



**HAL**  
open science

# Verification of the Three Step Model in Assessing the Pathogenicity of Mismatch Repair Gene Variants

Minttu Kansikas, Reetta Kariola, Minna Nystrom

► **To cite this version:**

Minttu Kansikas, Reetta Kariola, Minna Nystrom. Verification of the Three Step Model in Assessing the Pathogenicity of Mismatch Repair Gene Variants. *Human Mutation*, 2010, 32 (1), pp.107. 10.1002/humu.21409 . hal-00602305

**HAL Id: hal-00602305**

**<https://hal.science/hal-00602305>**

Submitted on 22 Jun 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**Verification of the Three Step Model in Assessing the  
Pathogenicity  
of Mismatch Repair Gene Variants**

Journal:	<i>Human Mutation</i>
Manuscript ID:	humu-2010-0329.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	24-Sep-2010
Complete List of Authors:	Kansikas, Minttu; University of Helsinki, Department of Biosciences Kariola, Reetta; University of Helsinki, Department of Biosciences Nystrom, Minna; University of Helsinki, Department of Biosciences
Key Words:	Colorectal cancer (CRC), Functional assessment, Hereditary nonpolyposis colorectal cancer (HNPCC), Lynch syndrome (LS), Variants of uncertain significance (VUS), MLH1, MSH2, MSH6

SCHOLARONE™  
Manuscripts

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18 **“Verification of the Three Step Model in Assessing the Pathogenicity**  
19 **of Mismatch Repair Gene Variants”**  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32

33 Minttu Kansikas<sup>1</sup>, Reetta Kariola<sup>1</sup> and Minna Nyström\*<sup>1</sup>  
34  
35  
36  
37

38  
39 <sup>1</sup>Department of Biosciences, Genetics, University of Helsinki, P.O.Box 56 (Viikinkaari 5),  
40 FI-00014 Helsinki, Finland;  
41

42  
43 \*Correspondence: Professor M Nyström;  
44

45 E-mail: minna.nystrom@helsinki.fi  
46  
47

48 Department of Biosciences, Genetics, University of Helsinki, P.O.Box 56 (Viikinkaari 5), FI-  
49 00014 Helsinki, Finland  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## ABSTRACT

In order to assess whether variations affecting DNA mismatch repair (MMR) genes are pathogenic and hence predisposing to Lynch syndrome (LS), a three step assessment model has been proposed. Where LS is suspected based on family history, STEP1 is dedicated to the identification of the causative MMR gene and the variation within it. Thereafter, in STEP2 of the assessment model, the effect of the variation on the function of the protein is assessed in an *in vitro* MMR and *in silico* assays. Where LS cannot be confirmed or ruled out in STEP2, the more specific biochemical laboratory assays such as analyzing the effect of the variation on expression, localization and interaction of the protein are required in STEP3. Here, we verified the proposed three step assessment model and its ability to distinguish pathogenic MMR variations from variants of uncertain significance (VUS) by utilizing the clinical as well as the laboratory and *in silico* data of 37 *MLH1*, 26 *MSH2* and 11 *MSH6* variations. The proposed model was shown to be appropriate and proceed logically in assessing the pathogenicity of MMR variations. In fact, for MMR deficient *MSH2* and *MLH1* variations the first two steps seem to be sufficient as STEP3 provides no imperative information concerning the variant pathogenicity. However, the importance of STEP3 is seen in the assessment of MMR proficient variations showing discrepant *in silico* results as their pathogenicity cannot be confirmed or ruled out after STEP2. *MSH6* variations may be applicable to the model if appropriate selection in terms of ruling out *MLH1* and *MSH2* variations and *MLH1* promoter hypermethylation is ensured prior to the completion of STEP2. In conclusion, taking into consideration the susceptibility gene the three step model can be utilized in an appropriate and efficient manner to determine the pathogenicity of MMR gene variations.

Deleted: are

Deleted: only

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

*KEYWORDS:* Colorectal cancer; CRC; Functional assessment; Hereditary nonpolyposis colorectal cancer; HNPCC; Lynch syndrome; LS; Variants of uncertain significance; VUS; *MLH1*; *MSH2*; *MSH6*

For Peer Review

## INTRODUCTION

Lynch syndrome (LS, often referred to as hereditary non-polyposis colorectal cancer syndrome; HNPCC; MIM# 120435) is highly associated with autosomal dominant inheritance of mutations in genes fundamental to the DNA mismatch repair (MMR) mechanism. The most frequently affected genes include *MLH1* (MIM# 120436, RefSeq NM\_000249.3), *MSH2* (MIM# 609309, RefSeq NM\_000251.1), *MSH6* (MIM# 600678, RefSeq NM\_000179.2), and *PMS2* (MIM# 600259, RefSeq NM\_000535) whose germline variations are reported in the LOVD database (<http://www.insight-group.org/>; <http://www.lovd.nl/>). Although, the majority of mutations affecting MMR genes are truncating, a significant proportion of mutations result in a single amino acid substitution or an in-frame deletion and are difficult to distinguish from harmless polymorphisms. Such alterations are often referred to as variants of uncertain significance (VUS) [Goldgar et al., 2008] due to the uncharacterized effect of the variation on the function of the polypeptide.

LS associated tumors generally occur in the colon, nevertheless a variety of extracolonic carcinomas, especially those of the endometrium are also frequently observed. The mean age of cancer onset in LS is significantly lower than that of sporadic colorectal cancer (CRC) [Lynch and de la Chapelle, 1999] based on the fact that in LS, an individual has already inherited susceptibility through a mutated allele and only needs a second hit in a somatic cell to lose MMR activity and start tumorigenesis. Hence, LS tumors are characterized by the lack or lowered level of a causative MMR protein as well as impaired DNA repair causing microsatellite instability (MSI) [Aaltonen et al., 1993]. The wide variety of clinical phenotypes complicates LS diagnostics and several clinical guidelines have been established to distinguish LS families from the general CRC burden. Currently, the clinical diagnosis of LS greatly relies on the Amsterdam criteria (AC) [Vasen et al., 1991, 1999] or the revised Bethesda guidelines [Umar et al., 2004], which take into account the age of cancer

Deleted: of which

Deleted: a

Deleted: due to the non-truncated nature of the protein

Deleted: such as

Deleted: Affected patients have inherited a mutated allele and subsequently after the

Deleted: may be lost

Deleted: s

Deleted: Furthermore, the mean age of onset in LS is significantly lower than that of sporadic colorectal cancer (CRC) [Lynch and de la Chapelle, 1999].

Deleted: S

Deleted: and assays

Deleted: the alteration underlying tumorigenesis. In fact

onset, the number and segregation of affected individuals in a family, and the level of MSI.

However, many putative LS families do not fit these criteria and could be confirmed as LS families only by characterizing a pathogenic germline MMR gene mutation in them.

**Deleted:** CRC families

**Deleted:** [Hampel et al., 2005].

**Deleted:** Overall, where a VUS is identified the characterization of its effect on the function of the polypeptide remains a challenge and calls for a unified assessment model.

**Deleted:** ,

The first clinical step in diagnosing LS associated tumors, includes immunohistochemistry (IHC) and MSI analysis followed by mutation analysis dictated by the IHC and MSI results. Hampel et al. [2005] have proposed a strategy for screening LS by

**Deleted:** patients for

analyzing all four MMR genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*) together with the potential

**Deleted:** taking

**Deleted:** into account

hypermethylation of the *MLH1* promoter region. When a variation with a known defect is

**Deleted:** Genetic sequencing dictated by the IHC and MSI results is carried out, and in the case of

found, LS can be confirmed or in the absence of a MMR gene variation, ruled out.

**Deleted:** effect

**Deleted:** either

**Deleted:** as likely

**Deleted:** in contrast ruled out

**Deleted:** s

Based on a similar approach (STEP1), Couch et al. [2008] have proposed a decision tree for the *in vitro* analysis of MMR VUS found in putative LS families. This model utilizes data from incompletely validated assays supplemented with data derived from other sources for classification of VUS for clinical purposes. More specifically, data derived from an *in vitro* MMR and in silico analyses should be considered upon the identification of a VUS (STEP2). Variations showing MMR deficiency in these assays indicate LS, whereas variations with no apparent MMR deficiencies require a selection of biochemical assays for further characterization of the effect of the variation on the protein expression or function (STEP3).

**Deleted:** In the case of a VUS, however, further analysis is required to determine the effect of the variation on the function of the protein.

**Deleted:** assay together with data from an

**Deleted:** analysis

**Deleted:** indicating

Here, we aim to verify the ability of the proposed three step model in assessing pathogenicity with the data of 74 MMR gene VUS including, results of tumor pathologic, genetic, biochemical and *in silico* analyses.

**Deleted:** by including altogether

**Deleted:** specific clinical data together with the

**Deleted:** assays to the model

## MATERIALS AND METHODS

## MLH1, MSH2 and MSH6 Variations and Clinical Data

This study comprises 37 *MLH1* (NM\_000249.3) [Raevaara et al., 2005; Christensen et al., 2009], 26 *MSH2* (NM\_000251.1) [Kariola et al., 2003; Ollila et al., 2006, 2008; Christensen et al., 2009] and 11 *MSH6* (NM\_000179.2) [Kariola et al., 2002, 2003, 2004] variations. The family history and the data of mutation, MSI and IHC analysis were mainly collected through an international LS collaboration. Because the 74 VUS included in the study were found by many research groups, different methods were used for mutation detection. The alterations are distributed over most of the known functional domains of the respective proteins as seen in the schematic representations presented in the results section. *MLH1* variations tend to cluster either at the amino terminal or carboxyl terminal of the protein, whereas *MSH2* and *MSH6* variations are dispersed throughout the length of the proteins, with preferential location in functional domain clusters seen particularly in the connector and ATPase domains of *MSH2*. The variations were constructed by site-directed mutagenesis and co-expressed with their native heterodimerization partners in *Spodoptera frugiperda* (Sf9) insect cells for protein production [Nyström-Lahti et al., 2002].

