

# Identifying mismatch repair-deficient colon cancer: near-perfect concordance between immunohistochemistry and microsatellite instability testing in a large, population-based series

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## Identifying mismatch repair-deficient colon cancer: near-perfect concordance between immunohistochemistry and microsatellite instability testing in a large, population-based series

**Aims:** Establishing the mismatch repair (MMR) status of colorectal cancers is important to enable the detection of underlying Lynch syndrome and inform prognosis and therapy. Current testing typically involves either polymerase chain reaction (PCR)-based microsatellite instability (MSI) testing or MMR protein immunohistochemistry (IHC). The aim of this study was to compare these two approaches in a large, population-based cohort of stage 2 and 3 colon cancer cases in Northern Ireland.

**Methods and results:** The study used the Promega pentaplex assay to determine MSI status and a four-antibody MMR IHC panel. IHC was applied to tumour tissue microarrays with triplicate tumour sampling, and assessed manually. Of 593 cases with available MSI and MMR IHC results, 136 (22.9%) were MSI-high (MSI-H) and 135 (22.8%) showed abnormal MMR

IHC. Concordance was extremely high, with 97.1% of MSI-H cases showing abnormal MMR IHC, and 97.8% of cases with abnormal IHC showing MSI-H status. Under-representation of tumour epithelial cells in samples from heavily inflamed tumours resulted in misclassification of several cases with abnormal MMR IHC as microsatellite-stable. MMR IHC revealed rare cases with unusual patterns of MMR protein expression, unusual combinations of expression loss, or secondary clonal loss of expression, as further illustrated by repeat immunostaining on whole tissue sections.

**Conclusions:** MSI PCR testing and MMR IHC can be considered to be equally proficient tests for establishing MMR/MSI status, when there is awareness of the potential pitfalls of either method. The choice of methodology may depend on available services and expertise.

**Keywords:** colon cancer, mismatch repair, immunohistochemistry, microsatellite instability

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## Introduction

The molecular classification of colorectal cancer (CRC) has, in recent years, been the subject of rapid interest and scientific progress.<sup>1</sup> The microsatellite instability (MSI) pathway remains the most consistent and best-characterised molecular pathogenic process leading to the development of CRC.<sup>2,3</sup> The identification of MSI-high (MSI-H) CRC is important for two distinct reasons. First, it is the hallmark cancer of Lynch syndrome, caused by constitutive mutation in one of the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, or *PMS2*, or in *EPCAM*, a key regulatory gene of *MSH2*.<sup>4,5</sup> Between 2% and 5% of all new diagnoses of CRC relate to underlying Lynch syndrome.<sup>6</sup> Second, MSI-H CRC is clinically, as well as biologically, distinct from microsatellite-stable (MSS) CRC, as MSI-H CRC patients have better stage-specific survival and may be non-responsive to conventional 5-fluorouracil-based chemotherapy.<sup>7</sup> Therefore, advanced-stage MSI-H CRC is now a target for potential alternative treatment with newer immunotherapies.<sup>8,9</sup> Although MSI-H CRCs include those related to Lynch syndrome, the group is predominantly composed of non-hereditary, 'sporadic' tumours, sharing a common pathway of MSI but caused most commonly by somatic hypermethylation of the *MLH1* promoter, associated with somatic *BRAF* mutation, rather than constitutive MMR gene inactivation. Sporadic MSI-H CRCs are much more common than those related to Lynch syndrome, accounting for ~15% of all new CRC diagnoses. Biallelic somatic mutations in MMR genes account for a significant proportion of MSI-H CRCs lacking constitutive mutation in MMR genes, somatic *BRAF* mutation, or *MLH1* promoter hypermethylation, especially among patients in older age groups.<sup>10–12</sup>

For these reasons, and driven primarily by health economics indicating the cost-effectiveness of global screening for Lynch syndrome, an increasing number of surgical or molecular pathology laboratories now offer reflex testing of all new diagnoses of CRC for MSI status, with some form of concurrent or subsequent algorithmic testing, notably tumour *BRAF* mutation status, to determine likely constitutive or somatic pathogenesis.<sup>13,14</sup> Such reflex testing is now advocated by many major national and international groups.<sup>15–20</sup> However, there is no clear consensus on the methods of tumour assessment to be employed or on the concurrent or sequential approach to testing.

Central to the development of a laboratory algorithm for determining MMR/MSI tumour status is the choice of polymerase chain reaction (PCR)-based MSI

testing, performed on extracted tumour DNA, or of MMR immunohistochemistry (IHC), performed on paraffin-embedded tumour tissue sections.<sup>13</sup> MMR IHC indirectly assesses MSI status by demonstrating the presence or absence, within tumour cell nuclei, of MMR protein expression. Complete loss of expression of one or more MMR proteins indicates an MMR-deficient (dMMR) tumour, and this generally equates to MSI-H status. For the vast majority of laboratories, MSI testing is now typically performed with PCR and fragment length analysis of the five quasimonomorphic mononucleotide repeat markers BAT25, BAT26, NR21, NR24, and MONO-27 (the Promega 'pentaplex assay'), which represents a modification of the original five-marker Bethesda panel (BAT-25, BAT-26, D2S123, D5S346, and D17S250).<sup>21–23</sup> Testing with MSI PCR and testing with MMR IHC both have inherent advantages and disadvantages, and this comparison has been the subject of previous excellent reviews.<sup>14,24,25</sup> The selection of testing modality by individual laboratories has generally been determined by the available technology and expertise.

