Most mutations are private mutations. We report on nine novel pathogenic germline mutations that have been found in families meeting either the Amsterdam or the Bethesda criteria. These findings include the mutations *MLH1*,c.884+4A>G, *MLH1*,c.1377_1378insA;p.Glu460ArgfsX19, *MLH1*,c.1415_1416delGA;p.Arg472ThrfsX5, *MSH2*,c.301G>T;p.Glu101X, *MSH2*,c.638_639delTG;p.Leu213GlnfsX18, *MSH2*,c.842C>A;p.Ser281X, *MSH2*,c.859G>T;p.Gly287X, *MSH6*,c.2503C>T;p.Gln835X and a large genomic deletion of exons 1–10 of the *PMS2* gene. The mutation *MLH1*,c.884+4A>G detected in two families results in a complete skipping of exon 10 on mRNA level and thus has been considered as pathogenic. In all cases the tumor tissue of the index patient revealed high microsatellite instability (MSI-H) and showed a complete loss of expression of the affected protein in the tumor cells by immunohistochemistry (IHC). The findings underline the importance of a pre-screening of tumor tissue for an efficient definition of conspicuous cases.

Lynch syndrome (hereditary non-polyposis colorectal cancer/HNPCC) is one of the most common cancer susceptibility syndromes with an autosomal dominant inheritance caused by germline mutations in DNA mismatch repair (MMR) genes, predominantly *MLH1* and *MSH2* [1]. In a minority of cases, mutations have been described in the MMR genes *MSH6* and *PMS2* [2–4]. Mutation carriers are at risk to develop a broad spectrum of malignancies including tumors of the colorectum, endometrium, stomach, small intestine, hepatobiliary system, ureter, renal pelvis, ovary and skin [1,5]. Here we report on nine novel pathogenic germline mutations identified in patients suspected of HNPCC. Among these, we demonstrate the functional relevance of an unclassified intronic variant in *MLH1*. Furthermore, the detection of a *PMS2* deletion in a large family extends the still limited knowledge on the tumor spectrum associated with *PMS2* defects.

Materials and methods

Patients

The patients included in this study were recruited at the University Hospital Bonn, Germany, within a project of the German HNPCC consortium. Patients were referred to this study from other hospitals, institutes of human genetics, private practice physicians, and private practice human geneticists or by self-referral. Patients were enrolled into the study if they fulfilled at least one of the clinical selection criteria shown in Table I. At this stage 762 families suspected as having Lynch syndrome were registered at our institute and in 224 families pathogenic germline mutations were identified. Descriptions of the mutation spectrum found in these families, genotype-phenotype correlations and pathology were reported elsewhere [6–8]. Here we report on nine novel germline mutations in ten patients who were recruited by the same selection criteria.

Table I. Definition of selection criteria.

	Definition
A2	Amsterdam II: family with at least 3 members in at least 2 successive generations who have histologically verified carcinoma of the colon, rectum, endometrium, small bowel, renal pelvis, or ureter and 1 of the 3 members is a first-degree relative of the other 2 and a familial adenomatous polyposis is excluded; one of the cancers was diagnosed at age <50 years
B2	Bethesda guideline 2: individual with 2 HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, small bowel or transitional cell carcinoma of the renal pelvis and ureter)
B3	Bethesda guideline 3: individual with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC related extracolonic cancer and/or a colorectal adenoma; one of the cancers was diagnosed at age <45 years and the adenoma was diagnosed at age <40 years
B4	Bethesda guideline 4: individual with colorectal or endometrial cancer diagnosed at age <45 years
B7	Bethesda guideline 7: individual with at least one adenoma diagnosed at age <40 years

All patients gave written informed consent authorizing data documentation, analysis of tumor tissue for HNPCC characteristics and mutation analysis in MMR genes. The study was approved by the Ethics Committee of the Bonn University Medical Faculty.

