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Tumour testing to identify Lynch syndrome in two Australian colorectal cancer cohorts

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Abstract

Background and Aim—Tumour testing of colorectal cancers (CRC) for mismatch repair (MMR) deficiency is an effective approach to identify carriers of germline MMR gene mutation (Lynch syndrome). The aim of this study was to identify MMR gene mutation carriers in two cohorts of population-based CRC utilising a combination of tumour and germline testing approaches.

Methods—CRCs from 813 patients diagnosed with CRC <60 years of age from the Australasian Colorectal Cancer Family Registry (ACCFR) and from 826 patients from the Melbourne Collaborative Cohort Study (MCCS) were tested for MMR protein expression using immunohistochemistry (IHC), microsatellite instability (MSI), *BRAF*^{V600E} somatic mutation and for *MLH1* methylation. MMR gene mutation testing (Sanger sequencing and MLPA) was performed on germline DNA of patients with MMR-deficient tumours and a subset of MMR-proficient CRCs.

Results—Of the 813 ACCFR probands, 90 probands demonstrated tumour MMR-deficiency (11.1%) and 42 had a MMR gene germline mutation (5.2%). For the MCCS, MMR-deficiency was identified in the tumours of 103 probands (12.5%) and 7 had a germline mutation (0.8%). All the mutation carriers were diagnosed prior to 70 years of age. Probands with a MMR-deficient CRC without *MLH1* methylation and a gene mutation were considered Lynch-like and comprised 41.1% and 22.3% of the MMR-deficient CRCs for the ACCFR and MCCS, respectively.

Conclusions—Identification of MMR gene mutation carriers in Australian CRC-affected patients is optimised by IHC screening of CRC diagnosed before 70 years. A significant proportion of MMR-deficient CRCs will have unknown aetiology (Lynch-like) proving problematic for clinical management.

Keywords

Colorectal cancer; Lynch syndrome; Mismatch repair protein expression; immunohistochemistry; microsatellite instability; *MLH1*; *MSH2*; *MSH6*; *PMS2*; *MLH1* methylation; *BRAF*^{V600E}

Introduction

Worldwide, colorectal cancer (CRC) is a leading cause of cancer related deaths[1]. Australia and New Zealand have the highest incidence of CRC with age-standardized rates of 44.8 and 32.2 per 100,000 in men and women, respectively[1]. The lifetime risk of CRC to age 75 years in Australia is 1 in 18 (5.5%) for men and 1 in 26 (3.9%) for women, and is the second most common cause of cancer-related death after lung cancer[2].

One way to reduce the incidence of CRC is to identify high risk individuals in the population and target them for screening and increased surveillance. Lynch syndrome is an autosomal dominant cancer predisposition disorder, defined by the identification of a pathogenic germline mutation in one of the DNA MMR genes *MLH1*, *MSH2*, *MSH6*, or *PMS2* or in *EPCAM*, a gene upstream of *MSH2*. MMR gene mutation carriers have a high risk of developing cancers within the large intestine and the endometrium, and also from the urinary tract, pancreas, hepatobiliary tract, stomach, small intestine and ovaries[3]. Up to 6% of all CRCs can be attributed to Lynch syndrome making it the most common hereditary CRC condition[4]. The identification of MMR gene mutation carriers enables appropriate risk management strategies that can improve patient outcomes.

Tumours arising in individuals with a MMR gene mutation demonstrate high levels of microsatellite instability (MSI) secondary to altered DNA MMR mechanisms. Testing tumours for evidence of this MMR deficiency by immunohistochemistry (IHC) is widely used by pathologists to screen for Lynch syndrome[5]. Two population-based studies have previously reported the identification of Lynch syndrome in Australia, one from Victoria in CRC patients diagnosed before 45 years[6] and one from Western Australia in CRC patients diagnosed before 60 years[7]. In this study, we describe the identification of Lynch syndrome in one early-onset CRC cohort and one later onset CRC cohort both derived from Victoria, Australia.

Methods

Study Participants

Participants were identified from two different Australian studies: the Australasian Colorectal Cancer Family Registry (ACCFR) and the Melbourne Collaborative Cohort Study (MCCS). In the ACCFR, population-based incident CRC cases were recruited, independent of family history of cancer, in Victoria between 1997 and 2007[8]. Of these, we identified 959 probands with a primary adenocarcinoma of the colon or rectum during two recruitment periods. Phase I recruitment (1997 to 2001) included all patients with a CRC diagnosed between 18 and 44 years of age and 50% of patients with CRC diagnosed between the ages of 45-59 years. Phase II recruitment (2001 to 2006) included all patients with a CRC diagnosed between 18 and 49 years of age. A proportion of these cases presented in this study (71 individuals from phase II recruitment) were reported in a previous study[6].

The MCCS is a prospective cohort study of 41,514 people (17,045 males and 24,469 females) recruited between 1990 and 1994[9]. Participants diagnosed with an incident CRC were aged 41 to 86 years. By 31 December 2009, 1046 participants had a first histopathological diagnosis of invasive adenocarcinoma of the colon or rectum in Victoria following the baseline study visit (a total of 1101 CRCs). A further 25 subjects had a clinical diagnosis only and were not considered as cases. Insufficient sample remained for 50 (5%) tumours, and the sample could not be obtained or was not sent for testing for 181 (17%) of the eligible tumours. In total, data were available for 851 of the eligible tumours from 826 of the MCCS participants.