The VUS chosen for this study are from putative LS families either fulfilling the AC, or from families not fulfilling AC but presenting LS tumors at an abnormally low age, or with an excessive amount of LS cancers occurring within the family. In fact, of the 37 *MLH1* variations, at least 27 are associated with a mean age of onset below 50 years even though the ACI are only fulfilled by families associated with 17 of the variations. Nineteen out of 26 *MSH2* variations are associated with a mean age of onset under 50 years and approximately half of all the *MSH2* variations were found in families fulfilling ACII. *MSH6* variations chosen for functional assessment were mainly picked from the LOVD database. The families

**Deleted:** previously

**Deleted:** and thereafter functionally characterized with the *in vitro* MMR assay as well as other functional and *in silico* analyses.

**Deleted:** Functional assessment of these variations has typically been mandated by clinical questions and ambiguities tracking the variation, susceptible tumor data or in some cases database information.

**Deleted:** All 74 MMR

**Deleted:** included fulfill the Amsterdam criteria

**Deleted:** (<http://www.insight-group.org>), and taken for functional assessment



1  
2 with *MSH6* variations do not fulfill AC, but instead, all but two variations have been linked to  
3  
4 the MSI-high (MSI-H) tumor phenotype. The mean age of tumor onset of *MSH6* VUS  
5  
6 carriers is 59.

Deleted: shown

Deleted: present

Deleted: Overall, the alterations are distributed over most of the known functional domains of the respective proteins as seen in the schematic representations presented in the results section (Figure 2). *MLH1* variations tend to cluster either at the amino- carboxyl terminal of the protein where as *MSH2* and *MSH6* variations are more dispersed throughout the length of the protein, with intra functional domain clusters seen particularly in the connector and ATPase domains of *MSH2*. ¶

### Functional analyses

7  
8  
9  
10 The 74 MMR gene VUS included in the verification study were functionally  
11 characterized in our previous studies [Kariola et al., 2002, 2003, 2004; Raevaara et al., 2005;  
12 Ollila et al., 2008; Christensen et al., 2009]. Studies were typically mandated by clinical  
13 questions and ambiguities tracking the variation, tumor pathology data, or in some cases  
14 database information. Functional assessment of each variation was evaluated in comparison  
15 to the performance of the corresponding wild type (wt) protein and functionally deficient  
16 negative controls. Assay results were composed of a minimum of three independent  
17 experiments and even a slight decrease in functionality compared to the corresponding result  
18 of WT protein indicated pathogenicity.

19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30 The applied *in vitro* MMR assay [Nyström-Lahti et al., 2002] uses a homologous  
31 human MMR system to study the ability of the variant protein to repair a G-T mispair. The  
32 standard deviation of the repair efficiency of the deficient variant protein remained below that  
33 of the WT protein. In order to assess the effect of the variation on protein expression, *MSH2*  
34 and *MSH6* variant proteins were expressed in *Sf9* insect cells [Kariola et al., 2002], where as  
35 *MLH1* proteins were expressed in 293T human cells. Thereafter, the expression levels of the  
36 VUS proteins were compared with that of WT protein by western blot analysis [Raevaara et  
37 al., 2003]. Results from interaction studies are based on coimmunoprecipitation and  
38 subsequent western blot analysis of the variant protein with its native heterodimerization  
39 partner [Kariola et al., 2002; Raevaara et al., 2003]. To study whether the *MLH1* variations  
40 affect the subcellular localization, VUS cDNAs were fused with the fluorescent protein  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

EGFP cDNA and transiently expressed in 293T cells [Raevaara et al., 2005]. The variants acting like WT MLH1 were classified as normal in the localization study.

### *In silico* analysis by **SIFT** and **MAPP-MMR**

*In silico* methods take a computational approach to identify highly conserved areas of a gene through a multiple sequence alignment analysis across numerous species and thereafter, deduce possible functional defects of a variation. Several prediction algorithms are available for *in silico* analyses. Here, the sorting intolerant from tolerant (SIFT) [Ng and Henikoff, 2001; <http://sift.jcvi.org/>] and the multivariate analysis of protein polymorphism (MAPP-MMR) [Stone and Sidow, 2005; Chao et al., 2008; <http://mendel.stanford.edu/SidowLab/>] programs were chosen for *in silico* assessment of the 74 VUS due to their high sensitivity and specificity [Tavtigian et al., 2008b]. The results from previously done SIFT analyses for *MLH1* and *MSH2* VUS [Raevaara et al., 2005; Ollila et al., 2006, 2008; Christensen et al., 2009] were complemented with results of *MSH6* VUS obtained from the LOVD database (<http://www.lovd.nl/>). The MAPP-MMR analysis was performed here for the *MLH1* and *MSH2* variations but was not available for *MSH6* variations. Neither of the programs can be applied to *in frame* deletions.

**Deleted:** The previously done *in silico* analysis was obtained through the program sorting intolerant from tolerant (SIFT) [Ng and Henikoff, 2001; <http://sift.jcvi.org/>] analysis. To supplement this data, here, the multivariate analysis of protein polymorphism (MAPP-MMR) [Stone and Sidow, 2005; <http://mendel.stanford.edu/SidowLab/>] was performed

**Deleted:** all

**Deleted:** The MAPP-MMR analysis is not available for *MSH6* variations and neither

### Verification of the three step model

In order to assess the pathogenicity of VUS, a three step model proposed by Couch et al. [2008] was applied (Figure 1). The model acknowledges the importance of appropriate VUS identification by emphasizing the use of family history, MSI and IHC data to ultimately identify the VUS by genetic testing analysis in STEP1. Upon the identification of a VUS, STEP2 consists of *in vitro* MMR and *in silico* analyses. MMR deficiency demonstrated by

**Deleted: The three step model**  
In order to assess the pathogenicity of VUS a three step model proposed by Couch et al. [2008] was applied (Figure 1). The model acknowledges the importance of appropriate VUS identification by emphasizing the use of family history, MSI and IHC data to ultimately identify the VUS by genetic testing. Upon the genetic testing of STEP1 and subsequent identification of a VUS, STEP2 consists of an *in vitro* MMR and an *in silico* analyses. MMR deficiency demonstrated by STEP2 confirms LS where as in the case of MMR proficient variations, a panel of biochemical assays is recommended in STEP3.

STEP2 confirms LS, whereas in the case of MMR proficient variations, a panel of biochemical assays is recommended in STEP3.

The 74 MMR VUS included in this study are found in families suspected to have LS and hence compose an appropriate and realistic panel of variations for the verification of the three step assessment model. Each variation is taken through the model and the assays constituting each of the steps. Results indicating pathogenicity are distinguished from ones indicating no effect of the variation. Discrepancies between individual tumor data (IHC and MSI) as well as between results from different *in silico* programs are marked.

Deleted: ,

STEP1 of the model proposed by Couch et al. [2008] is represented by the family history and tumor pathology data of the proband leading to mutation analysis. Tumors with 2 or more unstable Bethesda panel markers [Umar et al., 2004] were considered to have a high degree of MSI and the lack or reduction of the MMR protein in IHC was considered to indicate protein deficiency.

Deleted: up the identification of the VUS by sequencing

Upon the identification of a VUS, STEP2 of the assessment model [Couch et al., 2008] suggests to combine the results of a functional *in vitro* MMR assay with those of an *in silico* analysis. Here, STEP2 is composed of the results of the in vitro MMR assay and two separate *in silico* assays derived from the SIFT and MAPP-MMR programs. Pathogenicity of a VUS was indicated by the *in vitro* MMR assay alone or together with the deleterious results obtained *in silico* as well as by deleterious results obtained through both of the *in silico* methods even in the absence of such indication by the *in vitro* MMR assay. Where pathogenicity in STEP2 was suggested by only one in silico analysis, STEP3 with further assays were required. Non-pathogenic VUS were distinguished by fully completed STEP2 assays with no indication of pathogenicity.

Deleted: a functional

Deleted: results

Deleted: analyses

Deleted: The applied *in vitro* MMR assay [Nyström-Lahti et al., 2002] uses a homologous human MMR system to assay the ability of the variant protein to repair a GT mismatch built in a unique restriction site of a plasmid substrate. In contrast, the *in silico* methods take a computational approach to identify highly conserved areas of the gene through a multiple sequence alignment across numerous species and thereafter deduce possible functional effects of the variation.

Deleted: only

STEP3 of the assessment model [Couch et al., 2008] is composed of a set of laboratory experiments taken to further clarify the pathogenicity of the variations, where previous steps

1  
2 did not already do so. This panel of experiments were suggested to include studies of protein  
3 stability, protein interaction and subcellular localization. STEP3 assays study specific  
4 fragments of the repair process complementing the *in vitro* MMR assay, which was  
5 performed in nuclear extracts and optimized to reveal even the slightest repair and thus, not  
6 able to discover problems in subcellular localization or mild problems in protein stability or  
7 interactions. Here, the STEP3 assays differ slightly between the three genes but have been  
8 included for all variations for the verification of the three step model. Together with one  
9 deficient result from an *in silico* assay, a decrease in variant protein expression, interaction or  
10 localization was considered to be an indication of pathogenicity. Consequently, a single  
11 indication of pathogenicity in STEP3, although measuring different aspects of the protein  
12 function than the MMR assay, was deemed sufficient to confirm variant pathogenicity.

Deleted: have

Deleted: ne

Deleted: has been

Deleted: MSH2 and MSH6 variant protein expression was carried out in *Spodoptera frugiperda* (Sf9) insect cells [Kariola et al., 2002] where as the expression of MLH1 variants was assayed in human 293T cells [Raevaara et al., 2003]. Results from interaction studies are based on coimmunoprecipitation and subsequent western blot analysis of the variant protein with its native heterodimerisation protein partner [Kariola et al., 2002; Raevaara et al., 2003]. In addition, data indicating the nuclear localization ability of MLH1 variants with wild type-PMS2 is included [Raevaara et al., 2005].

Deleted: either

Deleted: in assessing the pathogenicity of the MMR gene VUS

Deleted: at the same time

13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26 In order to verify the three step model and determine the necessity and validity of all  
27 three steps in it, results and interpretations after STEP2 and STEP3 were compared. Finally,  
28 STEP1 data was considered together with these comparisons to further verify the indications  
29 of pathogenicity and to form a consensus for each step of the model.

