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Familial colorectal cancer: eleven years of data from a registry program in Switzerland

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Abstract Deleterious germ-line variants involving the DNA mismatch repair (MMR) genes have been identified as the cause of the hereditary nonpolyposis colorectal cancer syndrome known as the Lynch syndrome, but in numerous familial clusters of colon cancer, the cause remains obscure. We analyzed data for 235 German-speaking Swiss families with nonpolyposis forms of colorectal cancer (one of the largest and most ethnically homogeneous cohorts of its kind) to identify the phenotypic features of forms that cannot be explained by MMR deficiency. Based on the results of microsatellite instability analysis and immunostaining of proband tumor samples, the kindreds were classified as MMR-proficient ($n = 134$, 57%) or MMR-deficient ($n = 101$, 43%). In 81 of the latter kindreds, deleterious germ-line MMR-gene variants have already been found (62 different variants, including 13 that have not been previously reported), confirming the diagnosis of Lynch syndrome. Compared with MMR-deficient kindreds, the 134 who were MMR proficient were less likely to meet the Amsterdam Criteria II regarding autosomal dominant transmission. They also had primary cancers with later onset and colon-segment distribution patterns resembling those of sporadic colorectal cancers, and they had lower frequencies of metachronous colorectal cancers and extracolonic cancers in general. Although the predisposition to colorectal cancer in these kindreds is probably etiologically heterogeneous, we were unable to identify distinct phenotypic subgroups solely on the basis of the clinical data collected in this study. Further insight, however, is expected to emerge from the molecular characterization of their tumors.

Keywords Colorectum · Familial cancer · Lynch syndrome · Mismatch repair · Switzerland

Abbreviations

MSI	Microsatellite instability
IHC	Immunohistochemistry
AC II	Amsterdam criteria II
rBG	Revised Bethesda guidelines
MMR	Mismatch repair
MLPA	Multiplex-ligation dependent-probe amplification
FCC-X	Familial colorectal cancer type-X
HNPCC	Hereditary nonpolyposis colon cancer
FAP	Familial adenomatous polyposis
MMR-deficient or-proficient FCC	Mismatch repair deficient or proficient, familial colorectal cancer

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Introduction

Colorectal cancer is one of the most frequent cancers in humans, and its incidence is highest in the so-called developed countries. Given its high prevalence, it is not surprising that the disease sometimes strikes more than one member of a single family. Aggregations of cancer within kindreds are usually referred to as *familial*, which does not necessarily mean the disease is genetically inherited. Few familial clusters of colorectal cancer are believed to be associated with a single genetic alteration: in most cases, the cancer predisposition probably stems from some combination of genetic and/or epigenetic, environmental, and/or behavioral factors (in particular, physical activity and diet). The variable contributions of these factors might result in diverse types of cancer, each with relatively distinct clinical and molecular characteristics.

Colorectal cancer syndromes with predominantly genetic causes have been thoroughly described in many countries. Several of the syndromes associated with the extensive formation of colorectal polyps have been traced to alterations in specific genes (e.g., familial adenomatous polyposis, which is caused mainly by deleterious germ-line alterations in the *APC* gene), but these forms are relatively rare. In most hereditary colon cancer syndromes, the number of adenomatous polyps is by no means exceptional, which makes it less likely that the syndrome will be identified on the basis of clinical findings alone.

Thus far, only one of these syndromes has been linked to specific genetic alterations: the Lynch syndrome. In this case, the increased risk of cancer (which involves not only the colon but also the endometrium and other organs) stems from germ-line defects involving one of four genes involved in DNA mismatch repair (MMR) (reviewed in [1, 2]). Mismatch repair deficiency can be diagnosed with assays of tumor DNA for microsatellite instability (MSI) and immunostaining of tumor sections for the major MMR proteins. When defects are found, germ-line DNA can then be analyzed to find the deleterious variant causing the disease. Once this variant has been pinpointed, carriers within an affected kindred can be identified and measures taken to prevent them from developing colon cancer.

Unfortunately, in most families with familial colon cancer (FCC) syndromes, this work-up reveals no MMR deficiency, and the basis of their predisposition to colorectal cancer thus remains unknown. In the present study, we prospectively analyzed a large number of Swiss kindreds with FCC. Our aim was to identify the phenotypic features of MMR-proficient, non-polyposis, colon cancer syndromes (a subset that probably includes several etiologically distinct forms of disease) and to see how they differ from those of similar syndromes linked to MMR deficiency.

Patients and methods

The study was approved by the Cantonal Ethics Committee of Basel, Switzerland (No. 258/05), and each subject investigated provided written informed consent to genetic testing, collection and analysis of data, and publication of the findings. Unless, otherwise specified, all commercial products mentioned below were used in accordance with manufacturers' instructions.

Kindreds

From 1997 to 2008, we enrolled a total of 509 unrelated kindreds with FCC, all living in the German-speaking cantons of Switzerland (total resident population: $\sim$5 million). The probands were referred to our staff by private and hospital-based practitioners for various reasons. One of the most common was that the colorectal cancer was associated with multiple colorectal polyps (10 or more) because the Research Group in Human Genetics of the University of Basel (MK and KH) is also a referral center for patients with gastrointestinal polyposis. As a result, an unrepresentatively large proportion of the enrolled kindreds were ultimately found to have polyposis-related cancer syndromes.

