Assessment of the InSiGHT Interpretation Criteria for the Clinical Classification of 24 MLH1 and MSH2 Gene Variants

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ABSTRACT

Pathogenicity assessment of DNA variants in disease genes to explain their clinical consequences is an integral component of diagnostic molecular testing. The International Society for Gastrointestinal Hereditary Tumors (InSiGHT) has developed specific criteria for the interpretation of mismatch repair (MMR) gene variants. Here, we performed a systematic investigation of 24 *MLH1* and *MSH2* variants. The assessments were done by analyzing population frequency, segregation, tumor molecular characteristics, RNA effects, protein expression levels and *in vitro* MMR activity. Classifications were confirmed for 15 variants and changed for 3, and for the first time determined for 6 novel variants. Overall, based on our results we propose the introduction of some refinements to the InSiGHT classification rules. The proposed changes have the advantage of homogenizing the InSIGHT interpretation criteria with those set out by the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) consortium for the *BRCA1/BRCA2* genes. We also observed that the addition of only few clinical data was sufficient to obtain a more stable classification for variants considered as "likely pathogenic" or "likely non pathogenic". This shows the importance of obtaining as many as possible points of evidence for variant interpretation, especially from the clinical setting.

INTRODUCTION

Lynch syndrome (LS) (MIM# 120435) is the most common form of inherited colorectal and endometrial cancer, predisposing also to other gastrointestinal (GI) (stomach, small bowel, biliary tract, pancreas) and non-GI cancers (urinary tract, ovary and others). The syndrome is transmitted as an autosomal dominant trait, and caused by constitutional defects in the mismatch repair (MMR) genes *MLH1* (MIM# 120436), *MSH2* (MIM# 609309) *MSH6* (MIM# 600678) and *PMS2* (MIM# 600259) [Lucci-Cordisco et al., 2003; Lynch & De la Chapelle, 2003]. Detection of a constitutional loss-of-function variant in an MMR gene provides diagnostic confirmation of LS and is essential for the identification of at-risk members in LS families through predictive testing. This is especially important for LS care, since carriers of MMR gene pathogenic variants can benefit from different risk reducing options, including stringent surveillance protocols, prophylactic surgery, and chemoprevention [Vasen et al., 2013].

A major challenge in the diagnosis and management of LS is the frequent occurrence of variants of uncertain significance (VUS) in the MMR genes. Depending on the gene, about $1/5^{th}$ to $1/3^{rd}$ of DNA sequence variants identified during the course of LS clinical testing are of uncertain significance [Sijmons et al., 2013], limiting risk reduction and management options in probands and preventing their use in predictive gene testing in relatives.

The classification of DNA sequence variants identified in MMR and other cancer predisposition genes is recommended to be based on data from multiple sources, including clinical observations, tumor pathology studies and several RNA and protein based functional assays [Couch et al., 2008; Hofstra et al., 2008; Spurdle et al., 2008; Tavtigian et al., 2008a; Richards et al., 2015]. A number of *in silico*

programs have also been devised to assist with the prediction of functional consequences of inherited MMR gene alterations [Tavtigian et al., 2008b; Thompson et al., 2013b; Niroula & Vihinen, 2015].

Recently, the International Society for Gastrointestinal Hereditary Tumors (InSiGHT) has developed criteria for the interpretation of MMR gene variants, with the aim to improve the clinical utility of genetic testing for LS. A systematic clinical classification of all variants contained in InSiGHT locus specific databases (http://insight-group.org/variants/database/) was performed, based on a multifactorial bayesian quantitative approach and/or on stringent combinations of qualitative clinical and functional data [Thompson et al., 2014]. Variants were classified using a 5-tier system devised for cancer predisposing genes [Plon et al., 2008].

In the present study, we assessed the pathogenicity of 24 MMR gene variants using an extensive combination of RNA and protein-based functional assays, segregation studies, and tumor analyses. We were able to classify 6 novel variants as well as to confirm or refine the classification of 18 variants previously assessed by InSiGHT. Overall, we show the necessity of using different analyses in VUS classification and discuss their specific value and status in the interpretation process.

MATERIALS AND METHODS

Genetic variants, patients and samples

The variants assessed in this study were detected in a single laboratory in families fulfilling the

Bethesda criteria [Vasen, 2005] ascertained through cancer family clinics from 2002 to 2011. Overall, 57 MMR gene variants (25 *MLH1* and 32 *MSH2*), excluding well established polymorphisms, were detected in a total of 56 families. Variants that were clearly disease causing (ie truncating, splicing alterations, or large rearrangements), as well as established polymorphisms, were not considered. Exceptions were some alleles that, despite being currently considered polymorphic, were not clearly classified at the time of their detection; these included *MLH1* c.1558+14G>A, and *MSH2* c.380A>G and c.1511-9A>T, whose assessment was useful for the validation of the variables investigated for classification. Overall, we evaluated 24 MMR gene variants, 13 *MLH1* and 11 *MSH2*, identified in 37 unrelated families. All variants were single nucleotide substitutions at the genomic level. Based on their positions in the DNA sequence and predicted effects, they could be divided into the following groups: 12 aminoacid substitutions (8 in *MLH1* and 4 in *MSH2*), 1 *MLH1* potential splice site change affecting the first exonic position and also potentially causing an aminoacid substitution, 4 synonymous exonic nucleotide substitutions (1 *MLH1*, 3 *MSH2*), and 7 intronic changes outside the most conserved positions of the splice site consensus sequences (4 *MLH1* and 3 *MSH2*). Six variants (4 *MLH1* and 2 *MSH2*) were previously unreported and therefore not assessed by InSiGHT.

Peripheral blood leukocyte (PBL) samples were collected from 76 and 16 subjects for DNA and RNA extractions, respectively. Furthermore, lymphoblastoid cell lines (LCLs) were established from 7 variant carriers. Forty-nine paraffin-embedded tumor specimens were obtained from 42 carriers of 23 different variants.

The study was approved by the Institutional Ethical Board of the Careggi University Hospital, Florence. Informed consent was obtained from all patients for the use of specimens and clinical/pathological data for research purposes.

Molecular analyses

The complete coding sequence and flanking exon–intron borders of the *MLH1* and *MSH2* genes were investigated by direct sequencing on genomic DNA. The presence of *MLH1* and *MSH2*, as well as of *EPCAM* (MIM# 185535), genomic rearrangements and the methylation status of the *MLH1* promoter were investigated by Multiplex Ligation-dependent Probe Amplification (MLPA), as previously described [Crucianelli et al., 2014]. Values lower than 0.15 were assumed as a cut-off for normal methylation levels according to previous studies [Gylling A et al., 2007; Crucianelli et al., 2014].

The identified *MLH1* and *MSH2* variants have been submitted to the InSiGHT MMR gene variant database (http://www.insight-group.org/variants/database/). Variants were defined according to the Human Genome Variation Society (HGVS) recommendations [den Dunnen et al., 2016]. DNA variant numbering is based on the *MLH1* and *MSH2* cDNA sequences (GenBank accession numbers NM_000249.2 and NM_000251.1, respectively) with the A of the ATG translation–initiation codon numbered as +1. Aminoacid numbering starts with the translation initiator methionine as +1.

To investigate the presence of the p.Val600Glu (V600E) hotspot mutation, *BRAF* (MIM# 164757) exon 15 was directly sequenced in 7 tumor samples of *MLH1* variant carriers, using previously reported primers and conditions [Mancini et al., 2010].

Loss of heterozygosity (LOH) analysis of the regions containing the identified variants was performed on matched leukocyte and tumor tissues from 19 probands by analysis of direct sequencing electropherograms [Janssen et al., 2011; Janssen et al., 2012].

Microsatellite instability and immunohistochemical analyses

A total of 47 tumor samples and matched normal mucosa or PBLs from 40 patients were evaluated for microsatellite instability (MSI) using a 5-mononucleotide marker panel [Suraweera et al., 2002; Buhard et al., 2006; Giunti et al., 2009]. Tumors were classified into three categories according to the proportions of markers showing instability: MSI-H (high-level MSI), MSI-L (low-level MSI) and MSS (microsatellite stable), which have ≥ 30 – 40 %, > 0 % – < 30 – 40 %, and 0 % unstable markers, respectively [Boland et al., 1998]. Immunohistochemical (IHC) analysis of MMR protein expression was performed on paraffin-embedded tumor tissue sections from 42 samples, as previously described [Roncari et al., 2007].

