Expression Defect Size among Unclassified MLH1 Variants Determines Pathogenicity in Lynch Syndrome Diagnosis

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Abstract

**Purpose:** Lynch syndrome is caused by a germline mutation in a mismatch repair gene, most commonly the MLH1 gene. However, one third of the identified alterations are missense variants with unclear clinical significance. The functionality of these variants can be tested in the laboratory, but the results cannot be used for clinical diagnosis. We therefore aimed to establish a laboratory test that can be applied clinically.

**Experimental Design:** We assessed the expression, stability, and mismatch repair activity of 38 MLH1 missense variants and determined the pathogenicity status of recurrent variants using clinical data.

**Results:** Four recurrent variants were classified as neutral (K618A, H718Y, E578G, V716M) and three as pathogenic (A681T, L622H, P654L). All seven variants were proficient in mismatch repair but showed defects in expression. Quantitative PCR, pulse-chase, and thermal stability experiments confirmed decreases in protein stability, which were stronger in the pathogenic variants. The minimal cellular MLH1 concentration for mismatch repair was determined, which corroborated that strongly destabilized variants can cause repair deficiency. Loss of MLH1 tumor immunostaining is consistently reported in carriers of the pathogenic variants, showing the impact of this protein instability on these tumors.

**Conclusions:** Expression defects are frequent among MLH1 missense variants, but only severe defects cause Lynch syndrome. The data obtained here enabled us to establish a threshold for distinguishing tolerable (clinically neutral) from pathogenic expression defects. This threshold allows the translation of laboratory results for uncertain MLH1 variants into pathogenicity statements for diagnosis, thereby improving the targeting of cancer prevention measures in affected families. Clin Cancer Res; 19(9); 2432–41. ©2013 AACR.

Introduction

Lynch syndrome is a hereditary predisposition for cancer that accounts for 2% to 5% of all colorectal cancers (MIM #120435; refs. 1, 2). In addition, the tumor risk for some other organs, especially the endometrium, is increased. Lynch syndrome is a relatively common genetic disorder: approximately 1 in 660–2,000 individuals is affected (3). It is caused by heterozygous germline mutational inactivation of 1 of 4 mismatch repair (MMR) genes (MLH1, MSH2, MSH6 or PMS2). Somatic loss of the remaining wild-type allele leads to microsatellite instability (MSI), which is a hallmark of Lynch syndrome tumors (4, 5).

To establish a diagnosis of Lynch syndrome and offer predictive testing for family members, an inactivating germline mutation has to be identified. Mutations in the MLH1 gene (MIM #120436) account for the majority of Lynch syndrome cases. One-third of all alterations found in this gene are missense variants (6). The clinical significance of these variants is unknown a priori, and they are therefore termed unclassified variants (7) and cannot be used to establish a diagnosis. Consequently, relatives of carriers cannot be offered predictive testing, and preventive surveillance cannot be properly targeted.

The internationally growing awareness of Lynch syndrome is leading to increases in systematic screening. In conjunction with the improved affordability of sequencing, this will likely result in the identification of growing numbers of unclassified variants in the future (8). Therefore, methods to correlate these mismatch repair gene unclassified variants with their clinical consequences are urgently required (9). For this purpose, both clinical and functional laboratory evidence may be used.

Because few alterations occur recurrently, and sufficient cosegregation data are rare, the available clinical information is insufficient in most cases. Therefore, we and others have exerted significant efforts during the last decade to assess the effects of MLH1 unclassified variants using
Translational Relevance
Lynch syndrome is the most common type of heritable predisposition for cancers of the colon and endometrium. To establish diagnosis of this syndrome, an inactivating mutation in a DNA mismatch repair gene has to be found. However, the most commonly affected gene (MLH1) frequently shows missense variations of unknown relevance, and no diagnosis can therefore be established for affected families. Functional tests have not yet solved this problem because no correlation between test readouts and clinical consequences has been established. Here, we report a quantitative link between impaired protein stability among MLH1 variants and cancer risk. The described testing system allows diagnosis of Lynch syndrome to be made based on a laboratory test for a variant for the first time. We also show that stability constraints are the most frequent consequence of MLH1 missense variations and are therefore the most relevant molecular cause of carcinogenesis and the most sensitive parameter for diagnosis.

