Prevalence of mismatch repair deficiency and Lynch syndrome in a cohort of unselected small bowel adenocarcinomas

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ABSTRACT

Aims Previous estimates of the prevalence of mismatch repair (MMR) deficiency and Lynch syndrome in small bowel cancer have varied widely. The aim of this study was to establish the prevalence of MMR deficiency and Lynch syndrome in a large group of small bowel adenocarcinomas.

Methods To this end, a total of 400 small bowel adenocarcinomas (332 resections, 68 biopsies) were collected through the Dutch nationwide registry of histopathology and cytopathology (Pathologisch-Anatomisch Landelijk Geautomatiseerd Archief (PALGA)). No preselection criteria, such as family history, were applied, thus avoiding (ascertainment) bias. MMR deficiency status was determined by immunohistochemical staining of MMR proteins, supplemented by *MLH1* promoter hypermethylation analysis and next generation sequencing of the MMR genes.

Results MMR deficiency was observed in 22.3% of resected and 4.4% of biopsied small bowel carcinomas. Prevalence of Lynch syndrome was 6.2% in resections and 0.0% in biopsy samples. Patients with Lynch syndrome-associated small bowel cancer were significantly younger at the time of diagnosis than patients with MMR-proficient and sporadic MMR-deficient cancers (mean age of 54.6 years vs 66.6 years and 68.8 years, respectively, p<0.000).

Conclusions The prevalence of MMR deficiency and Lynch syndrome in resected small bowel adenocarcinomas is at least comparable to prevalence in colorectal cancers, a finding relevant both for treatment (immunotherapy) and family management. We recommend that all small bowel adenocarcinomas should be screened for MMR deficiency.

INTRODUCTION

Small bowel cancer is a rare form of cancer, with an incidence of less than 1.0/100 000,[1] and little is known about the risk factors for development of this rare disease. However, monogenic cancer predisposition syndromes, such as familial adenomatous polyposis (FAP) and Lynch syndrome, are known to be responsible for a proportion of small bowel adenocarcinomas.[2] While FAP, which is caused by a germline pathogenic variant in the *APC* gene, is characterised by the presence of polyposis coli, Lynch syndrome may be harder to recognise.[3,4]

Lynch syndrome is caused by germline pathogenic variants in one of four mismatch repair (MMR) genes (*MLH1, MSH2 (EPCAM), MSH6* and *PMS2*) and predisposes carriers to the development of mainly colorectal and endometrial cancer.[4] In addition, risk for several other malignancies is increased, including risk for small bowel adenocarcinomas, currently estimated to be between 0.4% and 12% for *MLH1* and *MSH2* variant carriers.[5] Unlike FAP, there are no overt clinical characteristics that distinguish a small bowel malignancy in a patient with Lynch syndrome from a sporadic case, although a personal or family history of a Lynch syndrome-associated cancer may be suggestive. Surveillance of the duodenum is generally not recommended in Lynch syndrome due to lack of evidence supporting its effectiveness.[6] Nonetheless, identification of a Lynch syndrome family via a small bowel cancer case may provide the patient and other family members with the opportunity for surveillance of the colon, which has proven value as a screening strategy.[7,8]

A hallmark of Lynch syndrome-related tumours is the presence of MMR deficiency, which results from biallelic inactivation of one of the MMR genes and can be demonstrated by immunohistochemical staining of tumour tissue for the MMR proteins and/or microsatellite instability (MSI) analysis.[9,10] Lack of nuclear staining of neoplastic cells or presence of MSI is indicative of MMR deficiency. MMR deficiency in Lynch syndrome occurs due to a second somatic hit in neoplastic cells, in addition to a germline variant. MMR deficiency may also occur in sporadic cases due to somatic inactivation of both alleles.[11] The presence of MMR deficiency might also be relevant to patient treatment, given that Programmed Death-Ligand 1 (PD-L1)-blockers produce a good response in MMR-deficient (colorectal) cancers regardless of sporadic or hereditary aetiology.[11,12] Universal screening for MMR deficiency in small bowel cancers, as introduced for colorectal cancer and endometrial cancer in many countries,[13,14] may therefore be warranted. The potential benefit of a comparable screening strategy can only be accurately assessed if the prevalence of MMR deficiency and Lynch syndrome in unselected small bowel cancer is first reliably estimated. Previous estimates of the prevalence of MMR deficiency were based on small cohorts and consequently

showed wide variability (0%–35%).[2 15] Few data are available on the prevalence of Lynch syndrome in these cohorts. In this study, a large, unbiased collection of small bowel cancers was used to reliably establish the prevalence of MMR deficiency and Lynch syndrome in this rare tumour group.