Although the relative performances of MSI PCR and MMR IHC in detecting MSI-H/dMMR status have been compared previously, some previous studies are limited by their historical nature, small study numbers, or case selection bias. Furthermore, many were conducted without an awareness of some technical limitations of MSI testing sensitivity or a current understanding of the full repertoire of possible MMR IHC staining patterns that can be encountered.<sup>13,26,27</sup> In the present article, we describe a modern appraisal of MSI PCR versus MMR IHC in a large, population-based study of colon cancer.

## Materials and methods

### STUDY COHORT

The study cohort was established as previously described (Northern Ireland Biobank ethical approval reference 13-0069/87/88).<sup>28–30</sup> In brief, the Northern Ireland Cancer Registry was used to identify a cohort of 661 patients with stage 2 and 3 colon cancer diagnosed between 2004 and 2008, for whom resection specimens were available to be retrieved via the Northern Ireland Biobank. Patients with rectal cancer were excluded, as neoadjuvant radiotherapy or chemoradiotherapy, which are often administered to these patients, can modify protein expression in these tumours, which is a well-recognised phenomenon in MMR IHC in particular.<sup>31</sup> Following pathological review, 1-mm diameter cores were sampled in

**Table 1.** Mismatch repair immunochemistry antibodies and conditions employed

Antibody	Clone	Company	Platform	Retrieval	Dilution	Detection
MLH1 mouse monoclonal	M1	Ventana	Ventana XT	Cell Conditioning Solution 1: 80 min	24 min (dilution as per container)	OptiView DAB Kit + amplification
MSH2 mouse monoclonal	G219-1129	Ventana	Ventana XT	Cell Conditioning Solution 1: 64 min	12 min (dilution as per container)	OptiView DAB Kit + amplification
MSH6 rabbit monoclonal	EP49	Dako	Ventana XT	Cell Conditioning Solution 1: 92 min	20 min (1:50)	OptiView DAB Kit + amplification
PMS2 rabbit monoclonal	EPR3947	Ventana	Ventana XT	Cell Conditioning Solution 1: 92 min	60 min (dilution as per container)	OptiView DAB Kit + amplification

triplicate from epithelium-rich areas of formalin-fixed paraffin-embedded primary tumour tissue for tissue microarray (TMA) construction. Approximately 100 cores were housed within each TMA block, so the entire cohort was represented by triplicate cores in 21 blocks. DNA was extracted from 5- $\mu$ m sections of representative whole tumour blocks following tumour annotation and macrodissection with the Maxwell 16 Instrument (Promega, Southampton, UK) and Promega DNA extraction kit (Promega).

#### MMR IHC

A 4- $\mu$ m section from each TMA block was used for IHC for each of the four MMR proteins, i.e. MLH1, MSH2, MSH6, and PMS2, with conditions as detailed in Table 1. All slides were digitised on an Aperio AT2 scanner at  $\times 40$  magnification. Each individual TMA core was evaluated by one observer (J.M.), using the open source whole slide image analysis software QuPath (version 0.1.2) to facilitate visualisation of cores, after application of the TMA dearray tool to each whole slide image.<sup>32</sup>

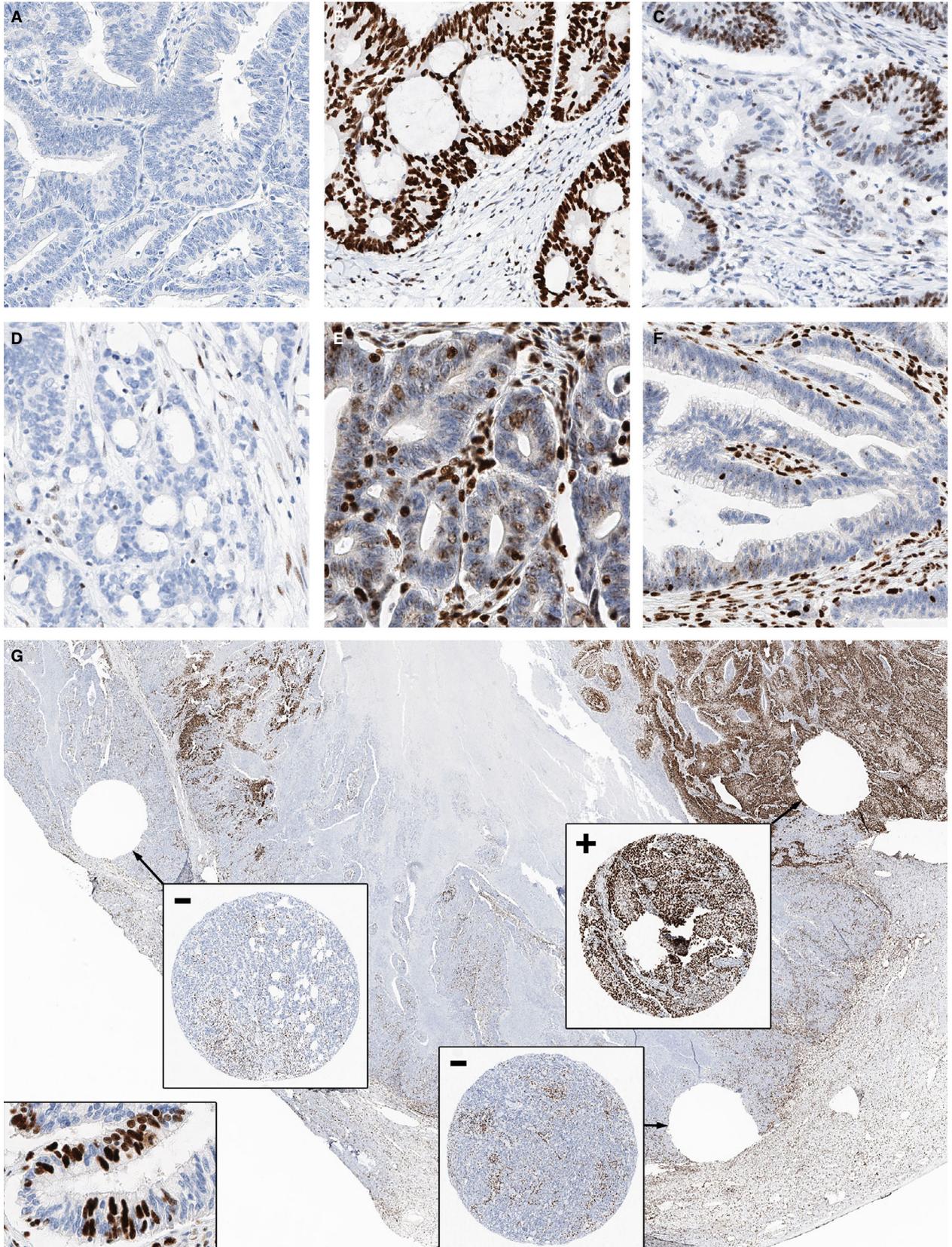
Individual cores considered to be not assessable were removed from the study if no interpretable tumour cells were present or if tumour cells were present but convincing nuclear staining in peritumoral stromal and/or lymphoid internal control cell populations was not seen (Figure 1A). For each biomarker, individual assessable cores were interpreted as either 'normal' or 'abnormal'. Normal cases showed intact immunoexpression, represented as convincing diffuse nuclear staining in the tumour cell population; staining was often variable in intensity, but comparable in intensity and pattern to that on internal control cells (Figure 1B,C). Abnormal cases typically showed