Allelic frequencies in control chromosomes

To assess frequencies of the 24 *MLH1* and *MSH2* variants in control chromosomes, one hundred and sixty DNA samples from anonymized healthy Italian blood donors with no history of colorectal cancer among 1st degree relatives and from the same region of origin (Tuscany) of most of the patients were analyzed by direct sequencing. In addition, the Exome Aggregation Consortium database (ExAC Browser (Beta), http://exac.broadinstitute.org/, 04/2016 accessed) was interrogated, excluding the Cancer Genome Atlas (TGCA) data.

Co-segregation with phenotype and multifactorial likelihood analysis

Co-segregation analysis was performed for 11 variants in 16 families; in these, 24 affected carrier relatives, in addition to probands, were identified. The variants were detected by direct sequencing. Multifactorial likelihood analysis was performed for 14 variants for which sufficient data were

available, as described previously [Thompson et al. 2013a; Thompson et al. 2013b]. Briefly, a probability of pathogenicity based on variant location or *in silico* scoring of missense substitutions is combined with likelihood ratios (LR) for segregation and tumour characteristics (MSI/BRAF status) to derive a posterior probability of pathogenicity.

mRNA splicing analysis

Total RNA was extracted from the 7 cycloheximide-treated and untreated LCLs established from *MLH1* or *MSH2* variant carriers, using RNeasy® Plus Mini Kit (Qiagen, Hilden, D)**.** Cycloheximide (Sigma-Aldrich, Saint Louis, MO, USA) was added at 10µg/ml to the medium 4 hr before harvesting the cells to prevent degradation of unstable transcripts by nonsense-mediated decay (NMD). cDNA was synthesized using TaqMan Reverse Trascription Kit (Applied Biosystems, Foster City, CA, USA). Primers and conditions used for cDNA amplification are available upon request. PCR products were analyzed on agarose gels, and individual bands, corresponding either to the full length or to the aberrantly spliced transcripts were excised and eluted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) before amplification and direct sequencing. All RT-PCR experiments were performed in duplicate. Since alternative splicing is commonly observed for *MLH1* and *MSH2* [Genuardi et al. 1998], to improve the detection and interpretation of splicing aberrations, eight control samples were also analyzed [Thompson et al., 2015].

Allele-specific expression (ASE) analysis

Allele-specific expression (ASE) was investigated by Single Nucleotide Primer Extension (SNuPE) in 10 patients heterozygotes for the coding SNPs rs1799977 (*MLH1* c.655G>A) or rs4987188 (*MSH2* c.965G>A, as previously described [Crucianelli et al., 2014]. Total RNA was extracted from blood samples collected into PAXgene Blood RNA tubes (PreAnalytiX, Qiagen, Hilden, Germany), using

the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples from heterozygotes for the same SNPs who had no additional *MLH1* and *MSH2* sequence change were used as controls [Perera et al., 2010; Pastrello et al., 2011]. ASE was calculated after measuring peak heights in heterozygous samples [Castellsagué et al., 2010]. Values included in the 0.8-1.2 range were assumed as a cut-off for normal ASE according to previous studies [Renkonen et al., 2003; Castellsagué et al., 2010; Perera et al., 2010]. All experiments were carried out in triplicate and two independent replicates of all experiments were performed. Control heterozygotes for the *MLH1* or *MSH2* exonic polymorphisms (rs1799977 and rs4987188, respectively) were included in each experiment.

Minigene splicing assay

Splicing assays were performed by comparing the splicing pattern of WT and mutant minigenes transiently expressed in HeLa cells [Soukarieh et al., 2016]. Two different vectors were used in the minigene splicing assay: pCAS2 or pSPL3m [Soukarieh et al., 2016], as specified. Except for 2 contructs (*MSH2* c.2006-6T>C and c.2081T>C), minigenes were prepared by first PCR-amplifying wild-type (WT) and mutant genomic segments from patients' DNA using forward and reverse primers mapping approximately at 150 nucleotides upstream and downstream the exon of interest, respectively. Primer sequences are available upon request. The PCR products were then inserted into the intron of pCAS2 to generate splicing reporter minigenes as previously described [Tournier et al., 2008]. Minigenes carrying the single variants *MSH2* c.2006-6T>C and *MSH2* c.2081T>C (present in *cis* in patient genomic DNA) were prepared by site-directed mutagenesis by using a two-stage overlap extension PCR method [Ho et al., 1989]. The psPL3m construct carrying *MLH1* c.301G>A was generated by transferring the insert from the previously described minigene pCAS1.MLH1.exon3.c.301G>A [Tournier et al., 2008].

227

228 *Protein stability and vitro MMR activity analyses*

229 Altogether 9 *MLH1* and *MSH2* missense variations were introduced into the *MLH1* and *MSH2*
230 cDNAs cloned into a pFastBac1 vector (Invitrogen, Carlsbad, CA, USA), using a PCR-based site-
231 directed mutagenesis kit according to manufacturer´s instructions (QuikChange Lightning®Site-
232 directed mutagenesis Kit, Stratagene, La Jolla, CA, USA). The mutated constructs were sequenced
233 (ABIPrism 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) prior to protein
234 production. Primer sequences and PCR parameters are available upon request.

235

236 Recombinant baculoviruses were generated by Bac-to-Bac system (Invitrogen, Carlsbad, CA, USA)
237 and proteins were produced in *Spodoptera frugiperda* (*Sf*9) insect cells as described earlier [Nyström-
238 Lahti et al., 2002; Kariola et al, 2002; Ollila et al., 2006]. For protein production *Sf9* cells were co-
239 infected with MLH1 and PMS2 viruses to yield MutLα heterodimers, or MSH2 and MSH6 viruses to
240 yield MutSα heterodimers. The total protein extracts (TE) were prepared as previously described
241 [Nyström-Lahti et al., 2002; Kariola et al., 2002].

242

243 The expression levels of produced protein variants were studied by Western blot analysis. The
244 proteins were blotted onto nitrocellulose membranes (Hybond, PVDF, Amersham Pharmacia biotech,
245 Uppsala, Sweden) and visualized with anti-MLH1 (BD Biosciences/Pharmingen, San Diego, CA,
246 USA, clone 168-15) (0.5 µg/ml), anti-PMS2 (Calbiochem/Oncogene Research, San Diego, CA, USA,
247 Ab-1) (0.2 µg/ml), anti-MSH2 (Calbiochem, San Diego, CA, USA, MSH2- Ab1, NA-26) (0.2 µg/ml)
248 and anti-MSH6 (BD Transduction Laboratories, Lexington, KY, USA, clone 44) (0.02 µg/ml)
249 monoclonal antibodies. To estimate the MMR protein level in the extracts, α-tubulin was used as a
250 loading control (anti-α-tubulin; Sigma, Louis, MO, USA, DM1A) (0.2 µg/ml).

The repair efficiencies (R%) of the recombinant protein variants were analyzed by complementing the MMR-deficient nuclear extracts (NE) of HCT116 or LoVo cells (American Type Culture Collection, Manassas, VA, USA) with the *Sf9* TEs containing overexpressed MLH1 or MSH2 proteins [Nyström-Lahti et al., 2002; Kantelinen et al., 2012] according to the protocol previously described [Kantelinen et al., 2012]. Seventy-five μg of NE was incubated with TE including in each sample comparable amounts of MutLα or MutSα, respectively, and with an excess amount (100 ng) of the heteroduplex DNA substrate. R% was calculated as an average of three independent experiments using GeneTools 3.08 (SynGene, Cambridge, England). The relative R% was calculated in respect to the WT control [Drost et al., 2010; Kantelinen et al., 2012].

Clinical classification of variants

The 5 class system for clinical classification recommended by the International Agency for Research on Cancer (IARC) working group on the interpretation of DNA sequence variants in cancer predisposition genes was used [Plon et al., 2008]. Class 5 and 4 include definitely pathogenic and likely pathogenic variants, respectively; when observed in a proband, they provide confirmation of LS diagnosis, so that relatives can be consequently offered predictive testing. Variants assigned to class 1 and 2 correspond to definitely and likely neutral/not pathogenic (or of low clinical impact) sequence changes, respectively; their detection is not followed by further clinical testing in the family. Finally, class 3 includes all those variants whose clinical and/or functional effects cannot be determined, due to either insufficient (e.g. detection in a single family) or contradictory evidence (ie discordant results

from similar *in vitro* assays performed in different laboratories); these are also called variants of uncertain (or unknown) significance (VUS).