## 30 31 32 33 34 35 36 RESULTS

### 37 38 39 40 41 42 Verification of the three step model with *MLH1* variations

43  
44 Our results show that, if strong evidence from the family history, MSI and IHC results  
45 give an indication of LS and an *MLH1* variation has been detected in STEP1, STEP2 is often  
46 sufficient to confirm the pathogenicity of the variation. As seen in Table 1, when STEP2  
47 results unanimously indicate pathogenicity (11/37), the expression and localization analyses  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

Deleted: Where

of STEP3 serve to confirm the results and are hence descriptive but not essential for assessment. This is also apparent when MMR proficiency is unanimously indicated by all STEP2 assays as seen in 7/37 variations. Assuming that pathogenicity can be shown with the *in vitro* MMR assay alone (5/37), or with two deleterious *in silico* indications (4/37), STEP2 is sufficient to confirm the pathogenicity of 20/37 *MLH1* VUS. The former requisite is supported by VUS for which the *in silico* results are not obtainable, since all the 5 *MLH1* deletions (p.T45\_I47delinsCF, p.E71del, p.I330del, p.P578\_E632del, and p.E633\_E663del) indicated to be pathogenic by the *in vitro* MMR assay, are confirmed to be so by STEP3, regardless of the lack of *in silico* results. Likewise, the 4 *MLH1* VUS (p.L550P, p.P648L, p.P648S, and p.654L) indicated to be pathogenic by both *in silico* analyses, but not with the MMR assay, are confirmed to be so by STEP3.

**Deleted:** Following our guidelines according to which

**Deleted:** ,

**Deleted:** This supposition

**Deleted:** further

**Deleted:** .

**Deleted:** In fact,

**Deleted:** one amino acid

**Deleted:** TSI45-47CF, Del171, Del330, Del578-632, and Del633-663

Nevertheless, in the absence of *in silico* results, or if pathogenicity is indicated by only one alignment analysis, the MMR proficiency is not sufficient to rule out pathogenicity. This is indicated by MMR proficient *MLH1* p.H329P, p.A589D, p.V612del, p.K616del, and p.K618T that display reduced expression and/or nuclear localization in STEP3. Generally, in such cases the expression assay suffices for STEP3. Especially results from *MLH1/PMS2* protein interaction analysis appear to have very little contribution towards the assessment of *MLH1* VUS. In contrast, four variants (p.K618A, p.Y646C, p.A681T, and p.R687W), in which the STEP2 differences are due to an indication of pathogenicity by only one *in silico* assay, the pathogenicity is not confirmed by STEP3. Similarly, for *MLH1* p.E460A, deficiency indicated by only SIFT is not confirmed by STEP3.

**Deleted:** where

**Deleted:** only

**Deleted:** of the two

**Deleted:** s

**Deleted:** as

**Deleted:** Del612, Del616, H329P, A589D and K618T

**Deleted:** In fact, where discrepancies arise between the *in vitro* MMR and *in silico* results indicating an MMR proficient alteration to be deleterious by *in silico* assays, STEP3 is able to further explain the pathogenicity by other functional deficiencies.

**Deleted:** variant protein

**Deleted:** as

**Deleted:** in particular

**Deleted:** . In effect, the pathogenicity of four MMR proficient *MLH1* variations with pathogenicity indicated by both *in silico* methods in STEP2 results (L550P, P648L, P648S, and P654L) is confirmed by expression analysis results.

**Deleted:** SIFT or MAPP-MMR

**Deleted:** indicated

**Deleted:** the assays of

**Deleted:** due to the lack of MAPP-MMR predictions

**Deleted:** STEP3 analyses are needed to rule out pathogenicity.

**Deleted:** is

The verification of the three step model with the *MLH1* variations demonstrates that STEP3 often supports the deductions, which, however, can be made from STEP2 analyses in the case of 27/37 variations. Only in the case of MMR proficient variations with either discrepant or no data from both *in silico* methods, STEP3 is required for interpretation (10/37).

Verification of the three step model with *MSH2* variations

As with the variations affecting *MLH1*, when the MMR assay results agree with those obtained *in silico*, STEP3 is not necessary. This is seen in the case of 12 pathogenic and 7 non-pathogenic *MSH2* variations (Table 2). Pathogenicity indicated by the *in vitro* MMR assay result or with two deleterious *in silico* results confirms the pathogenicity of 14 VUS raising the total number of successfully assessed VUS in STEP2 to 21 out of 26. Notably, no indication of pathogenicity is seen in STEP3 only.

Deleted: seen

Deleted: where

Deleted: STEP2

Deleted: *in vitro*

Deleted: as

Deleted: with

Deleted: alone

Deleted: ,

Deleted: indications

Deleted: in STEP2

Deleted: VUS

Deleted: by

Unlike in the case of some *MLH1* variations, the pathogenicity of *MSH2* variations (p.T44M, p.N127S, p.A636P, p.V722I and p.A834T) indicated by only one *in silico* assay cannot be confirmed by STEP3 assays. The only exception is *MSH2*-p.A636P, which has a strong clinical implication of pathogenicity and which has been predicted to be pathogenic in an alternative STEP3 assay due to a decrease in binding activity [Ollila et al., 2008]. *MSH2*-

Deleted: to be pathogenic

Deleted: , with the

Deleted: of

p.T44M has poor availability of clinical data, whereas the other three VUS (p.N127S, p.V722I, p.A834T) have been found in individuals with other MMR gene variations. Remarkably, a total of 8 of the 26 *MSH2* variations (p.N127S, p.I145M, p.G322D, p.M688V, p.V722I, p.A834T, p.E886G and p.V923E) have been found in carriers of other MMR gene variations. Furthermore, 7 of these variations do not appear pathogenic, although three (*MSH2*-p.N127S, *MSH2*-p.V722I and *MSH2*-p.A834T) are indicated to be deleterious by SIFT analysis. *MSH2*-p.M688V is assumed as pathogenic by both *in silico* results and decreased expression in STEP3 indicating protein instability even though *MSH2* protein was detectable by IHC. Overall, the IHC data linked to these 8 variations is either incomplete or in many cases in concordance with the additional variations found in the carriers.

Deleted:

Deleted: two

Deleted: from

Deleted: eight

Deleted: included in the verification of the three step model

Deleted: mutations

Deleted: /8

Deleted: ,

Deleted: in STEP2

Deleted: only

Deleted: on the other hand is shown to have a reduction in *Sf9* expression and hence indicates a problem in the variant

Deleted: the presence of

Deleted: is

Deleted: of STEP1

Deleted: other

Deleted: carriers of multiple MMR VUS

The verification of the three step model with the *MSH2* variations demonstrates that STEP2 is sufficient to assess the pathogenicity of 21/26 *MSH2* VUS. The confirming role of STEP3 is important in the 5 MMR proficient variations for which pathogenicity is suggested by only one *in silico* result. None of the pathogenicities indicated by only one *in silico* result is confirmed by STEP3.

Deleted: seen

Deleted: case of

Deleted: STEP2 indicates pathogenicity by one *in silico* method only and no indication of pathogenicity is seen in STEP3 assays either.

### Verification of the three step model with *MSH6* variations

Even though, none of the families carrying *MSH6* variations fulfilled the AC, the variations originate from suspected LS families with MSI-H tumor phenotypes. Only one of the 11 *MSH6* variations (p.E1193K) reliably indicates pathogenicity with the three step approach of assessment (Table 3), as seen by the lack of *MSH6* by IHC (STEP1), by *in vitro* MMR deficiency (STEP2, *in silico* results not available), and by reduced *MSH2* interaction capability (STEP3). Reduced expression of *MSH6*-p.G566R (STEP3) suggests pathogenicity, which is supported by assays measuring its ability to stimulate ATPase activity [Kariola et al., 2002; Cyr and Heinen, 2008]. Nevertheless, *MSH6*-p.G566R does not appear pathogenic in STEP2.

Deleted: the

Deleted: included in this study

Deleted: ,

No indication of pathogenicity is detectable for three *MSH6* variations (p.R128L, p.K728T, p.G881delinsKS), notably, all of which lack *MLH1* protein but not *MSH6* (or *MSH2*) according to the IHC results. The MMR deficiencies of these tumors as well as that of *MSH6*-p.P623L have indeed been shown to be due to *MLH1* promoter hypermethylation [Kariola et al., 2004], suggesting that these four VUS are non-pathogenic. Moreover, as seen with 8 *MSH2* VUS, an additional MMR gene variation, a non-pathogenic *MSH2*-p.I145M variation has been found in both *MSH6*-p.R1095H and *MSH6*-p.L1354Q carriers. There appears to be no indication of *MSH6*-p.S144I pathogenicity, however, the *in silico* results

Deleted: G881K+S

Deleted: ,

Deleted: area

Deleted: In addition, *MSH6*-S144I and *MSH6*-P1087T show no indication of pathogenicity in STEP2 or STEP3 of the assessment model and need to be reviewed more carefully, along with *MSH6*-P1087R which is indicated to be pathogenic by one SIFT analysis only.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

were not obtainable. *MSH6*-p.P1087T and *MSH6*-p.P1087R on the other hand, are predicted to be deleterious by SIFT analysis although the MMR *in vitro* and STEP3 analyses do not detect defects in protein function.

Even though the 11 *MSH6* variations do not compose an ideal data set for the verification of the three step model in assessing VUS pathogenicity, the importance of the interpretation of tumor IHC data prior to the identification of the VUS taken for further assessment is highlighted. Where the loss of MLH1 is detected by IHC, *MLH1* promotor hypermethylation analysis should be carried out prior to *MSH6* mutation analysis. The applicability of the model is further challenged by the lack of reliable *in silico* predictions for *MSH6* alterations and only if *MSH6* deficiency is indicated by STEP1, or by the exclusion of other causative mutations, the verification of the three step model is feasible.