complete loss of nuclear staining in the tumour cell population (Figure 1D). Much more rarely, but also considered to be abnormal, were cases with markedly weak/abnormal staining in comparison with internal controls (Figure 1E), 'clonal' loss of staining, with an abrupt transition from present to absent staining for one or more markers (Figure 1G), or punctate staining, seen uniquely for MLH1, associated with complete loss of PMS2 staining (Figure 1F). These patterns are all now considered to be variants of abnormal MMR IHC staining.<sup>14,26,33–36</sup>

Cases considered to be abnormal or equivocal for scoring on initial assessment by the primary observer were reviewed by an experienced gastrointestinal pathologist (M.B.L.) with expertise in MMR IHC interpretation. Patterns of MMR IHC abnormality were recorded for each core, and cases were dichotomised overall as either normal (staining was maintained for all four proteins within assessable cores) or abnormal (at least one protein in at least one core showed an abnormal pattern). Cases with any unusual staining pattern were subjected to repeat MMR IHC for all four proteins, performed on whole tumour sections. All scores were initially recorded within QuPath and then exported to Microsoft Excel for statistical analysis.

#### MSI TESTING

MSI analysis was performed within the Queen's University Belfast Precision Medicine Centre of Excellence (formerly the Northern Ireland Molecular Pathology Laboratory), with the standard five quasi-monomorphic mononucleotide repeat markers BAT25, BAT26, NR21, NR24 and MONO-27 in the Promega 'pentaplex assay' version 1.2 kit (Promega).<sup>22</sup> This assay does not require the inclusion of



**Figure 1.** Patterns of mismatch repair protein immunohistochemistry (A–E, applicable to all four proteins). A, Unassessable; no staining in tumour or stromal/lymphoid cells. B,C, Normal, either strong diffuse (B) or heterogeneous (C) staining in tumour cells, comparable to staining in control stromal/lymphoid cells. D, Commonest abnormal pattern; total loss of staining in tumour cells with intact stromal/lymphoid cell staining. E, Rare abnormal pattern; present but abnormally weak staining in tumour cells as compared with stromal/lymphoid cells. F, Rare abnormal pattern of weak, punctate staining, seen uniquely for MLH1, with normal stromal/lymphoid cell staining. G, Rare abnormal pattern of heterogeneous or clonal loss of staining, most commonly seen with MSH6 in association with complete loss of MLH1 and PMS2 staining, as a result of secondary somatic *MSH6* mutation. Loss of MSH6 staining in two of three cores is illustrated (central insets), alongside follow-up staining on a whole section, with holes indicating the sites of triplicate core sampling for the tissue microarray. This heterogeneous MSH6 tumour cell staining can also be seen at the level of individual tumour glands (lower left inset). Immunoperoxidase.

matched normal DNA from the same patient as control. PCR products were separated by capillary electrophoresis with an ABI 3500 Genetic Analyser (Fisher Scientific UK, Loughborough, UK). Tumours were categorised as either MSI-H, MSI-low (MSI-L), or MSS. Cases were considered to be MSI-H when two or more markers were unstable, as MSI-L when only one marker was unstable, and as MSS when all five markers were stable. For the most part, cases were excluded from analysis if all five markers were not readable, unless at least two of those that were readable could be interpreted as demonstrating instability, indicating MSI-H status. Equivocal cases (e.g. with one unstable marker out of four and one unreadable marker) were repeated and, if the same equivocal findings recurred, the case was excluded.