RESULTS

Population frequency

We first verified variant allele frequencies in control populations (Table 1). Previous classifications performed by InSiGHT relied on frequencies reported in the 1000 Genomes Project database (http://www.1000genomes.org/), in addition to data published or reported by single centers on local populations. In this study we used values from the Exome Aggregation Consortium (ExAC), which is a more comprehensive dataset and which also incorporates the 1000 Genomes data. We also tested 160 Italian control subjects for 20 variants.

Four variants (*MLH1* c.1558+14G>A; *MSH2* c.380A>G, c.1511-9A>T and c.2006-6T>C) that reached minor allele frequencies $\geq$ 0.01 had previously been classified as Class 1-not pathogenic based on population frequency data alone [Thompson et al., 2014], and were subjected to further analyses to verify consistency across different points of evidence. The frequency of *MSH2* c.380A>G in phase 1 of the 1000 Genomes Project, which had been originally used for classification, was 0.02, while the currently reported frequency in ExAC is slightly lower, 0.00692. Of note, the frequencies of *MLH1* c.1039-8T>A were 0.00155 in ExAC and 0.02187 in 160 Italian controls, respectively. This difference is likely accounted for by a low quality of calling in the ExAC population, as suggested by the following observations: (i) it is called in less than 80% of individuals in ExAC; (ii) the variant was

297 found to be in linkage disequilibrium with *MLH1* c.1558+14G>A in the Italian population; and (iii)
298 *MLH1* c.1558+14G>A had similar polymorphic frequencies in ExAC and in Italian controls (0.03948
299 versus 0.02187).
300
301 *Tumor pathology data*
302 Analyses performed on tumors included microsatellite instability (MSI), MMR
303 immunohistochemistry (IHC), *BRAF* p.Val600Glu somatic mutation, *MLH1* promoter methylation,
304 and loss of heterozygosity (LOH) (Table 1).
305
306 MSI and/or IHC data were available for 23/24 and 22/24 variants, respectively (Table 1). These
307 included 12/13 predicted missense changes, for 6 of which $\geq$ 2 tumors were investigated. Results
308 indicative of *in vivo* MMR inactivation (MSI-H status and/or lack of expression of the protein
309 encoded by the gene carrying the constitutional variant) were observed for 7 predicted missense
310 substitution variants: *MLH1* c.301G>A p.(Gly101Ser), c.779T>G p.(Leu260Arg), c.1421G>C
311 p.(Arg474Pro), c.1814A>C p.(Glu605Ala), and *MSH2* c.2081T>C p.(Phe694Ser) and c.2087C>T
312 p.(Pro696Leu), as well as for the potential splice variant *MSH2* c.2006G>T. Results indicative of *in*
313 *vivo and in vitro* MMR proficiency (MSS status, normal MMR protein expression and proficient
314 functional assays) were obtained on 2 missense variants, *MLH1* c.1043T>C p.(Leu348Ser) and *MSH2*
315 c.244A>G p.(Lys82Glu). Discordant MSI and IHC results were observed for the missense variants
316 *MLH1* c.2041G>A p.(Ala681Thr) and c.2059C>T p.(Arg687Trp) in tumors from different
317 individuals; for both variants 1 MSI-H tumor showed normal IHC staining, while 1 MSS sample was
318 associated with lack of MLH1 expression.
319

BRAF and/or *MLH1* promoter analyses were performed on tumor samples for 9 variants (Table 1). The *BRAF* p.Val600Glu mutation was detected in two MLH1-negative tumors from carriers of the *MLH1* variants c.1421G>C p.(Arg474Pro) and c.1743G>A p.(Pro581=), respectively. *MLH1* epigenetic defects could be tested only for the tumor from the carrier of the c.1743G>A variant, which however did not show *MLH1* promoter hypermethylation. Four additional samples had both *BRAF* p.Val600Glu and *MLH1* promoter methylation tested: both analyses were negative in 3 MSI-H samples from carriers of *MLH1* c.779T>G p.(Leu260Arg) carriers and in 1 MSI-H sample from a c.2041G>A p.(Ala681Thr) carrier.

LOH analysis was performed for 14 variants, 8 *MLH1* and 6 *MSH2* (Table 1). Loss of the variant allele was detected in tumors from carriers of 3 different *MLH1* variants: c.1217G>A p.(Ser406Asn), c.1421G>C p.(Arg474Pro) and c.1732-19T>C.

RNA analyses

The *MSH2* variant c.2006G>T, which is currently assigned to Class 5 based on evidence of a major splicing defect, was associated with complete exclusion of exon 13 in the minigene assay (Fig. 1), but with only partial skipping in the LCL from a carrier (Fig. 2A and 2B). Both alleles at position 2006 were detected in the full-length cDNA product from the LCL (Fig. 2C and 2D). Partial exon 13 loss was also detected in blood samples drawn in PAXgene tubes from the patient above and from 2 additional individuals carrying *MSH2* c.2006G>T investigated in another laboratory in France, as well as in a LCL established from one of these French carriers (data not shown).

Three other variants (*MLH1* c.301G>A and c.1039-8T>A; *MSH2* c.2006-6T>C) were associated with partial exon skipping (Table 2; Fig. 1; Supp. Fig. S1-S2) in patient samples, in the *ex vivo* minigene assay, or in both. These involved in all cases known alternatively spliced transcripts [Genuardi et al., 1998; Thompson et al., 2015]. Interestingly, for *MSH2* c.2006-6T>C partial exon 13 exclusion was only detected by the minigene assay (Fig. 1) but not in the patient sample (Tournier et al., 2008), further suggesting that the minigene assay may overestimate the splicing defect for this exon. On the other hand, in this study partial skipping of exon 12 was observed in lymphoblastoid cells (data not shown) but not with the minigene assay for *MLH1* c.1039-8T>A (Supp Fig. S2). The latter result is in accordance with previous findings obtained for this variant by minigene assay [Petersen et al., 2013].

In addition, the minigene assay showed that Class 5-pathogenic *MLH1* c.301G>A p.(Gly101Ser) is associated with loss of the use of an alternative splice site (Supp Fig. S3); while the clinical significance of this finding cannot be established based on the minigene result only, the variant allele should produce only the canonical transcript.

None of the 10 variants tested by the SNuPE assay showed evidence of allelic expression imbalance, consistent with the splicing assay results (Table 2).

Mismatch repair activity and protein expression analyses

An *in vitro* MMR complementation assay based on the synthesis of MMR protein in *Spodoptera frugiperda* (*Sf*9) insect cells was performed in MMR-defective human cell lines for 9 coding variants (Table 2). Three of the five MMR defective protein variants (MLH1 p.Leu260Arg; MSH2 p.Phe694Ser and p.Pro696Leu were found to be unstable *in vitro* (Fig. 3). Loss of MMR activity

(relative activity < 1%) was demonstrated for 5 variants: MLH1 p.Gly101Ser and p.Leu260Arg, and MSH2 p.Gly669Val, p.Phe694Ser and p.Pro696Leu. The remaining four variants, MLH1 p.Leu348Ser, p.Arg474Pro, p.Glu605Ala, and MSH2 p.Lys82Glu, were all stable in the transient expression assay and MMR proficient.

Four other missense variants, MLH1 p.Ser406Asn, p.Ala681Thr, p.Arg687Trp, and MSH2 p.Asn127Ser, had been previously shown to be proficient in the MMR activity assay, although two of them, MLH1 p.Ala681Thr and p.Arg687Trp, showed discordant protein instability results across different studies (Table 2).

Clinical data, multifactorial analysis and variant classification

Family history types, co-occurrence of other MMR gene variants, and the components and results multifactorial analysis are shown in Supp. Tables S1-S2.

