Inactivation of DNA Mismatch Repair by Variants of Uncertain Significance in the *PMS2* Gene

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ABSTRACT

Lynch syndrome (LS) is a common cancer predisposition caused by an inactivating mutation in one of four DNA mismatch repair (MMR) genes. Frequently a variant of uncertain significance (VUS), rather than an obviously pathogenic mutation, is identified in one of these genes. The inability to define pathogenicity of such variants precludes targeted healthcare. Here, we have modified a cell-free assay to test VUS in the MMR gene *PMS2* for functional activity We have analyzed nearly all VUS in *PMS2* found thus far and describe loss of MMR activity for five, suggesting the applicability of the assay for diagnosis of LS.

LS predisposes to various cancers, most notably to colon and ovarian cancer [de la Chapelle, 2004]. LS is caused by heterozygous inactivating mutations in one of the MMR genes *MSH2* (MIM# 609309), *MSH6* (MIM# 600678), *MLH1* (MIM# 120436) or *PMS2* (MIM# 600259) [de la Chapelle, 2004]. Inadvertent loss of the second, wild type, allele in somatic cells results in MMR deficiency, which underlies the accumulation of spontaneous genomic mutations and the rapid development of cancer [de la Chapelle, 2004]. Confirmed LS patients enroll in lifelong preventive surveillance programs and may benefit from personalized chemoprevention and chemotherapy [Hewish et al., 2010; Burn et al., 2011]. Unfortunately, genetic diagnosis of LS patients is complicated by the fact that a significant fraction of all MMR gene alterations found are so-called Variants of Uncertain Significance (VUS) [de la Chapelle, 2004]. In the absence of data on the impact of the VUS on gene function, it is often difficult to interpret their pathogenicity. Moreover, the incidence of VUS is believed to increase steeply with the advent of personalized genomics [Rasmussen et al., 2012]. To enable personalized healthcare for carriers of pathogenic variants and to liberate unaffected relatives from the burden associated with the uncertain pathogenicity of the VUS, it is of great importance to develop procedures to evaluate their pathogenicity [Rasmussen et al., 2012].

Of all MMR genes, VUS in *PMS2* have the highest incidence, comprising ~49% of all alterations described in this gene (http://www.med.mun.ca/MMRvariants/statistics.aspx). We have recently described a cell-free assay to measure the functional activity of VUS in the MMR genes *MSH2*, *MLH1* and *MSH6* [Drost et al., 2010, 2012]. To facilitate the assessment of pathogenicity of VUS in *PMS2* we have modified the cell-free assay to analyze their functional activity. In this assay the mutated cDNA is recreated by PCR, followed by *in vitro* transcription/translation of the variant PMS2 protein and of its wild type heterodimeric partner MLH1 (Figure 1*A*). To serve as template for the generation of the variant *PMS2* alleles we used a wild type PMS2 fused to short S and thrombin tags, as this fusion protein displayed higher *in vitro* expression than the native PMS2 (Supp. Figure S1A, compare the first two lanes), while it did not affect its activity (Supp. Figure S1B). The variant MLH1/PMS2 heterodimer is added to an MLH1/PMS2-deficient cell extract and tested for its ability to restore a *Hin*DIII restriction enzyme recognition site that is disrupted by an embedded G·T mismatch (Figure 1*B*). The inability to restore repair of the mismatch is indicative of the pathogenicity of a *PMS2* VUS. All experimental procedures are described in the Supp. Materials & Methods. The assay appeared relatively insensitive to the amount of PMS2 included in the reaction, contributing to its robustness (Supp. Figure S1). The absolute repair efficiency of tagged wild type PMS2 under the conditions used in this assay is 43.5%±4.2 (mean±S.E.M.) and this is highly reproducible (Supp. Figure 1*B*). As the substrate concentration is in excess, absolute repair efficiencies are not a relevant measure for defects of a VUS. For this reason the *in vitro* MMR assay data is expressed as percentage of repair relative to wild type.