Deleted: sequencing

Deleted: in fact,

Deleted: where

Deleted: directly

Deleted: is

### The necessity of STEP3 in the three step model

Only with 3/74 variations (*MLH1*-p.V612del, *MLH1*-p.K616del and *MSH6*-p.G566R) STEP3 results provided information not already indicated by STEP2. Remarkably, all three variations lack *in silico* results. Hence, STEP3 is useful for the verification of the STEP2 assays, yet descriptive but often not necessary for the assessment of VUS pathogenicity. The verification of the model is indicated in terms of the amount of steps required for pathogenicity assessment of each VUS. Figure 2 demonstrates the collected results of each required assessment step for each gene and its variations. STEP3 of the assessment model across the variations of all three genes serves to confirm differences between the results of the previous assessments. Furthermore, STEP3 can be utilized to confirm or clarify causes of variant pathogenicity indicated by STEP2. STEP3 of the three step model confirms the pathogenicity of 36 variations of which 31 is indicated by a reduction in expression. Where STEP3 is required, expression analysis should be the assay of choice as only 4 variations

Deleted: Del612

Deleted: Del616

Deleted: of the assessment model

Deleted: Notably

Deleted: assay

Deleted: Nevertheless



1  
2 indicate pathogenicity in STEP3 by localization or interaction analyses and not by the  
3  
4 expression analysis.  
5  
6  
7  
8

## 9 10 DISCUSSION

11  
12 Based on the application of 74 MMR gene variations and clinical data, the three step  
13 assessment model seems to be a valuable tool for correctly identifying pathogenic MMR gene  
14 mutations, which in turn permits predictive gene testing in the family and enables targeted  
15 cancer surveillance. The identification of the MMR gene for mutation analysis greatly relies  
16 on the comprehensive use of the patient's family history, MSI and IHC data. The absence of  
17 an MMR protein in IHC gives a good but not an absolute indication of the causative gene  
18 responsible for the MSI phenotype and subsequent tumorigenesis as indicated by the  
19 variations included in this study. Pathogenic *MSH2* mutations are shown to be highly  
20 associated with the lack of protein expression in IHC analyses [Mangold et al., 2005; Ollila et  
21 al., 2008], which is also frequently characterized by the absence of MSH6, the  
22 heterodimerization partner of MSH2 [Chang et al., 2000]. Furthermore, the sensitivity of IHC  
23 in predicting pathogenic *MSH6* mutations has been said to be as high as 90% [Hendriks et al.,  
24 2004]. However, IHC results demonstrating the lack of MLH1 expression may be misleading  
25 as MLH1 expression is often lost due to the hypermethylation of its promoter region [Kane et  
26 al., 1997]. In addition, the presence of a protein cannot be implied to indicate its  
27 functionality as pathogenicity can be caused by functional problems not affecting the stability  
28 of the protein [Mangold et al., 2005; Raevaara et al., 2005].  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45

Deleted: is

Deleted: LS susceptibility

Deleted: terms of clinical relevance mirror the efficiency and appropriate delivery of treatment, counseling and patient follow up.

Deleted: sequencing

Deleted: heterodimerisation

Deleted: et

46  
47 The application of the 74 variants to the three step assessment model suggests that  
48 pathogenicity is reliably indicated by the STEP2 *in vitro* MMR assay as supported by other  
49 functional assays in STEP3 (49/74, Figure 2). Nonetheless, when no indication of  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1 pathogenicity is seen in the *in vitro* MMR assay the importance of computational methods  
 2  
 3 become apparent. *In silico* methods have been shown to have a high predictive value (88.1%)  
 4 when four different methods are in agreement [Chan et al., 2007] and alignments are  
 5 manually revised [Tavtigian et al., 2008a]. As the model proposed by Couch et al. [2008]  
 6 considers the *in vitro* MMR and *in silico* assay results in a single step, we combined results  
 7 from two *in silico* approaches previously shown to be appropriate for MMR gene variations  
 8 [Ollila et al., 2006; Chao et al., 2008; Tavtigian et al., 2008b], with those obtained from the *in*  
 9 *vitro* MMR assay in order to verify the model and the necessity of STEP3. A total of 28/63  
 10 *MLH1* and *MSH2* variations are indicated as pathogenic by both *in silico* methods and in 24  
 11 of them pathogenicity is further supported by STEP3 results. Our results also suggest that in  
 12 most cases where discrepancies between SIFT and MAPP-MMR results are seen, the *in vitro*  
 13 MMR proficiency should be assumed correct. A single *in silico* result suggesting deficiency  
 14 is often linked to other ambiguities associated to the variation and is not sufficient to  
 15 characterize the MMR variation alone as seen in the case of 7 *MLH1*, 5 *MSH2*, and 2 *MSH6*  
 16 variations. Of these, only 3 *MLH1* variations were confirmed to be pathogenic by STEP3  
 17 assays. Overall, STEP3 of the assessment model is not required in cases where it does not  
 18 provide information not already revealed by STEP2. Variant deficiency can be indicated by  
 19 the *in vitro* MMR assay alone or by both *in silico* analyses, hence STEP2 suffices for the  
 20 characterization of most *MLH1* (27/37) and *MSH2* (21/26) variations. The 11 *MSH6*  
 21 variations applied to verify the model present with atypical family background and should  
 22 hence be assessed with scrutiny. Regardless of the limited selection of *MSH6* variations the  
 23 applicability of the three step model to the assessment of *MSH6* variations is not ruled out –  
 24 merely more attention to STEP1 is called for in order to eliminate phenotypes caused by other  
 25 MMR genes. As our results suggest, the MSI-H phenotype in 3/11 tumors from *MSH6* VUS  
 26 carriers is more likely to be linked to the *MLH1* promoter hypermethylation than *MSH6*

Deleted: are

Deleted: either STEP2 or

Deleted: when discrepancies between SIFT and MAPP-MMR analyses arise.

Deleted: contradictory to those obtained in the *in vitro* MMR assay

Deleted: does

Deleted: one

Deleted: 13 variations

Deleted: amino terminal

Deleted: shown

Deleted: Furthermore, 24/28 variations shown to be pathogenic by both SIFT and MAPP-MMR analyses could be confirmed by STEP3 assays, where as the remaining four variations were also MMR deficient in the *in vitro* MMR assay.

Deleted: caught

Deleted: For example, *MLH1* expression is lost its promoter hypermethylation should be assayed before the more challenging and time consuming other functional assays determining the pathogenicity of an *MSH6* VUS as in the case of 3/11 of the *MSH6* VUS presented here

variations found in the families and thus, if *MLH1* expression is lost in the tumor, its promoter hypermethylation should be assayed before the more challenging and time consuming functional assays.

Only two *MLH1* (p.V612del and p.K616del) and one *MSH6* (p.G566R) variations of the total 74 (4%) indicate pathogenicity in STEP3 for the first time. Of these, p.K616del pathogenicity is supported by the AC fulfillment, MSI and IHC data, whereas p.V612del pathogenicity is supported by only the AC fulfillment. Here, the lack of indication of pathogenicity already in STEP2 is probably due to the lack of *in silico* data, which is unfortunately the case for in frame deletions. We also want to acknowledge that the *in vitro* MMR assay was carried out using parameters, which maintain the amount of variant protein at optimal levels for detecting even the slightest repair. In the future, the assay could also be titrated to allow the detection of less prominent functional defects, possibly facilitating the assessment of carboxyl terminal *MLH1* variations, which nevertheless are currently recognized by the combination of two *in silico* analyses in STEP2.

When a variation does not appear to be causative of the LS phenotype, the presence of other predisposing variations should be considered. Carriers of 14 VUS (4 *MLH1*, 8 *MSH2* and 2 *MSH6*) included in this verification were reported to also carry other MMR gene variations. Unsurprisingly, only one (*MSH2*-p.M688V) of the 14 VUS could be considered as pathogenic, although the other mutations, *MLH1* (p.T117M) and *MSH6* (p.A1889V), identified in the *MSH2*-p.M688V carriers may also contribute towards the LS phenotype [Christensen et al., 2008, 2009]. Another problematic VUS in terms of assessment of pathogenicity is *MLH1*-p.K618A since 7 families carrying the variation show extremely variable phenotypes in terms of MSI and IHC. Even though, its slightly decreased ability to interact with PMS2 has been reported [Guerrette et al., 1999; Kondo et al., 2003], p.K618A

Deleted: three variations of the total 74 (4%),

Deleted: Del612

Deleted: Del616

Deleted: used to verify the three step assessment model

Deleted: Nevertheless it must be noted that o

Deleted: Del616

Deleted: all

Deleted: and Del612

Deleted: available clinical data, AC,

Deleted: by

Deleted: may be

Deleted: The verification of the three step model

Deleted: similar *in vitro* MMR assay

Deleted: for the functional assessment of all three genes

Deleted: T

Deleted: three step model through the

Deleted: LS susceptibility

Deleted: causing

Deleted: affecting MMR genes, and u

Deleted: included here

Deleted: likely

Deleted: . Nevertheless,

Deleted: affecting

Deleted: have been

Deleted: and

Deleted: as

Deleted: a

Deleted: suggested

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

does not appear to be pathogenic by STEP2 and STEP3 assays. Furthermore, one of the MLH1-p.K618A families was also reported to carry MLH1-p.R659Q missense mutation affecting a codon highly linked to the aberrant splicing and subsequent skipping of exon 17 (p.E633\_E663del) [Kohonen-Corish et al., 1996; Nyström-Lahti et al., 1999]. Interestingly MLH1-p.R659Q itself does not appear to be pathogenic, suchs as other VUS (p.R659P) in the same codon. Skipping of exon 17 (MLH1-p.E633\_E663del) is, however, shown to cause MMR deficiency [Nyström-Lahti et al., 2002].

Deleted: itself

Deleted: has been shown to harvest

Deleted: Del633-663

Deleted: regardless of a variation affecting

Deleted: (R659P) appearing to be so unanimately by the assays of STEP2 and STEP3.

Deleted: is

Deleted: (MLH1-Del633-663)

According to our verification, the three step assessment model is a logical and useful tool for the assessment of the pathogenicity of MMR variations as demonstrated with the 74 VUS and their clinical, laboratory and computational data. When both the *in vitro* MMR and the *in silico* assay results are available, STEP2 of the assessment model seems to be sufficient to assess the pathogenicity of most *MLH1* and *MSH2* variations. Furthermore, STEP2 also suffices to indicate non-pathogenicity to the same extent as STEP3 and is hence also important in guiding the reassessment of the cause of LS susceptibility in a family. Although, the results of this work are not yet sufficient to allow application of this specific approach in the clinical setting, they are promising and thus encourage the use of the model for a comprehensive validation study against a set of VUS that have been defined as clearly pathogenic or neutral.