Immunohistochemical analysis and microsatellite instability

One patient of each family (index patient) underwent the following diagnostic procedure: tumor material of the index patient was analyzed both for expression of the MMR proteins MLH1, MSH2, MSH6 and PMS2 by immunohistochemical staining and for microsatellite instabilities (MSI). For immunohistochemical analyses mouse anti-human monoclonal antibodies were used (G219-1129 for MSH2, G168-15 for MLH1, 610918 for MSH6 and 556415 for PMS2, BD PharMingen, San Diego, California, USA). Staining procedures and analysis for microsatellite instability (MSI) were performed as previously described [9]. Tumors were classified as MSI-H (high-level MSI) if at least two markers of the reference panel (BAT25, BAT26, D5S346, D2S123, D17S250) exhibited instabilities. Tumors showing no instabilities in these markers were classified as microsatellite-stable (MSS). A second panel of five alternative markers (BAT40, D10S197, D13S153, MYCL1, D18S58) was applied if only one marker of the first reference panel was unstable. In this case, the result was interpreted as MSI-H if at least 30% of all markers showed instabilities; otherwise it was regarded as MSI-L (low-level MSI).

Mutation analysis

Based on results of immunohistochemistry, mutation analysis started with *MSH2* in cases showing combined loss of MSH2 and MSH6 proteins, and with *MLH1* in cases of combined loss of MLH1 and PMS2 proteins in their tumors. Mutation analysis in the *MSH6* or *PMS2* genes was performed only in

those few patients showing an isolated loss of MSH6 or PMS2 proteins. No mutation analysis was conducted if the tumor was MSS or MSI-L and protein expression was normal. Peripheral blood was drawn from all index patients to extract genomic DNA by standard salting out procedure. Search for small germline mutations in *MSH2* and *MLH1* was performed as previously described [6]. Mutation analysis in *MSH6* was performed by direct sequencing of the entire coding region (primers available on request). For detection of large genomic deletions in *MSH2*, *MLH1*, *MSH6* and *PMS2* multiplex ligation-dependent probe amplification (MLPA) was applied by use of SALSA MLPA Kit P003 for *MLH1/MSH2* and P008 for *MSH6/PMS2* according to the manufacturer's protocol (MRC-Holland, Amsterdam, The Netherlands).

The mutation nomenclature is according to den Dunnen and Antonarakis (2000) [10].

mRNA analysis

To determine the effect of the mutation *MLH1*, c.884+4A>G on splicing, fresh venous blood samples (2.5 ml) were collected into PAXgene Blood RNA Tubes (PreAnalytiX, QIAGEN, Hilden, Germany) containing RNA stabilizing solution. Total RNA was extracted by use of the PAXgene Blood RNA Kit (QIAGEN) according to the manufacturer's protocol. First strand cDNA was synthesized from 2 to 3 µg of total RNA by random hexamer-primed reverse transcription with the Superscript first strand system for RT-PCR (Invitrogen GmbH) according to the manufacturer's protocol. RT-PCR fragments were obtained according to standard PCR protocols by use of different primers to generate the appropriate fragments (exon 8F 5'-C AAGGAGAG ACAGTAGCTGATG TT AGG-3' and exon 11R 5'-CCACATTCTGGGGA CTGATT TC-3'). Aliquots of the RT-PCR products were separated on a 2% agarose gel and visualized with ethidium bromide on an UV imaging

system (BIORAD). DNA fragments were excised from the gel and extracted by using the QIAGEN PCR Purification Kit. Part of RT-PCR product containing both the normal and the aberrant fragments were sequenced directly. Changes of splicing efficiencies due to single base substitutions were evaluated by use of the splice prediction program of the BDGP (http://www.fruitfly.org/seq_tools/splice.html).

Results

Here we report on nine novel pathogenic germline mutations in *MLH1*, *MSH2*, *MSH6* and *PMS2* not listed in the database of the International Collaborative Group on HNPCC (<http://www.insight-group.org>) or in the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/search.html>) (Table II). Medical and family histories are shown in Table III. Data were obtained from the patients during genetic counselling and/or from medical reports. In all cases the analysis of the index patient's tumor tissue revealed high MSI and a defect in MMR expression pointing to the MMR gene involved.

