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# Germline mutations in *PMS2* and *MLH1* in individuals with solitary loss of PMS2 expression in colorectal carcinomas from the Colon Cancer Family Registry Cohort

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## ABSTRACT

**Objectives:** Immunohistochemistry for DNA mismatch repair proteins is used to screen for Lynch syndrome in individuals with colorectal carcinoma (CRC). Although solitary loss of PMS2 expression is indicative of carrying a germline mutation in *PMS2*, previous studies reported *MLH1* mutation in some cases. We determined the prevalence of *MLH1* germline mutations in a large cohort of individuals with a CRC demonstrating solitary loss of *PMS2* expression.

**Design:** This cohort study included 88 individuals affected with a PMS2-deficient CRC from the Colon Cancer Family Registry Cohort. Germline *PMS2* mutation analysis (long-range PCR and multiplex ligation-dependent probe amplification) was followed by *MLH1* mutation testing (Sanger sequencing and multiplex ligation-dependent probe amplification).

**Results:** Of the 66 individuals with complete mutation screening, we identified a pathogenic *PMS2* mutation in 49 (74%), a pathogenic *MLH1* mutation in 8 (12%) and a *MLH1* variant of uncertain clinical significance predicted to be damaging by in silico analysis in 3 (4%); 6 (9%) carried variants likely to have no clinical significance. Missense point mutations accounted for most alterations (83%; 9/11) in *MLH1*. The *MLH1* c.113A> G p.Asn38Ser mutation was found in 2 related individuals. One individual who carried the *MLH1* intronic mutation c.677+3A>G p.Gln197Argfs*8 leading to the skipping of exon 8, developed 2 tumours, both of which retained MLH1 expression.

**Conclusions:** A substantial proportion of CRCs with solitary loss of PMS2 expression are associated with a deleterious *MLH1* germline mutation supporting the screening for *MLH1* in individuals with tumours of this immunophenotype, when no *PMS2* mutation has been identified.

## Strengths and limitations of this study

- Largest reported sample of colorectal cancers with solitary loss of PMS2 expression.
- Most comprehensive approaches used for testing for germline *PMS2* mutations.
- Multicentre setting which may affect the consistency in the formalin fixation conditions of tissue blocks and lead to immunostaining artefacts.
- No mutation screening data available for 20 cases (24%).
- Selected cases (young individuals with strong family history of colorectal carcinoma (CRC)) that may not reflect the actual rate of PMS2-deficient CRC in the general population and the mutation rates in *PMS2* and *MLH1*.

## INTRODUCTION

Lynch syndrome is an autosomal-dominant inherited condition defined by the identification of a germline mutation in a DNA mismatch repair (MMR) gene (*MLH1*, *MSH2*, *PMS2* or *MSH6*), or in the *EPCAM* gene, leading to constitutional epigenetic silencing of the downstream *MSH2* gene.[1] Individuals who carry a MMR gene mutation are at an increased risk of developing cancers at multiple sites, most notably colorectal and endometrial carcinomas, but also carcinomas from the upper urinary tract, pancreas, hepatobiliary tract, stomach, small intestine and ovaries.[2]

The current diagnostic approach for the identification of individuals with an MMR

gene mutation is a multistep process in which pathologists play an instrumental role. Tumours arising in individuals with an MMR gene mutation demonstrate high levels of microsatellite instability (MSI) secondary to altered DNA MMR mechanisms in tumour cells. Immunohistochemistry for DNA MMR proteins is widely used to identify MMR deficiency in colorectal carcinomas (CRCs) as a screen for MMR gene carriers.[3] Of all abnormal patterns of immunohistochemical results, loss of expression of MLH1 and PMS2 is the most common. MLH1 and PMS2 function as a stable heterodimer that, along with MSH2, MSH6 and EXO1, corrects small errors involving mispaired nucleotides which are introduced by DNA polymerase during DNA replication. A functional defect in *MLH1* results in the degradation of both MLH1 and PMS2, whereas a defect in *PMS2* results only in the degradation of PMS2. Consequently, loss of expression of MLH1 and PMS2 in CRC generally indicates an alteration in *MLH1*, either by somatic methylation of the *MLH1* promoter region (sporadic cases) or by a *MLH1* germline mutation (Lynch syndrome), and solitary loss of PMS2 expression generally indicates an underlying germline defect in *PMS2*.