To test the applicability of the assay we have determined repair efficiencies of 27 VUS in *PMS2*, representing the large majority of all VUS registered in the Leiden Open Variation Database (LOVD; Table 1). Of these, variant E705K served as an MMR-deficient, pathogenic, control [Deschênes et al., 2007; van Oers et al., 2010]. Additionally, variants

E541K and G857A were included as innocuous polymorphisms, as judged from their high allele frequencies (dbSNP rs2228006 and rs1802683, respectively). *PMS2* variants registered in the LOVD as *PMS2* pseudogene-derived were excluded from analysis. All alleles were recreated by PCR and protein was produced *in vitro* (Figure 1*C*).

As the polymorphic *PMS2* alleles enabled repair activities significantly higher than the known pathogenic control E705K, the assay has sufficient resolution to distinguish repair-proficient from repair-deficient VUS (Figure 1*D*). Variants with repair efficiencies not significantly higher than the pathogenic control (E41A, S46I, S46N and C843Y) were

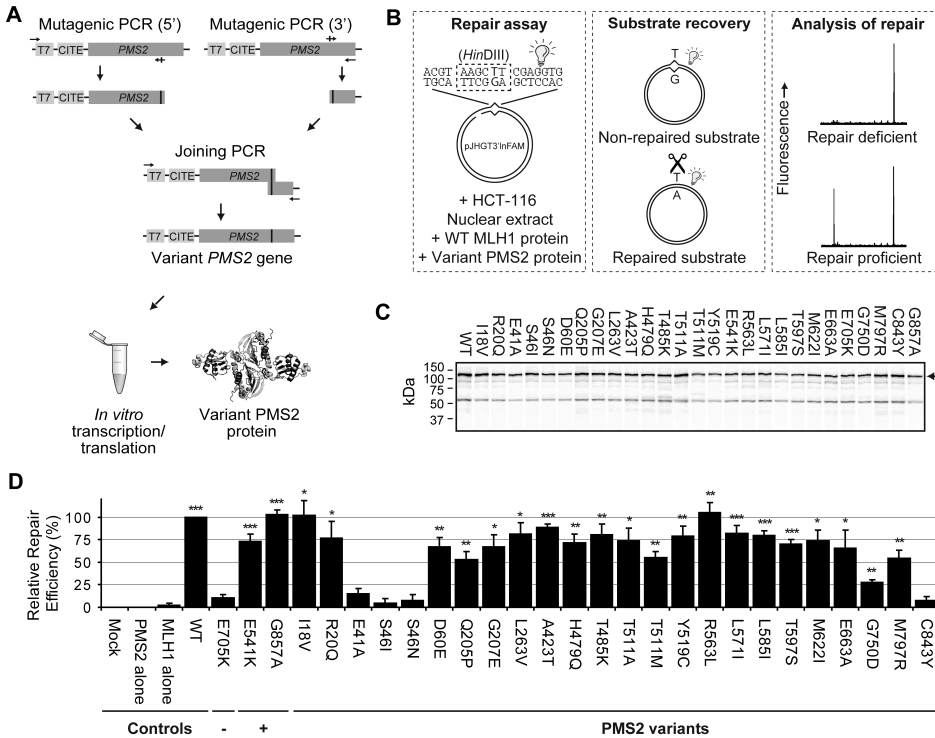

Figure 1. *Mismatch repair activity of PMS2 VUS. (A) Production of variant PMS2 alleles and proteins. All alleles, including template vector-derived T7 promoter and CITE sequences that are required for efficient transcription/ translation in vitro, are generated by two sequential site-specific mutagenic PCR reactions. Variant PMS2 alleles are then used as a template in an in vitro transcription/translation reaction to produce variant PMS2 proteins. (B) Flow scheme of the cell-free assay. Left: Fluorescently labelled (light bulb) substrate pJHGT3'lnFAM is incubated in HCT-116 nuclear extract and in vitro produced heterodimeric variant PMS2/wild type MLH1 protein. Middle: After incubation, the substrate is purified and digested. Right: Repair products are visualized by automated fragment analysis and quantified. (C) Representative expression of 35^S-Methionine-labeled variant PMS2 proteins, visualized after SDS-PAGE gel electrophoresis and autoradiography. Arrow: full-length PMS2 variants. (D)Relative repair efficiencies for PMS2 VUS. (–): Repair-deficient control, (+): Repair-proficient controls. Results are shown as mean±S.E.M. of 3–4 independent experiments for all VUS and >6 experiments for controls. Mock: Mock expression. Asterisks: Significantly higher than repair-deficient control E705K. * p<0.05; ** p<0.01; *** p<0.001 (Student's one-tailed t-test). For the "Mock" and "PMS2 only" reactions, no repair was detected in any of the experiments.*