Written informed consent was obtained from all participants to collect a blood sample and tumour pathology materials. The study protocols were approved by Human Research Ethics Committees at the University of Melbourne (ACCFR) and the Cancer Council Victoria (MCCS).

Family History of CRC and Extra-Colonic Cancers

For the ACCFR, information on personal and family history of CRC and other cancers in first- and/or second-degree relatives was obtained from completion of baseline and follow-up questionnaires completed at recruitment and 5 yearly thereafter, respectively. Cancers were verified, where possible using pathology reports, medical records, cancer registry reports, and/or death certificates[8]. Information on cancer family history was used to determine if the proband's family history met Amsterdam I or II criteria or the revised Bethesda guidelines[10, 11]. Information on family history of CRC was not available for the MCCS probands.

Pathology Review

Primary CRC tissue was available for 813 of the probands from the ACCFR Jeremy Jass Memorial Tissue Bank and 851 from the MCCS for standardised pathological review and molecular characterisation[12]. Tumours from the ileo-caecal junction through the caecum, ascending colon, hepatic flexure, and transverse colon were grouped as right-sided (proximal). Tumours from the splenic flexure, descending, sigmoid colon and recto-sigmoid junction were classified as left-sided (distal); tumours from the rectum were considered as a third distinct group. Tumour TNM stage was derived from the pathological stage of the tumour and of the lymph node status, and the clinical metastatic stage.

DNA Mismatch Repair Deficiency and Molecular Testing

MMR-deficiency was determined primarily by IHC staining for the four MMR proteins as previously described[13, 14]. Microsatellite instability (MSI) was determined from a ten-marker panel in 794 tumours collected from the MCCS and for 555 tumours collected from the ACCFR (predominantly Phase I recruited participants and for CRCs showing loss of MMR protein expression), as previously described[5, 13]. Tumours were categorised as: 1) MMR-deficient if they were MSI-H and/or showed loss of expression of one or more of the MMR proteins by IHC; or 2) MMR-proficient if tumours were MSS (microsatellite stable) or MSI-L (low-level MSI) and/or showed normal retained expression of all four MMR proteins by IHC. CRCs for which both MSI and MMR IHC testing were completed (ACCFR 555/823, 67.4%; MCCS 794/851, 93.3%) demonstrated 95.7% and 98.9% concordance for MMR-deficiency, respectively. For the discordant cases, CRCs were categorised as MMR-deficient if one of the tests was positive (MSI-H or abnormal IHC). In addition, tumours demonstrating loss of the MLH1 and PMS2 by IHC were characterised for methylation of the *MLH1* promoter region using the MethyLight assay as previously described[15, 16]. All CRCs were tested for the $BRAF^{V600E}$ somatic mutation using a fluorescent allele-specific PCR assay as previously described[17].

Germline Mutation Testing

Germline MMR gene mutation testing was performed using Sanger sequencing and Multiplex Ligation Dependent Probe Amplification (MLPA), including testing for 3′ deletions in *EPCAM* as previously described[8, 14, 15, 18]. For the ACCFR, the following probands were tested: 1) MMR-deficient CRCs (n=90), 2) a subset of MSI-L CRCs (n=40), and 3) a subset of probands with MMR-proficient CRC who met Revised Bethesda guidelines, Amsterdam I or II criteria or who had a family history of CRC (n=35). For MCCS, only probands with MMR-deficiency and negative for *MLH1* promoter methylation were screened for germline mutations (n=32). A subset of probands with *MLH1* methylation positive CRCs from both studies were tested for germline MMR gene mutations (n=19). Germline variants were classified for pathogenicity based on the InSiGHT database classifications (http://insight-group.org/variants/classifications/) where classes 4 and 5 were considered pathogenic[19]. For variants not yet classified by InSiGHT, we considered a variant as pathogenic if it resulted in a stop codon, frameshift, large deletion, or if it removed a canonical splice site. Individuals with a MMR-deficient CRC, negative for the $BRAF^{V600E}$ mutation and *MLH1* promoter methylation, but without an identified MMR gene germline mutation were considered as Lynch-like syndrome cases[20].

Statistical Analysis

Statistical analyses were conducted using SPSS statistics software version 19.0 (SPSS Inc., Chicago, IL). Categorical variables were compared using Fisher's exact test, and continuous variables were compared using non-parametric Wilcoxon-Mann-Whitney rank-sum test. All p-values were two-tailed and p-values less than 0.05 were considered to be statistically significant to test null hypothesis.

Results

Primary CRC tissue (n=823) was available for molecular characterisation from 813 ACCFR probands where MMR-deficiency was identified in 94 tumours from 90 probands (11.1%). For the MCCS CRC-affected probands (n=1046), molecular pathology results were obtained for 851 CRCs from 826 probands (79%). MMR-deficiency was identified in the tumours of 103 participants (12.5%). The characteristics of the ACCFR and MCCS study participants overall and by their tumour MMR status are shown in Tables 1 and 2, respectively.