Inconsistent immunohistochemical results have been reported, in particular the retained expression of MLH1 in tumours from individuals with a germline *MLH1* mutation.[4–8] This phenomenon can be misleading if PMS2 immunostaining is not performed. We sought to confirm that germline mutations in *MLH1* may underlie a substantial proportion of CRC with solitary loss of PMS2 expression. To address this question, we performed mutation analysis of the *MLH1* and *PMS2* genes in individuals from the Colon Cancer Family Registry Cohort whose tumours showed solitary loss of PMS2.

## MATERIALS AND METHODS

### Study participants

Participants were probands and relatives from families recruited between 1997 and 2012 to the Colon Cancer Family Registry Cohort via both population-based recruitment and clinic-based recruitment in Australasia and North America.[9] All CRC cases were reviewed by specialist gastrointestinal pathologists for histological type and grade.[10] Tumours from the caecum, ascending colon, hepatic flexure and transverse colon were considered proximal tumours. Immunohistochemistry for DNA MMR proteins MLH1, PMS2, MSH2 and MSH6 was performed as previously described.[3] A subset of tumours were analysed for MSI status from formalin-fixed paraffin-embedded tissue as previously described.[3] Individuals were eligible for this study if they had a histologically confirmed diagnosis of CRC with an immunohistochemical profile of the DNA MMR proteins, demonstrating presence of expression of the MLH1 protein and concomitant loss of expression of the PMS2 protein. The somatic T> A mutation at nucleotide 1799 in exon 15 of the *BRAF* gene (*BRAF*$^{V600E}$ mutation) was detected using fluorescent allele-specific PCR.[11] *MLH1* promoter methylation was analysed using the MLH1-M2 methylight reaction using an Arthobacter luteus (ALU) control reaction to normalise for bisulfite-converted input DNA.[12] Informed consent was obtained from all participants to collect a blood sample and tumour pathology materials (tumour blocks and slides). Ethics approval was obtained from the relevant institutional Human Research Ethics Committees at recruiting centres.

### Family history of cancer

Information on personal and family history of CRC and other cancers in first-degreeand second-degree relatives was obtained via standardised questionnaires at the time of baseline recruitment. Cancer diagnoses were verified, where possible, using pathology reports, medical records, cancer registry reports and death certificates. Probands and relatives were either actively or passively followed up approximately every 5 years from baseline enrolment, including the collection of updated information by linkage to tumour registries and death indices on the number, sex and birthdates of first-degree relatives, their cancer history, vital status and, if deceased, date of death. All cancers, except for non-melanoma skin cancers, were recorded with dates of diagnosis. The present study was based on all available baseline and follow-up data. Family history of cancer that fulfilled either the Amsterdam I or II criteria were determined.[13]

### Germline mutation testing

Germline mutation testing for the individuals in this study primarily involved testing for *PMS2* gene mutations and when a *PMS2* mutation was not identified, germline mutation testing of the *MLH1* gene was conducted. *PMS2* was screened for germline mutations using a DNA-based, best practice, approach combining long-range PCR and multiplex ligation-dependent probe amplification (MLPA). Briefly, for point mutation analysis, parts of the *PMS2* gene (exons 1–5, 9 and 11–15) were specifically targeted, while avoiding pseudogene sequences, via a set of three long-range PCRs (TaKaRa LA Taq; TaKaRa Bio Inc, Shiga, Japan). These long-range products are then used as the template for a set of *PMS2*-specific exonic PCRs (see online supplementary table S1 for primer sequences). To assess for large-scale (whole exon) deletions, we used the P008-B1 MLPA kit according to the manufacturer's instructions (MRC-Holland; Amsterdam, The Netherlands). To accurately call *PMS2* mutations at the 3′ end of the gene, the MLPA kit contains probes targeted to paralogous sequence variants which requires pseudogene-specific sequence data to interpret the findings. Pseudogene sequences were obtained as above, replacing the *PMS2*-specific long-range amplicon with a pseudogene-specific amplicon. Germline mutation testing for *MLH1* was performed by Sanger sequencing as previously described.[9 14] Large duplication and deletion mutations were detected by MLPA. Germline

variants within the *MLH1* and *PMS2* genes were classified for pathogenicity based on the InSiGHT database classifications[15] (http://insight-group.org/variants/classifications/). If no classification was available, the predicting effect of an unclassified variant (UV) to the protein function was assessed in silico using the 'Sorting Tolerant From Intolerant' (SIFT) and the 'Polymorphism Phenotyping v2' (PolyPhen-2) web-based algorithms.[16 17]