METHODS
Cohort
The nationwide network and registry of histopathology and cytopathology in the Netherlands, also known as Pathologisch-Anatomisch Landelijk Geautomatiseerd Archief (PALGA), were consulted in 2017 in a nationwide search of tumour samples from patients with small bowel cancer.[16] All excerpts labelled by the reporting pathologist as a neoplasm of the small bowel were extracted for the 5-year period, 2012–2016. The conclusions of the resulting pathology reports were then screened for:

1. All resected primary small bowel adenocarcinomas within the 5-year time frame. This resulted in the selection of 411 eligible tumour specimens.
2. The hundred most recent samples that included a biopsy of an adenocarcinoma with a (possible) primary origin in the small bowel. This second category of samples was added to ensure inclusion of unresectable cases (some duodenal adenocarcinomas present at an advanced stage and are not resectable due to the high morbidity of surgery).

Formalin-fixed paraffin-embedded (FFPE) material representative of these adenocarcinomas was then requested. Material from 332 resection specimens and 68 biopsy samples was obtained. Due to the anonymous nature of the samples and the rules and regulations of the PALGA network, obtaining consent was not possible or required.

Study procedures
The study flow is visualised in figure 1. On receipt, 4 µm sections were taken from the FFPE blocks and subjected to H&E staining and immunohistochemical staining of the MMR proteins. Additionally, depending on tumour size and histology, 10 µm sections or punches from the tumour were taken for later DNA isolation. Guided by a matching H&E slide, the 10 µm sections were microdissected to enrich for tumour. All samples were coded for complete anonymity according to Dutch guidelines. Anonymous basic personal data (age at diagnosis and gender) were available for each patient, in addition to historical pathology reports. No other clinical data were available.

All adenocarcinomas were initially immunohistochemically stained for PMS2 and MSH6 protein expression.[17] Subsequent immunohistochemical staining for MLH1 and/or MSH2 was performed if the tumour was PMS2 deficient or MSH6 deficient. This approach is more cost-effective than using a four-antibody panel and has good sensitivity. The rationale for this approach is that functionally, MLH1 forms a heterodimer with PMS2, while MSH2 forms a heterodimer with MSH6, and mutations in MLH1 or MSH2 result in degradation of their heterodimer partners. Hence, use of PMS2 and MSH6 antibodies as a first screening step will generally identify loss of protein expression of MLH1 or MSH2.[17 18] In cases with MLH1 deficiency, *MLH1* promoter hypermethylation analysis was performed. In cases with loss of expression of MLH1 in the absence of *MLH1* promoter hypermethylation or in cases with MSH2, MSH6 and solitary PMS2 expression loss, the MMR genes were further analysed using next generation sequencing (NGS). If NGS identified a variant with an allele frequency of >40%, DNA from matching non-neoplastic tissue (when available) was isolated to determine whether the variant was germline or somatic in origin.
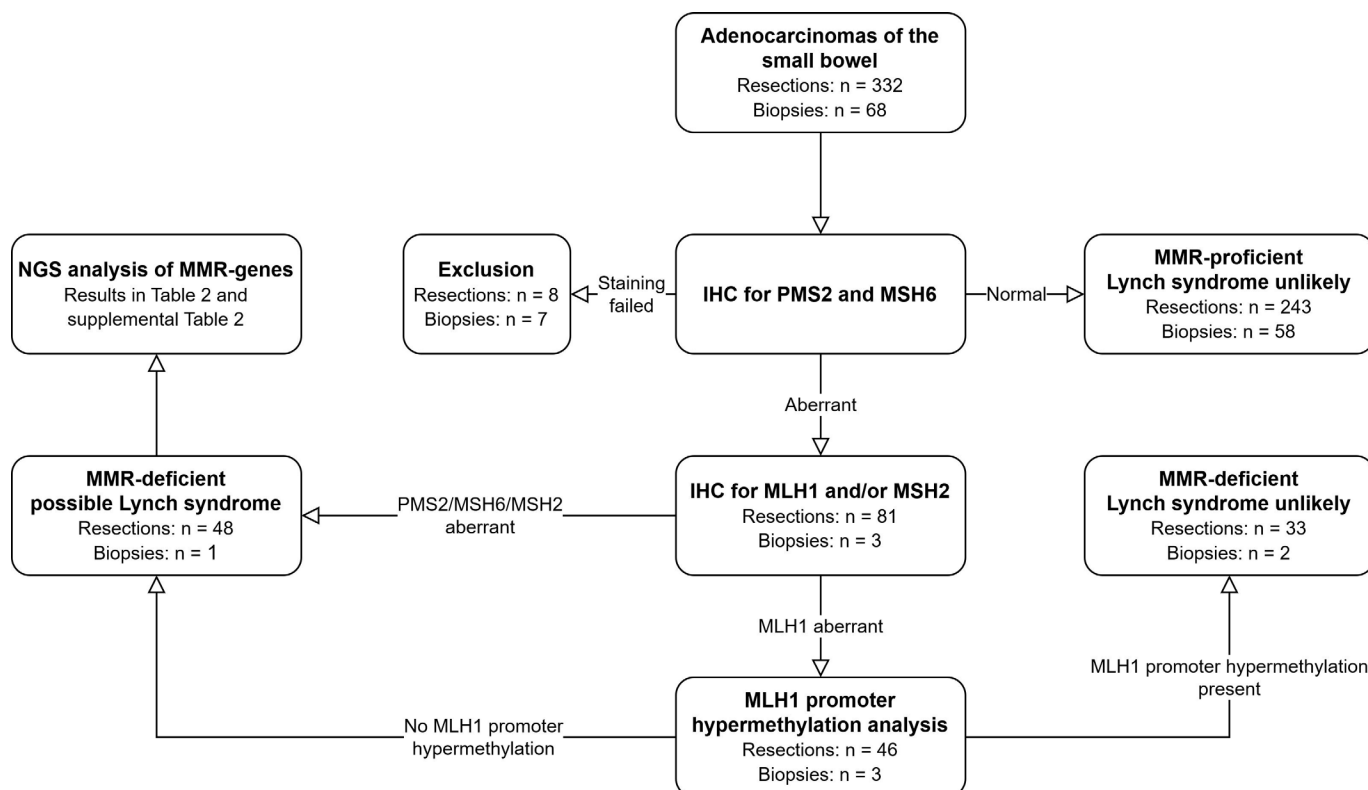