Three novel mutations were detected in four patients who exhibited loss of MLH1 and PMS2 proteins in their tumors (Table II):

The variant *MLH1*, c.884+4A>G in intron 10 of the *MLH1* gene was identified in two apparently unrelated index patients. Due to its position within the not highly conserved position +4 of the splice donor sequence of exon 10 this single base substitution had to be considered as an unclassified variant (UV). This variant segregated with the disease in family 841: the affected sister and the affected father of patient 841 also carry this mutation. According to the splice site prediction program a decrease of splicing efficiency at the splice donor site from 0.93 to 0.59 was predicted for this variant. In order to evaluate the functional relevance of this variant we assessed its influence on splicing by mRNA analysis on index patient 841. RT-PCR products obtained with primers localized in exon 8 and in exon 11, respectively, were separated on an agarose gel and visualized by ethidium bromide staining. In patient 841, a fragment of 219 bp was obtained in addition to the expected fragment of 313 bp (Figure 1). Sequencing of the 219 bp fragment revealed a loss of exon 10. In order to examine whether the mutant allele was retained in the full length fragment, we made use of the fact that the patient is heterozygous at polymorphic site c.655A>G;p.Val219Ile in exon 8 of the *MLH1* gene. The RT-PCR product obtained with a reverse primer localized in exon 10 contained only one allele (A) at nucleotide position

Table II. Mutations detected in ten index patients.

Patient number	Gene	Exon (intron)	Mutation	Consequence	MSI status [†]	IHC for MSH2	IHC for MSH6	IHC for MLH1	IHC for PMS2
841	<i>MLH1</i>	10	c.884+4A>G	splice defect*	MSI-H				ND
1165	<i>MLH1</i>	10	c.884+4A>G	splice defect*	MSI-H	+	+	-	-
1304	<i>MLH1</i>	12	c.1377_1378insA;p.Glu460ArgfsX19	frameshift	MSI-H	+	+	-	-
1289	<i>MLH1</i>	13	c.1415_1416delGAp.Arg472ThrfsX5	frameshift	MSI-H	+	+	-	-
1186	<i>MSH2</i>	2	c.301G>T;p.Glu101X	nonsense	MSI-H	-	-	+	NE
1209	<i>MSH2</i>	3	c.638_639delTGG;p.Leu213GlnfsX18	frameshift	MSI-H	-	-	+	+
1218	<i>MSH2</i>	5	c.842C>A;p.Ser281X	nonsense	MSI-H	-	-	+	+
1197	<i>MSH2</i>	5	c.859G>T;p.Gly287X	nonsense	MSI-H	-	-	+	+
1097	<i>MSH6</i>	4	c.2503C>T;p.Gln835X	nonsense	MSI-H	+	+	+	+
908	<i>PMS2</i>	1-10	del exon 1-10	large deletion	MSI-H	+	+	+	-

*leads to an in frame deletion of exon 10, *MLH1*;

[†]microsatellite analysis and immunohistochemical analysis were performed in tumor tissue of a colorectal carcinoma;

MSI: microsatellite instability;

IHC: immunohistochemistry;

ND: not done;

NE: not evaluable.

+: expression.

-: loss of expression.

Table III. Tumor histories and family tumor histories of ten HNPCC patients identified with novel MMR gene mutations.

Patient/family number	Patient's tumor history malignancy (age at diagnosis)	Family's tumor history relative: malignancy (age at diagnosis)	Criterion*
841	ovarian cancer (48 years) colorectal cancer (51 years)	sister: colorectal cancer (61 years); father: colorectal cancer (51 years); cousin on father's side: colorectal cancer (age unknown)	B2
908	colorectal cancer (32 years)	sister: colorectal cancer (42 years) brother: gastric cancer (53 years) aunt on mother's side: breast cancer (53 years)	B3
1097	testicular cancer (25 years) colorectal cancer (52 years)	sister: colorectal polyps; mother: kidney cancer (59 years), colorectal cancer (61 years); endometrial cancer (62 and 63 years); grandfather on mother's side: colorectal cancer (75 years)	B2
1165	colorectal cancer (38 years)	father: colorectal cancer (49 and 50 years); uncle on father's side: gastric cancer (40 years); grandfather on father's side: kidney cancer (70 years)	B3
1186	colorectal cancer (35 years)	father: duodenal cancer (32 years); "skin tumour" [‡] (63 years); grandfather on father's side: gastric cancer (37 years)	B3 [§]
1197	colorectal cancer (38 years)	sister: colorectal cancer (41 years); sister: colorectal cancer (41 years), "skin tumour" [‡] (age unknown); mother: colorectal cancer (age unknown); aunt on mother's side: colorectal cancer (28 years); aunt on mother's side: colorectal cancer (age unknown); grandmother on mother's side: lung cancer (63 years)	A2
1209	colorectal cancer (42 years)	mother: "cancer of the lower abdomen" [†] (age unknown)	B4
1218	endometrial cancer (34 years); colorectal cancer (46; 51 and 62 years)	mother: breast cancer (88 years)	B2
1289	synchronous colorectal cancer (39 years)	cousin on father's side: colorectal cancer (38 years); grandmother on father's side: colorectal cancer (81 years)	B2
1304	cervical cancer (45 years) colorectal cancer (47 years)	father: colorectal cancer (42 years) aunt on father's side: colorectal cancer (age unknown) uncle on father's side: colorectal cancer (age unknown)	A2