Deleted: is

Deleted: ¶

#### **ACKNOWLEDGEMENTS**

This study was supported by grants from the Sigrid Juselius Foundation; the European Research council 2008-AdG 232635; Finnish Cancer Organizations; Academy of Finland; Grant number: 110300; and the Helsinki Graduate Program in Biotechnology and Molecular Biology.

**REFERENCES**

Aaltonen LA, Peltomäki P, Leach FS, Sistonen P, Pylkkänen L, Mecklin JP, Järvinen H, Powell SM, Jen J, Hamilton SR, Petersen GM, Kinzler KW, Vogelstein B, de la Chapelle A. 1993. Clues to the pathogenesis of familial colorectal cancer. *Science* 260:812-6.

[Chan PA, Duraisamy S, Miller PJ, Newell JA, McBride C, Bond JP, Raevaara T, Ollila S, Nyström M, Grimm AJ, Christodoulou J, Oetting WS, Greenblatt MS. 2007. Interpreting Missense Variants: Comparing Computational Methods in Human Disease Genes \*CDKN2A\*, \*MLH1\*, \*MSH2\*, \*MECP2\*, and Tyrosinase \(\*TYR\*\). \*Hum Mutat\* 28:683-693.](#)

Chang DK, Ricciardiello L, Goel A, Chang CL, Boland CR. 2000. Steady-state regulation of the human DNA mismatch repair system. *J Biol Chem* 275:18424-18431.

[Chao EC, Velasquez JL, Witherspoon MSL, Rozek LS, Peel D, Ng P, Gruber SB, Watson P, Rennert G, Anton-Culver H, Lynch H, Lipkin SM. 2008. Accurate Classification of \*MLH1/MSH2\* Missense Variants With Multivariate Analysis of Protein Polymorphisms-Mismatch Repair \(MAPP-MMR\). \*Hum Mutat\* 29:852-860.](#)

Christensen LL, Madsen BE, Wikman FP, Wiuf C, Koed K, Tjønneland A, Olsen A, Syvänen AC, Andersen CL, Orntoft TF. 2008. The association between genetic variants in hMLH1 and hMSH2 and the development of sporadic colorectal cancer in the Danish population. *BMC Med Genet* 9:52.

Christensen LL, Kariola R, Korhonen MK, Wikman FP, Sunde L, Gerdes AM, Okkels H, Brandt CA, Bernstein I, Hansen TVO, Hagemann-Madsen R, Andersen CL, Nyström M, Orntoft TF. 2009. Functional characterisation of rare missense mutations in MLH1 and MSH2 identified in Danish colorectal cancer patients. *Familial Cancer* 8:489-500.

1  
2 Couch FJ, Rasmussen LJ, Hofstra R, Monteiro ANA, Greenblatt MS, de Wind N, IARC  
3 unclassified variants working group. 2008. Assessment of Functional Effects of Unclassified  
4 Genetic Variants. *Hum Mutat* 29:1314-1326.  
5  
6

7  
8  
9 Cyr JL, Heinen CD. 2008. Hereditary cancer-associated missense mutations in hMSH6  
10 uncouple ATP hydrolysis from DNA mismatch binding. *J Biol Chem* 283:31641-8.  
11

12  
13 Goldgar DE, Easton DF, Byrnes GB, Spurdle AB, Iversen ES, Greenblatt MS. 2008. Genetic  
14 Evidence and Integration of Various Data Sources for Classifying Uncertain Variants Into a  
15 Single Model. *Hum Mutat* 29:1265-1272.  
16  
17

18  
19  
20 Guerrette S, Acharya S, Fishel R. 1999. The interaction of the human MutL homologues in  
21 hereditary nonpolyposis colon cancer. *J Biol Chem* 274:6336-6341.  
22  
23

24  
25 Hampel H, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, Nakawaga H, Sotamaa  
26 K, Prior TW, Westman J, Panescu J, Fix D, Lockman J, Comeras I, de la Chapelle A. 2005.  
27 Screening for the Lynch Syndrome (Hereditary Nonpolyposis Colorectal Cancer). *N Engl J*  
28 *Med* 352:1851-1860.  
29  
30  
31

32  
33 Hendriks YMC, Wagner A, Morreau H, Menko F, Stormorken A, Quehenberger F, Sandkuijl  
34 L, Møller P, Genuardi M, Van Houwelingen H, Tops C, Van Puijenbroek M, Verkuijlen P,  
35 Kenter G, Van Mil A, Meijers-Heijboer H, Tan GB, Breuning MH, Fodde R, Winjen JT,  
36 Bröcker-Vriends AHJT, Vasen H. 2004. Cancer Risk in Hereditary Nonpolyposis Colorectal  
37 Cancer Due to MSH6 Mutations: Impact on Counseling and Surveillance. *Gastroenterology*  
38 127:17-25.  
39  
40  
41  
42  
43  
44

45  
46 Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, Jessup JM, Kolodner R.  
47 1997. Methylation of the hMLH1 Promoter Correlates with Lack of Expression of hMLH1 in  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2 Sporadic Colon Tumors and Mismatch Repair-defective Human Tumor Cell Lines. *Cancer*  
3  
4 *Res* 57:808-811.

5  
6  
7 Kariola R, Raevaara TE, Lönnqvist KE, Nyström-Lahti M. 2002. Functional analysis of  
8  
9 MSH6 mutations linked to kindreds with putative hereditary non-polyposis colorectal cancer  
10  
11 syndrome. *Hum Mol Genet* 11:1303-1310.

12  
13 Kariola R, Otway R, Lönnqvist KE, Raevaara TE, de la Chapelle A, Nyström-Lahti M. 2003.  
14  
15 Two mismatch repair gene mutations found in a colon cancer patient—which one is  
16  
17 pathogenic? *Hum Genet* 112:105-9.

18  
19  
20 Kariola R, Hampel H, Frankel WL, Raevaara TE, de la Chapelle A, Nyström-Lahti M. 2004.  
21  
22 MSH6 missense mutations are often associated with no or low cancer susceptibility. *Br J*  
23  
24 *Cancer* 91:1287-92.

25  
26  
27 Kohonen-Corish M, Ross VL, Doe WF, Kool DA, Edkins E, Faragher I, Wijnen J, Khan PM,  
28  
29 Macrae F, St John DL. 1996. RNA-based mutation screening in hereditary nonpolyposis  
30  
31 colorectal cancer. *Am J Hum Genet* 59:818-24.

32  
33  
34 Kondo E, Suzuki H, Horii A, Fukushige S. 2003. A yeast two-hybrid assay provides a simple  
35  
36 way to evaluate the vast majority of hMLH1 germ-line mutations. *Cancer Res* 63:3302-3308.

37  
38 Lynch HT, de la Chapelle A. 1999. Genetic susceptibility to non-polyposis colorectal cancer.  
39  
40 *J Med Genet* 36:801-818.

41  
42  
43 Mangold E, Pagenstecher C, Friedl W, Fischer HP, Merkelbach-Bruse S, Ohlendorf M,  
44  
45 Friedrichs N, Aretz S, Buettner R, Propping P, Mathiak M. 2005. Tumours from MSH2  
46  
47 mutation carriers show loss of MSH2 expression but many tumours from MLH1 carriers  
48  
49 exhibit weak positive MLH1 staining. *J Pathol* 207:385-95.

1  
2 Ng PC, Henikoff S. 2001. Predicting deleterious amino acid substitutions. *Genome Res*  
3  
4 11:863-874.  
5

6  
7 Nyström-Lahti M, Perrera C, Räschle M, Panyushkina-Seiler E, Marra G, Curci A,  
8  
9 Ouaresima B, Conzanzo F, D'Urso M, Venuta S, Jiricny J. 2002. Functional analysis of  
10  
11 MLH1 mutations linked to hereditary nonpolyposis colon cancer. *Genes Chromosomes*  
12  
13 *Cancer* 33:160-167.  
14

15  
16 Nyström-Lahti M, Holmberg M, Fidalgo P, Salovaara R, de la Chapelle A, Jiricny J,  
17  
18 Peltomäki P. 1999. Missense and nonsense mutations in codon 659 of MLH1 cause aberrant  
19  
20 splicing of messenger RNA in HNPCC kindreds. *Genes Chromosomes Cancer* 26:372-375.  
21

22  
23 [Ollila S, Sarantaus L, Kariola R, Chan P, Hampel H, Holinski-Feder E, Macrae F, Kohonen-](#)  
24  
25 [Corish M, Gerdes AM, Peltomäki P, Mangold E, de la Chapelle A, Greenblatt M, Nyström](#)  
26  
27 [M. 2006. Pathogenicity of MSH2 Missense Mutations Is Typically Associated With Impaired](#)  
28  
29 [Repair Capability of the Mutated Protein. \*Gastroenterology\* 131:1408-1417.](#)  
30

31  
32 Ollila S, Dermadi Bebek D, Jiricny J, Nyström M. 2008. Mechanisms of pathogenicity in  
33  
34 human MSH2 missense mutants. *Hum Mutat* 29:1355-63.  
35

36  
37 Raevaara TE, Vaccaro C, Abdel-Rahman WM, Mocetti E, Bala S, Lönnqvist KE, Kariola R,  
38  
39 Lynch HT, Peltomäki P, Nyström-Lahti M. 2003. Pathogenicity of the Hereditary Colorectal  
40  
41 Cancer Mutation hMLH1 del616 Linked to Shortage of the Functional Protein.  
42  
43 *Gastroenterology* 125:501-509.  
44

45  
46 Raevaara TE, Korhonen MK, Lohi H, Hampel H, Lynch E, Lönnqvist KE, Holinski-Feder E,  
47  
48 Sutter C, McKinnon W, Duraisamy S, Gerdes AM, Peltomäki P, Kohonen-Corish M,  
49  
50 Mangold E, Macrae F, Greenblatt M, de la Chapelle A, Nyström M. 2005. Functional  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60