### Statistical analysis

Statistical analyses were performed with SPSS statistics software V.17.0 (SPSS Inc, Chicago, Illinois, USA). Comparisons for categorical variables were performed using Pearson's $\chi^2$ test or Fisher's exact test where appropriate. Student t test was used for continuous variables. A two-tailed p value was used for all analyses and values less than 0.05 were considered to be significant.

## RESULTS

The study included 90 CRCs from 88 individuals demonstrating loss of PMS2 expression and normal retained MLH1 expression by immunohistochemistry. They had a mean age at CRC diagnosis of 51.7±SD 12.4 years and included 57% males. MSI status was available for 46/90 CRCs (51%), with high levels of MSI observed in 42/46 (91%) cases. *MLH1* methylation and/or a $BRAF^{V600E}$ mutation were present in 4 of the 90 CRCs that were excluded from the study. Six CRCs (7%) also showed loss of MSH6 protein expression. Four individuals were not tested for *PMS2* and *MLH1* mutations due to the unavailability of blood-derived DNA, and complete gene testing was not possible for a further 14 individuals (figure 1). The final study group consisted of 66 individuals with complete screening for germline mutations in the *PMS2* and *MLH1* genes. A pathogenic *PMS2* germline mutation was identified in 49 individuals (74%; see online supplementary table S2), some of which were reported previously.[18] Variants in the *MLH1* gene were identified in 11 individuals (17%). In eight individuals, the variants were classified as pathogenic mutations (class 5); in the other three individuals, variants were unclassified but predicted to be damaging by SIFT and PolyPhen-2 algorithms (table 1 and figure 2).

Immunostained slides were reviewed in 5 of these 11 cases, confirming the retained expression of MLH1 and the loss of PMS2 expression in carcinoma cells. No mutation within *PMS2* or *MLH1* could be found in the remaining six individuals (9%). The clinicopathological characteristics of the *PMS2* mutation carriers, the *MLH1* pathogenic mutation and UV carriers and those individuals tested but found not to have a mutation in *PMS2* or *MLH1* are shown in table 2.

The mean age at CRC diagnosis of the individuals with a *MLH1* mutation or UV was significantly younger than those individuals with a *PMS2* mutation (p=0.046). Amsterdam criteria I or II were less frequently found in *PMS2* mutation carriers compared with *MLH1* variant carriers (p=0.001).

Missense variants were the most common *MLH1* alteration identified, in eight individuals (83%). The *MLH1* c.113A>G p.Asn38Ser variant was found in two related individuals (cases 2 and 3). One individual who carried the intronic *MLH1* germline mutation c.677+3A>G p.Gln197Argfs*8, which leads to the skipping of exon 8, developed two CRCs both of which retained MLH1 expression (cases 5 and 6). One individual carried a splice site mutation leading to an in-frame deletion of two exons (case 4) and one individual carried a small insertion resulting in a frameshift mutation (case 7; table 1).

## DISCUSSION

To assess the possible role of *MLH1* mutations in CRCs showing solitary loss of PMS2 expression by immunohistochemistry, we studied a series of 90 CRCs from 88 individuals from the Colon Cancer Family Registry Cohort with this immunophenotype. Among the 66 individuals with complete germline mutation analysis, we identified a pathogenic *PMS2* mutation in 49 cases (74%) and a pathogenic *MLH1* mutation in 8 cases (12%). A further three cases (4%) had a variant of uncertain clinical significance in *MLH1* predicted to be damaging, and six cases (9%) had no identifiable variant likely to have clinical significance in either gene. Moreover, a high proportion of the *MLH1* variants identified resulted in missense changes, suggesting that a non-functional MLH1 protein that retains its MLH1 antigenicity is a conceivable explanation.

