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.Gin197Argfs*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.

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. 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. 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. All CRC cases were reviewed by specialist gastrointestinal pathologists for histological type and grade. 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. A subset of tumours were analysed for MSI status from formalin-embedded tissue as previously described. 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 (BRAFV600E mutation) was detected using fluorescent allele-specific PCR. MLH1 promoter methylation was analysed using the MLH1-M2 methylight reaction using an Arthrobacter luteus (ALU) control reaction to normalise for bisulphite-converted input DNA. 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-degree and 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.

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. 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 classifications15 (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 χ² 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 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 al4 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%).

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.

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).

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When restricted to patients with a CRC, a deleterious germline mutation in \textit{MLH1} was reported in two of nine tested patients (22%). Compared with these studies, our rate of \textit{PMS2} mutation in 66 tested individuals was higher at 74% and the rate of \textit{MLH1} deleterious mutation slightly lower at 12%. Two cousins (tumours 2 and 3) who carried the same \textit{MLH1} mutation both had CRC with solitary PMS2 loss. Similarly, one individual, who carried the \textit{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 \textit{et al.} described two sisters who carried the same \textit{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 \textit{PMS2} gene. Interestingly, we identified \textit{MLH1} methylation or the somatic \textit{BRAFV600E} mutation in four cases, indicating that isolated PMS2 loss of expression can occur outside Lynch syndrome. It may

![Figure 1](image-url) Flow diagram of the study. CRC, colorectal carcinoma.

### Table 1 Characteristics of the 11 individuals with a germline \textit{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.
In a large population-based study of the Colon and cryptic mutations, have been described in a recent fashion in these cases, including biallelic somatic mutations causes for the underlying loss of PMS2 protein expres-
suspected Lynch syndrome. A number of potential remain unexplained and are referred to as Lynch-like or

BRAFV600E

therefore be useful to test PMS2-deficient CRC for BRAFV600E 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 BRAFV600E 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.

In a large population-based study of the Colon Cancer Family Registry Cohort, 5.6% (271/4853) 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. 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 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, 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

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**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.
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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