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MSH6 and MUTYH Deficiency Is a Frequent Event in Early-Onset Colorectal Cancer

María Dolores Giráldez¹, Francesc Balaguer¹, Luis Bujanda², Miriam Cuatrecasas¹, Jenifer Muñoz¹, Virginia Alonso-Espinaco¹, Mikel Larzabal², Anna Petit¹, Victoria Gonzalo¹, Teresa Ocaña¹, Leticia Moreira¹, José María Enríquez-Navascués², C. Richard Boland³, Ajay Goel³, Antoni Castells¹, and Sergi Castellví-Bel¹

¹Gastroenterology Department, Institut de Malalties Digestives i Metabòliques, Hospital Clínic, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Institut d'Investigacions Biomèdiques August Pii Sunyer, (DIBAPS), University of Barcelona, Barcelona, Spain

²Departments of Gastroenterology and General Surgery, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, University of Country Basque, Donostia Hospital, Donostia, Spain

³Department of Internal Medicine, Division of Gastroenterology, Charles A. Sammons Cancer Center and Baylor Research Institute, Baylor University Medical Center, Dallas, Texas

Abstract

Purpose—Early-onset colorectal cancer (CRC) is suggestive of a hereditary predisposition. Lynch syndrome is the most frequent CRC hereditary cause. The *MUTYH* gene has also been related to hereditary CRC. A systematic characterization of these two diseases has not been reported previously in this population.

Experimental Design—We studied a retrospectively collected series of 140 patients ≤50 years old diagnosed with nonpolyposis CRC. Demographic, clinical, and familial features were obtained. Mismatch repair (MMR) deficiency was determined by microsatellite instability (MSI) analysis, and immunostaining for MLH1, MSH2, MSH6, and PMS2 proteins. Germline MMR mutations were evaluated in all MMR-deficient cases. Tumor samples with loss of MLH1 or MSH2 protein expression were analyzed for somatic methylation. Germline *MUTYH* mutations were evaluated in all cases. *BRAF* V600E and *KRAS* somatic mutational status was also determined.

Results—Fifteen tumors (11.4%) were MSI, and 20 (14.3%) showed loss of protein expression (7 for MLH1/PMS2, 2 for isolated MLH1, 3 for MSH2/MSH6, 7 for isolated MSH6, and 1 for MSH6/PMS2). We identified 11 (7.8%) germline MMR mutations, 4 in *MLH1*, 1 in *MSH2*, and 6 in *MSH6*. Methylation analysis revealed one case with somatic *MLH1* methylation. Biallelic *MUTYH* mutations were detected in four (2.8%) cases. *KRAS* and *BRAF* V600E mutations were present in 39 (27.9%) and 5 (3.6%) cases, respectively.

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Corresponding Authors: Francesc Balaguer and Sergi Castellví-Bel, Gastroenterology Department, Hospital Clínic, Villarroel 170, Barcelona 08036, Spain. Phone: 34-93-2275418; Fax: 34-93-2279387; fprunes@clinic.ub.es; sbel@clinic.ub.es.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Conclusions—Loss of MSH6 expression is the predominant cause of MMR deficiency in early-onset CRC. Our findings prompt the inclusion of *MSH6* and *MUTYH* screening as part of the genetic counseling of these patients and their relatives.

Colorectal cancer (CRC) is the third most common cancer and the second cause of cancer-related deaths in most western countries (1). Although aging is the major risk factor for CRC initiation and progression, up to 10% of the total CRC burden occurs in individuals who are ages ≤ 50 years (2,3). This population represents an important clinical problem because it is not usually included in the CRC screening programs. Moreover, epidemiologic data suggest that the incidence of early-onset CRC has increased during the past three decades (4,5). Finally, early-onset CRC is suggestive of a hereditary predisposition that may have important consequences not only for the index case but also for their relatives.

Recent studies have suggested that early-onset CRC represents a biologically distinct disease, with clinicopathologic and molecular differences compared with patients with older onset of disease (6–8). Indeed, early-onset CRC is more likely to present at advanced stages, to be poorly differentiated, and to be located in the distal colon, especially in the rectum. From a molecular point of view, it represents a heterogeneous disease, including known hereditary syndromes, familial cases, and apparently sporadic CRC. Lynch syndrome, which accounts for up to 2% to 3% of the total burden of CRC (9,10), is caused by germline mutations in DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) and is thought to account for up to 20% of early-onset CRC (11–13). These cases usually show microsatellite instability (MSI), the hallmark of MMR deficiency, and loss of expression of the corresponding mutated protein by immunohistochemistry. Current guidelines (i.e., revised Bethesda criteria) recommend doing a molecular prescreening by either MSI and/or immunohistochemistry in CRC patients < 50 years old, regardless of family history of CRC (10,14).