Expression Defect Size Determines MLH1 Variant Pathogenicity

**Protein expression and expression quantification**

pDNA3-MLH1, pSG5-PM2, and the HEK293 and HEK293T cell lines have been described previously (13, 24). Missense variants were generated via site-directed mutagenesis (QuickChange II Kit, Stratagene) and confirmed by direct sequencing. HEK293T cells were transiently transfected with 5 µg of vector DNA and 20 µl of polyethyleneimine (1 mg/mL, linear, 25 kDa, Polysciences) and extracted as described previously (24, 25). The extracts were analyzed via SDS-PAGE and immunoblotting (using anti-MLH1, G168-728, BD Biosciences, and anti-PM2, E-19, and anti-β-actin, C2, from Santa Cruz Biotechnologies). Chemiluminescence signals (Immobilon, Millipore) were detected in an LAS-4000 mini camera (Fuji) and quantified using Multi Gauge v3.2.

**qPCR analysis of MLH1 transcription**

MLH1 transcript levels were measured using quantitative PCR (qPCR) according to the MIQE guidelines (26). Total RNA was extracted from transfected HEK293T cells using TRIzol (Invitrogen). The RNA was then dissolved in 50 µL of RNase-free water, and the RNA content was quantified via UV spectrometry. The RNA was treated then with DNase (DNase RQ1, Promega) to remove potential residual plasmid DNA. The success of the DNase treatment was controlled via PCR analysis. cDNA was generated from 1 µg of total RNA. This RNA had either been prepared fresh or came from samples that had been stored at −80°C for less than one month and not thawed more than twice. Reverse transcription was conducted for 10 minutes at 25°C, followed by 50 minutes at 50°C, with M-MLV reverse transcriptase (50U, RNase H Minus point mutant, Promega) and 250 ng of random primers (Promega) according to the manufacturers’ recommendations in a total volume of 25 µL. The cDNA samples were stored at −20°C.

Primer and probe sequences were designed using FileBuilder software and produced by Applied Biosystems. A PCR product spanning exons 12–13 was used because this area corresponds to the unconserved linker region of the MLH1 protein where few genetic alterations have been reported; therefore, mRNA from all variant cDNA constructs could be quantified using the same primers and probe, without the interference of the individual genetic alterations. The primers used in these assays were as follows (mRNA sequence NM_000249.3): AGAGAGACCTACTTCCAGCAA (f), ATCTTCCACCATTTCCACATCAGAA (r) and CCCCAGAAAGAGACATC (hydrolysis probe). The obtained amplicon length was 71 bp. The hydrolysis probes contained fluorescein amidite (FAM) as a reporter dye and a nonfluorescent quencher. Calibration curves generated from dilutions of the MLH1 plasmid showed that the qPCR results were linear over a wide range (Supplementary Fig. S1). The only reference gene used was GAPDH (assay #Hs99999905_m1, Applied Biosystems) because all samples were from the same source material and of the same quality.

Control qPCR assays with RNA but without reverse transcription were conducted, and no amplification occurred. In addition, untransfected HEK293T cells were

Materials and Methods

**Selection of variants for analysis**

A total of 38 MLH1 missense variants were selected from public MLH1 variation databases; these variants included 7 repair-proficient and 3 repair-deficient recurrent alterations for which large amounts of clinical data are available. Additional variants were selected arbitrarily.
always analyzed in parallel and yielded much lower levels of amplification (Cq 10 cycles higher than the transfected samples on average). The qPCR assays were conducted in a total volume of 15 μL, which included TaqMan universal master mix, an assay mixture containing the primers and hydrolysis probe, and 1.5 μL of a sample. The cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 60 cycles of 15 seconds at 95°C and 1 minute at 60°C. qPCR was conducted in a StepOnePlus Realtime cycler (Applied Biosystems). The StepOne 2.0 software was used to generate qPCR curves and Cq values.

To calculate MLH1 transcript expression, the samples were normalized on the basis of the results for GAPDH

\[ \Delta C_q = C_q(MLH1) - C_q(GAPDH) \]

Subsequently, the variants were compared to the calibrator (wild-type MLH1) by calculating the \( \Delta \Delta C_q \) value

\[ \Delta \Delta C_q(\text{variant}) = \Delta C_q(\text{variant}) - \Delta C_q(\text{wild-type}) \]

Relative expression was calculated using the standard formula

\[ f = 2^{-\Delta \Delta C_q} \]