Detail for the patterns of abnormal MMR expression and germline mutations are given in Table 3, Supplementary Table 1 and in the flow diagrams (Figures 1 and 2). Methylation of the *MLH1* gene promoter was observed in 25% and 85.7% of MLH1/PMS2-deficient CRCs from the ACCFR and MCCS, respectively. Of the MLH1/PMS2-deficient CRCs that were positive for *MLH1* methylation, only 61.5% and 77.8% were also positive for the $BRAF^{V600E}$ somatic mutation (ACCFR and MCCS studies, respectively). No pathogenic *MLH1* gene mutations were identified out of 10 (ACCFR) and 9 (MCCS) probands with *MLH1* methylated CRCs that were tested. *MLH1* methylation was not observed in 47 MMR-proficient CRCs tested from the ACCFR.

A MMR gene mutation was identified in 42 ACCFR probands (5.2%), representing 46.5% of the MMR-deficient CRCs. Two of the mutation carriers were identified among individuals with MMR-proficient tumours, both carried *MSH6* mutations. None of the 40 ACCFR probands with an MSI-L CRC were found to have a germline MMR gene mutation. Comparing these results to the MCCS findings, only 7 probands had a pathogenic germline mutation (0.8%), representing 5.8% of the MMR-deficient CRCs. Of the 47 non-overlapping MMR gene mutation carriers identified in this study, 13 (27.7%) met Amsterdam I criteria, 4 (8.5%) met Amsterdam II criteria, 26 (55.3%) met Revised Bethesda guidelines and 1 (2.1%) had no clinical family history.

A number of atypical patterns of MMR-deficiency were observed across both studies namely, the loss of expression of 3 or more MMR proteins within a single CRC (Table 3). Four CRCs (all females, diagnosis ages were 59, 73, 75, and 77 years) demonstrated loss of MLH1 and PMS2 as well as loss of MSH6. All four tumours were right-sided, demonstrated *MLH1* promoter methylation, the $BRAF^{V600E}$ somatic mutation and the absence of *MLH1* or *MSH6* germline mutations. A single CRC demonstrated loss of MSH2 and MSH6 as well as loss of PMS2 expression while retaining MLH1 expression. A germline mutation in *PMS2* was identified while no *MSH2* or *MSH6* germline mutation was identified to account for the loss of MSH2/MSH6 expression. A female MCCS proband developed synchronous ascending colon tumours at 77 years of age where one CRC demonstrated loss of expression of all 4 MMR proteins while the other showed loss of MLH1/PMS2 expression only. Both of these CRCs demonstrated *MLH1* promoter hypermethylation and the $BRAF^{V600E}$ somatic mutation however no *MSH2* or *MSH6* germline mutation was identified. One additional MCCS proband developed a MLH1/PMS2 deficient CRC in the caecum at 72 years of age resulting from *MLH1* promoter methylation and was diagnosed 6 months later with a rectal adenocarcinoma that demonstrated loss of MSH2 and MSH6 protein expression however no *MSH2* or *MSH6* germline mutation was identified.

Probands with MMR-deficient CRCs that underwent germline mutation testing but had no pathogenic mutation identified, or had a VUS identified or did not show evidence of *MLH1* methylation were, therefore, considered suspected Lynch or Lynch-like cases. For the ACCFR 37/90 (41.1%) MMR-deficient CRCs were considered Lynch-like while for the MCCS 26/103 (25.2%) of the MMR-deficient CRCs were considered Lynch-like. The combined Lynch-like syndrome probands from the ACCFR and MCCS had a median age of diagnosis of 50 years which was older than the median age of MMR gene mutation carriers ($p=0.002$) and younger than the median age of MMR-proficient cases ($p=0.02$) and *MLH1* methylation cases ($p<0.001$) (Table 4). There was no evidence for a difference in the tumour characteristics of MMR gene mutation carriers and Lynch-like syndrome probands (Table 4).

The combined data from the two cohorts demonstrated that all the mutation carriers identified in this study were diagnosed prior to 70 years of age and that 95.7% of carriers were identified prior to a diagnosis age of 60 years (Table 5). When considering the group of individuals with MMR-deficient CRCs who were positive for *MLH1* methylation and, therefore, would not have germline mutation testing in the clinical setting, 56.5% and 83.5% of all the *MLH1* methylated MMR-deficient CRCs were identified in the age at CRC diagnosis groups of >70 years and >60 years, respectively. The yield of MMR mutation

carriers relative to the number of MMR IHC tests that were performed demonstrated that testing: 1) all CRCs, 2) only those diagnosed <70 years or 3) those diagnosed <60 years resulted in yields of 2.9%, 3.7% and 4.7%, respectively, for a total of 1639, 1267 and 951 MMR IHC tests performed for those same three scenarios. The sensitivity, specificity, positive and negative predictive values and positive likelihood ratio for MMR IHC testing overall and by differing age at CRC diagnosis thresholds are shown in Supplementary Table 2.