Biallelic mutations in the *MUTYH* gene, a member of the base excision repair system, represent another hereditary cause of early-onset CRC (15). Although biallelic inactivation of this gene usually predispose to an attenuated form of colonic polyposis (MAP or *MUTYH*-associated polyposis; ref. 16), population-based studies have shown that up to 30% of biallelic mutation carriers develop CRC in the absence of a polyposis phenotype (17,18). The absence of a specific clinicopathologic feature of nonpolyposis *MUTYH*-associated CRC makes it a diagnostic challenge. In this sense, it has recently been suggested that all early-onset CRC should be tested for *MUTYH* mutations (15). In addition, because *MUTYH*-associated tumors are characterized by the accumulation of somatic G:C $\rightarrow$ T:A transversions, which is the hallmark of base excision repair system deficiency, the analysis of this particular somatic mutation in *KRAS* has also been proposed as screening method (19,20).

Finally, the remaining 75% to 80% of early-onset CRCs have proficient MMR and a yet unidentified genetic predisposition. Although some studies have suggested that these cases are more often diploid than older onset CRC, it is still controversial if they represent a unique molecular form of CRC (6,7,21). Interestingly, several studies have reported a strong familial association in early-onset micro-satellite stable (MSS) CRC (6,7).

Understanding the molecular basis of early-onset CRC is critical to uncover yet unidentified genetic conditions and consequently tailor appropriate preventive strategies for this disease. A comprehensive and systematic approach to identify Lynch syndrome in this cohort with the combination of immunohistochemistry for all four MMR proteins and MSI analysis followed by genetic testing has not been attempted previously. In addition, the frequency of nonpolyposis *MUTYH*-associated CRC in this population remains poorly explored. The aim

of this study was to systematically assess the clinical, histologic, and molecular features of a large cohort of unselected early-onset CRC patients and to analyze the prevalence of known hereditary nonpolyposis syndromes.

Materials and Methods

We retrospectively recruited all patients ≤ 50 years old diagnosed with CRC who were surgically treated at two Spanish centers (Hospital Clínic of Barcelona and Hospital of Donostia) in 1995 to 2007 and from whom archival formalin-fixed paraffin-embedded samples were available. Patients with personal history of colorectal polyposis or inflammatory bowel disease were excluded. Clinicopathologic data were obtained from each patient's medical record. Family history of cancer, including at least first-degree and second-degree relatives, was obtained either from the medical record or by phone contact. Positive family history was considered if ≥ 1 first-degree or second-degree relative(s) had cancer. To compare the clinicopathologic features of the subjects included in this study with a cohort of older-onset CRC (>50 y), we used patients recruited in the EPICOLON project, a previously reported Spanish population-based cohort of CRC (10). The study was approved by the institutional ethics committee of each participating hospital. Written informed consent was obtained at CRC diagnosis on a systematic basis. In deceased cases in which the informed consent was missing, next-of-kin consent was obtained.

DNA isolation

Genomic DNA from each patient was extracted from formalin-fixed paraffin-embedded tumoral and corresponding normal colonic mucosa with the use of the QIAamp Tissue kit (Qiagen) according to the manufacturers' instructions. Peripheral blood DNA, when available, was extracted with the use of the QIAamp DNA blood Mini kit (Qiagen).

Tumor MMR protein expression

One block of formalin-fixed paraffin-embedded tumor tissue was selected per case, and immunostaining was done with the use of standard protocols. The following mouse monoclonal antibodies were used: anti-MLH1, anti-MSH2, anti-MSH6, and anti-PMS2 (BD Pharmingen). Tumor cells were considered to be negative for protein expression only if they lacked staining in a sample in which healthy colonocytes and stromal cells were stained. If no immunostaining of healthy tissue could be shown, the results were considered undetermined.

Tumor MSI analysis

MSI status was assessed with the use of five mononucleotide markers (22): BAT25, BAT26, NR21, NR24, and MONO27 (MSI Analysis System version 1.2, Promega) according to the manufacturers' instructions. Tumors with instability at ≥ 3 markers were classified as high (MSI) and those showing instability at ≤ 2 markers as MSS. Researchers scoring immunostaining were blinded to the MSI results, and vice versa.

Germline MMR mutational analysis

Patients found to have tumors with MSI and/or lack of MMR protein expression underwent germline genetic testing for MMR genes. Gene screening selection was based on the immunostaining results (*MLH1* for MLH1/PMS2 loss, *MSH2* for MSH2/MSH6 loss, and *MSH6* for isolated MSH6 loss). First, genomic rearrangements were analyzed by multiple ligation probe amplification (MRC-Holland, the Netherlands) according to the manufacturers' instructions. Multiple ligation probe amplification results were confirmed by the same technique with the use of a confirmation kit and by an independent method.