Table 1. Pathology data and references for *PMS2* VUS tested in this work.

Mutation			Pathology[c]			
				IHC		
Protein[a]	DNA[b]	*In vitro* MMR (This work)	MSI	PMS2	MLH1	References[d]
I18V	c.52A>G	+				1, 2
R20Q	c.59G>A	+	H[3]			1, 2, 3, 4, 5
E41A	c.122A>C	-				5
S46I	c.137G>T	-	H[3, 6]	Neg[3, 6]	Pos[6]	2, 3, 4, 6, 7, 8
S46N	c.137G>A	-				7, 8
D60E	c.180C>G	+				LOVD
Q205P	c.614A>C	+				LOVD
G207E	c.620G>A	+	H[9]	Neg[9]		9
L263V	c.787C>G	+				LOVD
A423T	c.1267G>A	+				10
H479Q	c.1437C>G	+				11, 12
T485K	c.1454C>A	+	H[6]	Neg[6]	Pos[6]	1, 2, 4, 6, 11
T511A	c.1531A>G	+	H[3]			2, 3, 6, 11, 12
T511M	c.1532C>T	+				LOVD
Y519C	c.1556A>G	+				LOVD
E541K	c.1621A>G	+				1, 2, 5, 6
R563L	c.1688G>T	+				1, 2, 8
L571I	c.1711C>A	+				2
L585I	c.1753C>A	+				1
T597S	c.1789A>T	+	L[3]			1, 3, 4, 5, 11
M622I	c.1866G>A	+				1, 2, 4, 6, 8, 11
E663A	c.1988A>C	+				8
E705K	c.2113G>A	-				2, 6, 8, 13, 14
G750D	c.2249G>A	+				8
M797R	c.2390T>G	+				2
C843Y	c.2528G>A	-				8
G857A	c.2570G>C	+				1, 2, 6, 12

[a] Amino acid numbering is based on the PMS2 reference sequence NP_000526.1 with +1 corresponding to the translation initiation amino acid.

[b] Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the translation initiation codon of the *PMS2* GenBank reference sequence (NM_000535.5).

[c] MSI=Microsatellite Instability. H=High, L=Low, IHC=Immunohistochemistry. Neg = Negative for staining, Pos = Positive for staining. In case cells are empty, the variant was not tested.

[d] Variants have been selected from the LOVD (http://chromium.liacs.nl/LOVD2/colon_cancer/variants.php?select_db=PMS2&action=view_all&view=Prot_sub). Appropriate references are shown. References: 1 [Hendriks et al., 2006], 2 [Clendenning et al., 2006], 3 [Pastrello et al., 2011], 4 [Thompson et al., 2012], 5 [Borràs et al., 2013], 6 [Nakagawa, 2004], 7 [Jackson et al., 2008], 8 [Senter et al., 2008], 9 [Montazer Haghighi et al., 2009], 10 [Ganster et al., 2010], 11 [Wang et al., 1999], 12 [Basil et al., 1999], 13 [Deschênes et al., 2007], 14 [van Oers et al., 2010].