Figure 1 Study procedures. IHC, immunohistochemistry; MMR, mismatch repair; NGS, next generation sequencing.

Immunohistochemical staining

Details on the immunohistochemical staining procedures are found in the online supplemental methods. The immunohistochemically stained samples were examined by an experienced pathologist (HM or AFS) using light microscopy to evaluate MMR status. MMR proficiency was defined as the presence of nuclear staining within neoplastic cells as well as within adjacent non-neoplastic cells. MMR deficiency was defined as an absence of nuclear staining within neoplastic cells, together with positive expression in non-neoplastic cells. A third category, subclonal loss of protein expression, was defined for those adenocarcinomas harbouring a subpopulation of cancer cells with loss of expression together with cells retaining expression of an MMR protein.

DNA isolation using the tissue preparation system

DNA was isolated using the Tissue Preparation System with VERSANT Tissue Preparation Reagents (Siemens Healthcare Diagnostics, Tarrytown, New York, USA), as previously described.[19]

MLH1 promoter hypermethylation analysis

Cases with loss of MLH1 expression were analysed for *MLH1* promoter hypermethylation by methylation-specific PCR (MSP).[20 21] Bisulphite conversion was carried out using the EZ DNA Methylation-Lightning Kit (D5031; Zymo Research) according to manufacturer's instructions.

Targeted NGS

Adenocarcinomas with aberrant expression of at least one of the MMR proteins in the absence of *MLH1* promoter hypermethylation underwent DNA variant analysis using an NGS panel. This panel consists of 20 colorectal cancer-associated and polyposis-associated genes and hotspot regions of the *CTNNB1* gene (see online supplemental table 1 for all genes and panel coverage). For the purposes of this study, analysis of NGS results was restricted to *MLH1*, *MSH2*, *MSH6* and *PMS2*. Sequencing was performed using the Ion Torrent platform according to the manufacturer's recommendations. Details are found in the online supplemental methods.

The unaligned sequence reads generated by the sequencer were mapped against a human reference genome (hg19) using the Burrows-Wheeler aligner. VarScan and ANNOVAR software were used for variant calling and annotation, respectively, and Integrative Genomics Viewer (IGV) software was used to visualise the read alignment and presence of variants. Additionally, the Leiden Open Variant Database, ClinVar and Alamut software were used whenever additional variant interpretation was needed.

Statistical analysis

Using IBM SPSS Statistics V.24, the χ^2 test and one-way analysis of variance (ANOVA) test were performed as appropriate to compare patient and tumour characteristics of MMR-proficient cases with sporadic MMR-deficient cases and Lynch syndrome-associated cases. A p value<0.05 was considered to be statistically significant. Cases with subclonal loss of one of the MMR proteins were excluded from these analyses.