Afterwards, we analyzed the coding sequencing and exon-intron boundaries of *MLH1*, *MSH2*, and *MSH6* by single-strand conformational polymorphism (SSCP) and direct sequencing of abnormal band shifts with the use of standard protocols (ABI 3100 Genetic Analyzer, Applied Biosystems). Primer sequences are available on request.

PolyPhen software (<http://genetics.bwh.harvard.edu/pph/index.html>) was used to test the potential pathogenic role of missense variants. This prediction is an *in silico* tool that predicts the possible effect of an amino acid substitution on the structure and function of a human protein with the use of physical and comparative considerations (23).

***MLH1* and *MSH2* methylation analysis**

In tumor samples with loss of *MLH1* or *MSH2* protein expression, DNA methylation status of the CpG island of these genes was established by PCR analysis of bisulfite-modified genomic DNA (EZ DNA Methylation-Gold kit, Zymo Research) with the use of pyrosequencing (PSQ HS 96A Pyrosequencing System, QIAGEN). Cases with somatic methylation in any of these genes were also tested for methylation in the germline (either normal colonic mucosa or blood). Primer sequences are available on request.

Germline *MUTYH* gene mutation analysis

All patients were screened for four *MUTYH* mutations prevalent in the Spanish population (Y176C, G393D, 1138delC, 1220_1221insGG; Genbank access NM_012222) by allele-specific TaqMan probes and resolved on a 7300 Real-Time PCR System (Applied Biosystems). In heterozygotes for any of these mutations, the coding region and exon-intron boundaries of the *MUTYH* gene were screened by SSCP and direct sequencing of abnormal band shifts, as previously described (17). Primer details are available on request.

Somatic *BRAF* V600E and *KRAS* mutation analysis

The *BRAF* V600E mutational analysis was done by allele-specific TaqMan probes as previously described. Primer details are available on request. The mutational hot spot of *KRAS* (codons 12 and 13) was analyzed by direct sequencing of both strands with the use of standard protocols (ABI 3100 Genetic Analyzer, Applied Biosystems). Amplification was done with the use of a coamplification-at-lower-denaturation-temperature PCR method (24). Primer details are available on request.

Statistical analysis

Data were analyzed with the use of the SPSS 13 statistical software. Quantitative variables were analyzed with the use of Student's *t*-test. Qualitative variables were analyzed with the use of either the chi-square test or Fisher's test. A two-sided *P*-value of <0.05 was regarded as significant.

Translational Relevance

Early-onset nonpolyposis colorectal cancer (CRC) is suggestive of a hereditary predisposition. Germline mutations in the mismatch repair (MMR; *MLH1*, *MSH2*, *MSH6*, and *PMS2*) and the base excision repair (*MUTYH*) genes have been associated with early-onset CRC. A systematic evaluation of these two diseases has not been reported previously in this population. We describe herein that mismatch MMR deficiency is present in ~15% of early-onset nonpolyposis CRC cases and is characterized by a high frequency of *MSH6* germline mutations. In addition, biallelic *MUTYH*-associated CRC accounts for 3% to 4% of early-onset MMR-proficient CRCs. The results of this study

prompt the inclusion of *MSH6* and *MUTYH* screening as part of the genetic counseling of early-onset CRC.

Results

Patient characteristics

We recruited 140 patients ≤ 50 years diagnosed with CRC. Clinicopathologic features are shown in Table 1. Mean age at diagnosis was 44.1 years (SD, 5.6 y); 105 (75%) tumors were located distal to the splenic flexure, and 46 (32.9%) were in the rectum. The majority of cases (65.7%) were diagnosed at advanced stages (III–IV); 10 (8.2%) showed poorly differentiated tumors, and 26 (18.6%) had mucinous features. Family history of CRC in either first-degree or second-degree relatives was present in 36 (26.3%) patients, and 8 (5.8%) cases fulfilled the Amsterdam II criteria.

Somatic MMR deficiency

MMR deficiency was evaluated by both immunohistochemistry and tumor MSI analysis in all cases. MMR deficiency was defined as loss of protein expression in any of the MMR proteins and/or having a MSI tumor. Fifteen tumors (10.7%) were MSI, and 20 (14.3%) showed loss of protein expression (7 for MLH1/PMS2, 2 for isolated MLH1, 3 for MSH2/MSH6, 7 for isolated MSH6, and 1 for MSH6/PMS2). Clinicopathologic features of patients with MMR deficiency are summarized in Table 2, and a summary of the molecular results of this study is shown in Table 3. All MSI cases had loss of protein expression; however, two cases with loss of MLH1 and two cases with loss of MSH6 showed MSS tumors. In one case with isolated loss of MSH6, MSI could not be determined. Accordingly, MMR deficiency in our cohort was present in 20 (14.3%) cases.