considered repair deficient and therefore presumably are pathogenic (Figure 1*D*). We conclude that this assay effectively identifies repair-deficient PMS2 variants. Variants such as Q205P, T511M and G750D that display repair efficiencies significantly higher than E705K but are compromised compared to wild type were not classified as repair deficient and we surmise that these variants might be pathogenic with reduced penetrance. Extensive calibration of the assay with clinical data is required to assess pathogenicity of alleles displaying such intermediate repair efficiencies.

For some of these VUS microsatellite instability (MSI), a hallmark of MMR deficient cancer, was previously investigated. Indeed, the MSI of a tumor carrying the S46I allele corresponds with its deficiency in MMR (Table 1). However, the MSI in tumors of carriers of the R20Q, G207E, T485K or T511A alleles is in apparent contrast with their normal MMR activity *in vitro* (Table 1). Possibly these patients carry another, yet unidentified MMR gene defect. Contrariwise, we cannot exclude that these alleles cause a defect that is only apparent *in vivo*. For this reason, all variants that are repair proficient in this assay cannot be classified as neutral, but may require additional analyses such as splicing assays, protein stability assays or nuclear localization assays [Rasmussen et al., 2012]. Ultimately, after the calibration of the assay, it may become a part of an integrated Bayesian analysis that determines pathogenicity of MMR gene VUS, as proposed by us and others [Goldgar et al., 2008; Rasmussen et al., 2012].

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SUPPLEMENTARY MATERIALS

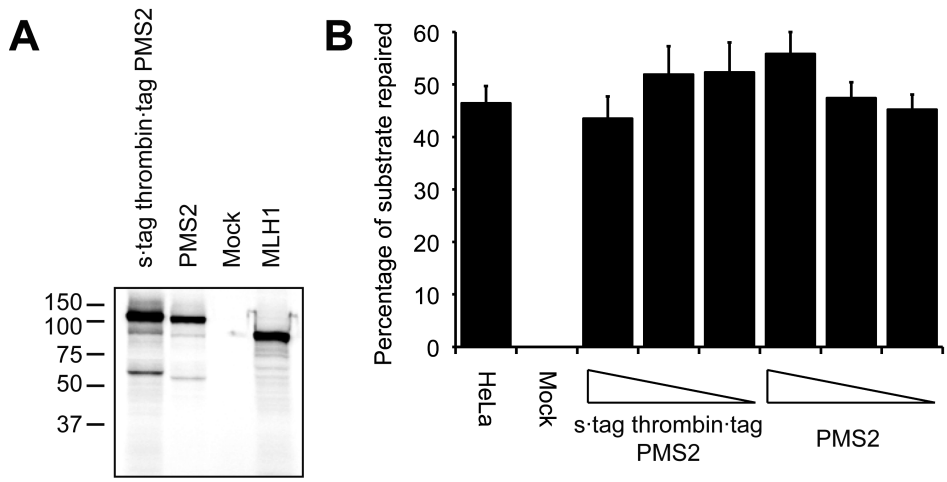
MATERIALS & METHODS

Cloning of PMS2. The human *PMS2* (GenBank NM_000535.5) cDNA was cloned into the pCITE4a expression vector (Novagen, Beeston, UK). *PMS2* cDNA was PCR-amplified using forward primer 5'-CGCGGATCCGCCACCATGGAGCGAGCTGAGAGCTCGAGTAC-3' and reverse primer 5'-GCGCGGCCGCGGTCAGTTCTGAGAAATGACAC-3'. The PCR fragment was cloned into pCITE4a after *Bam*HI and *Not*I (New England Bioland, Ipswich, MA, USA) digestion. The encoded PMS2 protein carries a 40 amino acid N-terminal tail, containing both an s tag and a thrombin tag. This tail increases protein expression without affecting protein activity (Supplementary Figure 1).