Determination of MMR activity

The MMR activity of MLH1 variants was scored in vitro as described previously (25). Briefly, protein extracts were mixed with 35 ng of DNA substrate containing a G-T single-strand nick at a distance of 83 bp with reaction buffer in a total volume of 15 μL. After incubation at 37°C, the DNA substrate was purified and digested with EcoRV and AseI. The restriction fragments were separated in agarose gels and analyzed using GelDoc XR plus detection and QuantityOne software (Bio-Rad). The repair efficiency (e) was calculated as:

\[ e = \frac{(\text{intensity of bands of repaired substrate})}{(\text{intensity of all bands of substrate})} \]

This result is independent of the amount of DNA recovered through plasmid purification. The typical total repair efficiencies ranged from 50% to 90%. The repair efficiency of MutLα variants was analyzed in direct comparison with a wild-type protein that had been produced in parallel, and calculated as e(relative) = e(variant)/e(wild-type)×100.

Collection of clinical data and in silico analyses

Publications addressing variants were identified using the Leiden Open Variation Database (LOVD; www.LOVD.nl/MLH1), the Mismatch Repair Gene Variants Database (www.med.mun.ca/mmrvariants), and the MMR Gene Unclassified Variants Database (http://www.mmrmis-sense.net). Moreover, searches were conducted for all alternative variant descriptions (e.g., MLH1 "V716M", "Val716-Met", "2146G>A") in PubMed and Google. Comprehensive clinical information was obtained from a recent publication by Hardt and colleagues from the German HNPPC consortium (15). Each reported patient carrying an alteration and exhibiting a Lynch-syndrome–associated tumor was listed in Supplementary Table S1 with all available information. Great care was taken to detect multiple reports of identical patients based on their patient/family identifiers, the reporting authors, the country of origin or other strikingly identical features; when such cases were found, all information was summarized in a single entry.

In some cases, the authors were contacted to resolve contradictions.

Structural and bioinformatic analyses

Function–structure evaluations were conducted with a model of human MLH1-PMS2 based on the structure of human PMS2-NTD (27) and homology models of MLH1-NTD and MLH1-PMS2-CTD (24, 25, 28). Figures were generated using PyMOL v.1.4.1 (Schrödinger LLC).

Results

Screening of the expression and MMR efficacy of MLH1 missense variants

A total of 38 MLH1 missense variants were selected from public databases of MLH1 variations. These included recurrent alterations (shown in gray in Fig. 1), for which large amounts of clinical data are available, and additional arbitrarily selected variants. We assessed 2 major functional protein parameters (expression and MMR activity), because missense alterations rarely affect transcriptional integrity (29, 30).

The obtained expression values covered the entire range from 0% to 100% of wild-type (Fig. 1A), whereas the variants formed 2 groups in terms of their repair capacity (Fig. 1B): 21 variants showed MMR efficiencies similar to wild-type (≥70% of wild-type activity), whereas MMR activity was largely absent in 15 variants (<30% of wild-type activity). These 2 groups comprised 53% and 40% of all variants, respectively (Fig. 1C). Overall, more variants showed low expression than low MMR activity (71% versus 45%; “low” here means <70% of wild-type).

Among the recurrent MLH1 variants, 7 were proficient in mismatch repair while displaying expression defects. These variants, hereafter referred to as validation variants, were (sorted by expression level): K618A>H718Y>V578G>V716M>A681T>L622H>P654L. Because they are catalytically active, the potential pathogenic effects of these variants are likely attributable to their expression defects. Therefore, they are suitable for assessing the correlation between expression levels and clinical outcomes.

Decreased protein stability underlies reduced MLH1 expression levels

To verify that the expression defects reflected reduced stability of the variant proteins, we quantified the levels of MLH1 transcripts in the expression system. Under the applied conditions, MLH1 transcript levels were quite robust to variations in the mass of transfected plasmid DNA. They decreased only when the amount of DNA transfected missed the standard amount by a factor ≥2 (Supplementary Fig. S2A). However, even this situation did not affect protein levels (Supplementary Fig. S2B), suggesting that MLH1 transcript abundance was not limiting for protein production. This was confirmed by the finding that the transcript levels in transfected HEK293T cells far exceeded those of endogenous MLH1 in HEK293 cells, whereas no corresponding increase in protein levels occurred (Supplementary Fig. S2C). Therefore, translation
was the rate-limiting step of the expression system. Consequently, small differences in the transfection efficiency or plasmid transcription did not influence the resulting protein levels, and expression level differences necessarily reflect properties inherent to the protein variant. Transcript quantification for the MLH1 validation variants additionally confirmed that their expression level defects were not caused by poor transfection or transcription (Supplementary Fig. S2D).