As shown in Table 1, compared with MMR-proficient tumors, cases with MMR deficiency were more likely to be proximal (70% versus 17.5%; $P = 0.0001$), and showed a trend toward having more mucinous features (35% versus 15.8%; $P = 0.059$) and being more frequently poorly differentiated (20% versus 6.5%; $P = 0.106$). The frequency of MMR deficiency according to patient age was as follows: 21 to 30 years, 25% (1 of 4); 31 to 40 years, 16.7% (4 of 24); and 41 to 50 years, 13.4% (15 of 112). On family history, patients with MMR-deficient tumors fulfilled more frequently the Amsterdam II criteria (21.1% versus 3.4%; $P = 0.013$) and showed a trend toward more frequently having a family history of CRC (42.1% versus 23.7%; $P = 0.1$). Compared with MLH1 or MSH2 deficiency, MSH6-deficient tumors were more frequently located in the distal colon (50% versus 16.7%; $P = 0.16$) and displayed less family history of CRC (14.3% versus 58.3%; $P = 0.14$), although the differences did not reach statistical significance.

Germline MMR mutations

We identified 11 (7.8%) cases with sequence variants in either *MLH1* (4 cases), *MSH2* (1 case), or *MSH6* (6 cases). Characteristics of these patients are shown in Table 2. For *MLH1*, three cases carried a nonsense mutation (p.R226X, p.R487X, p.Q700X), and a missense variant was present in one case (p.S692F). Based on an *in silico* approach, this change was predicted as “benign” but with a borderline pathogenic prediction. For *MSH2*, we identified a complete deletion of exon 7, whereas for *MSH6*, four of six corresponded to missense variants (p.S1279P, p.I1115T, p.P202A, p.P656L) and two to nonsense mutations (p.R732X and p.Q344X; Fig. 1). All four *MSH6* missense variants were predicted to be “possibly damaging” with the use of an *in silico* approach. Overall, the mutation detection rate was 55% (11 of 20), and MMR-deficient cases with or without identified germline mutation did not differ in any clinicopathologic or familial feature (data not shown). However, family

history of CRC was more frequently seen in *MLH1/MSH2* mutation carriers than in *MSH6* ones (100% versus 0%; $P = 0.02$).

***MLH1* and *MSH2* methylation analysis**

Among 12 patients showing loss of either *MLH1* or *MSH2* expression, we only found somatic *MLH1* methylation in one case (Table 2). This patient corresponded to a 30-year-old male with a stage II tumor located in the ascending colon and without family history of any cancer. In this case, neither a pathogenic *MLH1* germline mutation nor a germline *MLH1* methylation was evident.

Germline *MUTYH* mutations

Biallelic *MUTYH* mutations were found in 4 of 140 (2.8%) patients (Table 3). Clinicopathologic features of biallelic carriers are shown in Table 4. Overall, biallelic *MUTYH* mutation carriers did not show any specific clinicopathologic feature, although two tumors were located in the rectum and one in the sigmoid colon. Interestingly, three of four patients showed synchronous adenomas, but none of them showed a polyposis phenotype. On family history, two of them showed a recessive pattern of inheritance of either CRC or colorectal polyposis. We could determine the *KRAS* mutational status in two of the patients, and one of them showed a G:C→T:A transversion in codon 12, the hallmark of base excision repair deficiency.

Monoallelic *MUTYH* mutations were identified in four (2.85%) cases (Tables 3 and 4). Interestingly, one heterozygous carrier of the G393D mutation also carried a missense mutation in *MSH6*. This patient was a 40-year-old female with a stage IV ascending colon tumor with MSI and isolated loss of *MSH6*. The family history was unremarkable for CRC or any other Lynch syndrome-associated tumors.

Somatic *BRAF* and *KRAS* mutations

BRAF V600E mutation was detected in 5 of 140 (3.6%) cases (Table 3). We found no *BRAF* mutation in any MMR-deficient tumor, and the only clinical feature associated with the presence of the *BRAF* V600E mutation was proximal tumor location (80% versus 22.7%; $P = 0.014$). Somatic *KRAS* mutations were detected in 39 of 140 (27.9%) cases (Table 3) and were associated with females (64.1% versus 40.9%; $P = 0.016$). The frequency of found mutations was as follows: Gly12Asp (nine, 23.0%), Gly13Asp (eight, 20.5%), Gly12Cys (seven, 18.0%), Gly12Val (six, 15.4%), Gly12Ala (four, 10.2%), Gly12Ser (two, 5.1%), Gly13Ser (one, 2.6%), Gly13Trp (one, 2.6%), and Gly13Val (one, 2.6%).

Clinicopathologic features of early-onset MMR and base excision repair-proficient CRC

We next evaluated the clinicopathologic features of early-onset CRC with both MMR and base excision repair proficiency, and compared them with a population-based cohort of patients >50 years diagnosed with CRC and recruited in the EPICOLON project (Table 5). Compared with older-onset CRC, MMR-proficient and base excision repair-proficient early-onset CRC was more frequently located in the distal colon (82.7% versus 72%; $P = 0.021$), more frequently diagnosed at advanced stages (66.6% versus 44.1%; $P < 0.0001$), and was associated to a stronger family history of CRC in first-degree and/or second-degree relatives (23.7% versus 16.4%; $P = 0.06$).