Generation of variant PMS2 proteins by PCR and In Vitro Expression. The plasmid construct described above was used as a template in a PCR procedure to recreate variant PMS2 alleles, including the vector-derived T7 promoter and CITE sequences that are required for efficient transcription/translation *in vitro*. PCR reactions were performed as described (Figure 1*A*) [Drost et al., 2012]. In brief, overlapping 5' and 3' PCR fragments were generated in separate, PCR reactions. 5' PCR fragments were generated using a common pCITE forward primer and a reverse primer specific for the VUS. 3' PCR fragments were produced using a forward primer complementary to the reverse primer in the 5' PCR, specific to the VUS, and a common pCITE reverse primer. In a subsequent PCR reaction, the two mutant fragments were joined and reamplified using nested primers. Oligonucleotide sequences are available upon request and were ordered from Biolegio (Nijmegen, The Netherlands). All PCR reactions were performed in a total volume of 10 µl, containing 0.4 U of Pfx Platinum Polymerase (Invitrogen, Carlsbad, CA). Proper introduction of the variant codon was confirmed by direct sequencing of PCR fragments.

Following amplification, all final PCR products were purified using the PCR Purification Kit (Qiagen). These purified fragments were then used for *in vitro* protein expression in the TnT Quick Coupled in vitro Transcription/Translation System (Promega, Madison, WI), in the presence of PCR Enhancer with minor modifications to the manufacturer's protocol. Expression levels were verified by producing parallel ^{35}S-Methionine-labeled reactions according to the manufacturer's instructions. Labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and Phosphorimaging (Perkin Elmer, Waltham). Wild type MLH1, the heterodimeric partner of PMS2, was produced by large-scale *in vitro* expression from its pCITE4a clone [Drost et al., 2010]. The PMS2/MLH1 heterodimers were generated by allowing *in vitro*-expressed wild type or variant PMS2 and WT MLH1 (1:1 v/v) to dimerize for 30 minutes at room temperature.

In Vitro MMR Assays. *In vitro* MMR assays were performed as described (Figure 1B) [Drost et al., 2010, 2012]. Reactions are carried out in 25µl containing 75µg of nuclear extract. Nuclear extract was prepared from HCT-116 colon cancer cells that lack both PMS2 and MLH1, as described [Holmes et al., 1990]. Extracts were complemented with 12µl of dimerized PMS2/MLH1 and 100ng of fluorescent substrate pJHGT3'lnFAM. After



Supplementary Figure 1. *The 40 amino acid N-terminal tag on PMS2 does not influence MMR activity. (A) Representative expression of ^{35}S-Methionine-labeled proteins, visualized after SDS-PAGE gel electrophoresis and autoradiography. (B) Repair efficiencies of in vitro produced PMS2 with or without fused N-terminal tags. PMS2 proteins were sequentially diluted twofold and dimerized to MLH1. Repair assays were carried out as described. MMR-proficient HeLa nuclear extract serves as a positive control. No significant differences were found between any of the PMS2 measurements, supporting the robustness of the assay. Results are shown as mean±S.E.M. of 4 independent experiments.*

40 min of incubation at 37°C, substrate DNA was purified using the MinElute kit (Qiagen, Germantown, MD). Then, the substrate was digested with *Hin*DIII and *Bsr*BI (Fermentas, Burlington, ON, Canada). One-fifth (2 µl) of digested substrate was mixed with 7.8 µl Hi-Di Formamide and 0.2 µl GeneScan-500 ROX size standard (Applied Biosystems, Bedford, MA) and fluorescent fragment analysis was performed under standard conditions. Signal was quantified using GeneMarker software (Softgenetics, State College, PA). Repair levels were calculated by dividing the height of the MMR-specific peak by the total fluorescent signal, and then normalized to wild type levels.

SUPPLEMENTARY REFERENCES

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