Discussion

In this study, we report the results of tumour and germline mutation testing of CRC-affected individuals from two Australian cohorts to identify Lynch syndrome. From the ACCFR, where probands were diagnosed with CRC before age 60, we identified MMR deficiency in 11.1% of cases of which 14.4% were secondary to *MLH1* methylation. A MMR gene mutation was identified in 40 probands with a MMR-deficient CRC and in 2 probands with a MMR-proficient CRC for a total prevalence of 5.2%. From the MCCS, where all incident CRCs were recruited from probands aged 41 to 86, the proportion of MMR deficiency was 12.5% of which 85.2% were caused by *MLH1* methylation; Lynch syndrome was diagnosed in 7 probands (0.8%). Previous studies looking at Lynch syndrome in incident CRC probands from Australian cohorts described 17% (18/105) of CRC-affected individuals diagnosed under the age of 45 years[6] and 2.7% (36/1344) of CRCs diagnosed before 60 years of age[7]. Compared to these studies, our study included individuals older than 60 years at CRC diagnosis to address the potential benefit of universal CRC screening by MMR IHC. The results for the detection of Lynch syndrome in the ACCFR were twice as high compared with the Western Australian cohort (5.2% versus 2.6%) where both studies were restricted to investigating CRCs diagnosed before 60 years of age, however, the recruitment of only 50% of patients with CRC diagnosed between the ages of 45-59 years in Phase I of the ACCFR study may have led to some bias in the number of mutation carriers identified.

Interestingly, 3 ACCFR cases with normal MMR IHC expression were *MSH6* mutation carriers, although 2/3 did demonstrate high levels of MSI. This is in agreement with previous studies which reported that a substantial proportion of tumours in *MSH6* mutation carriers showed low level or absence of MSI and retained IHC expression of MSH6, in particular for missense mutations[21, 22]. These observations and our own findings showing that patients with *MSH6* mutations maybe missed by IHC and MSI testing suggests the prevalence of Lynch syndrome caused by *MSH6* mutations is probably underestimated in the population.

Most MLH1-deficient CRCs are sporadic, caused by somatic *MLH1* promoter methylation. Two molecular genetic tests are currently used to identify these cases: *MLH1* promoter methylation and *BRAF*^{V600E} mutation testing. In our study, *BRAF*^{V600E} was observed in 61.5% and 77.8% of CRCs positive for *MLH1* methylation from the ACCFR and MCCS studies, respectively, suggesting that using *BRAF*^{V600E} instead of *MLH1* methylation as a negative MMR mutation predictor would result in ~20-40% of CRCs with MLH1 and PMS2 loss of protein expression being incorrectly referred for germline mutation testing. However, in most pathology departments, *BRAF*^{V600E} testing is the most accessible and therefore

preferred strategy showing less variability in methodologies with non-quantitative results. Recently, IHC with a specific antibody for the BRAF V600E protein has become available to be used in as a promising surrogate marker for sequencing. However, despite some initial encouraging results,[23] BRAF V600E IHC should be used with caution due to the variable reliability for determining the *BRAF*^{V600E} status in CRC with sensitivity and specificity as low as 59% and 51%, respectively (reviewed in [24]), and the increasing recognition of other *BRAF* mutations (non V600E) in up to 23% of CRC that would not be identified with this mutation-specific antibody[25-27].

Universal testing, or reflex testing of all newly diagnosed CRC tumours for MMR protein status, has been recommended or endorsed by several organisations as the preferred approach to identify Lynch syndrome[28-31]_ENREF_37. Universal testing has been shown to reduce morbidity and mortality from Lynch syndrome in relatives[28]. Compared with selective strategies, universal testing is more sensitive to identify Lynch syndrome patients and is cost-effective[32-34]. In this study, when the results from both cohorts were combined, the number of MMR gene mutation carriers identified peaked in the 41-50 years age group. Based on our data, adopting a universal tumour testing approach compared with a strategy that tested only CRCs diagnosed ≤60 years would result in 688 additional MMR IHC tests and 65 additional *MLH1* methylation tests for the identification of two mutation carriers. An upper age threshold of 70 years would mean 372 fewer MMR IHC tests and 57 fewer *MLH1* methylation tests for no gains in identified mutation carriers. The use of upper age cutoffs to limit the testing of older CRC patients who are less likely to have Lynch syndrome has received support[35]. Although the study by Moreira and colleagues[36] found that universal testing was the most sensitive, a model where all CRC diagnosed <70 years and only those CRCs diagnosed >70 years that met Bethesda guidelines were tested resulted in a sensitivity of 95.1% (versus 100% sensitivity for universal MMR IHC) with ~35% fewer MMR IHC tests being performed.