#### MMR IHC/MSI DISCORDANT CASES

Discordant cases showing MSI-H status but classified as having normal MMR IHC, or abnormal MMR IHC but MSS status, were subjected to either repeat MSI testing or to repeat MMR IHC for all four proteins performed on whole tumour sections, to verify or correct the TMA IHC interpretation, as appropriate.

#### BRAF MUTATION TESTING

*BRAF* mutation screening was performed as part of the ColoCarta panel with a validated mass spectrometry-based targeted screening panel of 32 somatic mutations in six genes (Agena Bioscience, Hamburg, Germany), which includes *BRAF* D594V, V600E, V600K, V600L and V600R mutations.<sup>37</sup> Tumour DNA was extracted at the Genomics Core Technology Unit (Queen's University Belfast, Northern Ireland), and the mutation screening assays were performed by the Agena Custom Services Laboratory (Hamburg, Germany).

#### STATISTICAL ANALYSIS

The characteristics of tumours with abnormal and normal MMR IHC, or MSI-H, MSI-L and MSS

tumours, were compared by the use of chi-squared tests in STATA version 14.2 (StataCorp, College Station, TX, USA).

## Results

Within the potential cohort of 661 colon cancer cases, two cases were not tested for MSI, because of clearly insufficient tumour epithelial cells in the sample to meet the required testing threshold. Of the remaining 659 cases, 41 (6.2%) cases failed MSI testing or produced equivocal results after MSI testing, 24 (3.6%) cases did not have at least one TMA core assessable for each of the four MMR proteins, and one additional case (0.15%) failed MSI testing and was unassessable for all four MMR proteins. Only the 593 colon cancer cases with both MSI testing results and at least one core that could be scored for expression of each MMR protein were retained for further analysis. Details of the number of cores available for MMR protein scoring for these 593 cases are shown in Table 2, with >85% of cases having two or three cores available for scoring for each protein.

#### DEMOGRAPHICS

The clinical features of the cohort are summarised in Table 3, stratified for overall MSI and MMR IHC status. MSI-H cases and cases with abnormal MMR IHC were consistently more likely to be female, aged 70–

**Table 2.** Number of cores scored per case by immunohistochemistry for each mismatch repair protein

Number of scored cores per case (maximum 3)	MLH1 (N = 593), n (%)	PMS2 (N = 593), n (%)	MSH2 (N = 593), n (%)	MSH6 (N = 593), n (%)
1	69 (11.6)	88 (14.8)	73 (12.3)	77 (13.0)
2	189 (31.9)	197 (33.2)	197 (33.2)	214 (36.1)
3	335 (56.5)	308 (51.9)	323 (54.5)	302 (50.9)

**Table 3.** Clinicopathological characteristics of study patients according to microsatellite instability status and mismatch repair (MMR) immunohistochemistry (IHC)

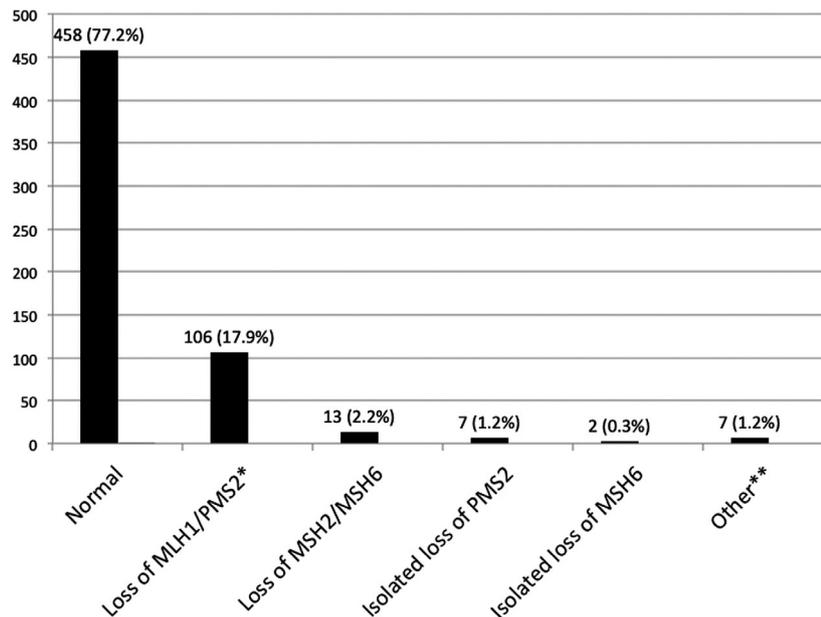
Characteristic	All cases ( <i>N</i> = 593) <i>n</i> (%)	MSS ( <i>N</i> = 447), <i>n</i> (%)	MSI-L ( <i>N</i> = 10), <i>n</i> (%)	MSI-H ( <i>N</i> = 136), <i>n</i> (%)	<i>P</i> - value	Normal MMR IHC ( <i>N</i> = 458), <i>n</i> (%)	Abnormal MMR IHC ( <i>N</i> = 135), <i>n</i> (%)	<i>P</i> - value
<b>Sex</b>								
Male	316 (53.3)	256 (57.3)	6 (60.0)	54 (39.7)	0.001	261 (57.0)	55 (40.7)	0.001
Female	277 (46.7)	191 (42.7)	4 (40.0)	82 (60.3)		197 (43.0)	80 (59.3)	
<b>Age category (years)</b>								
<50	32 (5.4)	21 (4.7)	0 (0.0)	11 (8.1)	0.26	20 (4.4)	12 (8.7)	0.03
50 to <60	56 (9.4)	58 (10.7)	0 (0.0)	8 (5.9)		49 (10.7)	7 (5.2)	
60 to <70	170 (28.7)	134 (30.0)	3 (30.0)	33 (24.3)		138 (30.1)	32 (23.7)	
70 to <80	211 (35.6)	150 (33.6)	4 (40.0)	57 (41.9)		154 (33.6)	57 (42.2)	
≥80	124 (20.9)	94 (21.0)	3 (30.0)	27 (19.8)		97 (21.2)	27 (20.0)	
<b>Stage</b>								
2	358 (60.4)	271 (60.6)	7 (70.0)	80 (58.8)	0.77	277 (60.5)	81 (60.0)	0.92
3	235 (39.6)	176 (39.4)	3 (30.0)	56 (41.2)		181 (39.5)	54 (40.0)	
<b>Tumour location</b>								
Right colon	338 (57.0)	218 (48.8)	5 (50.0)	115 (84.6)	<0.001	225 (49.1)	113 (83.7)	<0.001
Left colon	249 (42.0)	224 (50.1)	5 (50.0)	20 (14.7)		229 (50.0)	20 (14.8)	
Unspecified	6 (1.0)	5 (1.1)	0 (0.0)	1 (0.7)		4 (0.9)	2 (1.5)	
<b>Family history of CRC</b>								
No	294 (49.6)	229 (51.2)	5 (50.0)	60 (44.1)	0.56	237 (51.8)	57 (42.2)	0.08
Yes	75 (12.6)	52 (11.6)	1 (10.0)	22 (16.2)		52 (11.3)	23 (17.0)	
Unknown	224 (37.8)	166 (37.2)	4 (40.0)	54 (39.7)		169 (36.9)	55 (40.7)	
<b><i>BRAF</i> status</b>								
WT	494 (83.3)	418 (93.5)	9 (90.0)	67 (49.3)	<0.001	428 (93.4)	66 (48.9)	<0.001
MT	88 (14.8)	26 (5.8)	1 (10.0)	61 (44.8)		27 (5.9)	61 (45.2)	
Unknown/ equivocal	11 (1.9)	3 (0.7)	0 (0.0)	8 (5.9)		3 (0.7)	8 (5.9)	