Multifactorial analysis was performed for 14 variants. Using quantitative analysis (based on multifactorial posterior probability) and/or assessment of qualitative criteria, variants were classified according to the 5-tier system proposed by InSiGHT (Table 3) [Thompson et al., 2014]. Four of the six novel variants (*MLH1*, c.1732-19T>C and c.1743G>A; *MSH2* c. 244A>G and c.2442T>G) were assigned to Class 2-likely not pathogenic. Of note, one novel variant, *MLH1* c.1814A>C p.(Glu605Ala), is in class 4-likely pathogenic according to multifactorial analysis based only on 2 available values, a 0.7 prior probability calculated *in silico*, and a single MSI-H CRC not expressing the MLH1 protein. The remaining novel variant, *MLH1* c.1043T>C p.(Leu348Ser) is in Class 3-VUS due to insufficient evidence.

The previous InSiGHT assignment of *MSH2* c.2006G>T to Class 5-pathogenic based on RNA splicing data was confirmed by the results of multifactorial analysis in this study (Table 3).

The classification made by InSiGHT was changed for 3 variants after the addition of novel segregation and molecular tumor data. *MLH1* c.301G>A p.(Gly101Ser), originally in Class 4-likely pathogenic, was upgraded to Class 5-pathogenic, while variants *MSH2* c.1387-8G>T and c.1737A>G p.(Lys579=) were moved from Class 2-likely not pathogenic to Class 1-not pathogenic.

Previous classifications of the other 15 variants were supported by the data obtained. Results of novel RNA analyses performed in this study were in agreement with the assignment to Class 1-not pathogenic of intronic *MLH1* variants c.1039-8T>A and c.1558+14G>A. Insufficient evidence to attain a clinically actionable category was available for *MLH1* c.1421G>C p.(Arg474Pro), even though novel data from tumor studies brought down the posterior probability of pathogenicity from 0.51 to 0.094.

DISCUSSION

The ultimate purpose of genetic testing for LS and other cancer predisposition syndromes is to reduce cancer morbidity and mortality through the implementation of specific preventive options for carriers of disease causing variants. Interpreting the significance of DNA variants identified in the diagnostic

laboratory is an integral component of clinical DNA testing. The interpretation process is complex, as several independent datasets must be taken into account. Recently, recommendations for clinical classification of MMR gene variants have been formulated [Thompson et al., 2014]. We have performed a thorough investigation of 24 MMR gene sequence variants identified in a single center in order to assess their clinical relevance, using points of evidence that are included in the InSiGHT recommendations, as well as additional potential classification components. Our findings confirm the overall validity of the InSiGHT criteria and suggest that the interpretation process could be improved by introducing some adjustments.

Overall, our results provide support to or improve previous classifications for the 18 variants that had already been assessed by InSiGHT (http://insight-group.org/variants/classifications). For 3 of these variants (*MLH1* c.301G>A; *MSH2* c.1387-8G>T and c.1737A>G), a more stable classification, either from Class 4-likely pathogenic to Class 5-pathogenic or from Class 2-likely not pathogenic to Class 1-not pathogenic, was achieved using novel clinical and molecular data. These changes do not affect cancer prevention strategies, since the same clinical recommendations apply to Class 5 and 4 and Class 2 and 1, respectively [Plon et al., 2008]. However, assignments to Class 5 and 1 can be considered definitive, since the likelihood that a variant in either of these categories will be moved to a class associated with different clinical advice is very low [Plon et al., 2008]. The IARC/InSiGHT interpretation criteria advise to consider research testing of further samples/relatives to try and obtain definitive classifications for Class 4-likely pathogenic and Class 2-likely not pathogenic variants [Plon et al. 2008; Thompson et al. 2014], and our results demonstrate the practical importance of this recommendation. For all 3 variants the classification was based on multifactorial analysis, and in all cases the class switch was made possible by the incorporation of few novel data on tumor characteristics and/or segregation, highlighting the relevance of collecting these types of information.

The novel variants *MLH1* c.1814A>C p.(Glu605Ala) and *MSH2* c.244A>G p.(Lys82Glu) were in Class 4-likely pathogenic and Class 2-likely not pathogenic, respectively, following multifactorial analysis. For both variants only one clinical observation, that is, molecular information obtained on a single tumor sample (Table 1 and Supp. Table S2), is available. The Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) in the *BRCA1/BRCA2* (MIM# 113705 and MIM# 600185) genes recommends that variants attaining thresholds for assignment to clinically actionable classes by multifactorial analysis with limited contribution from clinical or laboratory evidence be considered of uncertain significance until further evidence is accrued (http://enigma.consortium.org/documents/ENIGMA_Rules_2015_03_26.pdf). We propose to adopt this recommendation also for the MMR genes, especially when there is apparent discordance between functional and clinical evidence, such as for *MLH1* p.(Glu605Ala). In particular, for *MLH1* variants additional evidence from *BRAF* and/or promoter methylation tumor testing could be used to reinforce the evidence in favor of pathogenicity.

The partially discordant RNA splicing results between the minigene assay and analyses of patient derived samples obtained in this study for *MSH2* c.2006G>T suggest that the splicing alteration may not be the only or the major inactivation mechanism for *MSH2* c.2006T. Indeed, the functional *in vitro* assay showed reduced repair activity of the protein encoded by the variant allele, p.669Val and the variant could be assigned to Class 5 also based on multifactorial analysis. However, complete absence of c.2006T allele in full-length transcript.in patient RNA, together with total exon 13 exclusion in a minigene assay, was observed in another study [van der Klift et al., 2015]. Therefore, further studies will be needed to clarify the mechanisms underlying pathogenicity of *MSH2* c.2006G>T. At the same time, the interpretation criteria for RNA analyses should be reconsidered based on these apparently inconsistent results.

Minor effects on splicing were observed either on patient RNA or by the minigene assay in this study for the Class 1-not pathogenic variants *MLH1* c.1039-8T>A and *MSH2* c.380A>G and c.2006-6T>C. All are in Class 1 based on population frequency only, confirming that they have no major clinical effects [Genuardi et al., 1998; Thompson et al., 2015].

None of the other variants were found to be associated with significant splicing anomalies. Lack of abnormal splicing products was important to assign *MLH1* c.307-19A>G, c.1732-19T>C and c.1743G>A, and *MSH2* c. 2442T>G p.(Leu814=) to Class 2-likely not pathogenic. Two of them, *MLH1* c.307-19A>G and c.1743G>A also had population frequencies ~1/5,000 and ~1/10,000, respectively. According to the InSiGHT criteria, synonymous or deep intronic variants for which splicing assays do not show alterations should be considered as Class 2-likely not pathogenic. One of the combinations required for assignment of a variant to Class 1-not pathogenic includes all of the following points of evidence: allelic frequency 0.01%-1%, lack of co-segregation with disease, estimated risk <1.5 determined by case-control studies, and presence of molecular features not compatible with involvement of the gene carrying the variant in ≥ 3 tumors; this criterion applies to all types of variants, regardless of their nature and prior probability of altering gene function. Since intronic and synonymous variants have a low *a priori* likelihood of affecting gene processing, combinations of any of the above evidences (ie, lack of segregation, population frequency, low estimated risk, and molecular characteristics) and normal splicing patterns could reasonably be considered sufficient for assignment to Class 1. Interestingly, the association of intronic location or synonymous coding nucleotide substitution and absence of mRNA aberrations demonstrated by *in vitro* assays has been proposed by the ENIGMA consortium as a criterion for assignment of variants in the *BRCA1/BRCA2* genes to Class 1-not pathogenic (http://enigmaconsortium.org/documents/ENIGMA_Rules_2015-03-26.pdf). Data from our study

indicate that it would be justified to consider homogenization of the Class 1 criteria for intronic/synonymous substitutions between the ENIGMA and InSiGHT consortia.

