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BACKGROUND & AIMS: Patients with bi-allelic germline mutations in mismatch repair (MMR) genes (*MLH1, MSH2, MSH6,* or *PMS2*) develop a rare but severe variant of Lynch syndrome called constitutional MMR deficiency (CMMRD). This syndrome is characterized by early-onset colorectal cancers, lymphomas or leukemias, and brain tumors. There is no satisfactory method for diagnosis of CMMRD because screens for mutations in MMR genes are noninformative for 30% of patients. MMR-deficient cancer cells are resistant to genotoxic agents and have microsatellite instability (MSI), due to accumulation of errors in repetitive DNA sequences. We investigated whether these features could be used to identify patients with CMMRD. **METHODS:** We examined MSI by PCR analysis and tolerance to methylating or thiopurine agents (functional characteristics of MMR-deficient tumor cells) in lymphoblastoid cells (LCs) from 3 patients with CMMRD and 5 individuals with MMR-proficient LCs (controls). Using these assays, we defined experimental parameters that allowed discrimination of a series of 14 patients with CMMRD from 52 controls (training set). We then used the same parameters to assess 23 patients with clinical

but not genetic features of CMMRD. **RESULTS:** In the training set, we identified parameters, based on MSI and LC tolerance to methylation, that detected patients with CMMRD vs controls with 100% sensitivity and 100% specificity. Among 23 patients suspected of having CMMRD, 6 had MSI and LC tolerance to methylation (CMMRD highly probable), 15 had neither MSI nor LC tolerance to methylation (unlikely to have CMMRD), and 2 were considered doubtful for CMMRD based on having only 1 of the 2 features. **CONCLUSION:** The presence of MSI and tolerance to methylation in LCs identified patients with CMMRD with 100% sensitivity and specificity. These features could be used in diagnosis of patients.

Keywords: Colon Cancer; Functional Tests; Predisposition; Tumor.

Individuals with Lynch syndrome (LS) harbor germline heterozygous mutations affecting one of the four major mismatch repair (MMR) genes (ie, *MLH1*, *MSH2*, *MSH6*, or *PMS2*) and are at greatly increased risk of developing colorectal and other epithelial tumors.[1] Typically, individuals with germline *MLH1* or *MSH2* defects develop MMR-deficient cancers during their 4th or 5th decade, whereas those with *MSH6* or *PMS2* mutations are affected less consistently. Patients with bi-allelic germline mutations in MMR genes suffer from constitutional MMR deficiency (CMMRD),[2–5] a distinct inherited cancer syndrome (Online Mendelian Inheritance in Man [OMIM] database accession no. 276300).[6] This syndrome is characterized by the development of childhood tumors such as early-onset colorectal cancers, lymphomas/leukemias, and brain tumors.[6–8] Because CMMRD is mainly due to bi-allelic inheritance of *PMS2* or *MSH6* germline mutations, the family history of patients shows only a low incidence of LS-related cancers in first- and second-degree relatives. To date, CMMRD has been reported in 146 patients from 91 distinct families. Because of variable clinical presentation, lack of unequivocal diagnostic features, and phenotypical overlap with other cancer syndromes (eg, neurofibromatosis type 1 (NF1), Li-Fraumeni, syndrome, familial adenomatous polyposis [FAP]), CMMRD syndrome is frequently unrecognized by clinicians and its incidence is almost certainly underestimated.

In the European Care for CMMRD consortium (C4CMMRD), we recently proposed clinical diagnostic criteria that should raise the suspicion of CMMRD when observed in a child or young adult cancer patient, based on the phenotypic presentation.[9] The suspected diagnosis then needs to be either confirmed or refuted. The current diagnosis of CMMRD requires identification of bi-allelic, deleterious germline MMR defects. Unfortunately, mutation analysis leads to non-informative results when variants of unknown functional significance (VUS) are detected, as observed in around 30% of patients. Moreover, the detection of *PMS2* alterations responsible for 60% of CMMRD families is complicated by the presence of numerous pseudogenes, resulting in a lack of sensitivity when performing mutation analysis only. Hence, although extensive mutation screening that includes comprehensive searches for large

genomic rearrangements of MMR genes remains crucial for identification of CMMRD patients and genetic counseling in CMMRD families, tests that can unequivocally confirm or refute a suspected diagnosis are highly desirable.

Because all CMMRD patients share a common and specific functional property (ie, MMR deficiency), we hypothesized that the detection of characteristic functional features of MMR-deficient blood cells from such patients could be used to diagnose this syndrome. Inactivation of MMR is known to increase cellular tolerance to specific genotoxic agents such as methylating and thiopurine drugs.[10–15] Moreover, MMR-deficient cancer cells specifically exhibit a microsatellite instability (MSI) phenotype due to accumulation of replication errors in repetitive DNA sequences.[16] In tissues derived from MMR-deficient neoplastic cells, MSI is easily detected through PCR amplification of microsatellites. However, earlier studies have shown that MSI cannot be detected in the germline DNA of CMMRD patients except by using the laborious technique of "small pool PCR."[6,17] The presence of somatic mutations within DNA repeats in MMR-deficient cells is related to cell division. We therefore hypothesized that in vitro culture of immortalized lymphoblastoid cells from CMMRD patients would eventually lead to the onset of both an MSI phenotype and tolerance to methylating/thiopurine agents.