CRC, colorectal cancer; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite-stable; MT, mutant; WT, wild-type.

80 years and have right-sided colonic tumours than cases without these features. MSI-high cases and cases with abnormal MMR IHC were also more likely to have *BRAF*-mutant tumours. No difference in tumour stage or family history was observed across MMR IHC or MSI status.

#### MMR IHC VERSUS MSI TESTING COMPARISON

Of the analytical cohort of 593 cases, 458 (77.2%) had normal MMR IHC, and 135 (22.8%) had abnormal MMR IHC, showing some evidence of dMMR. Abnormal cases most frequently showed loss of MLH1/PMS2



**Figure 2.** Relative frequencies of different patterns of mismatch repair immunohistochemical staining on tissue microarray evaluation ( $n = 593$ ). \*Three cases showed additional clonal loss of MSH6 staining and/or MSH2 staining; eight cases showed a punctate pattern of MLH1 staining, with complete loss of PMS2 staining. \*\*Two cases showed loss of MSH6 and PMS2 staining; one case showed loss of MLH1 and MSH6 staining; one case showed loss of MSH6 staining and clonal loss of MSH2 staining; one case showed loss of MLH1, PMS2 and MSH6 staining; and two cases showed loss of all four proteins (see text for subsequent findings on whole slide review).

staining ( $n = 106$ , 17.9%), followed by loss of MSH2/MSH6 staining ( $n = 13$ , 2.2%), isolated loss of PMS2 staining ( $n = 7$ , 1.2%), isolated loss of MSH6 staining ( $n = 2$ , 0.3%), or some other unusual combination of MMR staining loss ( $n = 7$ , 1.2%) (Figure 2). Of the 593 cases, 447 (75.4%) were MSS, 136 (22.9%) were MSI-H, and 10 (1.7%) were MSI-L.

When MSI testing and MMR IHC were combined, 149 cases (135 with abnormal MMR IHC plus 14 with MSI-H/L status; 25.1%) showed some abnormality of one or other assay (Table 4). These included 10 MSI-L cases, all of which showed normal MMR IHC. Concordance between MSI-H cases and those with abnormal MMR IHC was extremely high, with 97.1% of MSI-H

cases showing abnormal MMR IHC, and 97.8% of cases with abnormal MMR IHC showing MSI-H status. Four MSI-H cases showed no MMR IHC abnormality, on initial TMA triplicate core assessment or on subsequent repeat MMR IHC performed on a whole tumour section. Three cases with loss of MLH1/PMS2 staining were MSS on initial assessment. However, review of tumour morphology revealed these three cancers to have a high inflammatory cell component. MSI testing was repeated on two of the three cases after more selective slide annotation and macrodissection to enrich for a viable tumour epithelial cell population. Repeat MSI testing revealed both cancers to be MSI-H, suggesting that the original MSS assignment resulted from excessive contamination by non-tumour DNA. The percentage of viable tumour nuclei present in the third case was very low, and it was not possible to enrich the epithelial component through annotation. As a result, the case was considered to be unassessable for MSI status evaluation, and was removed from the overall analysis. Therefore, after repeat MSI testing, definitive careful analysis confirmed that MSI-H status was detected in all assessable cases with abnormal MMR IHC.