Nine missense variants were investigated by *in vitro* MMR assay based on the production of MSH2 or MLH1 proteins in *Sf9* insect cells and subsequent complementation of human MMR deficient cell lines. The same assay had been previously used for three other missense substitutions found in our series [Raevaara et al., 2005; Ollila et al., 2008; Christensen et al., 2009; Kansikas et al., 2011], while one variant - *MLH1* c.1217G>A p.(Ser406Asn) - had been tested with two different mammalian repair assays. All five MMR deficient variants (*MLH1* p.(Gly101Ser) and p.(Leu260Arg); *MSH2* p.(Gly669Val), p.(Phe694Ser) and p.(Pro696Leu)) are in Class 5-pathogenic, supporting the classifications based on multifactorial analysis. Of note, the aminoacids replaced in *MSH2* p.(Phe694Ser) and p.(Pro696Leu) are located nearby in the ATPase domain, indicating that this region is particularly sensitive to structural changes; this suggestion is reinforced by the observation that none of the 29 reported *MSH2* exon 13 missense changes have been so far assigned to Class 1-not pathogenic or Class 2-likely not pathogenic by InSiGHT (http://chromium.lovd.nl/LOVD2/colon_cancer/variants.php?select_db=MSH2&action=search_all&search_Variant%2FExon=13&search_MutCol=%3E&search_Variant%2FDNA=&search_Variant%2FRNA=&search_Variant%2FProtein=&search_Patient%2FPhenotype%2FDisease=&search_Patient%2FReference=).

MLH1 c.2041G>A p.(Ala681Thr) and c.2059C>T p.(Arg687Trp) are assigned to Class 5-pathogenic despite the results of the functional assays, which show inconclusive data on protein expression and normal MMR activity, with discordant observations across different studies. However, both are associated with an abundance of clinical data allowing them to overcome the Class 5-pathogenic

posterior probability threshold using multifactorial analysis. At the same time discordant tumor pathology findings, including samples that were MSS and/or expressed MLH1, have also been reported for both variants. It will be interesting to verify the degree of phenotypic expression associated with these two variants. By analogy with equivocal functional results obtained on the BRCA1 variant p.Arg1699Gln [Spurdle et al., 2012] they might be considered as candidate intermediate risk variants. Notably, other *MLH1* missense substitutions located in proximity of these variants are associated with proficient repair but reduced or inconclusive protein expression data; these include for instance the Class 5-pathogenic c.1942C>T p.(Pro648Ser) and c.1943C>T p.(Pro648Leu), and the Class 3-VUS c.1918C>T p.(Pro640Ser), c.1919C>T p.(Pro640Leu), c.1976G>A p.(Arg659Gln), c.2027T>G p.(Leu676Arg), and c.2027T>C p.(Leu676Pro). Therefore variants located in this region of the MLH1 protein may cause functional impairment through reduced expression/stability and/or other as yet to be determined mechanisms not directly affecting repair activity.

While LOH is an important silencing mechanism of the wild type allele [Alemayehu et al., 2007], so far it has not been considered as a point of evidence for MMR gene variant classification by InSiGHT. This is due to several reasons, including multiple observations of loss of variant pathogenic alleles in cancers from MMR gene carriers and technical difficulties, ie, due to the potential presence of MSI hampering analysis of LOH using microsatellite markers [Hofstra et al., 2008]. The findings from this study, especially the observation of loss of the variant allele in samples from carriers of Class 1-not pathogenic and Class 2–likely not pathogenic variants confirm that LOH should be considered with caution for the interpretation of variant pathogenicity in the MMR genes. Studies on large series are needed to assess the usefulness of this marker and its predictive value.

The evaluation of multiple clinical parameters and functional assays undertaken in this study allows refining the strategy for the clinical classification of MMR gene variants. Intronic and synonymous variants that cannot be tested in the *in vitro* MMR assay should be assessed for effects on RNA processing, by detection of aberrant transcripts (in the presence of NMD inhibitors) and allele-specific expression (in the absence of NMD inhibitors). We suggest that, when no major alteration is observed, the variant could be assigned to Class 1-not pathogenic, even without further evidence (from ie, segregation and tumor characteristics), as stated by ENIGMA for *BRCA1* and *BRCA2*. The underlying rationale is that the probability that an intronic variant with no documented splicing aberration will cause high tumor risk is very low, < 1/1,000.

For potential missense variants, concordant evidence in favour or against pathogenicity should be derived both from functional assays - RNA first, and if normal, protein - and clinical data. Given the importance of obtaining segregation and molecular tumor results for the purpose of variant classification, any attempt should be made to test additional patients and samples, especially from carriers of missense variants which are usually more difficult to classify compared to silent and intronic changes.

Finally, classifications obtained by multifactorial analysis should be supported by multiple data points; this could be achieved by requiring a minimum threshold or different points of evidence from clinical and tumor data to allow assignment to a clinically actionable class.

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Supplementary Information Figure S1. Identification of *MSH2* splicing effects by using a splicing minigene reporter assay. (A) Structure of the pCAS2-MSH2 minigenes. Boxes represent

exons and lines in between indicate introns. *MSH2* segments are shown in dark colour. Splicing events detected in the minigene assay are indicated by the dotted lines and further described on the right. (B) Analysis of the splicing pattern of wild-type and mutant pCAS2-MSH2 minigene transcripts. Wild-type and mutant minigenes, as indicated, were transfected into HeLa cells and the minigene transcripts were analyzed by RT-PCR. The image shows a 2% agarose gel stained with ethidium bromide, visualized by exposure to ultraviolet light under conditions of non-saturating exposure. The identities of the RT-PCR products are shown both on the left and on the right of the gel. Results are representative of 3 independent experiments. M, size marker (100 bp DNA ladder, New England Biolabs); pCAS2, empty vector; WT, wild-type.

Supplementary Information Figure S2. Analysis of the impact on splicing of *MLH1* variants by using a minigene reporter assay. (A) Structure of the pCAS2-MLH1 minigenes. Boxes represent exons and horizontal lines in between indicate introns. *MLH1* segments are shown in dark colour. Splicing events detected in the minigene assay are indicated by the dotted lines and further described on the right. (B) Analysis of the splicing pattern of wild-type and mutant pCAS2-MLH1 minigene transcripts. Wild-type and mutant minigenes, as indicated, were transfected into HeLa cells and the minigene transcripts were analyzed by RT-PCR. The image shows a 2% agarose gel stained with ethidium bromide, visualized by exposure to ultraviolet light under conditions of non-saturating exposure. Results are representative of 3 independent experiments. M, size marker (100 bp DNA ladder, New England Biolabs); pCAS2, empty vector; WT, wild-type.

Supplementary Information Fig. S3. *MLH1* c.301G>A alters the alternative splicing pattern of *MLH1* exon 3 in the minigene splicing assay. (A) Structure of the pSPL3m-MLH1ex3 minigene. Boxes represent exons and horizontal lines in between indicate introns. The *MLH1* segment is shown in dark colour. Splicing events detected in the minigene assay are indicated by the dotted lines. (B) Analysis of the splicing pattern of wild-type and mutant pSPL3m-MLH1ex3 minigene transcripts. Wild-type and mutant minigenes, as indicated, were transfected into HeLa cells and the minigene transcripts were analyzed by RT-PCR as described under Materials and Methods. The image shows a 2% agarose gel stained with ethidium bromide, visualized by exposure to ultraviolet light under conditions of non-saturating exposure. The identities of the RT-PCR products are shown on the left and below the gel. (C) Usage of the reference 5'ss and a5'ss of *MLH1* exon 3 (NM_000249.3 and NM_001167617.1, respectively) in the WT and mutant contexts. The upper panel shows *in silico* predictions for the effect of c.301G>A on the strength of the 5' splice site of *MLH1*exon 3 (predictions obtained with 5 different algorithms, as described in Soukarieh et al., 2016). The bottom panel shows the sequence of the RT-PCR products indicated by the star (heteroduplexes) and purified from the gel shown in B. 5'ss, 5'splice site; a5'ss, alternative 5' splice site; Δ5 nts, deletion of the last 5 nucleotides of *MLH1* exon 3.

Supplementary Information Table S1. Clinical data and co-occurrence of multiple variants in families with MMR gene variants.

Supplementary Information Table S2. Segregation and multifactorial likelihood analysis for the investigated MMR gene variants.

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FIGURE LEGENDS

Figure 1. Identification of *MSH2* exon 13 splicing alterations by using a splicing minigene reporter assay. (A) Structure of the pCAS2-MSH2ex13 minigene. Boxes represent exons and horizontal lines in between indicate introns. The *MSH2* segment is shown in dark colour. Splicing events detected in the minigene assay are indicated by the dotted lines and further described on the right. (B) Analysis of the splicing pattern of wild-type and mutant pCAS2-MSH2ex13 minigene transcripts. Wild-type and mutant minigenes, as indicated, were transfected into HeLa cells and the minigene transcripts were analyzed by RT-PCR as described under Materials and Methods. The image shows a 2% agarose gel stained with ethidium bromide, visualized by exposure to ultraviolet light under conditions of non-saturating exposure. The identities of the RT-PCR products are shown on the right. Results are representative of 3 independent experiments. Marker, 100 bp DNA ladder (New England Biolabs); pCAS2, empty vector; WT, wild-type; *, heteroduplexes.