RESULTS
Immunohistochemistry

The prevalence of MMR deficiency, as determined by immunohistochemical staining, was 22.3% in resected small bowel

Table 1 Prevalence of mismatch repair (MMR) deficiency and immunohistochemical staining patterns in resected and biopsied adenocarcinoma samples

Immunohistochemistry results	Resections N (%)	Biopsies N (%)
MMR proficient	243 (73.2)	58 (85.3)
MMR deficiency—complete tumour	74 (22.3)	3 (4.4)
MLH1/PMS2	42	3
PMS2 only	7	0
MSH2/MSH6	19	0
MSH6 only	6	0
Subclonal MMR deficiency	7 (2.1)	0 (0)
MLH1/PMS2	4	
MSH6 only	1	
All four deficient	2	
No tumour, excluded from further analysis	8 (2.4)	7 (10.3)
Total	332	68

adenocarcinomas and 4.4% in biopsies (table 1). Additionally, seven (2.1%) resected samples showed subclonal loss of at least one MMR protein. Eight resected adenocarcinomas and seven adenocarcinoma biopsy samples had to be excluded from further analysis because no (representative) tumour tissue was present in the available FFPE blocks.

Causes of mismatch repair deficiency

The most common cause of MMR deficiency was *MLH1* promoter hypermethylation (40.5% of MMR-deficient resections and 66.7% of MMR-deficient biopsies, table 2). In more than a quarter of MMR-deficient resection samples, the MMR deficiency was related to Lynch syndrome (27%, table 2 and online supplemental table 2). The prevalence of Lynch syndrome within the total resection cohort was therefore at least 20/324 (6.2%). The true number might in fact be higher because in six cases an MMR gene variant with a high allele frequency (>40% of reads) was identified within the tumour, but matched normal tissue was not available to confirm or refute germline origin of the variant.

Table 2 Causes of mismatch repair (MMR) deficiency

	MMR-deficient tumours		Subclonal loss N (%)
	Resections N (%)	Biopsies N (%)	Resections N (%)
MLH1 promoter hypermethylation	30 (40.5)	2 (66.7)	3 (42.9)
Two somatic hits	10 (13.5)	0	1 (14.3)
Lynch syndrome	20 (27.0)	0	0
MLH1 variant	6		
MSH2 variant	7		
PMS2 variant	2		
MSH6 variant	5		
MMR variants identified in tumour, normal tissue not available, but high variant allele frequency	6 (8.1)	0	0
MMR deficiency molecularly unexplained (no or only one somatic hit identified)	8 (10.8)	1 (33.3)	3 (42.9)
Total	74	3	7

Table 3 Cohort characteristics for Lynch syndrome versus mismatch repair (MMR) proficient versus MMR-deficient cases

		MMR-proficient N=243	Sporadic MMR-deficient carcinomas N=44	Lynch syndrome N=20	P value
Gender—male		126 (51.9%)	23 (52.3%)	13 (65.0%)	0.525
Mean age at diagnosis in years (range)		66.6 (27–91)	68.8 (43–90)	54.6 (35–77)	<0.000
Location (%)	Duodenum	126 (51.9%)	26 (59.1%)	12 (60.0%)	0.893
	Jejunum	51 (21.0%)	7 (15.9%)	3 (15.0%)	
	Ileum	33 (13.6%)	4 (9.1%)	3 (15.0%)	
	Small bowel not otherwise specified	33 (13.6%)	7 (15.9%)	2 (10.0%)	
Previous history of Lynch syndrome-associated cancer*		28 (11.5%)	8 (18.2%)	13 (65.0%)	<0.000
Previous history of other cancer type(s) (non-Lynch)†		27 (11.1%)	6 (13.6%)	6 (30.0%)	0.050
Crohn's disease—yes		8 (3.3%)	0 (0%)	0 (0%)	0.339
Coeliac disease—yes					

*Lynch syndrome-associated cancers: colorectal cancer, endometrial cancer, ovarian cancer, gastric cancer, cancer of the bile duct or gallbladder, pancreatic cancer or urothelial cancer (Møller et al[22]).
†Excluding basal cell cancer of the skin.

Comparison of patient and tumour characteristics

A comparison of patient and tumour characteristics of MMR-proficient, (apparently) sporadic MMR-deficient and Lynch syndrome-associated cases included only the resected adenocarcinoma cases, as they represent the largest subcohort and have a documented primary tumour location within the small bowel. The six cases carrying a high allele frequency variant but without available-matched normal tissue were excluded due to uncertainty regarding their status as Lynch syndrome or sporadic MMR-deficient cases. Cases with an unexplained MMR deficiency and those with subclonal MMR deficiencies were also excluded from this analysis.

Mean age at cancer diagnosis was significantly lower in the patients with Lynch syndrome (table 3), and a previous history of a Lynch syndrome-associated cancer was significantly elevated in patients with Lynch syndrome. Interestingly, coeliac disease (diagnosed based on pathology reports of small bowel biopsies unconnected to the small bowel cancer diagnosis) was significantly more common in sporadic MMR-deficient cases. No other significant associations were identified (eg, location, gender, other cancer history,[22] Crohn's disease).