The present analysis was restricted to the 235 kindreds who did not fall into this category and whose probands found to have *nonpolyposis* forms of FCC (Fig. 1). At the time of referral, all 235 probands had family histories satisfying at least one of the following criteria: (1) diagnosis at any age of colorectal, gastric, duodenal, small intestinal, endometrial, or ovarian cancer (the cancers most frequently associated with the Lynch and polyposis syndromes) in the proband and in at least one of his/her first-degree relatives; or (2) diagnosis of one or more of the cancers listed above in the proband only, but at an age of 50 years or younger.

Medical information for these families has been provided by probands and/or family members themselves or by the latters' physicians. Whenever possible, we also reviewed the actual medical charts, pathology reports, and death certificates. Detailed pedigrees (minimum: 2 generations) have been created with Cyrillic v2.1.3 software (Cherwell Scientific Publishing, Oxford, UK), and each has been carefully analyzed to determine whether it fulfilled the Amsterdam criteria II (AC II) and/or the revised Bethesda Guidelines (rBG) [1].

Protocol for diagnosing Lynch syndrome

DNA extraction from peripheral blood and tumor tissue

Germ-line DNA was isolated from the peripheral blood of the probands with the salting-out procedure described by

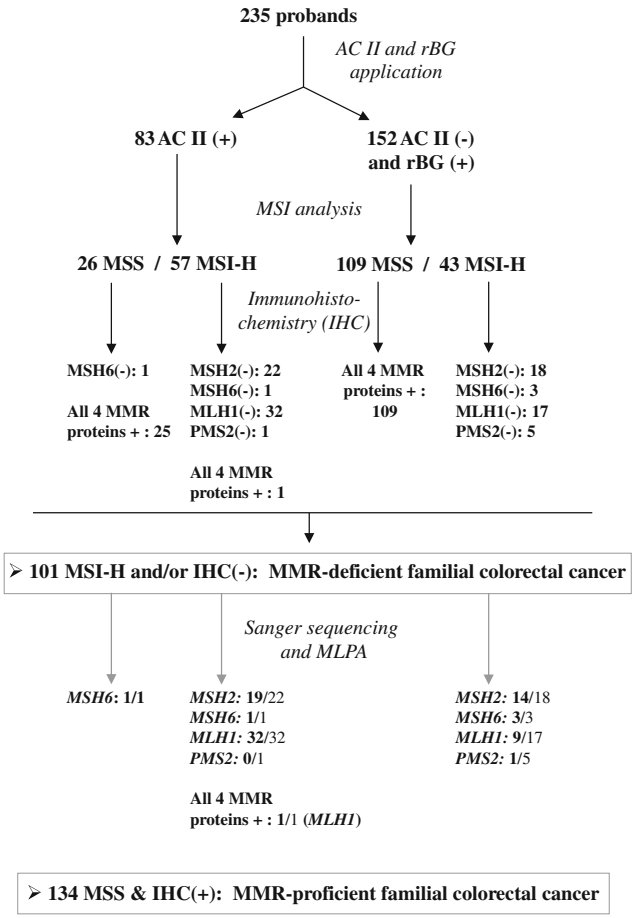

Fig. 1 The diagnostic flow chart used to classify the 235 probands' families having MMR-deficient or MMR-proficient familial colorectal cancer. See "Results" for details

Miller et al. [3]. Tumor DNA was extracted from formalin-fixed, paraffin-embedded tissues with the Ambion RecoverAll™ tissue kit (Applied Biosystems, Foster City, CA, USA). Hematoxylin and eosin-stained sections of the tissue block were examined to locate a representative portion of the tumor with an average epithelial content of >70%, and this portion was dissected for DNA extraction.

Analysis of microsatellite instability and immunohistochemistry

In accordance with National Cancer Institute recommendations [4], we analyzed tumor DNA for microsatellite instability (MSI) at 5 loci (BAT25, BAT26, D5S346, D17S250, and D2S123) using a capillary sequencing protocol that has been previously described [5]. Only those tumor phenotypes classified as MSI-high (instability at 2 or more of the 5 [40–100%] loci) were considered indicative of MMR deficiency. For the purposes of the present analysis, findings were classified as negative when they revealed instability at only 1 [20%] of the 5 loci (MSI-low phenotype) or absence of instability at all 5 loci (microsatellite-stable [MSS] phenotype).

MMR protein expression was assessed in tumor sections with standard immunohistochemical (IHC) techniques, as described elsewhere [6], and antibodies against each of the 4 major MMR proteins (MSH2, MSH6, MLH1, and PMS2) [7].

On the basis of MSI and IHC findings, probands were classified as having MMR-deficient tumors (MSI-high and/or loss of expression of one or more of the 4 MMR proteins) or MMR-proficient tumors (all others).