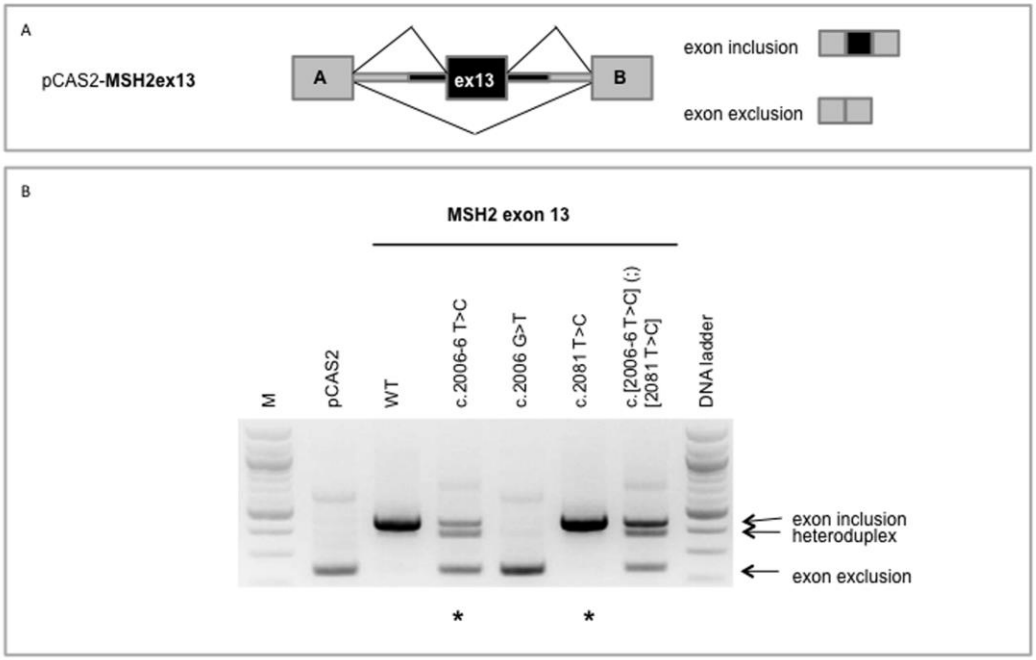


Figure 2. Splicing analysis by RT-PCR on cDNA from a LCL established from a carrier of *MSH2* variant c.2006G>T. (A) Gel electrophoresis of cDNA PCR products obtained using primers located in *MSH2* exons 12 and 14. MW: molecular weight marker (100 bp ladder). The upper band corresponds to the full length mRNA product, the fainter lower band to the isoform lacking exon 13. The arrow next to the asterisk shows the heteroduplex formed by the two PCR products. (B) Schematic representation of *MSH2* mRNA encompassing exons 12-14 and of the two splicing products detected in the LCL sample. (C) Electropherogram of the sequence of the Δ13 cDNA product. (D) Electropherogram of the sequence of the full-length cDNA product showing presnece of both alleles at c.2006G>T (the reverse strand is shown).

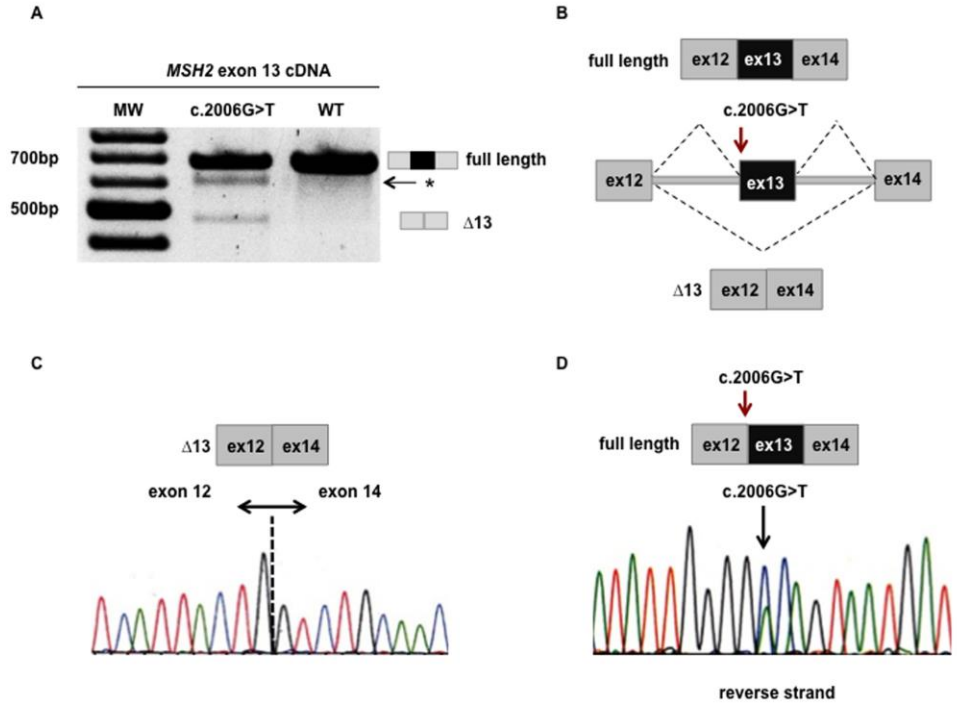
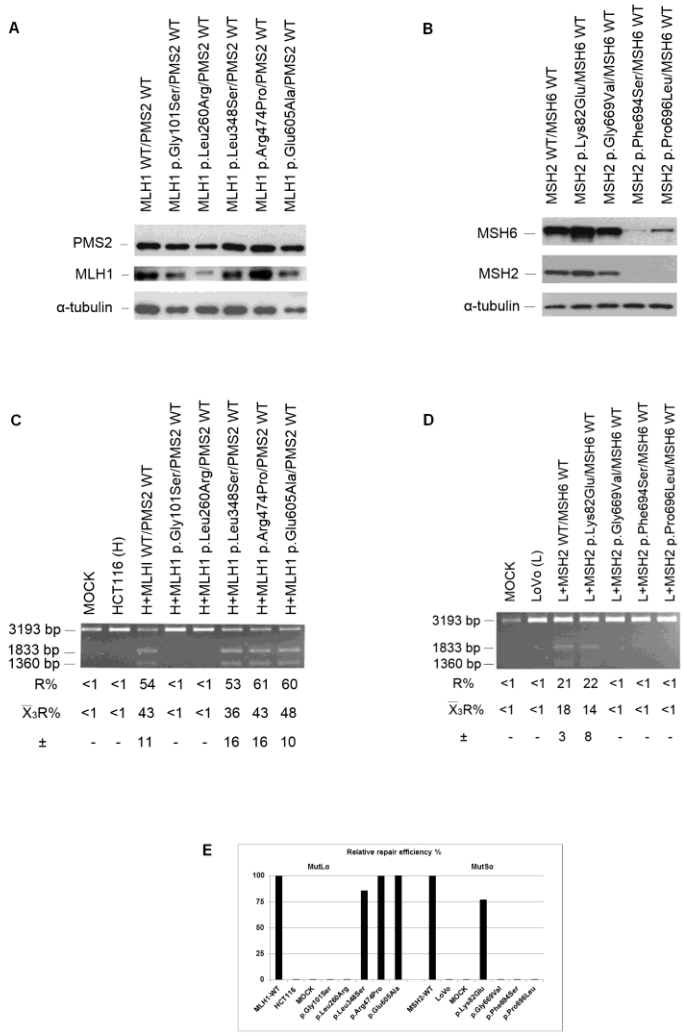


Figure 3. Expression and functional analyses of the 5 MLH1 and 4 MSH2 missense variants. (A-B) Western Blot analysis of total protein extracts from *Sf9* cells coinfected with baculovirus constructs expressing PMS2 wild-type protein (PMS2 WT) with either MLH1 WT or with MLH1 variant, and MSH6 WT with either MSH2 WT or MSH2 variant, and showing instability of proteins