1  
2 significance and clinical phenotype of nontruncating mismatch repair variants of MLH1.  
3  
4 Gastroenterology 129:537-49.  
5

6  
7 Stone EA, Sidow A. 2005. Physiochemical constraint violation by missense substitutions  
8  
9 mediates impairment of protein function and disease severity. Genome Res 15:978-986.  
10

11 [Tavtigian SV, Byrnes GB, Goldgar DE, Thomas A. 2008a. Classification of Rare Missense](#)  
12 [Substitutions, Using Risk Surfaces, With Genetic- and Molecular- Epidemiology](#)  
13 [Applications. Hum Mutat 29:1342-1354.](#)  
14  
15  
16

17  
18 [Tavtigian SV, Greenblatt MS, Lesueur F. 2008b. In silico analysis of missense substitutions](#)  
19 [using sequence-alignment based methods. Hum Mutat 29:1327-1336.](#)  
20  
21  
22

23  
24 Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lindor  
25  
26 NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT,  
27  
28 Peltomäki P, Ramsey SD, Rodriguez-Bigas MA, Vasen HFA, Hawk ET, Barrett JC,  
29  
30 Freedman AN, Srivastava S. 2004. Revised Bethesda Guidelines for hereditary nonpolyposis  
31  
32 colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 96:261-  
33  
34 268.  
35

36  
37 Vasen HFA, Mecklin J-P, Khan PM, Lynch HT. 1991. The International Collaborative Group  
38  
39 on Hereditary Non-polyposis Colorectal Cancer (ICG-HNPCC). Dis Colon Rectum 34:424-  
40  
41 425.  
42

43  
44 Vasen HFA, Watson P, Mecklin J-P, Lynch HT, the ICG-HNPCC. 1999. New clinical  
45  
46 criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed  
47  
48 by the international collaborative group on HNPCC. Gastroenterology 116:1453-1456.  
49  
50

**FIGURE LEGENDS**

**FIGURE 1.** A three step decision tree proposed to facilitate the functional assessment of VUS. (Modified from Couch et al. [2008])

**Deleted:** with three steps

**FIGURE 2.** Schematic illustration of (A) MLH1 (B) MSH2 and (C) MSH6 showing the known functional domains, locations of the studied variations and the amount of steps required for their assessment of pathogenicity. Each required step of the three step assessment model is represented with a circle. STEP1 is divided to indicate the accordance of the family history with the Amsterdam criteria I/II (lower half: Black, AC fulfilled; White, AC not fulfilled; Gray, AC fulfilled by some families; Diagonal line, data not available) and the MSI and IHC results of the tumor (upper half: Black, MSI-H and/or reduced protein expression (IHC); White, no MSI-H and no problems in protein expression (IHC); Gray, contradicting data between several families; Diagonal line, data not available). STEP2 is divided to indicate variant protein *in vitro* MMR activity (lower half: Black, deficient; White, proficient) and *in silico* results (upper half: Black, pathogenic effect predicted by SIFT and MAPP-MMR; White, neutral effect predicted by SIFT and MAPP-MMR; Gray, discordant or only one result, either pathogenic or nonpathogenic available; Diagonal line, data not available). STEP3 assay results are combined to assess VUS pathogenicity (Black, results indicating pathogenicity; White, results indicating non-pathogenicity; Gray, STEP inconclusive; Diagonal line, data not available.)

**Deleted:** :

**Deleted:** Grey

**Deleted:** Grey

**Deleted:** For

**Deleted:** and

**Deleted:** For each VUS only the steps required for the assessment of pathogenicity are included.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

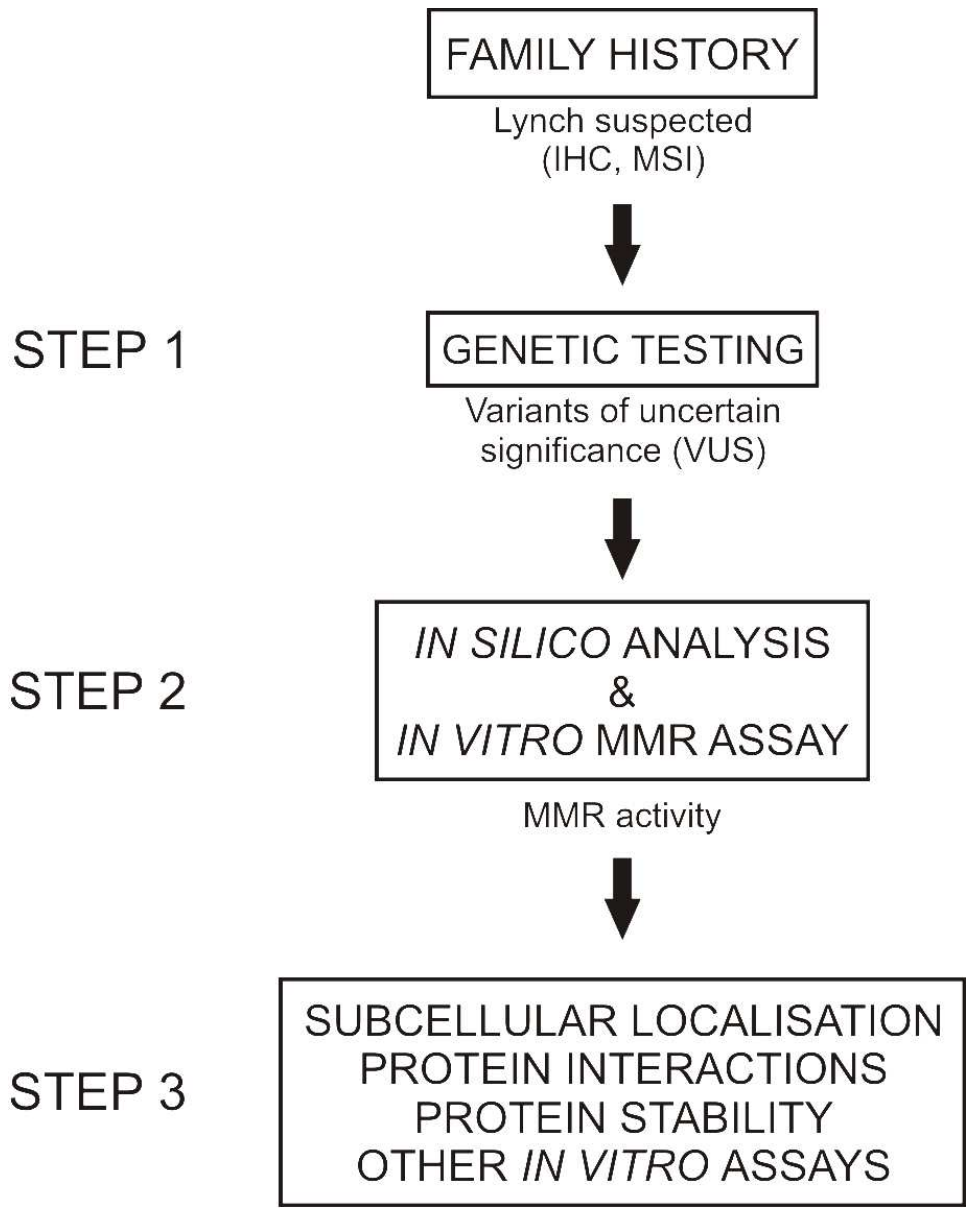


FIGURE 1. A three step decision tree proposed to facilitate the functional assessment of VUS.  
(Modified from Couch et al. [2008])  
77x97mm (300 x 300 DPI)