Analysis of MMR genes for deleterious variants

In patients with MMR-deficient tumors, MMR genes were analyzed in germ-line (leukocyte-derived) DNA. When possible, this analysis was restricted to the gene identified in IHC as the one most likely to harbor the primary alteration responsible for the disease (see "Results" for details). Otherwise, we analyzed all 4 MMR genes.

First, we sequenced the MMR gene(s) using BigDye Terminator chemistry (version 1.1, Applied Biosystems, Foster City, CA, USA). (Primer sequences and PCR conditions are available on request.) Results indicative of a deleterious variant were confirmed in a second, independently drawn blood sample. The probability that missense variants exerted damaging effects on the function of their protein products was estimated with the PolyPhen-2 scoring system, as described by Adzhubei et al. [8].

When sequencing findings were negative, the DNA sample was screened for copy number aberrancy with multiplex-ligation dependent-probe amplification (MLPA) kits P003 (for MLH1, MSH2, and EPCAM); P008 (MSH6 and PMS2); and P072 (for MSH6 only) (MRC Holland, Amsterdam, The Netherlands). GeneMarker software (SoftGenetics, State College, PA, USA) was used for identification and dosage quotient analysis of each specific amplicon. (Dosage quotients of $\sim$1 indicate the presence of 2 copies of a given gene; quotients of $\sim$0.5 indicate loss of 1 copy.) MLPA results indicative of a germ-line deletion were confirmed in a second, independently drawn blood sample. All apparently single-exon deletions were directly sequenced to identify possible sequence variations within the ligation-probe binding site, which can mimic single-exon deletions.

When positive findings emerged from either of the above studies, the kindred was definitively classified as having Lynch syndrome. If no deleterious variants were identified in the germ-line with these methods, kindreds whose tumors were MMR-deficient were classified as having probable Lynch syndrome and genetic testing continued to identify the causative alteration.

Statistical analysis

We compared the groups with MMR-deficient and MMR-proficient FCC to identify differences regarding sex, age at first cancer diagnosis, mutational status, etc. (variables shown in all tables). Differences were analyzed with the Fisher exact test (for categorical variables) or the Student *t* test (for continuous variables). All probabilities are reported as two-tailed *P* values, considering $P < 0.05$ to be statistically significant.

We then used multivariate analysis to identify clusters of patients within the two main groups (MMR-proficient and MMR-deficient FCCs) with similar clinical and molecular profiles. Thirteen clinical variables (sex; age at first cancer diagnosis; type of first cancer; type of second cancer; specific site of the first cancer; and occurrences of colorectal, small bowel, stomach, endometrial, ovarian, brain, urothelial cancer, and colorectal adenomatous polyps) and 2 molecular features of tumors (MSI and expression of MMR proteins) were considered in this analysis. Several multivariate data analysis algorithms (symmetric and nonsymmetric correspondence, redundancy analyses) [9–11] were used to detect patient subgroups within the two main groups and to predict membership of patients in a given molecular cluster based on their clinical profiles, or vice versa.

Results

From 1997 to the end of 2008, we enrolled 509 unrelated Swiss kindreds that met the minimal inclusion criteria described above. As noted in "Patients and methods", 274 were found to have hereditary polyposis syndromes or multiple colorectal adenomas (≥10 adenomatous polyps) and were excluded from the present analysis.

Figure 1 summarizes the results of our analysis of the 235 kindreds (703 affected individuals) with nonpolyposis forms of FCC. Pedigree data for 3 or more generations were available for 70% of these kindreds and for 2 generations in the remaining 30%. Eighty-three (35%) families fulfilled the AC II; the remaining 152 (65%) satisfied one or more of the inclusion criteria of the rBG.

On the basis of the results of MSI analysis and IHC of tumor samples from the probands, the FCC in 101 (43%) of these 235 kindreds was classified as MMR-deficient ($n = 101$, 43%). The other 134 (57%) were MMR-proficient [12, 13]. As shown in Fig. 1, MSI and IHC findings were concordant in 99 of the 101 MMR-deficient probands (i.e., MSI-H plus loss of expression of at least 1 MMR protein). In 79 of these cases, mutation screening of germline DNA revealed a deleterious variation in the unexpressed MMR gene (Supplementary Table 1), thereby confirming the diagnosis of Lynch syndrome. In two other cases, the classification of MMR deficiency was supported by only one of the two methods (MSI or IHC). In both cases, however, mutation screening confirmed the diagnosis of Lynch syndrome. One of these (case no. 2047/01) involved an MSI-H tumor in which all 4 MMR proteins were normally expressed. Screening of all 4 MMR genes revealed a missense variant at the *MLH1* locus [c.292G > C(p.Gly98Arg)], which was classified as "damaging" on the basis of the PolyPhen-2 score [8]. The second discrepancy involved case no. 2467/01. This individual had an MSS tumor that did not express MSH6, and a nonsense variant was indeed found in the gene that encodes this MMR protein (Supplementary Table 1).