MLH1 p.Leu260Arg, MSH2 p.Phe694Ser, and MSH2 p.Pro696Leu. α-tubulin was used as a loading control. (C-D) Repair efficiency (R%) of the recombinant MutLα (MLH1+PMS2) and Mutsα (MSH2+MSH6) protein variants measured in the *in vitro* MMR assay and calculated as the ratio of double digested DNA relative to total DNA added to the reaction, and showing functional deficiency in MLH1 p.Gly101Ser, MLH1 p.Leu260Arg, MSH2 p.Gly669Val, MSH2 p.Phe694Ser, and MSH2 p.Pro696Leu. R% corresponds to the assay results shown in the figure, $\bar{X}_3$R% denotes the average of three independent experiments with standard deviations (±). Nuclear extract free MOCK and uncomplemented MMR-deficient HCT116 NE and LoVo NE serve as negative controls and HCT116 and LoVo NE complemented by MutLα and Mutsα, respectively, serve as positive controls. The top fragment (3193 bp) shows the migration of unrepaired linearized G·T mismatch containing construct and the two smaller fragments (1833 bp and 1360 bp) represent the repaired and double digested fragments. (E) The relative repair % was calculated in respect to the wild type control (MutLα or MutSα respectively) set as 100 (100, $\bar{X}_3$R%) according to Drost et al., 2010, and Kantelinen et al., 2012.



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932 Tabel 1.Population frequency and tumor molecular and immunohistochemical characteristics of MMR gene variants.

	Sequence variant			Population frequency		Tumor data				
Gene	Location	Nucleotide change[1]	Predicted aminoacid change[1]	ExAC[2,3]	Italian control chromosomes[4]	MSI status[6]	IHC[6]	*BRAF* p.Val600Glu[6]	*MLH1 promoter methylation*[6,7]	LOH[6]
ML	3	c.301G>	p.(Gly101	nr	0	MSI	nt	nt	wt	nt

H1		A	Ser)			-H (1)			
	3i	c.307-19A>G		0.00020	0	MSS (1)	normal (1)	nt	nt	no (1)
	9	c.779T>G	p.(Leu260Arg)	nr	0	MSI-H (6); MSS (1)	MLH1 loss (5: all MSI-H tumors)	wt (3)	wt (3: all with MLH1 loss)	no (3)
	11i	c.1039-8T>A		0.00155	0.021875	MSI-H (1)	MLH1 loss (1: tumor with MSI status unknown); MSH2 loss (1: MSI-H tumor)	nt	nt	nt
	12	c.1043T>C	p.(Leu348Ser)	0.00002	0	MSS (1)	normal (1)	nt	nt	no (1)
	12	c.1217G>A	p.(Ser406Asn)	0.00089	0.00333	MSI-H (2)	MLH1 loss (1); MSH2/MSH6 loss (1)	nt	wt (1: tumor with MLH1 loss)	variant allele (1: tumor with MLH1 loss)

	13	c.1421G>C	p.(Arg474Pro)	nr	0	MSI-H	MLH1 loss	mut	nt	variant allele
	13i	c.1558+14G>A		0.03948	0.021876	MSI-H (1)	MLH1 loss (1: tumor with MSI status unknown); MSH2 loss(1: MSI-H tumor)	nt	nt	nt
	15i	c.1732-19T>C		nr	0	MSS (1)	normal	nt	nt	variant allele
	16	c.1743G>A	p.(Pro581=)	0.00008	0	MSI-H (1)	MLH1 loss	mut	wt	nt
	16	c.1814A>C	p.(Glu605Ala)	nr	0	MSI-H (1)	MLH1 loss	nt	nt	no
	18	c.2041G>A	p.(Ala681Thr)	nr	0	MSS (1); MSI-H (1)	MLH1 loss (1: MSS tumor); normal (1: MSI-H tumor)	wt (1: MSI-H tumor)	wt (1: MSI-H tumor)	no (1: MSI-H tumor)
	18	c.2059C>T	p.(Arg687Trp)	0.00003	0	MSI-H (2); MSS	normal (1: MSI-H tumor); MLH1	wt (1: MSI-H tumor)	nt	nt

						(1)	loss (1: MSS tumor)			
MSH2	2	c.244A>G	p.(Lys82Glu)	nr	0	MSS (1)	normal	nt	nt	no (1)
	3	c.380A>G	p.(Asn127Ser)	0.00692	nt	nt	nt	nt	nt	nt
	81	c.1387-8G>T		0.00194	nt	MSI-L (1); MSS (1)	normal (1: MSI-L tumor); MLH1 loss (1: MSS tumor)	nt	nt	no (1: MSS tumor)
	9i	c.1511-9A>T		0.08400	0.07333	MSI-H (5); MSS (2)	normal (3: 1 MSI-H and 2 MSS tumors); MSH2/MSH6 loss (4: all MSI-H tumors)	nt	meth (1: MSI-H tumor)	nt
	11	c.1666T>C	p.(Pro556=)	0.00437	0	MSI-H (1); MSS (1)	normal (2)	nt	wt (1: MSI-H tumor)	nt

	11	c.1737A>G	p.(Lys579=)	0.00192	0	MSI-L (1); MSS (1)	normal (1: MSI-L tumor); MLH1 loss (1: MSS tumor)	nt	nt	no (1: MSS tumor)
	12i	c.2006-6T>C		0.11500	nt	MSI-H (2)	MSH2/MSH6 loss (2)	nt	nt	nt
	13	c.2006G>T	reported as p.(Pro670Leufs*) (predicted missense change: p.(Gly669Val)	nr	0	MSI-H (1)	MSH2/MSH6 loss (1)	nt	nt	no (1)
	13	c.2081T>C	p.(Phe694Ser)	nr	0	MSI-H (3)	MSH2/MSH6 loss (3)	nt	nt	nt
	13	c.2087C>T	p.(Pro696Leu)	nr	0	MSI-H (3)	MSH2/MSH6 loss (1)	nt	nt	no (1)
	14	c.2442T>G	p.(Leu814=)	nr	nt	MSI-H (1)	MSH2/MSH6 loss (1)	nt	nt	no (1)

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934 [1] Previously unclassified variants are indicated in bold.

935 [2] nr = not reported.

936 [3] ExAC: http://exac.broadinstitute.org/; TGCA allele frequencies are excluded.

937 [4] nt = not tested.

938 [5] c.1039-8T>A and c.1558+14G>A are in linkage disequilibrium in the Italian population.

939 [6] In brackets number of samples; nt = not tested.

940 [7] wt = not methylated, meth = methylated.

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944 **Table 2.** Effects of MMR gene variants on RNA processing, protein stability and *in vitro* MMR activity.

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Gene	Sequence variant			mRNA analysis[2,3]			Functional analysis[3,4]	
	Location	Nucleotide change[1]	Predicted aminoacid change[1]	Studies on patient samples		Minigene splicing assay[4]	Mammalian protein stability	Mammalian mmr activity
				Splicing analysis	SNUPE assay			
MLH1	3	c.301G>A	p.(Gly101Ser)	nt	no allelic imbalance	total inclusion of reference exon 3 (Tournier et al., 2008; this study) with concomitant loss of alternative 5'ss five nucleotides upstream the reference5' ss	Stable	Deficient

						(this study)		
	3i	c.307-19A>G		nt	nt	no effect (Tournier et al. 2008)	na	na
	9	c.779T>G	p.(Leu260Arg)	no effect (Montera et al., 2000; this study)	no allelic imbalance	no effect	Unstable	Deficient
	11i	c.1039-8T>A		partial loss of exon 12	nt	no effect (Petersen et al., 2013; this study)	na	na
	12	c.1043T>C	p.(Leu348Ser)	no effect	no allelic imbalance	no effect	Stable	Proficient
	12	c.1217G>A	p.(Ser406Asn)	no effect	no allelic imbalance (Pastrello et al. 2011 and this study)	no effect	Stable (Takahashi et al. 2007)	Proficient (Takahashi et al. 2007; Drost et al. 2010)
	13	c.1421G>C	p.(Arg474Pro)	nt	nt	no effect	Stable	Proficient
	13i	c.1558+14G>A		nt	nt	no effect	na	na
	15i	c.1732-		no effect	nt	no effect	na	na