Because these results suggested that the substitutions caused decreased stability in the MLH1 proteins, we analyzed the protein degradation rates of the validation variants using an in vitro pulse-chase method developed for this purpose (31). Four of the validation variants (K618A, V716M, H718Y, and E578G) showed similar reductions of the half-life time to 64% on average. The A681T variant was clearly less stable (half-life time decreased to 43%, Supplementary Fig. S3A). The expression of the L622H and P654L variants was too low to assess the degradation rate in this system, but this finding also corroborates the notion that low stabilities underlie the expression defects observed in the transfection system.

Proteolytic stability of missense variants usually correlates with thermal stability (32). Therefore, we tested refolding temperatures via differential scanning fluorimetry (33). Thermal stability was slightly compromised in the K618A variant but was decreased more strongly in V716M, H718Y, and E578G (Supplementary Fig. S3B). The low-stability variants were again poorly expressed (in a different expression system) which prevented their purification for this analysis.

In conclusion, all of the obtained data confirmed that the decreases in expression levels observed in the transfection system reflected protein stability constraints due to missense substitutions. Although the precise sorting of the stability of the variants differed slightly between the experimental systems, the following consensus was obtained: wt > K618A > E578G ≥ V716M ≥ H718Y > A681T > L622H > P654L.

Clinical phenotypes of recurrent MLH1 variants

To investigate the correlation between MLH1 variant expression level and clinical phenotypes, we conducted a comprehensive analysis of clinical data for the recurrent variants (the 7 MLH1 validation variants and 3 additional, repair-deficient pathogenic control variants: T117M, R659P, and L749P). Altogether, reports from 350 patients carrying these 10 variants in the germline were evaluated (Table 1 summarizes the complete data presented in Supplementary Table S1). For the purpose of comparison, a Lynch syndrome control group composed of carriers of pathogenic mutations was formed. In parallel, all published information on the frequency of these variants in unaffected control individuals was collected.

The average age at diagnosis, frequency of tumor MSI, and fulfillment of the Amsterdam criteria displayed a clear gradient in carriers of the analyzed variants (Fig. 2). In carriers of K618A, E578G, V716M, and H718Y, the average age at diagnosis was more than in the Lynch syndrome control group (47.4 vs. 38.8 y, P = 0.00006). In addition, few carriers of these 4 alterations were positive for the Amsterdam criteria (27% vs. 74%) or showed tumor MSI (35% vs. 97%).

We evaluated 4 additional clinical parameters that can provide evidence of pathogenicity or neutrality: (i) the frequency of the alteration in control populations (which
Table 1. Evaluation of clinical parameters of MLH1 variant carriers

<table>
<thead>
<tr>
<th>MLH1 variant</th>
<th>Conservation score* (residues in this position)</th>
<th>MAPP-MMRscore</th>
<th>Total case #</th>
<th>Average age at diagnosis/cases</th>
<th>Amsterdam-positive% /cases</th>
<th>MSI% /cases</th>
<th>Loss of MLH1 in IHC% /cases</th>
<th>Frequency in controls% /number of controls</th>
<th>Cosegregation</th>
<th>Homozygosity%</th>
<th>Co-occurrence% /cases</th>
<th>Case-control analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>K618A</td>
<td>7 (FHKMNRTWY)</td>
<td>5.06</td>
<td>123</td>
<td>49.3 /P = 0.001/35</td>
<td>22/49</td>
<td>30/54</td>
<td>26/35</td>
<td>0.67/4652</td>
<td>No (1, [26])</td>
<td>1 case compound</td>
<td>10% (13)</td>
<td>Not pathogenic [53, 62, 66, 74]</td>
</tr>
<tr>
<td>E578G</td>
<td>4 (DENPOSTV)</td>
<td>4.85</td>
<td>11</td>
<td>51.6 /P = 0.03/10</td>
<td>56/9</td>
<td>20/10</td>
<td>0/6</td>
<td>0.00/340</td>
<td>No (2/2, [68])</td>
<td>Yes (2, [26–29])</td>
<td>18% (2)</td>
<td>Slight risk increase for multiple adenomas [57]</td>
</tr>
<tr>
<td>V716M</td>
<td>6 (EFILMTV)</td>
<td>2.78</td>
<td>55</td>
<td>45.2 /P = 0.02/25</td>
<td>25/24</td>
<td>44/18</td>
<td>38/21</td>
<td>0.32/1747</td>
<td>Yes (30)</td>
<td>2 controls [92]</td>
<td>20% (11)</td>
<td></td>
</tr>
<tr>
<td>H718Y</td>
<td>7 (DERHKNSV)</td>
<td>3.45</td>
<td>22</td>
<td>44.0/8</td>
<td>25/4</td>
<td>80/5</td>
<td>0/1</td>
<td>0.84/1134</td>
<td>1 case [102]</td>
<td>1 control [94]</td>
<td>18% (4)</td>
<td></td>
</tr>
<tr>
<td>Repair-deficient pathogenic control variants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T117M</td>
<td>9 (ST)</td>
<td>21.05</td>
<td>38</td>
<td>40.5/20</td>
<td>85/27</td>
<td>91/11</td>
<td>100/9</td>
<td>3% (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L749P</td>
<td>9 (L)</td>
<td>37.37</td>
<td>8</td>
<td>38.8/6</td>
<td>100/6</td>
<td>100/2</td>
<td>50/4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R659P</td>
<td>6 (ACEHKLQRST)</td>
<td>11.70</td>
<td>5</td>
<td>38.5/2</td>
<td>100/3</td>
<td>100/5</td>
<td>100/1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carriers of variants classified neutralb</td>
<td>166</td>
<td></td>
<td></td>
<td>47.4 /P = 0.0006/77</td>
<td>27/86</td>
<td>35/87</td>
<td>27/55</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carriers of variants classified pathogenicc</td>
<td>79</td>
<td></td>
<td></td>
<td>38.8/55</td>
<td>74/62</td>
<td>97/32</td>
<td>na/na</td>
<td></td>
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</tbody>
</table>