In the latest 2012 edition of the Cancer Protocol for CRC, recommendation from the Royal College of Pathologists of Australasia (RCPA) is to perform IHC for MMR protein expression in all CRC cases diagnosed in patients less than 50 years of age and in patients meeting the revised Bethesda criteria[37]. Despite low numbers of identified Lynch syndrome in individuals older than 50 years, our findings suggest that the RCPA could increase their age limit recommendations to test all CRCs diagnosed <70 years of age, and be performed in conjunction with *MLH1* promoter methylation or *BRAF*^{V600E} mutation testing for MLH1-deficient CRCs, as the optimal strategy to identify MMR gene mutation carriers. A cost-effectiveness study in Australia would be useful to support this suggestion. If laboratory resources are limited, MMR IHC tumour testing of all CRC cases diagnosed ≤60 years of age in conjunction with *MLH1* promoter methylation testing would also be an efficient strategy, although less sensitive and has been recommended for Lynch syndrome screening in endometrial cancer[16].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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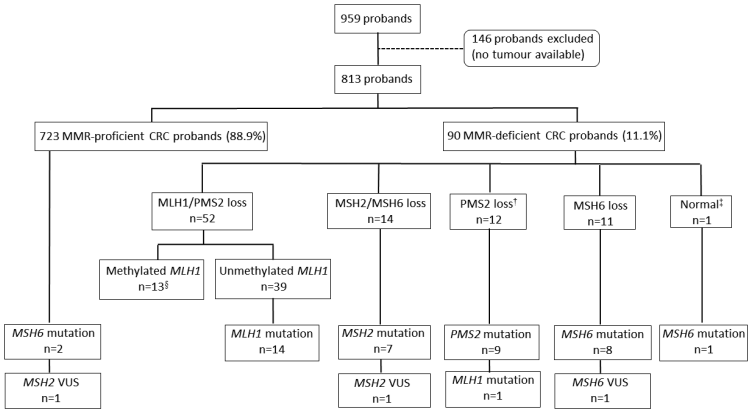

Figure 1.

Flow diagram of the study with results of germline mismatch repair gene mutations by pattern of abnormal immunohistochemical expression in the ACCFR.

† One tumour showed loss of PMS2 and MSH2/MSH6 expression.

‡ One tumour showed normal expression of MMR proteins but an MSI-H phenotype.

§ One tumour showed loss of MLH1/PMS2 and MSH6 expression. From the 13 tumours with methylation of *MLH1*, 8 were also positive for the $BRAF^{V600E}$ somatic mutation.

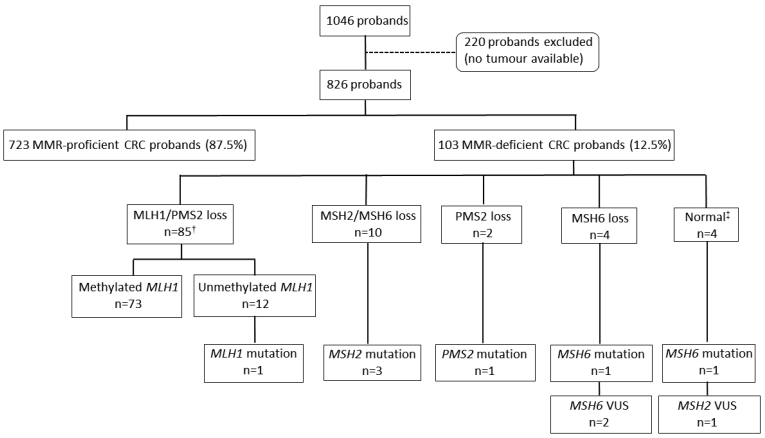

Figure 2.

Flow diagram of the study with results of germline mismatch repair gene mutations by pattern of abnormal immunohistochemical expression in the MCCS.

† One tumour loss of MLH1/PMS2 and MSH2/MSH6 expression and was *MLH1* methylated.

‡ These tumours showed high levels of MSI (MSI-H) but showed normal expression of all four MMR proteins

Table 1

Proband characteristics and tumour histological features from the Australian Colon Cancer Family Registry (ACCFR)

	Number (%)		
	All (823 CRC, 813 probands)	**MMR-Proficient (729 CRC, 723 probands)**	**MMR-Deficient (94 CRC, 90 probands)**
Gender			
Female	395 (49)	352 (49)	43 (48)
Male	418 (51)	371 (51)	47 (52)
Age at diagnosis [†] (years)			
Median (range)	47 (18-59)	47 (18-59)	43 (18-59)
Mean ± Standard deviation	46.3 ± 8.0	46.7 ± 7.7	43.1 ± 9.1
18 – 30	25 (3)	18 (2.5)	7 (7.4)
31 – 40	153 (18.6)	129 (17.7)	24 (25.5)
41 – 50	374 (45.4)	332 (45.5)	42 (44.7)
51- 60	268 (32.6)	249 (34.2)	19 (20.2)
61- 70	3 (0.4)	1 (0.1) [a]	2 (2.1) [b]
71-80	0	0	0
>80	0	0	0
Family History			
Amsterdam I	38 (5)	26 (4)	12 (13)
Amsterdam II	11 (1)	5 (1)	6 (7)
Amsterdam I or II	49 (6)	31 (4)	18 (20)
Revised Bethesda	602 (74)	514 (71)	90 (100)
Tumour location			
Proximal colon	214 (28)	155 (23)	59 (66)
Distal colon	231 (30)	216 (32)	15 (17)
Rectum	317 (42)	302 (45)	15 (17)
Missing	61	56	5
Histological type			
Adenocarcinoma	724 (91)	658 (93)	66 (74)
Mucinous carcinoma	70 (9)	47 (7)	23 (26)
Signet ring cell carcinoma	4	4	0
Missing	25	20	5
Histological grade (adenocarcinoma)			
Low grade	591 (82)	543 (83)	48 (73)
High grade	126 (18)	108 (17)	18 (27)
Missing	7	7	0
Stage (TNM)			
Stage I	134 (18)	118 (17)	16 (19)