On the whole, 81 (80%) of the 101 MMR-deficient probands were confirmed as having the Lynch syndrome by sequencing and/or MLPA-based documentation of a deleterious variants at one of the major MMR loci. A total of 62 different variants were found, including 8 carried by two or more probands. Thirteen of the mutations appeared to be novel in that they were not listed in the Memorial University of Newfoundland MMR-Gene Variant Database [14] or in the Leiden Open Variation Database [15]. As shown in Supplementary Figure 1, the 33 variants detected in *MSH2* included 9 nonsense alterations, 1 missense variant, 9 in-frame deletions, and 14 small insertions, deletions, or splice site variants that caused reading-frame shifts resulting in premature termination of translation. All 5 of the *MSH6* alterations were nonsense variants. The 42 variants detected in *MLH1* included nonsense ($n = 6$) and missense ($n = 5$) variations, in-frame deletions (p.Lys616del [$n = 5$], p.Glu578_Glu633del [$n = 2$], p.Val664_Gln701del [$n = 7$]), and 17 small insertions, deletions, or splice site variants that shifted the translation frame. As for *PMS2,* only 1 nonsense variant has been identified thus far. The family is 1 of the 6 with PMS2-deficient tumors. All 6 of the missense variants we have found (5 involving *MLH1* and 1 *MSH2*) were considered to be deleterious on the basis of their PolyPhen-2 scores (Supplementary Table 1).

In the remaining 20 probands with MMR-deficient tumors (7 with loss of MSH2, 8 with loss of MLH1, and 5 with loss of PMS2), no pathogenic germ-line variants in MMR genes could be identified by Sanger sequencing or gene copy number analysis by MLPA. Technically speaking then, the presence of Lynch syndrome in these kindreds cannot be confirmed at this time. Further testing, however, might well reveal germ-line alterations of a genetic nature in regions of the MMR genes not covered by our analyses (e.g., intronic changes or those involving the 5′ or 3′ UTR) and/or epigenetic changes (e.g., promoter hypermethylation).

The next step was to identify phenotypic differences between the MMR-deficient and MMR-proficient FCC subsets. Table 1 shows the clinical characteristics of the *probands* in these two groups. The MMR-deficient group

exhibited: (1) a higher probability of fulfilling the AC II (57.4% [58/101] versus 18.6% [25/134] of the MMR-proficient FCC group; Fischer's exact P value < 0.001); (2) earlier onset of the primary cancer (by ~ 4 years compared with MMR-proficient FCC probands; $P = 0.013$); (3) a higher proportion of primary colon cancers that were located in the proximal colon (52.5% [53/101] versus 18.6% [25/134] of the probands with MMR-proficient FCC; $P < 0.001$); (4) a higher frequency of metachronous cancers of the colorectum ($P = 0.036$); and (5) a higher frequency of cancers in extracolonic organs specified in the rBG ($P < 0.001$), particularly the endometrium. MMR-deficient probands also exhibited a trend toward better survival at 5 years. (Only 5 of the 27 probands who died within 5 years of diagnosis were members of this group.) The two groups of probands were similar in other respects, including sex ratios and the presence of synchronous colorectal cancers or synchronous and metachronous colorectal adenomas.

Next, we analyzed the clinical features reported in Table 1 in the total population investigated in this study (i.e., all 703 *affected* individuals, including the 235 probands in Table 1 and their 468 relatives) (Table 2). For the purpose of this analysis, tumors that could not be analyzed for MMR-deficiency (specimens unavailable) were presumptively classified as having the same MMR status as the corresponding proband's tumors. The accuracy of this classification is obviously less than 100%, but its general reliability is supported by two findings. First, all of the non-proband tumors that were available for MSI analysis and IHC (approximately 15% of all those reported) had MMR statuses identical to those of the corresponding proband's tumors. Second, the affected family members were almost all first- ($n = 239$; 51.1%) or second-degree ($n = 143$; 30.5%) relatives of their respective probands; only 86 (18.4%) were third-degree relatives.

Using this approach, we estimated that 395 (56.2%) of the 703 individuals considered (101 probands and 294 of their relatives) had MMR-deficient tumors, and 308 (134 probands, 174 of their relatives) had MMR-proficient FCC. As shown in Table 2, the clinical features that distinguished MMR-deficient and MMR-proficient groups in the extended population were the same ones that differentiated these 2 groups in the proband population. In this case, however, the MMR-deficient group was also characterized by a significantly lower age at the diagnosis of colorectal *adenomas* [$P = 0.025$ versus the MMR-proficient FCC subgroup]. This difference had not been statistically significant when the probands were compared (probably owing to the smaller size of these groups), but it came as no surprise because both the proband and extended MMR-deficient FCC populations exhibited significantly earlier onset of primary colorectal *cancers* than their counterparts with MMR-proficient FCC. Comparison of Tables 1 and 2 shows that the ages at diagnosis of primary cancers in both extended populations were ~ 5 years higher than those observed among the respective proband populations. This difference reflects a selection bias related to the documented tendency to enroll younger individuals in cancer screening programs, and it is consistent with previous reports [16]. More details on the clinical features of the 235 probands and the extended population of affected individuals are reported in Supplementary Tables 2 and 3, respectively, where AC II(+) and AC II(−) subgroups are compared.