Immunohistochemistry for the DNA MMR proteins MLH1, PMS2, MSH2 and MSH6 in CRC is a highly sensitive test to screen for Lynch syndrome, with 93–100% concordance with MSI testing.[3 4] However, false-negative results for MLH1 immunohistochemistry have been reported in small series. In a study evaluating the benefit of adding PMS2 to MLH1 staining, de Jong *et al*[4] found eight *MLH1* mutations (42%) compared with only three *PMS2* mutations (16%) out of 19 CRCs demonstrating solitary loss of PMS2 expression. When considering all the *MLH1* mutations identified in their study, a high proportion (8/35; 23%) showed loss only of PMS2 expression while retaining expression of MLH1. A large deletion of exons 14–19 of *MLH1* was also reported in 2 of 8 (25%) CRC with solitary PMS2 loss of expression in a separate study.[5] A recent study of 16 CRCs and 16 endometrial carcinomas from 31 individuals, all with solitary loss of PMS2 expression, explored the frequency of *MLH1* mutations in this group.[19] Of the 17 individuals who subsequently had germline mutation testing of the *MLH1* and *PMS2* genes, six had pathogenic mutations in *PMS2* (35%), two had variants of uncertain clinical significance in *PMS2* (12%), four had *MLH1* pathogenic mutations (24%) whereas five had no mutation identified in either gene (29%).

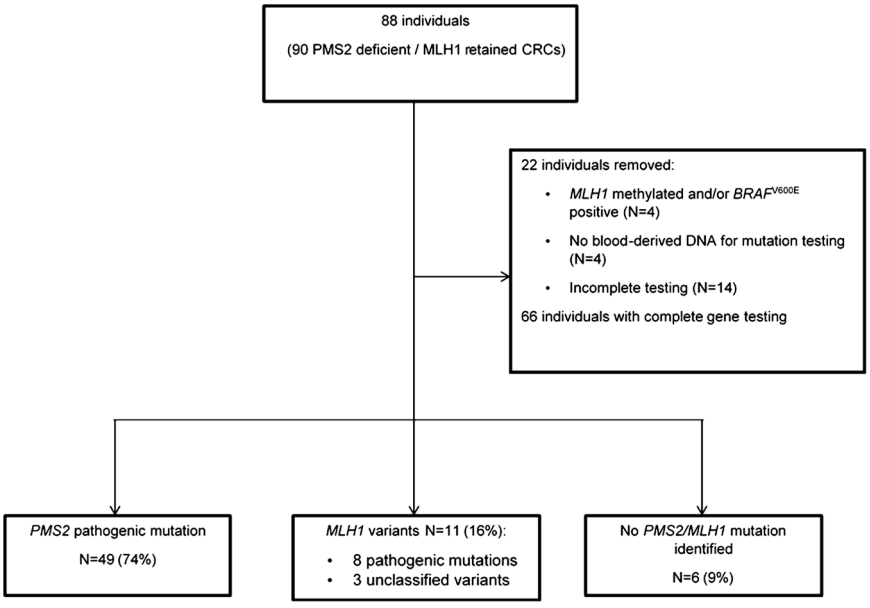



**Figure 1** Flow diagram of the study. CRC, colorectal carcinoma.

When restricted to patients with a CRC, a deleterious germline mutation in *MLH1* was reported in two of nine tested patients (22%). Compared with these studies, our rate of *PMS2* mutation in 66 tested individuals was higher at 74% and the rate of *MLH1* deleterious mutation slightly lower at 12%. Two cousins (tumours 2 and 3) who carried the same *MLH1* mutation both had CRC with solitary PMS2 loss. Similarly, one individual, who carried the *MLH1* c.677+3A>G p.Gln197Argfs*8 mutation, developed two CRCs with solitary PMS2 loss. Both these examples suggest that it is the nature of the mutation rather than a technical anomaly associated with tissue fixation or staining quality that is the cause of this differential staining pattern. In support of this, Zighelboim *et al*[20] described two sisters who carried the same *MLH1* mutation: one developed endometrial cancer at 48 years and the other CRC at 45 years and endometrial cancer at 53 years; all tumours showed solitary loss of PMS2 expression and the presence of MLH1 expression.