Discussion

In this study we have systematically assessed the clinicopathologic, molecular, and familial features of a large cohort of early-onset nonpolyposis CRC showing that, overall, the frequency of known hereditary CRC syndromes in this population is ~10%. This cohort

depicts a distinct molecular profile of MMR deficiency characterized by a high frequency of germline mutations in *MSH6*. Biallelic *MUTYH* mutations account for 3% of early-onset CRC and are clinically indistinguishable from non-*MUTYH* mutation carriers. Early-onset CRC with MMR and base excision repair proficiency, the predominant group, is characterized by distal location, advanced stage, low frequency of mutations in the *BRAF-KRAS-MEKK* pathway, and frequent family history of CRC, suggesting a distinct biological entity.

Lynch syndrome, caused by mutations in the MMR genes (i.e., *MLH1*, *MSH2*, *MSH6*, and *PMS2*) is the most frequent monogenic hereditary CRC syndrome (9,25). Accurate diagnosis of this syndrome is of great benefit for the management of both patients and relatives because surveillance has proven to be highly effective (26). The current guidelines, based on the revised Bethesda criteria (14), recommend doing tumor MMR deficiency testing to preselect those cases with higher probability of being germline mutation carriers in one of these genes (10). Previous studies have revealed that CRC diagnosed at early ages have a high probability of having MMR deficiency, ranging from 17% to 73% (11,12,27–30). The reason for this wide range is the heterogeneity of the studies. First, the threshold to consider “early-onset” CRC varies between different studies, ranging from 24 to 50 years. Second, the methods used to assess the MSI status are highly heterogeneous, and the proteins analyzed by immunohistochemistry are usually restricted to *MLH1* and *MSH2*. Finally, the majority of studies have analyzed either high-risk or general populations of CRC, and it is known that the rate of MSI is much lower in the latter (29). Our study, based on a large unselected population of CRC ≤ 50 years, shows that the overall frequency of MMR deficiency in this population is ~15%. The rate of MSI was slightly lower than previous reports, consistent with a lower frequency of MSI previously reported in a Spanish population (10). We have found a distinct profile of MMR deficiency characterized by a high frequency of isolated loss of *MSH6*, accounting for 40% of the MMR-deficient tumors. This frequency is much higher than previously reported because germline mutations in *MSH6* are usually responsible for only 10% to 15% of all cases of Lynch syndrome (9). Interestingly, compared with tumors with loss of *MLH1* or *MSH2* expression, those with loss of *MSH6* were predominantly located in the distal colon (4 of 6) and lacked family history of CRC (31). Of note, 25% of them were MSS and would be missed if MSI is used as a single screening approach. In clinical practice, all these features make these cases a diagnostic challenge, and they probably remain unrecognized. These results highlight the significance of a systematic evaluation of MMR deficiency in an unselected population of CRC to recognize unusual features of hereditary syndromes. In this sense, these results also underline the need to include *MSH6* deficiency evaluation in the assessment of early-onset CRC.

In our population of early-onset CRC, the overall frequency of Lynch syndrome, defined by the presence of a germline mutation in one of the MMR genes, was 7.8%, which is similar to a previous report of the frequency of *MLH1/MSH2/MSH6* mutations in a series of CRC < 55 years old (32). Although we did not do MMR genetic testing in the whole cohort, it is unlikely that we missed any unidentified germline mutations due to our systematic approach to MMR deficiency. On the *MSH6* germline mutations, the predominant mutated gene in this population, two changes corresponded to pathogenic nonsense mutations (p.R732X and p.Q344X), and four were missense variants (p.P202A, p.P656L, p.I1115T, and p.S1279P). The decreased frequency of frameshift mutations and increased levels of point mutations associated with *MSH6* deficiency, compared with *MLH1* or *MSH2* deficiency, is a well-documented fact in the literature (33). All four missense variants were predicted to be pathogenic with the use of an *in silico* approach, and neither the nonsense mutation nor missense variants have been previously reported in any Lynch syndrome mutation database. The finding of a high number of *MSH6* mutations in an early-onset CRC cohort is in

agreement with a recent report that found germline *MSH6* mutations in 7 of 38 (18.4%) patients without family history of cancer who were diagnosed with CRC before the age of 45 years. On *MLH1* and *MSH2* germline mutations, we only identified one carrier of a *MLH1* missense variant (p.S692F). This case was a 32-year-old patient with a stage IV MSS rectal tumor with loss of MLH1/PMS2 and a positive family history of CRC. This missense variant is novel, and it is likely pathogenic because it had a borderline prediction by PolyPhen.