DISCUSSION

In a large group of resected primary small bowel adenocarcinomas, we found complete MMR deficiency in 22.3% and subclonal deficiency in 2.1% of cases, while biopsied small bowel adenocarcinomas showed a lower prevalence of MMR deficiency (4.4%). To the best of our knowledge, this is the first study to systematically screen a large, consecutive group of small bowel adenocarcinomas for the prevalence of MMR deficiency. Previous studies were either smaller and/or used selected cases with a higher a priori chance of being related to Lynch syndrome. Furthermore, many of these studies did not include molecular analysis to verify whether MMR deficiency was Lynch syndrome-related or sporadic.[2 15 23]

A recently published French study by Aparicio et al[24] reported a Lynch syndrome prevalence of 6.9% in a large cohort of small bowel adenocarcinomas, in line with a prevalence of at least 6.2% in our cohort. MMR deficiency prevalence could not be compared because this French cohort was not systematically screened for MMR deficiency.

Of particular note, the prevalence of MMR deficiency in our study differed considerably between the resected and biopsied specimens. A higher prevalence of MMR deficiency in resected versus biopsied samples might be related to the association of MMR deficiency with a better prognosis in other cancers,[25] so resections may represent patients with cancer with a relatively good prognosis, whereas biopsies may represent patients with a poor prognosis who are less likely to undergo resection. Interestingly, the prevalence of MMR deficiency identified in biopsied samples, 4.4%, is close to the 5.0% prevalence identified in a metastatic colorectal cancer cohort.[26] However, as no further clinical data were available to verify that a biopsied sample was a confirmed primary small bowel cancer, our cohort may also have included cancers with a different primary location (where MMR deficiency prevalence is lower). Further validation of the prevalence of MMR deficiency in a cohort of small bowel cancers that were not resected is therefore required.

The relevance of subclonal loss of MMR protein expression is still poorly understood. While it seems unlikely that these patients have Lynch syndrome, the relevance of subclonal loss for prognosis and/or therapy will require further investigation.[18 27]

A significant overrepresentation of patients with coeliac disease was noted among cases with sporadic MMR deficiency. An association of coeliac disease with sporadic MMR deficiency (particularly with *MLH1* promoter hypermethylation) has been described previously,[28 29] and two out of three MMR-deficient cases from our cohort also showed *MLH1* promoter hypermethylation. A limitation of our study was the lack of accompanying clinical data, which meant that we had no information on treatment/diet and could not verify whether the pathological signs of coeliac disease correlated with patient symptoms. These results should therefore be interpreted with caution, because there are other conditions that mimic the histological signs of coeliac disease.[30]

Another drawback of anonymous data is that it precludes verification of the number of Lynch syndrome cases, knowledge that might otherwise be used to establish how many patients are missed using current practices. Nevertheless, from pathology reports we could deduce that 13 out of 20 patients with Lynch were likely already identified, either because MSI and/or immunohistochemical testing was described (in the small bowel tumour or a previous tumour) or a previous diagnosis of Lynch syndrome was mentioned (online supplemental table 3).

There is an ongoing discussion whether a two-antibody panel for immunohistochemical staining of the MMR proteins has sufficient sensitivity to detect MMR-deficient cases. Although a small number of MMR-deficient cases may be missed with a

two-antibody panel, it is not expected that the results of a four-antibody approach would alter our conclusions.

A molecular cause of MMR deficiency could not always be identified (n=12). This is likely partly explained by the fact that we did not perform multiplex ligation-dependent probe amplification analysis to screen samples for deletions and/or insertions (germline or somatic) of the MMR genes or *EPCAM* (table 2 and online supplemental table 2). Nonetheless, NGS data were manually checked using the IGV for evidence suggesting a deletion, which led to the identification of deletions in three samples (online supplemental table 2, eg, study ID 33). Although this approach lowers the risk of missing copy number variants, not all deletions/insertions will be identified. As *EPCAM* was not sequenced, deletions of this gene will have been missed by definition. However, as only 1%–3% of all Lynch syndrome families carry an *EPCAM* deletion and deletions/insertions of the MMR genes explain a minority of Lynch syndrome families,[4 31] Multiplex Ligation-dependent Probe Amplification (MLPA) analysis is unlikely to have altered our conclusions and recommendations. Another possible explanation for the failure of NGS results to resolve all MMR deficiency cases is that some cases lacked the informative single nucleotide polymorphisms required to determine whether loss of heterozygosity has occurred.

The analysis of *PMS2* is complicated by the presence of pseudogenes. Nevertheless, researchers from our group have shown that it is possible to reliably detect variants in *PMS2*, even when using DNA isolated from FFPE material, as long as the correct amplicons are selected.[32] Exceptions include variants in exon 12–15 due to gene conversion. The two germline variants identified in our cohort are found in exons 1–11.