*Amsterdam or Bethesda criteria (see Table I).

[†]precise data on the affected organ are not known, only rough information on localisation of the tumor (lower abdomen) is available;

[‡]precise data on the affected organ are not known, only rough information on localisation of the tumor (skin) is available;

[§]The family history of patient 1186 does not meet the Amsterdam criteria since gastric cancer is not included in these criteria. Nevertheless gastric cancer is considered to be associated with HNPCC.

655, suggesting the complete skipping of exon 10 in one allele due to the intronic mutation. The deletion of exon 10 is predicted to result in a frameshift and a premature stop after two codons. We therefore concluded this splice mutation being pathogenic and offered predictive testing for persons at risk in families 841 and 1165.

In addition the frameshift mutations c.1377_1378insA;p.Glu460ArgfsX19 and c.1415_1416delGA;p.Arg472ThrfsX5 in *MLH1* were found in patients 1304 and 1289, respectively.

We further identified four novel germline mutations in the *MSH2* gene. In these patients tumor tissues showed loss of expression of the MSH2/MSH6 protein complex. The transversions c.301G > T;p.Glu101X, c.842C > A;p.Ser281X, c.859G > T;p.Gly287X and the small deletion c.638_639delTG;p.Leu213GlnfsX18 in *MSH2* resulting in a premature stop codon were identified in index patients 1186, 1218, 1197 and 1209, respectively.

The novel nonsense mutation c.2503C > T; p.Gln835X in exon 4 of the *MSH6* gene was detected in patient 1097. His colorectal cancer revealed an isolated loss of expression of the MSH6 protein. The patient suffered from testicular cancer at age 25 that is not part of the typical tumor spectrum of Lynch syndrome. Unfortunately the tumor tissue of the testicular cancer was not available for analysis. We performed predictive testing on all five of the patient's siblings. One sister inherited the *MSH6* mutation as well and now undergoes the HNPCC surveillance program on a yearly basis.

In patient 908 a selective loss of PMS2 expression pointed to a germline mutation in this gene. A large deletion of exons 1 to 10 of the *PMS2* gene was found in this patient. The isolated loss of PMS2 was found in the tumor tissue of his affected sister as well. She is also carrier of the large deletion in the *PMS2* gene. Our index patient was at age 32 when his colorectal cancer was diagnosed and his affected sister was at age 42. One older brother died