The multivariate analysis described in "Patients and methods" was performed in the proband and extended populations. In both cases, it readily discerned the MMR-deficient FCC and MMR-proficient FCC clusters, as well as those defined by sex (because of the high incidence of endometrial cancer associated with colorectal cancer), but no evident sub-clusters could be identified within either these two obvious groups (data not shown).

Discussion

Apart from the rare inherited colorectal polyposis syndromes, the only hereditary colorectal cancer syndrome that has been linked to a specific genetic alteration is the Lynch syndrome, which stems from germ-line defects in the DNA MMR genes. Using a combination of MSI analysis of tumor DNA and IHC assessment of MMR protein expression in tumor sections, we found that 101 of the 235 probands we studied had MMR-deficient tumors, and the presence of Lynch syndrome was confirmed in 81 of the cases by the demonstration of deleterious variants at a major MMR locus in the germ-line DNA.

The two-pronged approach we used revealed 2 cases of MMR-deficiency (both of which were later confirmed as Lynch syndrome) that would have been missed if MSI analysis or MMR IHC had been used alone. In general, however, the two methods yielded concordant results. This is largely due to the fact that we perform immunostaining for all 4 MMR proteins, a practice dictated by the relative stability of the MMR proteins within the heterodimers they form (MSH2/MSH6 and MLH1/PMS2) [6, 7]. It also reflects the quality of tumor-block fixation and paraffin embedding procedures in most of Switzerland's pathology laboratories, an absolute prerequisite for reliable IHC assessment.

IHC was also highly sensitive in detecting tumors harboring damaging missense variants at MMR loci. A single amino-acid substitution does not always produce protein destabilization that is detectable with this approach (as we saw in the patient with the missense variant in *MLH1*

Table 1 Clinical features of 134 MMR-proficient and 101 MMR-deficient probands

Variables (no. probands available for the analysis)	No. of cases	MMR-P probands			MMR-D probands			MMR-P vs. MMR-D[d]
		MSS 132	MSI Low 2	All 134 (57)	MSI-High 100	MSS 1[c]	All 101 (43)	
Clinical criteria (235)								
AC II(+)	83	24	1	**25** (30.1)	57	1	**58** (69.9)	**<0.001**
AC II(−)	152	108	1	**109** (71.7)	43	0	**43** (28.3)	
Sex (235)								
Male	111	60	0	**60** (54)	50	1	**51** (46)	0.429
Female	124	72	2	**74** (59.7)	50	0	**50** (40.3)	
Age at diagnosis of primary cancer (235)								
Mean (SD)		46.6 (12.1)	50 (12.8)	**46.6** (12)	42.7 (11.7)	43	**42.7** (11.7)	**0.013** (0.492)
Median (IQR)		44.5 (14)	50 (18)	**45.5** (13.5)	41.5 (15)	43	**42** (15.2)	
Range		21–90	41–59	**21–90**	19–70		**19–70**	
Primary cancer site (235)								
Colon and rectum	214	126	2	**128** (59.8)	85	1	**86** (40.2)	**0.0096**
Small intestine	3	1	0	**1** (33.3)	2	0	**2** (66.7)	0.578
Stomach	4	1	0	**1** (25)	3	0	**3** (75)	0.316
Endometrium[e]	12	3	0	**3** (25)	9	0	**9** (75)	**0.033**
Ovary[e]	2	1	0	**1** (50)	1	0	**1** (50)	1
Site of primary colorectal cancer (201)								
Proximal colon	78	25	0	**25** (32)	53	0	**53** (68)	**<0.001**
Distal colon and rectum	123	95	2	**97** (78.9)	25	1	**26** (21.1)	
Synchronous colorectal cancer (229[a])								
Yes	12	8	0	**8** (66.7)	4	0	**4** (33.3)	0.765
No	217	123	2	**125** (57.6)	91	1	**92** (42.4)	
Metachronous colorectal cancer (229[a])								
Yes	26	10	0	**10** (38.5)	16	0	**16** (61.5)	**0.036**
No	203	121	2	**123** (60.6)	79	1	**80** (39.4)	
Synchronous and/or metachronous colorectal adenomas (229[a])								
Yes	65	31	1	**32** (49.2)	33	0	**33** (50.8)	0.137
No	164	99	1	**100** (61.0)	63	1	**64** (39.0)	
Age at diagnosis of colorectal adenomas (62 polyps[b])	62	29	1	30 (48.4)	32	0	32 (51.6)	
Mean (SD)		49.8 (12.3)	41	**49.5** (12.2)	45.1 (10.6)		**45.1** (10.6)	0.136 (0.459)
Median (IQR)		46 (15)	41	**46** (14)	45 (18.5)		**45** (18.5)	
Range		30–75		**30–75**	21–63		**21–63**	