A trend towards universal CRC tumour immunohistochemistry will increase the detection of abnormal staining patterns that require interpretation. This allows the most probable cause to be decided and thus the most appropriate management instituted. A solitary loss of PMS2 expression is suggestive of Lynch syndrome with a primary defect in the *PMS2* gene. Interestingly, we identified *MLH1* methylation or the somatic $BRAF^{V600E}$ mutation in four cases, indicating that isolated PMS2 loss of expression can occur outside Lynch syndrome. It may

**Table 1** Characteristics of the 11 individuals with a germline *MLH1* variant from 12 colorectal carcinomas with loss of PMS2 expression and retained MLH1 expression

| Tumour # | Gender | Age, years | Amsterdam criteria | Tumour location | Variant | Protein | InSiGHT classification |
|---|---|---|---|---|---|---|---|
| 1 | Female | 40 | None | Descending | c.230G>A | p.Cys77Tyr | Class 5 |
| 2* | Male | 44 | None | Descending | c.113A>G | p.Asn38Ser | Class 5 |
| 3* | Male | 40 | I | Rectum | c.113A>G | p.Asn38Ser | Class 5 |
| 4 | Female | 51 | I | Descending | c.790+1G>A | p.Glu227_Ser295del | Class 5 |
| 5† | Male | 34 | II | Cecum | c.677+3A>G | p.Gln197Argfs*8 | Class 5 |
| 6† | Male | 34 | II | Rectum | c.677+3A>G | p.Gln197Argfs*8 | Class 5 |
| 7 | Male | 63 | I | Caecum | c.2195_2198dup | p.His733Glnfs*14 | Class 5 |
| 8 | Male | 49 | None | Unknown | c.230G>A | p.Cys77Tyr | Class 5 |
| 9 | Female | 33 | None | Rectum | c.199G>A | p.Gly67Arg | Class 5 |
| 10 | Male | 62 | II | Transverse | c.374C>A | p.Ala125Glu | UV |
| 11 | Male | 24 | None | Ascending | c.187G>C | p.Asp63His | UV |
| 12 | Male | 38 | I | Cecum | c.187G>C | p.Asp63His | UV |

UV: unclassified variant by InSiGHT. These UVs were predicted to be damaging through in silico analysis.
*Cousins.
†2×colorectal carcinomas from the same individual.

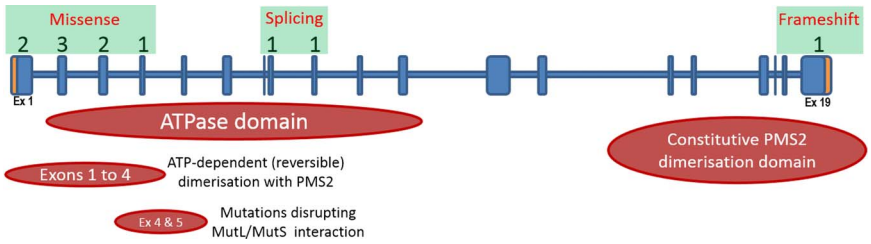



**Figure 2** Graphical overview of the location of the 11 *MLH1* mutations identified. Numbers above the gene schematic denote the amount of mutations identified in the corresponding exons. Mutation subtypes are boxed in green and the predicted functional domains of the MLH1 protein are displayed below the gene schematic.