		19T>C						
	16	c.1743G>A	p.(Pro581=)	nt	nt	no effect	na	na
	16	c.1814A>C	p.(Glu605Ala)	nt	no allelic imbalance	no effect	Stable	Proficient
	18	c.2041G>A	p.(Ala681Thr)	no effect (Jakubowska et al., 2001; Betz et al., 2010)	no allelic imbalance	no effect (Tournier et al., 2008)	Discordant results (Raevaara et al., 2005; Takahashi et al., 2007; Xie et al., 2010; Hardt et al., 2011; Hinrichsen et al., 2013)	Proficient (Raevaara et al., 2005; Takahashi et al., 2007; Hinrichsen et al., 2013)
	18	c.2059C>T	p.(Arg687Trp)	no effect (Jakubowska et al., 2001; Furukawa et al., 2002; Auclair et al., 2006; Arnold et al., 2009; this study)	no allelic imbalance	no effect	Discordant results (Takahashi et al., 2007; Christensen et al., 2009)	Proficient (Takahashi et al., 2007; Christensen et al., 2009)
MS	2	c. 244A>G	p.(Lys82Glu	nt	no	no effect	Stable	Proficie

H2					allelic imbalance			nt
	3	c.380A>G	p.(Asn127Ser)	nt	nt	partial exclusion of exon 3	Stable (Kansikas et al., 2011)	Proficient (Ollila et al., 2008)
	8i	c.1387-8G>T		nt	nt	no effect (Tournier et al., 2008)	na	na
	9i	c.1511-9A>T		nt	nt	no effect (Tournier et al., 2008)	na	na
	11	c.1666T>C	p.(Pro556=)	no effect (Auclair et al., 2006)	nt	no effect (Tournier et al., 2008)	na	na
	11	c.1737A>G	p.(Lys579=)	no effect (Auclair et al., 2006)	nt	no effect (Tournier et al., 2008)	na	na
	12i	c.2006-6T>C5		no effect (Tournier et al., 2008)	na	partial exon 13 exclusion (Tournier et al., 2008; this study)	na	na
	13	c.2006G>T	reported as p.(Pro670Leufs*) (predicted missense	partial exon 13 exclusion (this study)	nt	complete exon 13 exclusion (van der Klift	Stable	Deficient

			change p.(Gly669Val)	complete exon 13 exclusion (van der Klift et al., 2015)		et al., 2015; this study)		
	13	c.2081T>C [5]	p.(Phe694Ser)	nt	no allelic imbalance	no effect	Unstable	Deficient
	13	c.2087C>T	p.(Pro696Leu)	nt	no allellic imbalance	no effect (Tournier et al., 2008)	Unstable	Deficient
	14	c.2442T>G	p.(Leu814=)	nt	nt	no effect	na	na

[1] Previously unclassified variants are shown in bold. [2] nt = not tested. [3] The results shown are from this study, unless otherwise indicated. [4] In italics: results of studies using *in vitro* mammalian assays different from that used in the present study. [5] These two variants (*MSH2* c.2006-6T>C and c.2081T>C) were also tested in combination in the minigene assay, since they were found in linkage disequilibrium.

Table 3. Clinical classification of *MLH1* and *MSH2* variants.

GENE	DNA and predicted protein change[1]	InSiGHT classification[2,3]	Proposed classification	Rationale for classification[4]	
				Posterior probability of pathogenicity by multifactorial analysis[3]	Qualitative criteria
MLH1	c.301G>A; p.(Gly101Ser)	4	5	0.99740	– Co-segregation – MSI/IHC data – Deficient MMR

					function – Allelic frequency: 0 (this study)
	c.307-19A>G	2	2	na	– Intronic location – Normal minigene splicing assay – MSI/IHC data – Allelic frequency: 0.0002 (ExAc); 0 (this study)
	c.779T>G; p.(Leu260Arg)	5	5	1	– Co-segregation – MSI/IHC data – Deficient MMR function – Allelic frequency: 0 (this study)
	c.1039-8T>A	1	1	na	– Intronic location – Allelic frequency: 0.00155 (ExAc); 0.02187 (this study) – MSI/IHC data – No major splicing abnormalities
	c.1043T>C; p.(Leu348Ser)		3	0.64637	Insufficient data (proficient MMR function; 1 MSS tumor; no major splicing alteration)
	c.1217G>A; p.(Ser406Asn)	1	1	< 0.00100	– Allele frequency: 0.00089 (ExAc);

					0.00333 (this study) – MSI/IHC data – Lack of co-segregation with phenotype (combined segregation likelihood ratio < 0.01) – Co-occurrence of MSH2 truncating variant that segregates with the phenotype in the family – Proficient MMR function – No major splicing abnormalities – Estimated risk from case-control studies (1.5)
	c.1421G>C; p.(Arg474Pro)	3	3	0.09448	Insufficient data (no major splicing alteration; proficient MMR function; 1 tumor MSI-H BRAF p.Val600Glu positive)
	c.1558+14G>A	1	1	na	– Intronic location – Allelic frequency: 0.03948 (ExAc); 0.02187 (this study) – MSI/IHC data

					– No major splicing abnormalities
	c.1732-19T>C	na	2	0.01386	– Intronic location – 1 MSS tumor – No major splicing abnormalities
	c.1743G>A; p.(Pro581=)	na	2	na	– Synonymous coding change – No major splicing abnormalities by minigene assay – Allelic frequency: 0.00008 (ExAc); 0 (this study) – 1 MSI-H MLH1-neg BRAF p.Val600Glu-pos tumor
	c.1814A>C; p.(Glu605Ala)	na	4	0.95294	MSI/IHC data
	c.2041G>A; p.(Ala681Thr)	5	5	0.99708	– Co-segregation – MSI/IHC data
	c.2059C>T; p.(Arg687Trp)	5	5	0.99999	– Co-segregation – MSI/IHC data – Homozygosity associated with constitutional mismatch repair deficiency syndrome
MSH 2	c. 244A>G; p.(Lys82Glu)	na	2	0.00980	– MSI/IHC data – Proficient MMR function

					– No major splicing abnormalities
	c.380A>G; p.(Asn127Ser)	1	1	na	– Allelic frequency: 0.0692 (ExAc) – No major splicing abnormalities by minigene assay – Proficient MMR function
	c.1387-8G>T	2	1	0.00088	– Intronic location – Allelic frequency: 0.00194 (ExAc) – MSI/IHC data (> 3 tumors not showing features of MMR deficiency and/or MSH2 inactivation) – No major splicing abnormalities
	c.1511-9A>T	1	1	na	– Intronic location – Allelic frequency: 0.08400 (ExAc); 0.07333 (this study) – MSI/IHC data – No major splicing abnormalities by minigene

					assay
	c.1666T>C; p.(Pro556=)	1	1	< 0.00010	– Synonymous coding change – Allele frequency: 0.00437 (ExAc); 0 (this study) – MSI/IHC data – No major splicing abnormalities
	c.1737A>G; p.(Lys579=)	2	1	0.00021	– Synonymous coding change – Allelic frequency: 0.0019 (ExAc); 0 (this study) – MSI/IHC data (> 3 tumors not showing features of MMR deficiency and/or MSH2 inactivation) – No major splicing abnormalities
	c.2006-6T>C	1	1	na	– Intronic location – No major splicing abnormalities
	c.2006G>T; reported as p.(Pro670Leufs*) (predicted missense change p.(Gly669Val)	5	5	0.99906	– Co-segregation – MSI/IHC data – Deficient MMR functional test – Contrasting results of RNA splicing analyses

	c.2081T>C; p.(Phe694Ser)	5	5	0.99990	– Co-segregation – MSI/IHC data – Deficient MMR function
	c.2087C>T; p.(Pro696Leu)	5	5	1	– Co-segregation – MSI/IHC data – Deficient MMR function
	c.2442T>G; p.(Leu814=)	na	2	na	– Synonymous coding change – No major splicing abnormalities by minigene splicing assay – Co-observation of MSH2 Class 5-pathogenic variant (phase unknown)

[1]Variants not yet classified by InSiGHT are shown in bold. [2] For previously classified variants, the classification corresponds to that reported on http://insight-group.org/variants/classifications/.

[3] na = not available. [4] Classification was achieved by multifactorial analysis, qualitative criteria or both; in italics data obtained at least in part from the present study.