Table 1 continued

Variables (no. probands available for the analysis)	No. of cases	MMR-P probands			MMR-D probands			MMR-P vs. MMR-D[d]
		MSS 132	MSI Low 2	All 134 (57)	MSI-High 100	MSS 1[c]	All 101 (43)	
All rBG cancers (235)								
Colon and rectum	271	146	2	**148** (54.6)	122	1	**123** (45.4)	**<0.001**
Small bowel	7	1	0	**1** (14.3)	6	0	**6** (85.7)	0.121
Stomach	6	2	0	**2** (33.3)	4	0	**4** (66.7)	0.684
Endometrium[e]	20	3	0	**3** (15)	17	0	**17** (85)	**<0.001**
Ovary[e]	4	1	0	**1** (25)	3	0	**3** (75)	0.622
Ureter/renal pelvis	6	0	0	**0** (0)	6	0	**6** (100)	**0.029**
Brain[f]	2	1	0	**1** (50)	1	0	**1** (50)	1
Probands with extracolonic rBG cancers (235)								
Yes	39	8	0	**8** (20.5)	31	0	**31** (79.5)	**<0.001**
No	196	124	2	**126** (64.3)	69	1	**70** (35.7)	
Probands with 2 or more rBG cancers (235)								
Yes	48	14	0	**14** (29.2)	34	0	**34** (70.8)	**<0.001**
No	187	118	2	**120** (64.2)	66	1	**67** (35.8)	
Five-year survival (218)								
Yes	191	101	1	**102** (53.4)	89	0	**89** (46.6)	0.064
No	27	22	0	**22** (81.5)	5	0	**5** (18.5)	

Data for the two groups are presented as counts with row percentages in parentheses, except for *age at diagnosis*, which is expressed in years with SD and IQR in parentheses

MSS microsatellite stability; *MSI* microsatellite instability; *MMR-P* mismatch repair proficient; *MMR-D* mismatch repair deficient; *AC II* Amsterdam criteria II; *SD* standard deviation; *IQR* interquartile range; *rBG cancers* those included in the Lynch syndrome cancer spectrum, as defined by the revised Bethesda Guidelines

P values indicate significance for all the values highlighted in bold

[a] The probands analyzed ($n = 229$) included 214 whose first malignancy was colorectal cancer and 15 whose colorectal cancer was the second or third malignancy diagnosed. All synchronous and metachronous cancers and adenomas were histologically verified

[b] In 45 of these 62 probands, adenomatous polyps and colorectal cancer were detected synchronously

[c] This proband had a germ-line variant *MSH6* c.718C > T (p.Arg240X) (see Supplementary Table 1)

[d] Fisher's exact *P* values; *F* values for variance comparison are reported in parentheses. *Primary cancer site* and *All rBG cancers*: Cancers in a given site were compared with those diagnosed in other sites

[e] Analyzed only in the female probands

[f] Includes 1 astrocytoma and 1 with unknown histology (unavailable)

Table 2 Clinical features of the 703 affected individuals investigated in this study (235 MMR-proficient and -deficient probands, and their 468 relatives)

Variables (no. probands available for the analysis)	No. of cases	MMR-P probands and their relatives[c]			MMR-D probands and their relatives[c]			MMR-P vs. MMR-D[e]
		MSS 304	MSI Low 4	All 308 (43.8)	MSI-High 392	MSS 3[d]	All 395 (56.2)	
Clinical criteria (703)								
AC II(+)	391	89	3	**92** (23.5)	296	3	**299** (76.5)	**<0.001**
AC II(−)	312	215	1	**216** (69.2)	96	0	**96** (30.8)	
Sex (703)								
Male	377	159	1	**160** (42.5)	216	1	**217** (57.5)	0.445
Female	326	145	3	**148** (45.4)	176	2	**178** (54.6)	
Age at diagnosis of primary cancer (652)	652	282	4	286 (43.9)	363	3	366 (56.1)	
Mean (SD)		52 (13.3)	53.5 (6.1)	**52** (13.3)	46.8 (12.7)	53.7 (9.7)	**46.8** (12.7)	**<0.001** (0.376)
Median (IQR)		50 (18)	52.5 (20)	**50** (18)	46 (17)	56 (14.2)	**46** (17)	
Range		17–91	41–68	**17–91**	11–84	43–62	**11–84**	
Primary cancer site (703)								
Colon and rectum	576	264	4	**268** (46.5)	307	1	**308** (53.5)	**0.002**
Small intestine	7	2	0	**2** (28.6)	5	0	**5** (71.4)	0.475
Stomach	39	19	0	**19** (48.7)	20	0	**20** (51.3)	0.868
Endometrium[f]	59	13	0	**13** (22)	44	2	**46** (78)	**<0.001**
Ovary[f]	6	2	0	**2** (33.3)	4	0	**4** (66.7)	0.7
Ureter/renal pelvis	4	1	0	**1** (25)	3	0	**3** (75)	0.635
Brain[g]	12	3	0	**3** (25)	9	0	**9** (75)	0.245
Site of primary colorectal cancer (234)								
Proximal colon	94	28	0	**28** (29.8)	66	0	**66** (70.2)	**<0.001**
Distal colon and rectum	140	101	2	**103** (73.6)	36	1	**37** (26.4)	
Synchronous colorectal cancer (604[a])								
Yes	20	8	0 (0)	**8** (40)	12	0	**12** (60)	0.82
No	584	261	4	**265** (45.4)	318	1	**319** (54.6)	
Metachronous colorectal cancer (604[a])								
Yes	32	11	0	**11** (34.4)	21	0	**21** (65.6)	0.274
No	572	258	4	**262** (45.8)	309	1	**310** (54.2)	
Synchronous and/or metachronous colorectal adenomas (604[a])								
Yes	86	42	1	**43** (50.0)	43	0	**43** (50.0)	0.351
No	518	227	3	**230** (44.4)	287	1	**288** (55.6)	
Age at diagnosis of colorectal adenomas (84 polyps[b])	84	40	1	41 (48.8)	43	0	43 (51.2)	
Mean (SD)		51.3 (12.4)	41	**51** (12.4)	45.5 (9.6)		**45.5** (9.6)	**0.025** (0.105)
Median (IQR)		48 (15)	41	**48** (15)	46 (9.7)		**46** (9.7)	
Range		30–86		**30–86**	21–63		**21–63**	