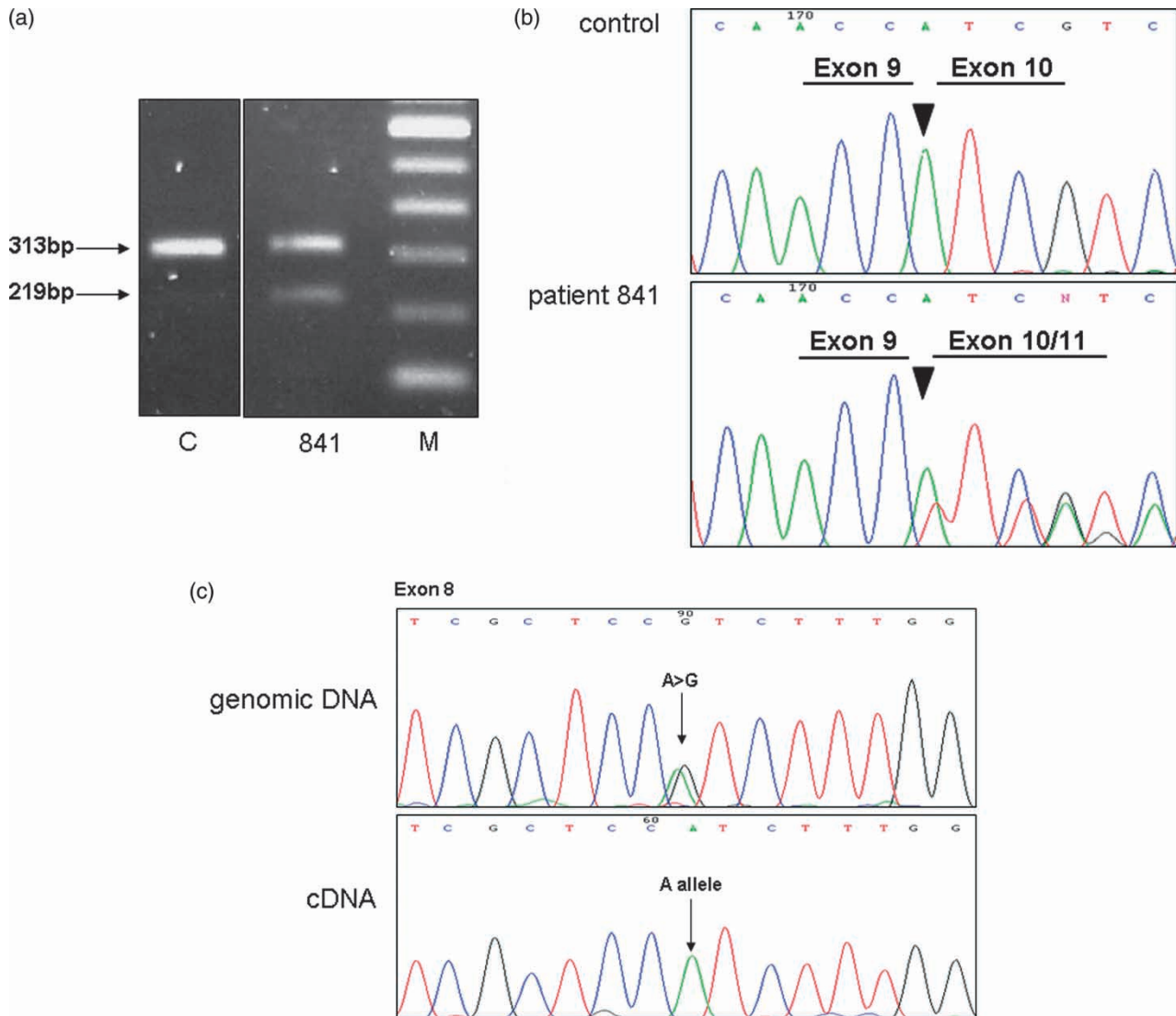


Figure 1. Characterization of variant *MLH1*, c.884+4A >G. a. Agarose gel showing RT-PCR products obtained in cDNA of index patient 841 and a control (C) by use of a forward primer in exon 8 and a reverse primer in exon 11 of the *MLH1* gene. An additional 219 bp fragment due to a deletion of exon 10 was observed in patient 841. DNA ladder with a spacing of 100bp (M). b. Sequencing pattern of the exon 9 – exon 10/11 junction of RT-PCR products of patient 841 showing skipping of exon 10. c. The full-length RT-PCR product of patient 841 contained only one allele (A) at nucleotide position 655, suggesting the complete skipping of exon 10 in one allele due to the intronic mutation.

on gastric cancer at age 53; it remains unknown whether he carried the mutation as well. Both affected siblings inherited the mutation from their mother who is 80 years old now. To our knowledge she has never undergone any surveillance program and is healthy until today.

Discussion

We considered the eight novel frameshift, nonsense or large deletion mutations described above as being pathogenic and offer predictive testing to family members at risk.