Although we identified 20 patients with MMR deficiency, a germline mutation in the MMR genes could only be detected in 11 patients (mutation detection rate of 55%). This frequency is consistent with previous reports in which even when patients were selected from high-risk CRC clinics, the mutation detection rate was no more than 50% to 60% (10,27–30,34). This fact constitutes a real problem in clinical practice, and in preventive strategies, these cases are usually considered as mutation carriers. Possible causes for these cases without an identified molecular alteration could be undetected mutations by current analytical methods or somatic events that affect both alleles of a MMR gene.

Another relevant finding of our study is that biallelic mutations in the *MUTYH* gene account for ~3% of early-onset CRC without apparent polyposis. Base excision repair deficiency due to biallelic inactivation of *MUTYH* was initially associated with the development of an attenuated form of colonic polyposis (16). However, several subsequent population-based studies showed that ~30% of biallelic mutation carriers develop a CRC in the absence of a polyposis phenotype (17,18,35). Accordingly, it has been suggested that *MUTYH* testing should be considered in early-onset CRC patients with intact DNA MMR, regardless of family history or number of colonic polyps (15,17). We previously showed that in a population-based cohort of early-onset MMR-proficient CRC without polyposis, the frequency of biallelic mutation carriers was 4.6% (17). Because our mutational screening strategy was based on the *MUTYH* mutational profile in the Spanish population (17), and we did not do systematic whole-gene sequencing, the described frequencies are likely to be underestimated. Considering that biallelic *MUTYH* mutation carriers are at risk for multiple cancers (36), our results highlight the fact that *MUTYH* should be tested in early-onset CRC regardless of family history or colonic polyps.

Finally, our study emphasizes that the genetic basis in the majority of early-onset CRC, if any, remains unknown. Eighty-three percent of patients included in this study showed no evidence of either MMR or base excision repair deficiency. Compared with older patients, we found that this subset of early-onset CRC patients have more frequent distal tumors (especially in the rectum), they are usually diagnosed at advanced stages, and more interestingly, they are associated with a higher frequency of family history of CRC. These features are consistent with previous reports suggesting the uniqueness of these patients. Boardman et al. analyzed the frequency of chromosomal instability in a group of 84 patients ≤50 years old with MSS CRC and compared them with a series of 90 patients ≥65 years old with MSS CRC. MSS tumors in the young group were more often diploid (46%) than those in older patients (26%; $P = 0.006$; ref. 6). Other studies have found similar results (7), although it is still a controversial issue (21). We found that the frequency of mutations in the *BRAF-KRAS-MEKK* pathway in this population is much lower than the one reported in unselected CRC (37,38), suggesting that this pathway does not play a central role in early-onset CRC carcinogenesis. Future studies focused on the understanding of the molecular mechanisms involved in the pathogenesis of early-onset MMR-proficient tumors are needed, and the higher frequency of family history of CRC in this subgroup suggests the presence of yet unidentified cancer susceptibility alleles.

In summary, our study represents the first systematic attempt to describe the frequency of known hereditary CRC syndromes in an unselected cohort of nonpolyposis early-onset CRC. Our results show that MMR deficiency accounts for ~15% of this population and is characterized by a high frequency of germline *MSH6* mutations, which frequently shows unusual features for Lynch syndrome. Accordingly, in addition to MLH1/MSH2 immunohistochemistry, MSH6 should not be overlooked in the molecular prescreening of these patients. If no MMR is found, biallelic *MUTYH* mutations are responsible for ~3% to 4% of cases, and screening for the most common mutations in this gene is recommended. Finally, a better understanding of the molecular basis of early-onset CRC neither due to MMR nor base excision repair deficiency is critical to be able to tailor appropriate preventive strategies for this disease and improve genetic counseling of these patients and their relatives.

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Fig. 1. MSH6 mismatch repair deficiency. Top, isolated loss of MSH6 expression by immunohistochemistry; left shows normal expression of MSH2, and right shows loss of MSH6 expression. Middle, MSI testing in case 75 showing instability for all five mononucleotide repeats. Penta D and Penta D correspond to pentanucleotide repeats used as controls. Bottom, MSH6 sequence electropherogram showing the p.S1279P mutation.