	Number (%)		
	All (823 CRC, 813 probands)	**MMR-Proficient (729 CRC, 723 probands)**	**MMR-Deficient (94 CRC, 90 probands)**
Stage II	259 (34)	208 (31)	51 (61)
Stage III	323 (42)	307 (45)	16 (19)
Stage IV	48 (6)	47 (7)	1 (1)
Missing	59	49	10
Tumour margin			
Expanding	447 (63)	376 (60)	71 (89)
Infiltrating	261 (37)	252 (40)	9 (11)
Missing	115	101	14
Peritumoural lymphocytes			
Present	320 (43)	266 (41)	54 (67)
Absent	412 (57)	385 (59)	27 (33)
Missing	91	78	13
Crohn's-like reaction			
Present	145 (20)	101 (16)	44 (55)
Absent	578 (80)	542 (84)	36 (45)
Missing	100	86	14
Tumour-infiltrating lymphocytes			
Present	166 (22)	100 (15)	66 (76)
Absent	605 (78)	584 (85)	21 (24)
Missing	52	45	7
***BRAF*^{V600E} mutation**			
Positive	57 (7)	44 (6)	13 (15)
Negative	735 (93)	659 (94)	76 (85)
Missing	31	26	5

†Age at diagnosis of first CRC for metachronous CRC

^{a}proband had MMR-proficient CRC diagnosed at 53 years and metachronous MMR-proficient CRC diagnosed at 61 years.

^{b}proband had MMR-proficient CRC diagnosed at 58 years and two metachronous MMR-deficient CRCs diagnosed at 62 years.

Table 2

Proband characteristics and tumour histological features from the Melbourne Collaborative Cohort Study (MCCS)

	Number (%)		
	All (851 CRC, 826 probands)	**MMR-Proficient (740 CRC, 723 probands)**	**MMR-Deficient (111 CRC, 103 probands)**
Gender			
Female	398 (48)	334 (46)	64 (62)
Male	428 (52)	389 (54)	39 (38)
Age at diagnosis† (years)			
Median (range)	70 (41-86)	69 (42-86)	72 (41-83)
Mean ± Standard deviation	68.5 ± 8.2	68.2 ± 8.2	70.3 ± 8.2
18 – 30	0	0	0
31 – 40	0	0	0
41 – 50	23 (2.7)	17 (2.3)	6 (5.4)
51- 60	117 (13.8)	111 (15)	6 (5.4)
61- 70	327 (38.4)	291 (39.3)	36 (32.4)
71-80	347 (40.8)	293 (39.6)	54 (48.7)
>80	37 (4.3)	28 (3.8)	9 (8.1)
Tumour location			
Proximal colon	301 (37)	212 (30)	89 (84)
Distal colon	215 (26)	208 (29)	7 (7)
Rectum	299 (37)	289 (41)	10 (9)
Missing	36	31	5
Histological type			
Adenocarcinoma	749 (90)	671 (93)	78 (72)
Mucinous carcinoma	66 (8)	42 (6)	24 (22)
Signet ring cell carcinoma	10 (2)	5 (1)	5 (5)
Undifferentiated	4 (0)	3 (0)	1 (1)
Missing	22	19	3
Histological grade (adenocarcinoma)			
Low grade	669 (81)	605 (84)	64 (60)
High grade	155 (19)	113 (16)	42 (39)
Undifferentiated	4 (0)	3 (0)	1 (1)
Missing	23	19	4
Tumour margin			
Expanding	522 (71)	444 (69)	78 (80)
Infiltrating	214 (29)	195 (31)	19 (20)
Missing	115	101	14
Peritumoural lymphocytes			

	Number (%)		
	All (851 CRC, 826 probands)	**MMR-Proficient (740 CRC, 723 probands)**	**MMR-Deficient (111 CRC, 103 probands)**
Present	160 (22)	114 (18)	46 (46)
Absent	583 (78)	528 (82)	55 (54)
Missing	108	98	10
Crohn's-like reaction			
Present	130 (18)	83 (13)	47 (48)
Absent	593 (82)	542 (87)	51 (52)
Missing	128	115	13
Tumour-infiltrating lymphocytes			
Present	185 (23)	113 (16)	72 (69)
Absent	608 (77)	576 (84)	32 (31)
Missing	58	51	7
***BRAF*^{V600E} mutation**			
Positive	134 (17)	74 (11)	60 (58)
Negative	657 (83)	613 (89)	44 (42)
Missing	60	53	7

†Age at diagnosis of first CRC for metachronous CRC

Table 3

Results from MMR tumour and germline testing from both ACCFR and MCCS.