Table 2 continued

Variables (no. probands available for the analysis)	No. of cases	MMR-P probands and their relatives[c]			MMR-D probands and their relatives[c]			MMR-P vs. MMR-D[e]
		MSS 304	MSI Low 4	All 308 (43.8)	MSI-High 392	MSS 3[d]	All 395 (56.2)	
All rBG cancers (703)								
Colon and rectum	657	285	4	**289** (44.0)	367	1	**368** (56.0)	**<0.001**
Small bowel	16	2	0	**2** (12.5)	14	0	**14** (87.5)	**0.021**
Stomach	43	20	0	**20** (46.5)	23	0	**23** (53.5)	0.426
Endometrium[f]	72	14	0	**14** (19.4)	56	2	**58** (80.6)	**<0.001**
Ovary[f]	11	3	0	**3** (27.3)	8	0	**8** (72.7)	0.539
Ureter/renal pelvis	12	1	0	**1** (8.3)	11	0	**11** (91.7)	**0.033**
Brain[g]	14	4	0	**4** (28.6)	10	0	**10** (71.4)	0.422
Patients with extracolonic rBG cancers (703)								
Yes	148	40	0	**40** (27)	106	2	**108** (73)	**<0.001**
No	555	264	4	**268** (48.3)	286	1	**287** (51.7)	
Patients with 2 or more rBG cancers (703)								
Yes	89	18	0	**18** (20.2)	71	0	**71** (79.8)	**<0.001**
No	614	286	4	**290** (47.2)	321	3	**324** (52.8)	

Data for the two groups are presented as counts with row percentages in parentheses, except for *age at diagnosis*, which is expressed in years with SD and IQR in parentheses

MSS microsatellite stability; *MSI* microsatellite instability; *MMR-P* mismatch repair proficient; *MMR-D* mismatch repair deficient; *AC II* Amsterdam criteria II; *SD* standard deviation; *IQR* interquartile range; *rBG cancers* those included in the Lynch syndrome cancer spectrum as defined by the revised Bethesda Guidelines

P values indicate significance for all the values highlighted in bold

[a] The probands analyzed ($n = 604$) included 576 whose first malignancy was colorectal cancer and 28 whose colorectal cancer was the second or third malignancy diagnosed. All synchronous and metachronous cancers and adenomas were histologically verified

[b] In 64 of these 84 probands, adenomatous polyps and colorectal cancer were detected synchronously

[c] The MSI status of most relatives' tumors was inferred from that of the corresponding proband (see text for details)

[d] Three affected members of the family with a germline variant *MSH6* c.718C > T (p.Arg240X) (see Supplementary Table 1)

[e] Fisher's exact *P* values; *F* values for variance comparison are reported in parentheses. *Primary cancer site* and *All rBG cancers*: Cancers in a given site were compared with those diagnosed in other sites

[f] Analyzed only in the female probands and relatives

[g] Includes 6 glioblastoma multiforme, 1 astrocytoma, and 7 with unknown histology (unavailable)

described above). However, the other 5 germ-line missense variants found in our MMR-deficient probands thus far (4 involving *MLH1,* 1 in *MSH2*) were all associated with negative staining for the protein encoded by the affected gene (MLH1 or MSH2) and for the affected protein's heterodimeric partner (PMS2 or MSH6, respectively). Using antibodies against all 4 MMR proteins provides intrinsic controls that allowed us to restrict our initial mutational analysis to a single MMR gene. In 80% of the cases tested thus far, this approach has successfully disclosed pathogenic germ-line variants, including 13 that have not been previously reported. A positive "side effect" of our study is that most pathology laboratories in Switzerland are now using MMR protein IHC to investigate tumors when FCC is suspected.