By mRNA analysis we could demonstrate that the mutation *MLH1*, c.884+4A >G at the less conserved position of the splice donor site in intron

10 leads to a complete deletion of exon 10 in the mutant allele, predicting a frameshift and thus a non-functional *MLH1* protein. Therefore we offered predictive testing to the two families with this mutation as well. All mutation carriers now undergo the HNPCC surveillance program on a yearly basis (from the age of 25 onwards but at least 5 years before the onset of the earliest tumor case in the family: annual physical examination, colonoscopy, abdominal sonography, gynaecological examination including transvaginal sonography and gastroscopy from the age of 35 regardless of occurrence of gastric cancer in the family history).

Until now only 14 truncating *PMS2* mutations have been described [3,4,11–13]. To our knowledge we present the second largest deletion in *PMS2*

ever described in patients with Lynch syndrome. Hendriks et al. (2006) [4] reported on four genomic rearrangements in *PMS2*, one of them being a deletion of exon 1–15. Their index patient had four colorectal carcinomas at age 26 and more than 14 adenomas. Unfortunately no more information on other tested family members was given.

It has recently been suggested that mutations in the *PMS2* gene do not lead to an autosomal dominant disorder [14] until Worthley et al. (2005) [11] reported on a kindred with autosomal dominant HNPCC due to a familial c.1021delA mutation in the *PMS2* gene. Nakagawa et al. (2004) [13] reported on a patient having a deletion of exon 8 of *PMS2*. The index patient described had a colorectal carcinoma at age 28 but had a healthy father at age 60 who also carries the deletion. In other cases of *PMS2* mutations individuals who were heterozygous for a pathogenic *PMS2* mutation showed non-penetrance or low penetrance as well [15,16].

In our family with the deletion of *PMS2* exon 1–10, a variable penetrance of tumor disease is also noticed.

For investigation of alterations in the MMR genes we have included patients meeting at least one criterion of the Bethesda guidelines [17]. As shown in Table III only two of the ten new families fulfilled the Amsterdam criteria. Thus, the Bethesda criteria combined with examination of tumor tissue perform very well in achieving the intended goal to help guide which families should undergo molecular evaluation for HNPCC. Tumors of all index patients exhibited MSI-H and showed loss of expression of at least one of the MMR proteins. In a previous study on 1 119 unrelated index patients who underwent the standardized screening for MSI-H and IHC a good correlation of these two markers has been described [18]. Further investigations on smaller series confirmed the nearly perfect positive predictive value and specificity as well [19,20]. The advantage of immunohistochemical analysis is that it may direct mutation analysis because the pattern of staining is specific for the underlying gene defect. The proteins MSH2 and MSH6 bind to form a heterodimer. In absence of MSH2, MSH6 is unstable. Thus, loss of both MSH2 and MSH6 points to a mutation in *MSH2*. If only MSH6 is absent, the MSH2 protein is still stable and reveals positive staining in immunohistochemistry. Tumors from MSH6 mutation carriers therefore usually show a selective lack of MSH6 staining [4,21]. Similarly, MLH1 binds with *PMS2* to form a heterodimer. Combined loss of MLH1 and *PMS2* is characteristic for patients with mutations in *MLH1*, while an isolated loss of *PMS2* protein in tumor tissue points to a germline mutation in *PMS2*. Our data support these findings as

shown in Table II. The identification of the novel germline mutations described underlines the advantage of pre-selection by immunohistochemical staining in tumor tissue for all four MMR proteins in conspicuous families.

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Databases

International Collaborative Group on HNPCC: <http://www.insight-group.org>

Human Gene Mutation Database: <http://www.hgmd.cf.ac.uk/ac/search.html>

Splice Site Prediction Program of the Berkeley Drosophila Genome Project (BDGP): http://www.fruitfly.org/seq_tools/splice.html

OMIN: #120435; <http://www.ncbi.nlm.nih.gov/entrez/dispmim.cgi?id=120435>
 #609310; <http://www.ncbi.nlm.nih.gov/entrez/dispmim.cgi?id=609310>
 #600678; <http://www.ncbi.nlm.nih.gov/entrez/dispmim.cgi?id=600678>
 #600259; <http://www.ncbi.nlm.nih.gov/entrez/dispmim.cgi?id=600259>

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