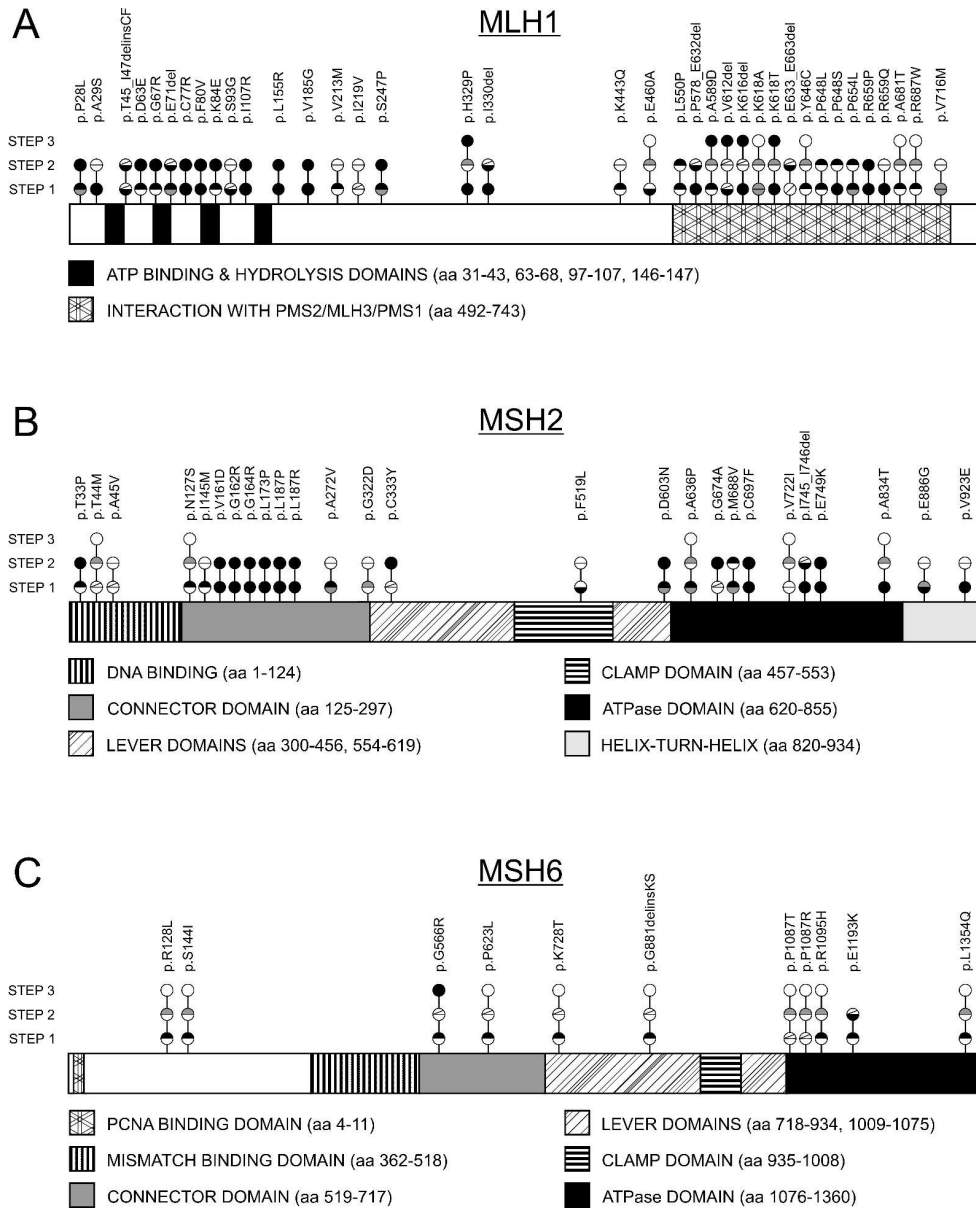


FIGURE 2. Schematic illustration of (A) MLH1 (B) MSH2 and (C) MSH6 showing the known functional domains, locations of the studied variations and the amount of steps required for their assessment of pathogenicity. Each required step of the three step assessment model is represented with a circle. STEP1 is divided to indicate the accordance of the family history with the Amsterdam criteria I/II (lower half: Black, AC fulfilled; White, AC not fulfilled; Gray, AC fulfilled by some families; Diagonal line, data not available) and the MSI and IHC results of the tumor (upper half: Black, MSI-H and/or reduced protein expression (IHC); White, no MSI-H and no problems in protein expression (IHC); Gray, contradicting data between several families; Diagonal line, data not available). STEP2 is divided to indicate variant protein in vitro MMR activity (lower half: Black, deficient; White, proficient) and in silico results (upper half: Black, pathogenic effect predicted by SIFT and MAPP-MMR; White, neutral effect predicted by SIFT and MAPP-MMR; Gray, discordant or only one result, either pathogenic or nonpathogenic available; Diagonal line, data not available). STEP3 assay results are combined to assess VUS pathogenicity (Black, results indicating pathogenicity; White,

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

results indicating non-pathogenicity; Gray, STEP inconclusive; Diagonal line, data not available.)

200x248mm (600 x 600 DPI)

For Peer Review

TABLE 1. Verification Data of *MLH1* Variations

STEP 1			<i>MLH1</i> (NM_000249.3)		STEP 2		STEP 3				
AC <sup>a</sup>	MSI <sup>b</sup>	IHC <sup>b</sup>			VUS		MMR ASSAY <sup>d</sup>	IN SILICO <sup>e</sup>	EXPRESSION <sup>d</sup>	LOCALIZATION <sup>d</sup>	INTERACTION <sup>d</sup>
		MLH1	MSH2	MSH6	NUCLEOTIDE CHANGE <sup>c</sup>	AMINO ACID CHANGE					
					c.83C>T	p.P28L					
					c.85G>T	p.A29S <sup>f</sup>					
					c.133_141delinsTGTTTT	p.T45_147delinsCF					
					c.189C>A	p.D63E					
					c.199G>A	p.G67R					
					c.211_213del	p.E71del					
					c.229T>C	p.C77R					
					c.238T>G	p.F80V					
					c.250A>G	p.K84E					
					c.277A>G	p.S93G					
					c.320T>G	p.I107R					
					c.464T>G	p.L155R					
					c.554T>G	p.V185G					
					c.637G>A	p.V213M					
					c.655A>G	p.I219V					
					c.739T>C	p.S247P					
					c.986A>C	p.H329P					
					c.988_990del	p.I330del					
					c.1327A>C	p.K443Q					
					c.1379A>C	p.E460A <sup>f</sup>					
					c.1649T>C	p.L550P					
					c.1734_1896del	p.P578_E632del					
					c.1766C>A	p.A589D					
					c.1834_1836del	p.V612del					
					c.1846_1848del	p.K616del					
					c.1852_1853AA>GC	p.K618A <sup>f</sup>					
					c.1853A>C	p.K618T					
					c.1897_1989del	p.E633_E663del					
					c.1937A>G	p.Y646C					
					c.1943C>T	p.P648L					
					c.1942C>T	p.P648S					
					c.1961C>T	p.P654L					
					c.1976G>C	p.R659P					
					c.1976G>A	p.R659Q <sup>f</sup>					
					c.2041G>A	p.A681T					
					c.2059C>T	p.R687W					
					c.2146G>A	p.V716M					

<sup>a</sup>Black, ACI fulfilled; White, ACI not fulfilled; Gray, ACI fulfilled in some families; A diagonal line, data not available.

<sup>b</sup>Black, results indicating pathogenicity; White, results indicating non-pathogenicity; Gray, discrepancies between individual tumor data (MSI & IHC); A diagonal line, data not available.

<sup>c</sup>Nucleotide numbering reflects cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen).

<sup>d</sup>Black, results indicating pathogenicity; White, results indicating non-pathogenicity; A diagonal line, data not available.

<sup>e</sup>Black, deleterious by SIFT and MAPP-MMR; White, neutral by SIFT and MAPP-MMR; Gray, deleterious by SIFT or MAPP-MMR only; A diagonal line, data not available; Vertically divided field indicates the availability of one result only.

<sup>f</sup>Families with additional MMR gene mutations: p.A29S, *MLH1*:c.-27C>A; p.E460A, *MSH2*:deletion of exon 8, *MSH2*:p.M663fs; p.K618A, *MLH1*:c.1976G>A (p.R659Q), p.R659Q, *MLH1*:c.1852\_1853AA>GC (p.K618A).

179x265mm (600 x 600 DPI)

TABLE 1. Verification Data of *MLH1* Variations

STEP 1			<i>MLH1</i> (NM_000249.3)		STEP 2		STEP 3				
AC <sup>a</sup>	MSI <sup>b</sup>	IHC <sup>b</sup>			VUS		MMR ASSAY <sup>d</sup>	IN SILICO <sup>e</sup>	EXPRESSION <sup>d</sup>	LOCALIZATION <sup>d</sup>	INTERACTION <sup>d</sup>
		MLH1	MSH2	MSH6	NUCLEOTIDE CHANGE <sup>c</sup>	AMINO ACID CHANGE					
					c.83C>T	p.P28L					
					c.85G>T	p.A29S <sup>f</sup>					
					c.133_141delinsTGTTTT	p.T45_I47delinsCF					
					c.189C>A	p.D63E					
					c.199G>A	p.G67R					
					c.211_213del	p.E71del					
					c.229T>C	p.C77R					
					c.238T>G	p.F80V					
					c.250A>G	p.K84E					
					c.277A>G	p.S93G					
					c.320T>G	p.I107R					
					c.464T>G	p.L155R					
					c.554T>G	p.V185G					
					c.637G>A	p.V213M					
					c.655A>G	p.I219V					
					c.739T>C	p.S247P					
					c.986A>C	p.H329P					
					c.988_990del	p.I330del					
					c.1327A>C	p.K443Q					
					c.1379A>C	p.E460A <sup>f</sup>					
					c.1649T>C	p.L550P					
					c.1734_1896del	p.P578_E632del					
					c.1766C>A	p.A589D					
					c.1834_1836del	p.V612del					
					c.1846_1848del	p.K616del					
					c.1852_1853AA>GC	p.K618A <sup>f</sup>					
					c.1853A>C	p.K618T					
					c.1897_1989del	p.E633_E663del					
					c.1937A>G	p.Y646C					
					c.1943C>T	p.P648L					
					c.1942C>T	p.P648S					
					c.1961C>T	p.P654L					
					c.1976G>C	p.R659P					
					c.1976G>A	p.R659P <sup>f</sup>					
					c.2041G>A	p.A681T					
					c.2059C>T	p.R687W					
					c.2146G>A	p.V716M					

<sup>a</sup> Black, ACI fulfilled; White, ACI not fulfilled; Gray, ACI fulfilled in some families; A diagonal line, data not available.

<sup>b</sup> Black, results indicating pathogenicity; White, results indicating non-pathogenicity; Gray, discrepancies between individual tumor data (MSI & IHC); A diagonal line, data not available.

<sup>c</sup> Nucleotide numbering reflects cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)).

<sup>d</sup> Black, results indicating pathogenicity; White, results indicating non-pathogenicity; A diagonal line, data not available.

<sup>e</sup> Black, deleterious by SIFT and MAPP-MMR; White, neutral by SIFT and MAPP-MMR; Gray, deleterious by SIFT or MAPP-MMR only; A diagonal line, data not available; Vertically divided field indicates the availability of one result only.

<sup>f</sup> Families with additional MMR gene mutations: **p.A29S**, *MLH1*:c.-27C>A; **p.E460A**, *MSH2*:deletion of exon 8, *MSH2*:p.met663fs; **p.K618A**, *MLH1*:c.1976G>A (p.R659Q), **p.R659Q**, *MLH1*:c.1852\_1853AA>GC (p.K618A).