**Table 4.** Comparison of mismatch repair (MMR) immunohistochemistry (IHC) and microsatellite instability testing

	MMR IHC normal ( $N = 458$ , $n$ (%))	MMR IHC abnormal ( $N = 135$ , $n$ (%))
MSS	444 (96.9)	3 (2.2)
MSI-L	10 (2.2)	0 (0.0)
MSI-H	4 (0.9)	132 (97.8)

MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite-stable.

#### UNUSUAL VARIANTS OF MMR IHC STAINING

Rare cases in the cohort showed unusual patterns of MMR IHC staining. Three cases with loss of MLH1

and PMS2 staining also showed clonal loss of MSH6 and MSH2 staining, with retention of staining in internal control cells and abrupt transition to adjacent intact staining. All three were MSI-H. Eight cases showed the abnormal punctate pattern of MLH1 staining, either throughout the tumour tissue examined or associated with regions of complete loss of MLH1 staining. All eight cases were MSI-H and showed complete loss of PMS2 staining. This pattern of abnormality has been described in more detail in another publication relating to this cohort.<sup>36</sup>

Seven cases showed unusual combinations of loss of expression as determined with MMR IHC on assessment of triplicate cores: two cases showed loss of MSH6 and PMS2 staining; one case showed loss of MLH1 and MSH6 staining; one case showed loss of MSH6 staining and clonal loss of MSH2 staining; one case showed loss of MLH1, PMS2 and MSH6 staining; and two cases showed loss of staining for all four proteins. All seven cases were MSI-H. Repeat MMR IHC on whole sections confirmed these findings, with two exceptions. The case initially assessed as having loss of MLH1 and MSH6 staining showed loss of MLH1 and PMS2 staining on whole section testing, along with clonal loss of MSH6 staining (MSH2 staining was normal). One of the two cases initially assessed as having loss of staining for all four proteins showed loss of MLH1 and PMS2 staining with clonal loss of MSH2 and MSH6 staining on whole section testing. The clonal nature of loss of expression in these cases was missed by random core sampling.

## Discussion

Given health economic assessments indicating the cost-effectiveness of testing all new CRC diagnoses for underlying Lynch syndrome, and the growing importance of immune checkpoint inhibitor therapy for treating advanced-stage dMMR/MSI-H CRC, the MMR/MSI status of CRC is now established as a matter of routine.<sup>6,8,13,15,18–20,38</sup> In this study, we conducted a thorough comparison of MSI testing and MMR IHC in a large population-based series of colon cancers, to help determine the suitability of one methodology or the other as a primary screening test.

We found near perfect correlation between assessment of MSI status and MMR IHC when the amount of tumour DNA was above the threshold for the sensitivity of the MSI test and when unusual patterns of abnormal MMR IHC were considered. With respect to identifying cases that were either MSI-H or dMMR on initial testing, the sensitivities of MSI testing and

MMR IHC were 97.8% and 97.1%, respectively. Repeat MSI testing after careful assessment of tumour morphology and exclusion of more inflammatory regions at macrodissection brought the MSI sensitivity close to 100%. This high rate of concordance is comparable to, or higher than, those reported previously. Earlier studies comparing MSI testing and MMR IHC tended to focus on the detection of patients with constitutive mutations (Lynch syndrome) rather than MSI-H/dMMR tumours, and often lacked PMS2 IHC, which is necessary for detection of some *MLH1* mutations (reviewed in Shia<sup>24</sup>). These previous studies suggested that MMR IHC was inferior to the better-established MSI testing. With the addition of PMS2 IHC to the testing panel, the sensitivity of MMR IHC in predicting constitutive mutations exceeded 90% in multiple studies, which is comparable to the sensitivity of MSI testing. The main limitation was in the detection of patients with *MSH6* constitutive mutations, whose tumours were often MSI-L rather than MSI-H.<sup>24,39–41</sup>

The 10 MSI-L cases in our series all showed normal MMR IHC, supporting the general consensus that MSI-L colon cancers should be grouped with MSS cancers rather than MSI-H.<sup>25</sup> None of the MSI-L cases in our cohort showed abnormal *MSH6* staining, which would have suggested that underlying *MSH6* constitutive mutation is likely.<sup>41</sup> Previous studies analysing the original Bethesda panel microsatellites showed that the frequency of MSI-L status was high, particularly in early CRC.<sup>42</sup> By comparison, when analysis was performed with the quasimonomorphic mononucleotide panel of microsatellites in the present study, the number of MSI-L cases in this large population cohort was only 10 of 593 (1.7%). This lends further support for true MSI-L status being an infrequent finding in colon cancers, and being of questionable clinical significance.