NOTE: Clinical data summary from Supplementary Table S1, see there for detailed information and references [in square brackets]. Bold values indicate percent values; italic values are total case numbers.

*Scored from 1 (no conservation) to 9 (highly conserved) using ConSeq.

See Supplementary Table S1, columns AF-AI.

If "Yes", the number in brackets gives the number of related alteration carriers affected by a Lynch syndrome tumor. If "No", the number in brackets gives the number of individuals related to index patient who are affected by a tumor from the Lynch syndrome spectrum but do not carry the alteration, plus number of further related carrier individuals without Lynch syndrome tumor.

Reports on individuals carrying the alteration homozygously in the germline ("case" refers to cancer patient, "control" refers to an unaffected individual).

Frequency of carrier individuals who carry a second, damaging germline mutation.

Carriers of variants classified with the damaging mutation K618del, but this patient had Lynch syndrome and not MMRCS.

A Student t test (2-sided) was applied to assess whether the age at diagnosis significantly differs from the group of carriers of pathogenic alterations.

Summary of the information on carriers of K618A, E578G, V716M, and H718Y; see columns J, AP-AU in Supplementary Table S1.

Summary of the information on carriers of repair-deficient control variants (T117M, R659P, L749P) and all other individuals who carried (additional) pathogenic mutations; see columns K and AK-AO of Supplementary Table S1.
is expected to be low for pathogenic alterations); (ii) cosegregation of the alteration with disease; (iii) the identification of homozygous variant carriers (who, in case of pathogenic variants, be affected not by Lynch syndrome but by mismatch repair cancer syndrome, MMRCS, MIM #276300, a severe condition that presents with malignancies in early childhood; ref. 34); and (iv) co-occurrence with other pathogenic mutations (frequent co-occurrence means that pathogenicity of the variant is unlikely).

The alterations K618A, E578G, V716M, and H718Y were found in control populations. They mostly did not cosegregate with disease, and homozygosity was observed in patients with cancer as well as in healthy controls, none of whom displayed MMRCS. Finally, co-occurrence with pathogenic mutations was frequent (10%–20%). Furthermore, 4 case–control analyses showed that there was no association of K618A with increased cancer risk (refs. 35–38; Table 1).

In summary, all of these findings provide strong evidence that K618A, E578G, V716M, and H718Y, which all resulted in MLH1 expression levels above 60%, represent neutral variants. In contrast, the variants A681T, L622H, and P654L, which were associated with lower expression levels
(52%, 42%, and 25%, respectively) are causative for Lynch syndrome.