therefore be useful to test PMS2-deficient CRC for $BRAF^{V600E}$ mutation or *MLH1* methylation to exclude sporadic tumour. Screening for *PMS2* mutations has been problematic due a large number of homologous sequences within pseudogenes that closely flank the functional gene and most likely accounts for the lower proportion of *PMS2* mutations reported in previous studies. The recent development of new methods incorporating long-range PCR and MLPA has eliminated most of the previous problems, such that the identification of large-scale deletions of exons 3 and/or 4 are now the only difficulty. The results from this study, representing the largest number of CRC with solitary loss of PMS2, support germline mutation screening of *MLH1* when no mutation in *PMS2* has been found. However, a substantial proportion of MMR-deficient CRCs with no evidence of *MLH1* methylation or $BRAF^{V600E}$ mutation remain unexplained and are referred to as Lynch-like or suspected Lynch syndrome. A number of potential causes for the underlying loss of PMS2 protein expression in these cases, including biallelic somatic mutations and cryptic mutations, have been described in a recent review.[21] In a large population-based study of the Colon Cancer Family Registry Cohort, 5.6% (271/4 853) of all CRCs were classified as Lynch-like syndrome, representing 56% of all MMR-deficient CRCs not secondary to *MLH1* methylation. In our study, six CRCs showed concurrent loss of MSH6 and PMS2. The most likely explanation for the loss of MSH6 expression in these six cases is the somatic frameshift mutation in the (C)8 microsatellite in exon 5 of the *MSH6* gene secondary to the loss of MMR function resulting from the PMS2 defect.[22] The use of panel testing rather than a single-gene approach would be useful; this is of particular interest clinically, where the *PMS2* gene has lower penetrance than other MMR genes[23] and family history is a suboptimal way of finding potentially high-risk families, where risk assessment and risk management has improved outcomes. However, *PMS2* testing remains challenging even by next generation sequencing due to its complex structure.

Our study included the largest reported sample of CRCs with solitary loss of PMS2 to date. Testing for germline *PMS2* mutations used in this study employed the most up-to-date and comprehensive approaches described,[18 24] as demonstrated by the high rate of identified *PMS2* mutations. One limitation of this study is the multicentre setting which may affect the consistency in the formalin fixation conditions of tissue blocks and lead to immunostaining artefacts. Other limitations include the absence of other Lynch syndrome-associated tumours, and the lack of mutation screening data for 20 (24%) cases. Moreover, our results may not reflect the

**Table 2** Characteristics of 66 individuals with 68 colorectal carcinomas (CRCs) showing loss of PMS2 expression and retained MLH1 expression

| Group | All (68 CRCs from 66 individuals) | *PMS2* mutations (N=50 CRCs from 49 individuals) | *MLH1* variant (N=12 CRCs from 11 individuals) | P value *PMS2* mutations vs *MLH1* variant | No *PMS2* or *MLH1* mutation (N=6) |
|---|---|---|---|---|---|
| Mean age at diagnosis±SD (range) in years | 51.7±11.7 (24–80) | 52.1±11.3 (35–80) | 43.5±12.0 (24–63) | 0.046 | 52.2±11.7 (33–69) |
| Gender male, N (%) | 36 (54.5) | 26/49 (53.1) | 8/11 (72.7) | 0.32 | 2 (33.3) |
| Amsterdam criteria I, N (%) | 6 (9.1) | 0 (0) | 6 (54.5) | 0.001* | 0 (0) |
| Amsterdam criteria II, N (%) | 10 (15.2) | 4 (8.2) | 6 (54.5) | | 0 (0) |
| Proximal CRC location, N (%) | 42/64 (65.6) | 35/47 (74.5) | 5/10 (50) | 0.14 | 2/6 (33.3) |
| Histological type, N (%) | | | | 1 | |
| Adenocarcinoma | 50/63 (79.4) | 36/47 (76.6) | 9/11 (81.8) | | 6/6 (100) |
| Mucinous carcinoma | 13/63 (20.6) | 11/47 (23.4) | 2/11 (18.2) | | 0/6 (0) |
| Histological grade, N (%) | | | | 0.024 | |
| Well/moderate | 42/62 (67.7) | 36/46 (78.3) | 4/10 (40) | | 1/5 (20) |
| Poor | 20/62 (32.3) | 10/46 (21.7) | 6/10 (60) | | 4/5 (80) |

*p Value for Amsterdam criteria I or II.

actual rate of PMS2-deficient CRC in the general population and the mutation rates in *PMS2* and *MLH1*, as these cases were selected in young individuals with strong family history of CRC.

In conclusion, the findings from this study suggest that CRCs in *MLH1* mutation carriers can demonstrate a normal pattern of MLH1 expression and justify the testing for *MLH1* germline mutation in individuals with a CRC showing solitary loss of PMS2 expression when a *PMS2* mutation is not identified.

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