There are very few published population-based data on the comparison of MSI testing and MMR IHC in the overall detection of MSI-H/dMMR CRC (of either constitutive or somatic aetiology). One large multi-centre international study reported very good overall concordance between MSI-H and dMMR status, with MSI testing detecting 749 (99.7%) and MMR IHC detecting 667 (88.8%) of 751 MSI-H/dMMR CRCs.<sup>27</sup> Notably, however, 11.2% of MSI-H CRCs were reported as showing normal MMR IHC, highlighting the need for awareness of the full spectrum of abnormal IHC patterns now appreciated, many of which are illustrated in this study.<sup>14,26</sup>

The available evidence suggests that either MSI testing or MMR IHC can be safely used for primary

screening of colon cancer in order to determine dMMR/MSI-H status. In practice, laboratory choice will probably be decided by access to testing pathways and availability of relevant expertise. As IHC is now widely available in almost all cellular pathology laboratories in the developed world, MMR IHC is likely to be the test of choice for many laboratories without ready access to molecular diagnostic services. However, caution regarding overreliance on MMR IHC is advised, given the occurrence of rare and unusual variants of abnormal staining encountered, including unusual combinations of global loss, weak or punctate MLH1 staining, and secondary clonal loss of MSH6 and/or MSH2 staining, as described in small case numbers in this study and previously.<sup>14,26</sup> Four MSI-H cancers in our series showed normal MMR IHC, on repeat testing of whole tumour sections, potentially because of a non-truncating MMR gene mutation affecting protein function but not translation, stability, or antigenicity.<sup>25</sup> A further potential explanation, not explored in this study, for MSI-H cancer with intact MMR expression is a genetic variant in one of the DNA polymerase genes, *POLE* or *POLD1*, resulting in an ultramutated phenotype.<sup>43</sup> Other unusual patterns of staining that have been reported but that were not encountered in this study include nucleolar staining and nuclear membrane staining.<sup>14,26,44</sup>

MMR IHC has an advantage over MSI testing in that it allows the opportunity to immediately begin the testing algorithm for Lynch syndrome, according to the MMR IHC result. Furthermore, MMR IHC may provide a reliable result if only limited tumour tissue is available or if the tumour is heavily contaminated by non-epithelial cells, as illustrated by several cases in this study. Awareness of tumour morphology and of assay sensitivity is therefore crucial to avoid potentially misclassifying a tumour as MSS. Given rare cases of MSI and MMR IHC discordance, some authors have recommended routine application of both assessment methods in all cases, to ensure the greatest assurance in assignment.<sup>13</sup>

It is questionable whether MMR IHC should be performed on endoscopic biopsy or surgical resection tumour tissue, or on both when available. Although TMA core and endoscopic tumour sampling are quite different, the overall reliability of applying MMR IHC to limited TMA samples in this study provides indirect support for performing MMR IHC on diagnostic biopsy specimens. This approach is generally favoured by most authors.<sup>45,46</sup> Better fixation of biopsy material greatly helps IHC interpretation, and overcalling dMMR on surgical resection material is well

recognised, owing to poor fixation and failure by the reporting pathologist to ensure acceptable staining of internal control inflammatory and stromal cell populations. Reporting on biopsy specimens generally yields a more timely result. However, if there is any uncertainty regarding the interpretation of MMR IHC on the biopsy specimen, or if any unusual staining pattern or combination of loss is encountered, repetition on whole tumour sections from the surgical specimen is recommended.

Some reports have advocated a two-antibody panel, rather than a four-antibody panel, for MMR IHC testing, on the premise that abnormal MSH2 or MLH1 staining is almost invariably accompanied by loss of MSH6 or PMS2 staining, respectively.<sup>47,48</sup> Indeed, none of the 135 cases with abnormal MMR IHC in our study would have been missed with the two-antibody panel approach, as all showed loss of staining for either MSH6 or PMS2, which would have triggered further assessment. However, despite this finding, we consider that a four-antibody panel approach is more likely to avoid false-normal reporting of MMR IHC, given some of the subtleties of interpretation described in this article and other studies. Furthermore, rare cases with loss of MSH2 staining but intact MSH6 staining have been described recently.<sup>49</sup>

Secondary clonal loss of expression of MSH6 and/or MSH2 was identified in five cases in this study (two only after whole section review), all of which were associated with loss of MLH1/PMS2 staining. This is a previously reported, but probably under-recognised, finding, the clinical significance of which remains unclear.<sup>33,34</sup> It is distinct from the better recognised more diffusely lost or weak MSH6 expression in rectal cancers following neoadjuvant chemotherapy or chemoradiotherapy, which is considered to be secondary to the hypoxic effects of cytotoxic therapy on the DNA repair system in tumour cells.<sup>31,34</sup> Clonal loss of MSH6 expression has been ascribed to secondary mutation in a coding mononucleotide tract in *MSH6*, in tumours with MLH1/PMS2 deficiency.<sup>33</sup> It is postulated this may subsequently affect expression of the heterodimer partner MSH2 in the same tumour population, accounting for the association between secondary clonal loss of MSH6 and MSH2 expression in the same tumours. Previous studies have not identified constitutive *MSH6* mutations in association with clonal loss of MSH6 expression, but one has reported a single case with an associated constitutive *PMS2* mutation, indicating the need for thorough clinical and laboratory evaluation of such cases.<sup>33,34</sup> This clonal loss of expression was restricted to MSH6 and/or MSH2 in this study, and

was not encountered for MLH1 or PMS2 expression in any tumours, as in previous reports of this phenomenon. The incidence reported in this study is probably an underestimate, given the TMA approach to immunostaining adopted. Examining one or more whole sections of all tumours showing loss of MLH1/PMS2 or MSH2/MSH6 staining may reveal a higher proportion of cancers with this phenomenon, and facilitate further investigation. This is illustrated by whole section review of several such cases in this study, which showed clonal loss that was not evident on initial assessment of cores.