In the present work we first validated the proof of concept that MSI and tolerance to methylating/thiopurine agents could be detected in lymphoblastoid cell lines (LCLs) derived from several CMMRD patients, but not in LCLs from MMR-proficient controls including LS patients. In a case-control study, we next determined the experimental conditions that allowed accurate discrimination of a series of CMMRD patients from MMR-proficient controls. Finally, we tested our functional approach using the same experimental conditions in a series of patients who showed clinical characteristics of CMMRD but for whom the standard diagnostic method was non-informative. This was performed within a European Consortium "Care for CMMRD" (C4CMMRD) that allowed us to collect a unique series of confirmed CMMRD cases and at-risk individuals for this syndrome.

Methods

Patients

At the first workshop of the European Consortium, "Care for CMMRD" (C4CMMRD), held in Paris on June 9, 2013, a call was

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made to contribute blood samples or LCLs from definite or possible CMMRD patients. Eligible subjects included patients already diagnosed with CMMRD (ie, with bi-allelic deleterious germline mutations in any of the 4 major MMR genes, as well as patients with a strong clinical suspicion of CMMRD (ie, with a clinical score ≥3 according to Wimmer et al[9]). LCLs were available (n = 10) or were established (n = 27) for 37 of the 42 eligible patients. MMR-proficient LCLs used as controls originated from 47 LS patients and 15 subjects considered free of MMR germline defects including patients with FAP or NF1 syndrome. All patients gave written informed consent. This study was approved by the institutional review boards/ethics committees of the participating centers.

Mutation screening of MMR genes

All analyses were performed in clinically approved laboratories. Analysis of the *MLH1*, *MSH2*, and *MSH6* genes was performed across different laboratories whereas analysis of *PMS2* was performed in the Rouen, Lille, or Innsbruck laboratories. Bi-directional Sanger sequencing from genomic DNA or direct cDNA sequencing[18] was performed to identify point mutations in exonic and flanking intronic regions. Sequencing reactions were performed using the ABI PRISM Kit (Applied Biosystems, Waltham, MA) and sequences were analyzed on an automated sequencer (ABI 3130XL Genetic Analyzer; Applied Biosystems) using sequencing analysis software version 5.2 (Applied Biosystems).[19] Screening for large rearrangements in the *MLH1*, *MSH2*, and *MSH6* genes was performed using multiplex ligation-dependent probe amplification and/or quantitative multiplex PCR of short fluorescent fragments. Rearrangements of the *PMS2* gene were analyzed by quantitative multiplex PCR of short fluorescent fragments for exons 6, 7, 8, and 10, or by multiplex ligation-dependent probe amplification using the SALSA MLPA kit P008 (MRC-Holland, Amsterdam, the Netherlands) together with appropriate reference DNAs that have an equal (2:2) distribution of gene- and pseudogene-derived sequences in exons 13-15.[20] In patient C26, *PMS2* exon 12 deletion escaped detection by multiplex ligation-dependent probe amplification but was identified by direct cDNA sequencing. Screening for the *NF1* gene was performed by using a variety of methodologies including DNA and RNA sequencing for small lesions, polymorphic microsatellite marker analysis, and multiplex ligation-dependent probe amplification or real-time PCR-based gene dosage analysis to allow assessment of microdeletions, as previously described.[21] Mutation analysis of the *APC* gene was performed by direct sequencing and Multiplex Ligation-dependent Probe Amplification.[22]

Lymphoblastoid Cell Lines

LCLs obtained following standard Epstein-Barr virus infection were grown in RPMI 1640 medium with stable glutamine, supplemented with 20% fetal calf serum, 100 IU/mL penicillin, and 100mg/mL streptomycin (PAA). Only LCLs with comparable growth rates and with viability greater than 85% were included.

Ex Vivo Microsatellite Instability Analysis

PCR products following amplification of the NR27, NR21 and BAT26 microsatellites were separated by capillary electrophoresis on an ABI 3100 genetic analyzer and quantified using Gene Mapper software v3.7 (Applied Biosystems). In

order to confidently detect allelic shifts of as little as 1 base pair in size, DNA from LCL and peripheral blood lymphocytes (PBLs) were analyzed concurrently in octuplicate.

Chemicals

All chemicals were obtained from Sigma unless otherwise indicated. Cells were exposed to 6-Thioguanine (6-TG) and *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine (MNNG) (TCI Europe, Zwijndrecht, Belgium). To exclude differences in MNNG cytotoxicity due to variations in O^6-methylguanine methyltransferase enzyme activity, the latter was abrogated by exposure to O^6-benzylguanine (20 μM final concentration) during the entire experiment. All chemicals were dissolved in DMSO to a concentration of 20 mmol/L, protected from light and stored at $-20°C$ until used.