In our cohort, the prevalence of MMR deficiency in resected cases (22.3%) was higher than the reported prevalence of MMR deficiency in colorectal cancer (15%).[33] This finding has implications for daily clinical practice in relation to three important issues: prognosis, treatment and surveillance. In (early stage) colorectal cancer, MMR deficiency has been linked to a better prognosis,[25 34 35] an association that may also hold true for MMR-deficient small bowel cancers. Indeed, the aforementioned study by Aparicio *et al* reported a trend towards better prognosis for Lynch-associated small bowel adenocarcinomas versus those related to Crohn's disease.[24] Furthermore, with the advent of immunoblockade therapy and its proven efficacy in MMR-deficient cancers,[36] MMR status is relevant when formulating treatment strategies regardless of germline or sporadic status. Finally, due to the high prevalence of Lynch syndrome, small bowel cancer as an entity may facilitate the identification of new Lynch syndrome families and consequently allow surveillance measures to be offered.

In light of the high prevalence of MMR deficiency and Lynch syndrome, together with associated relevance and benefits, we recommend the implementation of universal screening of all primary small bowel adenocarcinomas for the presence of MMR deficiency. An age limit of 70 years is often used in the universal screening of colorectal cancers for mismatch repair deficiency. However, as the Lynch syndrome-associated cases included in our study showed a very broad age range (35–77 years, table 3) at diagnosis, we suggest that age limits on universal screening for small bowel cancer may be detrimental.

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Take home messages

► Prevalence of mismatch repair deficiency (22.3%) and Lynch syndrome (6.2%) in resected small bowel adenocarcinomas is high.
► No clear clinical or histological predictors of mismatch repair deficiency were identified.
► We recommend the implementation of universal screening for mismatch repair deficiency in all small bowel adenocarcinomas.

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Supplemental Methods

Immunohistochemical staining

4μm FFPE sections were deparaffinized with xylene and rehydrated in ethanol. A 0.3% H_2O_2 solution was used to block endogenous peroxidase, and microwave-mediated antigen retrieval was performed in Tris-EDTA, pH 9.0. Sections were incubated overnight with primary antibodies against MLH1 (clone ES05, 1:50; Agilent, USA), MSH2 (clone FE11, 1:200, Agilent, USA), MSH6 (clone EPR3945, 1:200, Genetex, USA) or PMS2 (clone EP51, 1:40, Agilent, USA) at 4°C. After washing, they were then incubated for 30 minutes with poly-HRP (VWRKDPVM110HRP, ImmunoLogic), visualised using a DAB+ substrate chromogen system (K3468; Agilent) and counterstained with haematoxylin. Finally, the sections were dehydrated and mounted with coverslips.

Targeted Next Generation Sequencing (NGS)

Sequencing was performed using the Ion Torrent platform according to the manufacturer's recommendations. In brief, 21 ng/14 µl isolated DNA was used to prepare two primer pools. After the first PCR, the pools were combined and a new PCR run was performed to digest the primers. A third PCR was then performed to add barcodes to the samples. After purification using AMPureXP beads (A63882; Beckman Coulter), the NGS libraries were pooled, diluted to 60 pmol/L and loaded on a chip using the Ion Chef. Sequencing was subsequently performed in an Ion GeneStudio S5 Series sequencer.

Supplemental table 1 – msCRC panel genes and coverage

Name	Chromosome	Exons	Coverage (%)
APC	5	16	100
BMPR1A	10	11	94.3
BRCA1	17	23	100
BRCA2	13	26	100
ENG	9	15	100
MLH1	3	21	100
MSH2	2	17	100
MSH3	5	24	99.8
MSH6	2	12	100
MUTYH	1	16	100
NTHL1	16	6	100
PALB2	16	42	100
PMS2	7	15	96.8
POLD1	19	26	100
POLE	12	40	100
PTEN	10	10	98.9
RNF43	17	9	99.9
SMAD4	18	11	98.5
STK11	19	9	100
TP53	17	12	100
KRAS	12	2,3,4	Hotspots
HRAS	11	2,3	Hotspots
NRAS	1	2,3,4	Hotspots
BRAF	7	11,15	Hotspots
CTNNB1	3	8	Hotspots
MYC	8	CNV	Hotspots

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Supplementary Table 2 – Next Generation Sequencing (NGS) result of MMR-deficient cases (excluding cases with *MLH1* promoter hypermethylation)

Variants are either likely pathogenic (class 4) or pathogenic (class 5) unless otherwise specified.