Despite its relatively high prevalence, Lynch syndrome accounted for only ~35% of the nonpolyposis forms of FCC in our registry (81 MMR-deficient probands in whom a deleterious MMR gene variants has been detected so far). Deleterious variants might also conceivably be discovered in some or all of the 20 MMR-deficient probands with probable Lynch syndrome, who are undergoing additional tests for the detection of genetic and epigenetic variations (see "Results"). In the other kindreds (~60% of those studied), the inherited (genetic, epigenetic) and noninherited causes of the predisposition to colorectal cancer are currently unknown. This type of disease is often referred to as *familial colorectal cancer type-X* (FCC-X) when the inheritance pattern is Mendelian or *common familial risk colorectal cancer* when it appears to be associated with intermediate-penetrance alleles [12, 13, 17]. For the sake of simplicity, we will refer to both forms hereafter as MMR-proficient FCC.

The clinical phenotypes of MMR-deficient and MMR-proficient FCC have been compared in various parts of the world, including Europe [12, 18–25], North America [12, 26], and Australasia [12, 27, 28]. Lindor et al. reported another large, well-characterized series of FCC kindreds from different countries (all fulfilling the original AC, also called AC I [29]) [12]. The phenotypic differences they observed between Lynch syndrome families ($n = 90$) and those with FCC-X ($n = 71$) were clearly present in the 235 Swiss kindreds we examined. In our series, individuals with inherited defects involving DNA MMR presented with colorectal cancer earlier in life (~5 years) than those whose MMR system was intact, and their cancers were much more likely to be located in the proximal colon. Their phenotype was also patently syndromic with an increased tendency to develop second primary tumors in the large intestine and extracolonic cancers (mainly endometrial) as well. These differences were still evident when we extended our analysis to the probands' affected relatives (Table 2).

The overall incidence of cancer (colorectal and other types) was also higher in the MMR-deficient kindred subset, which comprised 294 affected blood relatives in addition to the 101 probands. (By comparison, the MMR-proficient FCC group included 134 probands but only 174 affected relatives.) This substantially higher penetrance for colorectal and extracolonic cancers explains why MMR-deficient families were more likely to meet the AC II (57.4% versus 18.6% of those with MMR-proficient FCC).

Optimal clinical management strategies for these two subsets of familial colorectal cancer will naturally differ in several respects, including the starting age and frequency of surveillance colonoscopy in family members and the extension of preventive diagnostics to organs other than the colon. Last but by no means least, members of Lynch syndrome families who do not harbor the deleterious MMR gene variant can be spared the ordeal of the high-frequency surveillance recommended for the carriers (although they should still follow population screening guidelines). This is not possible in MMR-proficient FCC families. Until the inherited genetic alteration(s) responsible for these cancers are identified, all members of these families need to undergo frequent check-ups.

Stringent clinical criteria like the AC are the starting point in the search for genetic loci that might be responsible for cancer predisposition. This approach has led to the identification of numerous Mendelian disorders, but the discovery of the cause of the Lynch syndrome was much more fortuitous. It stemmed from the observation of MSI in the tumors associated with this syndrome, a phenomenon that had already been linked to DNA MMR in lower organisms (reviewed in [30]). The germ-line variants in the *MUTYH* gene that cause colorectal polyposis were also identified thanks to clues gleaned through the molecular characterization of somatic alterations in tumors (reviewed in [31]).

What can these experiences teach us regarding the search for the cause(s) of MMR-proficient FCC? For one thing, if the genetic approach is to be used, it is essential to remember that MMR-proficient FCC is not a single entity. It almost certainly encompasses several different conditions, some of which are classic Mendelian disorders while others are more complex, multifactorial diseases with variable genetic, epigenetic, behavioral, and environmental components. As shown in Fig. 1, 25 of our kindreds had tumors that were microsatellite-stable and appeared to express all four of the major MMR proteins. And yet their pedigrees satisfied the AC II. It is in this group of families that the possibility of a predominantly genetic etiology should be explored using Mendelian genetics, even though efforts along these lines conducted thus far have not (to our knowledge) been successful.

In other MMR-proficient FCC kindreds, multiple factors presumably contribute to the onset of colorectal cancer. Ferreting these out will undoubtedly prove to be a much more complicated task. The tools of quantitative genetics are better suited to this purpose since they also take into consideration behavioral and environmental contributions, which are thought to be highly important in the inheritance of such cancers. Multivariate analysis based on the clinical variables documented by our team failed to identify different subgroups among our MMR-proficient FCC kindreds. This suggests that greater effort should go into the clinical characterization of probands and their families during recruitment for such studies.

The search for etiologic factors in MMR-proficient FCC could also be jump-started by a more complete biological characterization of the *tumors* themselves. The molecular phenotype(s) of these cancers (like those associated with the Lynch syndrome and colorectal polyposis) hold important clues that could point the search in the right direction. A high-throughput, –omics-based analysis of these phenotypes might provide productive hints on their etiology. A systems biology approach that analyzes data on the MMR-proficient FCC tumors' (epi)genome, transcriptome, proteome, and metabolome could allow us to make an informed guess as to their cause(s), which could then be experimentally tested. The value of this approach has been confirmed by previous experience with the MMR-deficient colorectal cancers, where a striking phenotype was accompanied by peculiar genomic [32] and transcriptomic patterns [33].

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