**Effect of MLH1 protein concentrations on MMR activity**

The analysis suggested that the 4 MMR-proficient variants (K618A, E578G, V716M, and H718Y) moderately destabilize the MLH1 protein but are not causative for Lynch syndrome, whereas the strongly destabilizing variants (A681T, L622H, and P654L) are pathogenic. In the tumor cells of carriers of these variants, the low intracellular MLH1 protein concentration (after loss of the wild-type allele) likely caused an MMR defect. We therefore investigated the minimal MLH1 protein level required for efficient mismatch repair. We used the cell line HEK293 and its clone HEK293T, which are MLH1- and repair-proficient and -deficient, respectively. We confirmed the mismatch repair defect by testing microsatellite instability in these cells: HEK293T, but not HEK293, displayed strong MSI (Supplementary Fig. S4).

Dilution experiments showed that reduction of the MLH1 concentration to 50% does not affect MMR activity, though it is reduced at lower concentrations (Fig. 3). Notably, this finding is in good agreement with observations regarding MSI in healthy tissues of Lynch syndrome individuals: normally, both MLH1 alleles contribute equally to MLH1 expression (41). Consequently, individuals heterozygous for an inactivating MLH1 mutation constitutively present cellular MLH1 levels that are 50% of normal levels. In the healthy tissues of these individuals, MSI is not prominent, but is detectable in in-depth analyses (42, 43). This confirms that the intracellular MLH1 concentration in mutation carriers is just sufficient to retain (almost normal) MMR activity.

**Pathogenicity threshold for expression defects in MLH1 variants**

Taken together, the data suggest that decreases in MLH1 protein stability are compatible with normal health to some degree, but below a certain threshold, MMR function is insufficient, and carriers display typical traits of Lynch syndrome (Fig. 4). This threshold, established on the basis of the neutral V716M and pathogenic A681T variants, is corroborated by clinical and functional data for 6 additional missense alterations (Fig. 4, see also Supplementary Table S2). Furthermore, it is consistent with the immunohistochemistry of MLH1 in tumors of affected patients: the tumors of carriers of variants showing stability above the threshold level were mostly positive for MLH1 immunostaining, whereas MLH1 was consistently undetectable in carriers of variants with a stability below the threshold (Fig. 4 and Table 1).

**Analysis of stability defects in silico and the relationships with protein structure**

Several in silico algorithms have been developed to predict the effect of missense variants. Of these algorithms, MAPP-MMR (44) was most consistent with the experimental data. It correctly predicted a high probability of a damaging effect for most repair-deficient variants (Supplementary Fig. S5), whereas repair-proficient variants were mostly scored as "neutral" or "borderline", irrespective of their stability defect. Other algorithms specifically designed for predicting...
Expression Defect Size Determines MLH1 Variant Pathogenicity

In our analysis, 7 MMR-proficient MLH1 variants displayed defects of stability whose degree was correlated with the clinical phenotype. This allowed us to establish a threshold defining what degree of stability reduction is associated with Lynch syndrome pathogenicity (cosegregation, homozygosity, co-occurrence, tumor MSI). This is, thus, a much rarer event. In investigating MLH1 missense unclassified variants, it hence seems to be reasonable to first test their expression (Fig. 5A) and to then analyze additional functional parameters (Fig. 5B) only when the level of a variants’ expression is above the threshold provided by the A681T variant.

In this analysis, the average expression of half of the variants (53%) was below this threshold, and statistical significance was achieved for 24% of the variants (P < 0.05; Fig. 4 and Supplementary Table S3). These variants can, thus, be considered pathogenic with high certainty.

In conclusion, an analysis showed that stability constraints of the MLH1 protein are a major consequence of missense alterations in MLH1 at the cellular level (Fig. 5B).
Precise determination of the expression defects associated with catalytically active variants allowed us to establish a threshold below which an expression defect is associated with Lynch syndrome. This threshold is compatible with the minimal cellular MLH1 protein concentration required for unimpaired MMR activity. It is also compatible with data obtained from MLH1 immuno-staining in tumors of variant carriers. For future efforts to classify uncertain MLH1 missense variants, we therefore propose first determining expression in direct comparison with clinically validated standard variants (A681T and V716M).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: S. Zeuzem, G. Plotz
Development of methodology: I. Hinrichsen, G. Plotz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Hinrichsen, A. Brieger, J. Trojan, M. Nilbert, G. Plotz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Hinrichsen, A. Brieger, M. Nilbert, G. Plotz
Writing, review, and/or revision of the manuscript: I. Hinrichsen, A. Brieger, J. Trojan, S. Zeuzem, G. Plotz

References
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