<u>Abbreviations:</u> VAF = Variant allele frequency. LOH = Loss of heterozygosity. SNP = Single Nucleotide Polymorphism. NP = not performed. VAF = Variant allele freque

<u>Immunohistochemistry results:</u> + = normal nuclear staining. - = loss of staining in neoplastic cells with positive internal controls. +/++ = weak staining in neoplastic c

* since germline variants may be unique to a family/person, only a general description of the germline variant type is given to protect privacy and maintain data an

Study ID	Resection or biopsy	Immunohistochemistry pattern				NGS results neoplastic tissue				Variant detected in non-neoplastic tissue
		PMS2	MLH1	MSH6	MSH2	Gene	Variant	VAF : coverage	LOH	
3	Resection	+	np	+/++	-	MSH2	Nonsense variant*	0.779:715	Probable based on 1 SNP and VAF	Yes
18	Biopsy	-	-	+	np		No relevant variants detected			
33	Resection	+	np	-	+/++	MSH2	NM_000251.2:c.1777C>T	0.480:125	No	No
							Deletion exon 1			No
46	Resection	-	+	+	np		NGS data of insufficient quality			
48	Resection	-	+	+	+	MLH1	NM_000249.3:c.112A>C	0.48:448	No informative SNPs	Normal tissue not available
71	Resection	+	np	-	+/++		No relevant variants detected			
85	Resection	Failed	np	-	+	MLH1	Missense variant classified as pathogenic by InSiGHT	0.479:1308	No informative SNPs	Yes
							NM_000249.3:c.1513_1520dup	0.168:1985		No
						MSH6	C-deletion			
94	Resection	-	-	+	np	MLH1	NM_000249.3:c.676C>T		yes	Not performed
98	Resection	+	np	-	+/++	MSH6	Frameshift variant*	0.483:1989	No	Yes
							NM_000179.2:c.3743del	0.329:1989		No
118	Resection	+	np	-	-		NGS data of insufficient quality			
119	Resection	-	+	+	np	PMS2	Frameshift variant*	0.498:1933	yes	Yes

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ID	Type					Gene	Variant	VAF	SNP/LOH	Normal tissue
119	Resection	-	+	+	np	MSH2	NM_000251.2:c.187dup	0.204:1967		No
124	Resection	+	np	-	failed		No relevant variants detected			According to PA-report this is a Lynch
156	Resection	+	np	-	-	MSH2	NM_000251.2:c.2027C>G	0.219:283	No informative SNPs	Not performed
206	Resection	-	-	+	np	MLH1	Frameshift variant*	0.429:1919	No informative SNPs	Yes
211	Resection	-	-	+	np	MLH1	NM_000249.3:c.454-13A>G	0.918:244	No informative SNPs, VAF is however	Normal tissue not available
214	Resection	subclonal -	+	+	np	MSH2	NM_000251.2:c.1414C>T (class 3 VUS)	0.156:257		Not performed
236	Resection	+	np	-	-	MSH6	Frameshift variant*	0.511:1621		Yes
							NM_000179.2:c.3172G>T (class 3 VUS)	0.313:1995		No
249	Resection	-	+	+	np	PMS2	NM_000535.5:c.2287G>T	0.159:1233	No informative SNPs	Normal tissue not available
							NM_000535.5:c.1882C>T	0.397:315		
316	Resection	+	np	+/++	-	MSH2	Exon deletion*	Not applicable	Yes	Yes
325	Resection	+	np	-	-		No relevant variants detected			
333	Resection	+	np	-	-	MSH6	NM_000179.2:c.3128del	0.185:352	No informative SNPs	Not performed
335	Resection	+	np	-	-	MSH2	Missense variant classified as likely pathogenic by InSiGHT*	0.520:1997	LOH probable based on 1 informative SNP	Yes
344	Resection	-	-	+	np	MLH1	NM_000249.3:c.94_110del	0.341:1510	No informative SNPs	Not performed
363	Resection	+	np	-	-	MSH2	Frameshift variant*	0.499:914	No based on 1 SNP	Yes
379	Resection	-	+	+	np	PMS2	Nonsense variant*	0.500:1225	No	Yes
							NM_000535.5:c.1802C>G	0.421:680		No
414	Resection	+	np	-	+/++	MSH6	Frameshift variant*	0.481:1795	No based on 1 SNP	Yes
							NM_000179.2:c.3533del	0.239:1980		No