Table 1

Clinicopathologic features of the early-onset CRC

Characteristic	Total cohort (n = 140)	MMR-deficient tumors (n = 20)	MMR-proficient tumors (n = 120)	P
Age, mean (SD)	44.1 (5.6)	42.6 (5.9)	44.3 (5.6)	0.218
Sex, n (%)				
Females	66 (47.1)	8 (40)	58 (48.3)	0.489
Males	74 (52.9)	12 (60)	62 (51.7)	
Tumor location *, n (%)				
Proximal	35 (25)	14 (70)	21 (17.5)	0.0001
Distal	59 (42.1)	4 (20)	55 (45.8)	
Rectum	46 (32.9)	2 (10)	44 (36.7)	
TNM tumor stage †, n (%)				
0	1 (0.7)	0 (0)	1 (0.8)	0.956
I	12 (8.6)	1 (5)	11 (9.3)	
II	33 (23.6)	5 (25)	28 (23.7)	
III	62 (44.3)	9 (45)	53 (44.9)	
IV	30 (21.4)	5 (25)	25 (21.2)	
Degree of differentiation ‡, n (%)				
Well/moderate	112 (91.8)	12 (80)	100 (93.5)	0.106
Poor	10 (8.2)	3 (20)	7 (6.5)	
Mucin production, n (%)	26 (18.6)	7 (35)	19 (15.8)	0.059
Synchronous CRC §	4 (2.9)	1 (5)	3 (2.5)	0.469
Family history of CRC //	36 (26.3)	8 (42.1)	28 (23.7)	0.1
Family history of endometrial cancer //	8 (5.8)	2 (10.5)	6 (5.1)	0.307
Family history of Lynch spectrum extracolonic tumors //	27 (19.7)	5 (26.3)	22 (18.6)	0.533
Fulfillment of Amsterdam II criteria //	8 (5.8)	4 (21.1)	4 (3.4)	0.013

* With respect to the splenic flexure.

† Based on 138 patients.

‡ Based on 122 patients.

§ Based on 138 patients.

// Based on 137 patients; includes first-degree and second-degree relatives.

Table 2

Clinicopathologic and molecular features of patients with MMR deficiency

Case	Age/Sex	Location	Stage	MSI	Immunohistochemistry*													
					MLH1	PMS2	MSH2	MSH6	Other tumors	Family history [†]	KRAS status	BRAF status	MLH1 meth [‡]	MSH2 meth [‡]	MMR germline mutation			
69D	32M	Rectum	IV	MSS					No	Colon (father, 69)	G12A	wt	1%	—	MLH1 p.S692F			
84D	44F	Ascending	III	MSI					Endometrium + ovary (40)	Colon (brother, 36)	wt	wt	2%	—	MLH1 p.Q700X			
111D	42M	Caecum	III	MSI					No	Rectum (father, 30) Colon (brother, 30) Colon (three cousins; 36, 39, 40)	G13D	wt	1%	—	MLH1 p.R226X			
98	41F	Rectum	III	MSI					Colon (38)	Colon (father, 55) Colon (uncle, 40) Stomach (grandmother, 50)	wt	wt	3%	—	MLH1 p.R487X			
16	50F	Caecum	II	MSS	ND				No	Renal (brother, 50) Adenoma hypophisis (aunt, 50) Endometrium (grandmother, 52) Colon (uncle, 60)	wt	wt	3%	—	No			
6	31F	Transverse	III	MSI					No	Endometrium (mother, 52)	G13D	wt	1%	—	No			
25	30M	Ascending	II	MSI					No	No	wt	wt	31%	—	No			
89	45F	Caecum	I	MSI					No	Endometrium (brother, 62) Ovarium (mother, 72) Stomach (grandmother, 70)	G13D	wt	1%	—	No			
85D	46F	Ascending	III	MSI					Colon (41)	Colon (mother, 67) Colon (grandmother, 50) Ovarium (cousin, 45) Metastatic tumor of unknown origin (father, 60) Ovarium (sister, 46)	wt	wt	—	2%	MSH2 del exon 7			
17	41M	Ascending	IV	MSI					No	No	wt	wt	—	2%	No			
35D	45M	Transverse	IV	MSI					No	Colon (48) Skin (40) [§]	wt	wt	—	3%	No			
86	48M	Caecum	III	MSI														
75	48M	Descending	IV	MSI														

Case	Age/Sex	Location	Stage	MSI	Immunohistochemistry*										
					MLH1	PMS2	MSH2	MSH6	Other tumors	Family history [†]	KRAS status	BRAF status	MLH1 meth [‡]	MSH2 meth [‡]	MMR germline mutation
101	37M	Transverse	III	MSS					No	No	wt	wt	—	—	MSH6 p.S1279P
112	46M	Sigmoid	ND	MSS					No	Unknown	wt	wt	—	—	MSH6 p.I1115T
117	46F	Sigmoid	ND	ND	N/D				No	Skin tumor (father, 93)	G12V	wt	—	—	MSH6 p.P202A
154D	40M	Ascending	IV	MSI					No	No	wt	wt	—	—	MSH6 p.R732X
									No	Leukemia (father, 62)	ND	wt	—	—	MSH6 p.P656L
										Prostate (grandfather, 70)					
40D	43F	Transverse	III	MSI					No	Stomach (aunt, 60)	wt	ND	—	—	MSH6 p.Q344X
74	48M	Sigmoid	II	MSI					No	Colon (mother, 91)	wt	wt	—	—	No
91	45M	Transverse	ND	MSI					No	CLL (father, 80)	wt	wt	—	—	No

Abbreviations: ND, not determined; wt, wild-type; CLL, chronic lymphocytic leukemia.