	ACCFR	%	MCCS	%
Probands recruited	959		1046	
Probands with CRC tissue for MMR IHC	813	100	826	100
Probands with MSI tested CRC	551	67.8	794	96.1
Probands with MMR-proficient CRC	723	88.9	723	87.5
Probands with MMR-deficient CRC (All MMR-deficient CRCs i.e. synchronous/metachronous)	90 (94)	11.1	103 (111)	12.5
Germline MMR gene mutation testing results				
MLH1/PMS2 loss[†]	52 (53)	57.8	84 (90)	81.6
MLH1 mutation-neg, *MLH1* methylated (*BRAF* V600E mutation positive)[‡]	13 (8)	25	72 (56)	85.7
MLH1 mutation-pos, *MLH1* unmethylated	14	26.9	1	1.2
MLH1 VUS-pos, *MLH1* unmethylated	0	0	0	0
MLH1 mutation-neg, *MLH1* unmethylated	24	46.2	11	13.1
MLH1 mutation-unknown, *MLH1* unmethylated[§]	1	1.9	0	0
MSH2/MSH6 loss	14	15.6	10	9.7
MSH2 mutation-positive	7	50	3	30
MSH2 VUS-positive	1	7.1	0	0
MSH2 mutation-negative	5	35.8	7	70
MSH2 mutation-unknown[§]	1	7.1	0	0
MSH6 only loss	11	12.2	4	3.9
MSH6 mutation-positive	8	72.7	1	25
MSH6 VUS-positive[¶]	1	9.1	2	50
MSH6 mutation-negative	2	18.2	1	25
MSH6 mutation-unknown[§]	0	0	0	0
PMS2 only loss	11	12.2	2	1.9
PMS2 mutation-positive[b]	8	72.7	1	50
MLH1 mutation-positive	1	9.1	0	0
PMS2 mutation-negative	1	9.1	0	0
PMS2 mutation-unknown[§]	1	9.1	1	50
PMS2 and MSH2/MSH6 loss	1	1.1	0	0
PMS2 mutation-positive, *MLH1* unmethylated, *MLH1, MSH2, MSH6* mutation-negative	1	100	0	0
MLH1/PMS2 and MSH2/MSH6 loss	0	0	1	0.9
MLH1, PMS2, MSH2, MSH6 mutation-	0	0	1	100

	ACCFR	%	MCCS	%
negative, *MLH1* methylated				
MSI-H and normal MMR protein expression	1	1.1	4	3.9
MSH6 mutation-positive, *MLH1*, *MSH2*, and *PMS2* mutation-negative[a]	1	100	1	25
MSH2 VUS-positive, *MLH1* and *MSH6* mutation negative	0	0	1	25
MLH1, *MSH2* and *MSH6* mutation-negative	0	0	2	50
MSI-L and normal MMR protein expression	53		NT	
MLH1 and *MSH2* mutation-negative	25			
MLH1, *MSH2* and *MSH6* mutation-negative	15			
MMR mutation testing not performed	13			
MSS and/or normal MMR protein expression	670		NT	
MSH6 mutation-positive	2			
MSH2 VUS-positive	1			
MLH1, *MSH2* and *MSH6* mutation-negative	32			
MMR mutation testing not performed	635			

NT= none tested

[†] 1x tumour showed loss of MLH1/PMS2 and MSH6 protein expression in ACCFR and 3x tumours showed loss of MLH1/PMS2 and MSH6 expression in the MCCS.

[‡] 10/13 individuals were tested for *MLH1* mutations and 3/13 had no blood-derived DNA available for MLH1 mutation testing for the ACCFR. For the MCCS, 9 out of 72 CRCs demonstrating *MLH1* methylation were tested for *MLH1* germline mutations. Neither ACCFR nor MCCS probands with *MLH1* methylated CRCs were found to carry a germline *MLH1* mutation in those who were screened.

[§] no blood-derived DNA available for germline mutation testing and therefore mutation carrier status is unknown

[¶] *MSH6* c.2341C=T p.Pro781Ser is classified as a class 3 variant with insufficient evidence for pathogenicity. CRC demonstrated solitary loss of MSH6 expression and MSI-H.

[a] *MSH6* c.3107_3108delTG p.(Phe1037Leufs*2) identified in proband from ACCFR and from MCCS that were subsequently identified as the same individual with MSI-H CRC.

[b] *PMS2* c.802dup p.(Tyr268Leufs*31) identified in proband from ACCFR and from MCCS that were subsequently identified as the same individual with CRC showing solitary loss of PMS2 expression.

Table 4

Comparison of proband and tumour features between cases with a MMR-proficient CRC, a MMR-deficient CRC with *MLH1* methylation, MMR gene mutation carrier (Lynch syndrome) and Lynch-like syndrome from the ACCFR and MCCS probands combined.