TABLE 2. Verification Data of MSH2 Variations

STEP 1				MSH2 (NM_000251.1)		STEP 2		STEP 3		
AC <sup>a</sup>	MSI <sup>b</sup>	IHC <sup>b</sup>			VUS		MMR ASSAY <sup>d</sup>	IN SILICO <sup>e</sup>	EXPRESSION <sup>d</sup>	INTERACTION <sup>d</sup>
		MLH1	MSH2	MSH6	NUCLEOTIDE CHANGE <sup>c</sup>	AMINO ACID CHANGE				
					c.97A>C	p.T33P				
					c.131C>T	p.T44M				
					c.134C>T	p.A45V				
					c.380A>G	p.N127S <sup>f</sup>				
					c.435T>G	p.I145M <sup>f</sup>				
					c.482T>A	p.V161D				
					c.484G>A	p.G162R				
					c.490G>A	p.G164R				
					c.518T>C	p.L173P				
					c.560T>C	p.L187P				
					c.560T>G	p.L187R				
					c.815C>T	p.A272V				
					c.965G>A	p.G322D <sup>f</sup>				
					c.998G>A	p.C333Y				
					c.1555T>C	p.F519L				
					c.1807G>A	p.D603N				
					c.1906G>C	p.A636P				
					c.2021G>C	p.G674A				
					c.2062A>G	p.M688V <sup>f</sup>				
					c.2090G>T	p.C697F				
					c.2164G>A	p.V722I <sup>f</sup>				
					c.2235_2240del	p.I745_I746del				
					c.2245G>A	p.E749K				
					c.2500G>A	p.A834T <sup>f</sup>				
					c.2657A>G	p.E886G <sup>f</sup>				
					c.2768T>A	p.V923E <sup>f</sup>				

<sup>a</sup> Black, ACII fulfilled; White, ACII not fulfilled; Gray, ACII fulfilled in some families; A diagonal line, data not available.

<sup>b</sup> Black, results indicating pathogenicity; White, results indicating non-pathogenicity; Gray, discrepancies between individual tumor data (MSI & IHC); A diagonal line, data not available.

<sup>c</sup> Nucleotide numbering reflects cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen).

<sup>d</sup> Black, results indicating pathogenicity; White, results indicating non-pathogenicity; A diagonal line, data not available.

<sup>e</sup> Black, deleterious by SIFT and MAPP-MMR; White, neutral by SIFT and MAPP-MMR; Gray, deleterious by SIFT or MAPP-MMR only; A diagonal line, data not available.

<sup>f</sup> Families with additional MMR gene mutations: p.N127S, MSH2:c.982G>C, MSH2:c.1264G>T, MLH1:c.1877del, MLH1:c.IVS15\_5T>C, MLH1:c.395T>C; p.I145M, MSH6:c.3284G>A (p.R1095H), MSH6:c.4061T>A (p.L1354Q); p.G322D, MSH2:c.1552C>T, MLH1:c.350C>T; p.M688V, MLH1:c.350C>T, MSH6:c.4016C>T; p.V722I, MLH1:c.1039 -8 T>A; p.A834T, MSH2:deletion of exon 8; p.E886G, MSH6: n/a; p.V923E, MSH6:c.3563G>A

178x224mm (600 x 600 DPI)



TABLE 2. Verification Data of *MSH2* Variations

STEP 1			<i>MSH2</i> (NM_000251.1)		STEP 2		STEP 3			
AC <sup>a</sup>	MSI <sup>b</sup>	IHC <sup>b</sup>			VUS		MMR ASSAY <sup>d</sup>	IN SILICO <sup>e</sup>	EXPRESSION <sup>d</sup>	INTERACTION <sup>d</sup>
		MLH1	MSH2	MSH6	NUCLEOTIDE CHANGE <sup>c</sup>	AMINO ACID CHANGE				
					c.97A>C	p.T33P				
					c.131C>T	p.T44M				
					c.134C>T	p.A45V				
					c.380A>G	p.N127S <sup>f</sup>				
					c.435T>G	p.I145M <sup>f</sup>				
					c.482T>A	p.V161D				
					c.484G>A	p.G162R				
					c.490G>A	p.G164R				
					c.518T>C	p.L173P				
					c.560T>C	p.L187P				
					c.560T>G	p.L187R				
					c.815C>T	p.A272V				
					c.965G>A	p.G322D <sup>f</sup>				
					c.998G>A	p.C333Y				
					c.1555T>C	p.F519L				
					c.1807G>A	p.D603N				
					c.1906G>C	p.A636P				
					c.2021G>C	p.G674A				
					c.2062A>G	p.M688V <sup>f</sup>				
					c.2090G>T	p.C697F				
					c.2164G>A	p.V722I <sup>f</sup>				
					c.2235_2240del	p.I745_I746del				
					c.2245G>A	p.E749K				
					c.2500G>A	p.A834T <sup>f</sup>				
					c.2657A>G	p.E886G <sup>f</sup>				
					c.2768T>A	p.V923E <sup>f</sup>				

<sup>a</sup> Black, ACII fulfilled; White, ACII not fulfilled; Gray, ACII fulfilled in some families; A diagonal line, data not available.

<sup>b</sup> Black, results indicating pathogenicity; White, results indicating non-pathogenicity; Gray, discrepancies between individual tumor data (MSI & IHC); A diagonal line, data not available.

<sup>c</sup> Nucleotide numbering reflects cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)).

<sup>d</sup> Black, results indicating pathogenicity; White, results indicating non-pathogenicity; A diagonal line, data not available.

<sup>e</sup> Black, deleterious by SIFT and MAPP-MMR; White, neutral by SIFT and MAPP-MMR; Gray, deleterious by SIFT or MAPP-MMR only; A diagonal line, data not available

<sup>f</sup> Families with additional MMR gene mutations: p.N127S, *MSH2*:c.982G>C, *MSH2*:c.1264G>T, *MLH1*:c.1877del, *MLH1*:c.IVS15\_5T>C, *MLH1*:c.395T>C; p.I145M, *MSH6*:c.3284G>A (p.R1095H), *MSH6*:c.4061T>A (p.L1354Q); p.G322D, *MSH2*:c.1552C>T, *MLH1*:c.350C>T; p.M688V, *MLH1*:c.350C>T, *MSH6*:c.4016C>T; p.V722I, *MLH1*:c.1039\_8T>A; p.A834T, *MSH2*:deletion of exon 8; p.E886G, *MSH6*: n/a; p.V923E, *MSH6*: c.3563G>A

TABLE 3. Verification Data of *MSH6* Variations

STEP 1			<i>MSH6</i> (NM_000179.2)		STEP 2		STEP 3			
AC <sup>a</sup>	MSI <sup>b</sup>	IHC <sup>b</sup>			VUS		MMR ASSAY <sup>d</sup>	IN SILICO <sup>e</sup>	EXPRESSION <sup>d</sup>	INTERACTION <sup>d</sup>
		MLH1	MSH2	MSH6	NUCLEOTIDE CHANGE <sup>c</sup>	AMINO ACID CHANGE				
					c.383G>T	p.R128L <sup>f</sup>				
					c.431G>T	p.S144I				
					c.1696G>A	p.G566R				
					c.1868C>T	p.P623L <sup>f</sup>				
					c.2183A>C	p.K728T <sup>f</sup>				
					c.2641delinsAAAA	p.G881delinsKS <sup>f</sup>				
					c.3259C>A	p.P1087T				
					c.3260C>G	p.P1087R				
					c.3284G>A	p.R1095H <sup>g</sup>				
					c.3577G>A	p.E1193K				
					c.4061T>A	p.L1354Q <sup>g</sup>				

<sup>a</sup> Black, ACII fulfilled; White, ACII not fulfilled; Gray, ACII fulfilled in some families; A diagonal line, data not available.

<sup>b</sup> Black, results indicating pathogenicity; White, results indicating non-pathogenicity; Gray, discrepancies between individual tumor data (MSI & IHC); A diagonal line, data not available.

<sup>c</sup> Nucleotide numbering reflects cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)).

<sup>d</sup> Black, results indicating pathogenicity; White, results indicating non-pathogenicity; A diagonal line, data not available

<sup>e</sup> Black, deleterious by SIFT and MAPP-MMR; White, neutral by SIFT and MAPP-MMR; Gray, deleterious by SIFT or MAPP-MMR only; A diagonal line, data not available; Vertically divided field indicates the availability of one result only.

<sup>f</sup> *MLH1* promoter hypermethylation found in tumors.

<sup>g</sup> Families with additional MMR gene mutations: p.R1095H, *MSH2*:c.435T>G (p.I145M); p.L1354Q, *MSH2*:c.435T>G (p.I145M)

170x161mm (600 x 600 DPI)



TABLE 3. Verification Data of *MSH6* Variations

STEP 1			<i>MSH6</i> (NM_000179.2)		STEP 2		STEP 3			
AC <sup>a</sup>	MSI <sup>b</sup>	IHC <sup>b</sup>			VUS		MMR ASSAY <sup>d</sup>	IN SILICO <sup>e</sup>	EXPRESSION <sup>d</sup>	INTERACTION <sup>d</sup>
		MLH1	MSH2	MSH6	NUCLEOTIDE CHANGE <sup>c</sup>	AMINO ACID CHANGE				
					c.383G>T	p.R128L <sup>f</sup>				
					c.431G>T	p.S144I				
					c.1696G>A	p.G566R				
					c.1868C>T	p.P623L <sup>f</sup>				
					c.2183A>C	p.K728T <sup>f</sup>				
					c.2641delinsAAAA	p.G881delinsKS <sup>f</sup>				
					c.3259C>A	p.P1087T				
					c.3260C>G	p.P1087R				
					c.3284G>A	p.R1095H <sup>g</sup>				
					c.3577G>A	p.E1193K				
					c.4061T>A	p.L1354Q <sup>g</sup>				

<sup>a</sup> Black, ACII fulfilled; White, ACII not fulfilled; Gray, ACII fulfilled in some families; A diagonal line, data not available.

<sup>b</sup> Black, results indicating pathogenicity; White, results indicating non-pathogenicity; Gray, discrepancies between individual tumor data (MSI & IHC); A diagonal line, data not available.

<sup>c</sup> Nucleotide numbering reflects cDNA with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)).

<sup>d</sup> Black, results indicating pathogenicity; White, results indicating non-pathogenicity; A diagonal line, data not available.

<sup>e</sup> Black, deleterious by SIFT and MAPP-MMR; White, neutral by SIFT and MAPP-MMR; Gray, deleterious by SIFT or MAPP-MMR only; A diagonal line, data not available; Vertically divided field indicates the availability of one result only.

<sup>f</sup> *MLH1* promoter hypermethylation found in tumors

<sup>g</sup> Families with additional MMR gene mutations: p.R1095H, *MSH2*:c.435T>G (p.I145M); p.L1354Q, *MSH2*:c.435T>G (p.I145M)