* Solid cells indicate loss of protein expression.

[†] Affected relative and age at diagnosis are indicated between parentheses. Only the affected side of the family is described.

[‡] Somatic methylation analysis done by pyrosequencing.

[§] Keratoachantomas.

Table 3

Summary of molecular results

	<i>n/N (%)</i>
Loss of MMR protein expression, <i>n (%)</i>	20/140 (14.3)
MLH1/PMS2	7 (5)
MLH1	2 (1.4)
MSH2/MSH6	3 (2.1)
MSH6	7 (5)
MSH6/PMS2	1 (0.7)
Undetermined	3 (2.1)
MSI testing, <i>n (%)</i>	
MSI	15 (10.7)
MSS	116 (82.9)
Undetermined	9 (6.4)
Germline MMR mutations*, <i>n (%)</i>	11/140 (7.8)
<i>MLH1</i>	4 (2.8)
<i>MSH2</i>	1 (0.7)
<i>MSH6</i>	6 (4.3)
Germline <i>MUTYH</i> mutations, <i>n (%)</i>	8/140 (5.7)
G393D/G393D	2 (1.4)
Y176C/1138delC	1 (0.7)
G393D/T474fs488X	1 (0.7)
G393D/–	3 (2.1)
Y176C/–	1 (0.7)
Somatic <i>KRAS</i> mutations, <i>n (%)</i>	39/140 (27.9)
Codon 12	28 (20)
Codon 13	11 (7.9)
Undetermined	11 (7.9)
Somatic <i>BRAF</i> V600E mutation, <i>n (%)</i>	5/140 (3.6)
Undetermined	3 (2.1)

* Germline testing done only in MMR-deficient patients.

Table 4

Clinicopathologic and molecular features of *MUTYH* mutation carriers

Case	Age	Sex	Tumor location	Stage	Differentiation	Mucinous production	MSI status	MMR IHC	Other tumors	Synchronous adenomas (no.)	Family history*	KRAS status	MUTYH mutation
52	46	M	Rectum	III	Moderately	No	MSS	Normal	Colon (46)	Yes (5)	Colon (brother, 43) Larynx (father, 33) Breast (mother, 50) Thyroid (mother, 70) Breast (maternal aunt, 60)	wt	Y176C/1138delC
95D	43	F	Rectum	III	Well	No	MSS	Normal	No	No	Breast (mother, 67)	ND	G393D/G393D
137D	47	F	Sigmoid	II	Well	No	MSS	Normal	No	Yes (1)	ND	ND	G393D/G393D
31D	45	M	Ascending	II	Well	No	MSS	ND	No	Yes (2)	Colonic polypsis (brother, ?) Colonic polypsis (sister, ?) Colon (uncle, 80) Colon (cousin, 48)	G12C	G393D/T474fs488X
38D	48	F	Rectum	I	ND	No	MSS	Normal	No	No	No	G12C	Y176C/-
154D	40	M	Ascending	IV	Well	No	MSI	Loss of MSH6	No	No	Leukemia (father, 62) Prostate (grandfather, 70)	ND	G393D/-
31	50	M	Rectum	IV	Poorly	Yes	MSS	Normal	No	No	No	wt	G393D/-
2	33	F	Rectum	III	Moderately	No	MSS	Normal	No	No	Endometrium (sister, 42) Endometrium (grandmother, 42)	wt	G393D/-

Abbreviation: IHC, immunohistochemistry.

* Affected relative and age at diagnosis are indicated between parentheses.

Table 5

Clinicopathologic features of MMR and base excision repair–proficient early-onset and older-onset CRC

Characteristic	CRC ≤50 y	CRC >50 y	P
Females, <i>n/N</i> (%)	56/116 (48.2)	438/1,059 (41.4)	0.166
Tumor location [*] , <i>n/N</i> (%)			0.021
Proximal	20/116 (17.2)	297/1	
Distal	54/116 (46.5)	388/1	
Rectum	42/116 (36.2)	374/1	
TNM tumor stage, <i>n/N</i> (%)			<0.0001
I–II	38/114 (33.3)	572/1,024 (55.9)	
III–IV	76/114 (66.6)	452/1,024 (44.1)	
Poor degree of differentiation, <i>n/N</i> (%)	7/103 (6.8)	72/961 (7.5)	1
Mucin production, <i>n/N</i> (%)	19/116 (16.4)	113/958 (11.8)	0.176
Family history of CRC [†] , <i>n/N</i> (%)	27/114 (23.7)	174/1,059 (16.4)	0.06

^{*} With respect to the splenic flexure.

[†] Includes first-degree and second-degree relatives.