	Number (%)			
	MMR-Proficient 1469 CRC, 1446 probands)	***MLH1* methylation (91 CRC, 85 probands)**	**MMR-gene mutation (49 CRC, 45 probands)**	**Lynch-like syndrome (63 CRC, 63 probands)**
Gender				
Female	686 (47.4)	61 (71.8)	15 (33.3)	31 (49.2)
Male	760 (52.6)	24 (28.2)	30 (66.7)	32 (50.8)
Age at diagnosis (years)				
Mean ± Standard deviation	57.5 ± 13.4	69.6 ± 8.8	43.1 ± 8.8	52.9 ± 16.3
Mean (range)	57 (18-85)	72 (46-83)	43 (22-69)	50 (18-81)
18-30	18 (1.2)	0	3 (6.1)	4 (6.3)
31-40	129 (8.8)	0	11 (22.4)	13 (20.6)
41-50	349 (23.8)	4 (4.4)	28 (57.2)	15 (23.8)
51-60	360 (24.5)	10 (10.9)	5 (10.2)	9 (14.3)
61-70	292 (19.9)	26 (28.6)	2 (4.1)	10 (15.9)
71-80	293 (19.9)	43 (47.3)	0	11 (17.5)
>80	28 (1.9)	8 (8.8)	0	1 (1.6)
Family History †				
Amsterdam I	24 (3)	0 (0)	13 (31)	1 (3)
Amsterdam II	5 (1)	1 (8)	4 (10)	1 (3)
Amsterdam I or II	29 (4)	1 (8)	17 (40)	2 (5)
Revised Bethesda	512 (71)	13 (100)	42 (100)	37 (100)
Tumour location				
Proximal colon	367 (26.5)	76 (90.5)	32 (69.6)	39 (61.9)
Distal colon	424 (30.7)	5 (5.9)	8 (17.4)	9 (14.3)
Rectum	591 (42.8)	3 (3.6)	6 (13)	15 (23.8)
Missing	87	7	3	0
Histological type				
Adenocarcinoma	1329 (93)	58 (67.4)	32 (69.6)	52 (82.5)
Mucinous carcinoma	89 (6.2)	22 (25.6)	14 (30.4)	10 (15.9)
Signet ring cell carcinoma	9 (0.6)	5 (5.8)	0	0
Undifferentiated	3 (0.2)	1 (1.2)	0	1 (1.6)
Missing	39	5	3	0
Histological grade (adenocarcinoma)				
Low grade	1148 (83.7)	45 (52.9)	32 (69.6)	46 (73)
High grade	221 (16.1)	39 (45.9)	14 (30.4)	17 (27)

	Number (%)			
	MMR-Proficient 1469 CRC, 1446 probands)	***MLH1* methylation (91 CRC, 85 probands)**	**MMR-gene mutation (49 CRC, 45 probands)**	**Lynch-like syndrome (63 CRC, 63 probands)**
Undifferentiated	3 (0.2)	1 (1.2)	0	0
Missing	26	6	3	0
Stage (TNM) †				
Stage I	117 (17)	2 (17)	8 (21)	7 (20)
Stage II	207 (31)	8 (66)	22 (56)	22 (63)
Stage III	307 (45)	2 (17)	9 (23)	5 (14)
Stage IV	47 (7)	0	0	1 (3)
Missing	49	1	6	0
Tumour margin				
Expanding	820 (64.7)	65 (81.3)	36 (90)	46 (83.6)
Infiltrating	447 (35.3)	15 (18.7)	4 (10)	9 (16.4)
Missing	202	11	9	8
Peritumoral lymphocytes				
Present	380 (29.4)	37 (44.6)	24 (60)	37 (64.9)
Absent	913 (70.6)	46 (55.4)	16 (40)	20 (35.1)
Missing	176	8	9	6
Crohn's-like reaction				
Present	184 (14.5)	40 (48.2)	17 (43.6)	34 (61.8)
Absent	1084 (85.5)	43 (51.8)	22 (56.4)	21 (38.2)
Missing	201	8	10	8
Tumour-infiltrating lymphocytes				
Present	213 (15.5)	60 (70.6)	30 (68.2)	47 (78.3)
Absent	1160 (84.5)	25 (29.4)	14 (31.8)	13 (21.7)
Missing	96	6	5	3

† Results include only ACCFR probands as no data was available for the MCCS probands

Table 5

The number of MMR gene mutation carriers identified within groups defined by age at first CRC diagnosis relative to the number of *MLH1* methylated CRCs and the number of MMR-deficient and MMR-proficient CRCs identified from both ACCFR and MCCS studies combined. The proportion of carriers identified by the total number of MMR IHC tumour tests for each age group are presented.

Age at first CRC diagnosis (years)	MMR mutation carriers†	*MLH1* methylated CRCs	Lynch-like	MMR-deficient CRCs	MMR-proficient CRCs	Total	Proportion of carriers from total tested
Mean ± Standard deviation	43.1 ± 8.8	69.6 ± 8.8	52.9 ± 16.3	57.7 ± 16	57.5 ± 13.4		
18-30	3 (6.7%)	0	4 (6.3%)	7 (3.6%)	18 (1.2%)	25	**12%**
31-40	11 (24.4%)	0	13 (20.6%)	24 (12.4%)	127 (8.8%)	151	**7.2%**
41-50	25 (55.6%)	4 (4.7%)	15 (23.8%)	44 (22.8%)	347 (24.0%)	391	**6.4%**
51-60	4 (8.9%)	10 (11.8%)	9 (14.3%)	23 (11.9%)	359 (24.8%)	382	**1.0%**
61-70	2 (4.4%)	23 (27.1%)	10 (15.9%)	35 (18.1%)	281 (19.4%)	316	**0.6%**
71-80	0	40 (47.1%)	11 (17.5%)	51 (26.4%)	286 (19.8%)	337	**0**
81-86	0	8 (9.4%)	1 (1.6%)	9 (4.7%)	28 (1.9%)	37	**0**
Total Probands	45	85	63	193	1446	1639	

† VUS were excluded