ID	Type					Gene	Variant	VAF		
426	Resection	-	-	+	np	MLH1	Frameshift variant*	0.539:1990	No informative SNPs	Yes
453	Resection	-	-	+	np	MLH1	NM_000249.3:c.791-2A>C (class 3 VUS)	0.634:1994	No informative SNPs	No
460	Resection	+	np	+/++	-	MSH2	NM_000251.2:c.2557G>T	0.241:1312	Unlikely based on 1 SNP	Not performed
466	Resection	-	-	+	np		No relevant variants detected			
474	Resection	+	np	-	-	MSH2	Frameshift variant*	0.691:676	Probable based on 1 SNP	Yes
480	Resection	-	-	Subclonal -	np	MLH1	Nonsense variant*	0.744:1999	yes	Yes
						MSH6	No relevant variants detected		yes	
526	Resection	+	np	-	-		No relevant variants detected			
551	Resection	-	-	+	Np	MLH1	NM_000249.3:c.2145_2168del	0.578:211	No informative SNPs	Normal tissue not available
558	Resection	-	+	Subclonal -	+/++	PMS2	NM_000535.5:c.638del	0.378:1995	Possibly based on 3 SNPs	No
568	Resection	-	+/++	+	np	PMS2	NM_000251.2:c.2458+1G>A	0.381:1998	No	Not performed
595	Resection	+	np	-	+/++	MSH6	Frameshift variant*	0.453:1190	No	Yes
							NM_000179.2:c.2232G>T (class 3 VUS)	0.169:349		No
596	Resection	+	np	-	-	MSH2	NM_000251.2:c.1861C>T	0.491:1611	No	Normal tissue not available
							NM_000251.2:c.2458+1G>A	0.271:399		
601	Resection	+	np	-	-	MSH2	NM_000251.2:c.1601G>A	0.346:619	No informative SNPs	Not performed
						MSH6	NM_000179.2:c.1436_1440del	0.35:1980	Yes	
687	Resection	-	np	-	-		NGS data of insufficient quality			
698	Resection	+	np	Subclonal	+		NGS data of insufficient quality			
710	Resection	-	+	+	np	PMS2	NM_000535.5:c.1405A>T	0.522:1994	No	Normal tissue not available

720	Resection	+	np	-	-	MSH2	Nonsense variant*	0.678:1772	No	Yes
722	Resection	+	np	-	+/++	MSH6	Frameshift variant*	0.532:342	no	Yes
							NM_000179.2:c.1444C>T	0.285:895		No
723	Resection	-	-	+	np	MLH1	Frameshift variant*	0.937:1449	Probable based on one SNP and VAF of	Yes
746	Resection	-	-	+	np	MLH1	Frameshift variant*	0.594:1721	Yes	Yes
748	Resection	+	np	-	-		NGS data of insufficient quality			
760	Resection	-	-	+	np	MLH1	NM_000249.3:c.252del	0.498:601	yes	No

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Supplemental Table 3 - clinical details of Lynch syndrome patients

Abbreviations: n.o.s. = not otherwise specified

Study ID	Gene	Sex	Age decade at small bowel cancer diagnosis (years)	Location of tumour	Differentiation grade as reported in PA-report	Aberrent IHC, MSI or Lynch diagnosis in pathology report	History of Lynch-associated malignancy	Number of Lynch-associated malignancies (excluding small bowel)	History of other malignancy (non-Lynch associated)	Number of other malignancies (non-Lynch associated)
3	MSH2	m	40-49	small bowel n.o.s.	moderate	yes	yes	1	no	-
85	MLH1	m	30-39	ileum	moderate	yes	yes	1	no	-
98	MSH6	m	70-79	duodenum	moderate	no	no	-	yes	1
119	PMS2	m	70-79	duodenum	moderate	yes	no	-	no	-
124	MSH2	v	60-69	jejunum	moderate	yes	yes	4	no	-
206	MLH1	f	30-39	duodenum	moderate	no	no	-	no	-
236	MSH6	m	40-49	duodenum	moderate	yes	yes	1	no	-
316	MSH2	f	50-59	jejunum	could not be assessed	yes	yes	2	no	-
335	MSH2	f	60-69	ileum	moderate	yes	yes	1	yes	1
363	MSH2	m	40-49	duodenum	moderate to high	yes	no	-	no	-
379	PMS2	m	50-59	duodenum	moderate	no	no	-	no	-
414	MSH6	f	50-59	duodenum	moderate	yes	yes	1	yes	1
426	MLH1	m	40-49	duodenum	poorly/high grade	yes	no	-	no	-
474	MSH2	f	50-59	duodenum	moderate	no	yes	1	no	-
480	MLH1	m	60-69	duodenum	moderate	yes	yes	2	no	-
595	MSH6	m	50-59	jejunum	poorly/high grade	no	no	-	no	-
720	MSH2	m	50-59	duodenum	moderate	no	yes	1	no	-

722	MSH6	f	50-59	ileum	could not be assessed	no	yes	2	yes	1
723	MLH1	m	50-59	small bowel n.o.s.	moderate	yes	yes	1	yes	1
746	MLH1	m	50-59	duodenum	moderate	yes	yes	3	yes	1