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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.

Keywords

Lynch Syndrome; DNA Mismatch Repair; VUS; *PMS2*

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

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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 1A). 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 *HinDIII* restriction enzyme recognition site that is disrupted by an embedded G-T mismatch (Figure 1B). 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 $43.5\% \pm 4.2$ (mean $\pm$ S.E.M.) and this is highly reproducible (Supp. Figure 1B). 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 1C).

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 1D). Variants with repair efficiencies not significantly higher than the pathogenic control (E41A, S46I, S46N and C843Y) were considered repair deficient and therefore presumably are pathogenic (Figure 1D). 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].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

LS	Lynch syndrome
MMR	DNA Mismatch Repair
PMS2	Postmeiotic Segregation Increased 2
VUS	Variant of Uncertain Significance

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'InFAM 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 35S-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

Protein ^a	Mutation DNA ^b	In vitro MMR (This work)	Pathology ^{c,d}			References ^d
			MSI	IHC	MLH1	
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

Mutation		In vitro MMR (This work)		Pathology ^{c,d}		References ^d
Protein ^a	DNA ^b			MSI	IHC	
				PMS2 MLH1		
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 [Borrás 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 [Descêhènes et al., 2007], 14 [van Oers et al., 2010].