Although those with limited molecular diagnostic capability or access to such services could consider IHC as the primary method of screening for MMR loss, it may also be beneficial for large centres performing reflex testing on significant numbers of CRCs to consider making TMAs to deal with the workload. Alternatively, a TMA approach could be employed as a second-line quality assurance measure for verification and validation of primary MSI testing results.<sup>50</sup> On the basis of the current study, and our internal previous use of TMAs, we would recommend at least triplicate sampling TMA construction. With this triplicate approach, MMR IHC was reportable in at least one core for each protein in 96% of the cases in our cohort. We consider that this would be a safe, accurate and efficient alternative approach to high-throughput testing. A similar high-throughput approach has been previously suggested for the assessment of oestrogen receptor and HER2 status in breast cancer.<sup>51</sup>

If a laboratory has access to molecular pathology services, MSI PCR testing for primary screening has some potential advantages over MMR IHC. In our hands, MSI testing by use of a kit format is cost-effective when compared with the cost of four-antibody IHC, and more so when the time needed for assessment is considered. There is a clinical need to test CRC in the metastatic setting for specific somatic mutations, in order to determine the probability of responsiveness to anti-epidermal growth factor receptor therapy.<sup>52</sup> Further requirements for mutation testing are likely to emerge. Such somatic mutation analysis, e.g. of *BRAF*, *KRAS*, and *NRAS*, can utilise previously extracted tumour DNA, should MSI testing be the primary test of choice. Additionally, if diagnostic endoscopic biopsy specimens are employed for testing, MSI testing rather than MMR IHC will maximise the amount of DNA available for molecular analysis, as at least four tissue sections will not have been used for IHC. However, some biopsy samples may be sub-optimal for MSI testing, in relation to the quantity or

quality of diagnostic material. We have found that highly inflamed tumour samples may compromise the proportion of viable tumour epithelium available for testing with the Promega MSI kit, and advise repeat analysis on a larger sample from any subsequent surgical resection should one become available. Alternatively, MMR IHC may be appropriate in this setting, being performed on a limited biopsy specimen for which a resection block is not available. The finding of several cases in this study that were erroneously deemed to be MSS on initial MSI testing but were associated with abnormal MMR IHC, flagging the need for review, reminds us of the critical importance of test sensitivity and the minimum percentage of tumour necessary to avoid a potential false-negative result.

The use of an MSI PCR pathway could also facilitate future up-front extensive gene panel testing with next-generation sequencing (NGS), which will abrogate the need for separate MSI testing and serial single-gene testing.<sup>14,53</sup> There is no doubt that the ongoing technological revolution means that NGS will transform the approach to evaluation of MSI status, as it has been shown that MSI testing is readily performed with such platforms.<sup>54</sup> In addition, performing MSI testing and MMR gene mutation analysis in the same NGS targeted assay will remove the need for a phased approach to Lynch syndrome screening. A single test result will indicate which patients should be referred to clinical genetics services with a high likelihood of having Lynch syndrome, for confirmation of diagnosis by constitutive mutation screening after appropriate counselling. However, it is important to note from recent research that NGS testing in the real-life setting may be unsuccessful in as many as 30% of CRC specimens.<sup>55</sup> This suggests that the approaches described in this article will still be very necessary, and will coexist with broader NGS approaches in molecular diagnostic laboratories for the foreseeable future.

The present study has some limitations. First, MMR IHC was conducted initially on triplicate TMA cores, rather than on whole sections, and it is therefore accepted that focal areas of abnormality may have been missed, in particular areas of clonal loss of expression. Second, the cohort was restricted to colon cancers, so the results may not be generalisable to rectal cancer. This is the likely explanation for the overall relatively high prevalence (~23%) of dMMR/MSI-H cases in this series, as compared with reports describing cohorts including both colonic and rectal cancers.<sup>56</sup> Restriction to stage 2 and stage 3 cancers will also have enhanced this proportion, as MMR

deficiency is less common at stage 1 and stage 4.<sup>57–59</sup> A further limitation of this study is the unavailability of either constitutive or somatic (tumour) MMR gene mutation status, making an accurate evaluation of the contributions of Lynch syndrome and somatic biallelic MMR gene mutations to colon cancer in this population not possible.<sup>10–12</sup>

This study represents a modern appraisal of MMR IHC versus MSI PCR testing, building on previous studies. It benefits from a population-based approach to case identification, avoiding the biases of selected case cohorts, and from large study numbers. The assays were conducted with laboratory methodologies and MMR IHC antibodies that are commonplace in routine clinical practice. Although MMR IHC was originally assessed on triplicate TMA sections, it was repeated on whole sections for all cases with any unusual staining patterns, or any unusual combination of expression loss or discordance with the MSI test result.

In summary, in establishing dMMR/MSI-H status in a large, population-based series of colon cancers, we have found near perfect concordance between MSI testing and MMR IHC. Therefore, either methodology is acceptable as a first-line screening test for this purpose. However, caution is advised to ensure adequate tumour DNA representation in samples for MSI testing, and adequate awareness of the numerous potential pitfalls in MMR IHC interpretation. For these reasons, having both assays available is desirable to facilitate the interpretation of difficult or unusual cases, and to maximise accuracy in the designation of this important molecular subtype of CRC.

## Conflicts of interest

All authors declare no conflicts of interest.

## Author contributions

Overall study design: M. B. Loughrey and J. A. James. Laboratory procedures, validation, and quality control: S. McQuaid, V. Bingham, C. McGready, and P. Maxwell. Data collection: J. McGrath, P. Bankhead, and H. G. Coleman. Assimilation and analysis of data: J. McGrath, M. P. Humphries, and S. G. Craig. Manuscript draft: M. B. Loughrey. Approval of final draft: all authors. M. B. Loughrey and J. A. James are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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