Methylation Tolerance Assay

Exponentially growing lymphoblastoid cells were seeded into 96-well round-bottom plates at a density of $0.15\text{-}1×10^4$ cells/well. After 24-hour incubation, extemporaneously reconstituted MNNG solution was added at 1.25, 2.5, and 5 μmol/L final concentration. Because of the short half-life of MNNG in aqueous solution (1 hour), the medium was not replaced after drug treatment and 1, 2, or 3 rounds of treatment separated by 24 hours were performed. Cell growth was evaluated after a total incubation time of 10 days and all samples were tested in triplicate. Each experiment was conducted at least in duplicate. Cytotoxicity was examined by the WST kit according to the supplier's recommendations (Roche, Indianapolis, IN). Absorbance was read at 450 nm using a microplate reader (Tecan Infinite F500; Tecan, Männedorf, Switzerland) and analyzed using Xfluor4GENiosPro software (Tecan). Percentage of cell survival was represented as the absorbance of treated sample relative to control.

Statistical Analyses

A Metropolis-Hastings algorithm was used to estimate the sensitivity and specificity of the three different diagnostic methods (MMR gene sequencing, functional testing, gMSI testing). This algorithm was applied to results obtained from genetically confirmed CMMRD patients, control patients, and patients with a strong clinical suspicion of CMMRD but without a molecularly confirmed diagnosis. Further information is available in Supplementary Materials and Methods.
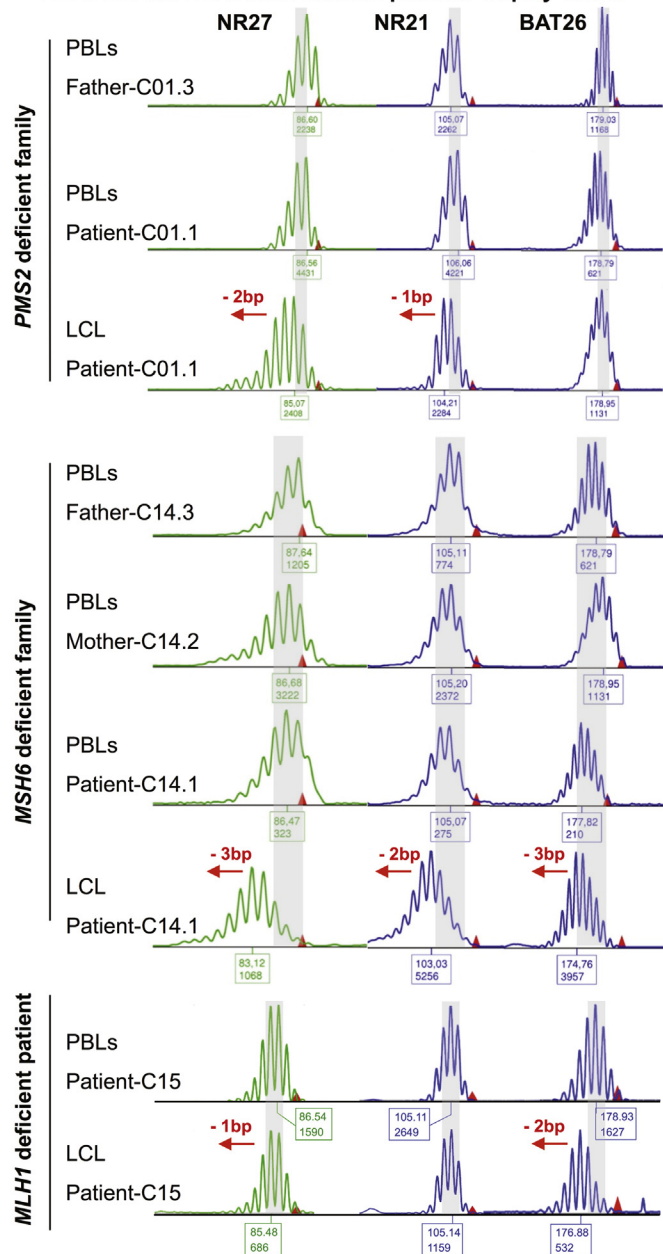
Results

Proof-of-Concept Study

We first investigated whether MSI and methylation/thiopurine tolerance could be detected in LCLs from 3 CMMRD patients with bi-allelic deleterious mutations in *MSH6*, *PMS2* or *MLH1*, but not in LCLs from 5 negative controls comprising 4 LS patients (*MSH6*, *MSH2*, *PMS2* or MLH1 heterozygous mutations) and one individual with wild-type MMR status.

MSI screening. As expected, MSI was not detected in PBLs from CMMRD patients (Figure 1A) following the analysis of 3 mononucleotide microsatellite markers (NR27, NR21, BAT26) that are used routinely to assess MSI

A LCLs but not PBLs from CMMRD patients display *ev*MSI
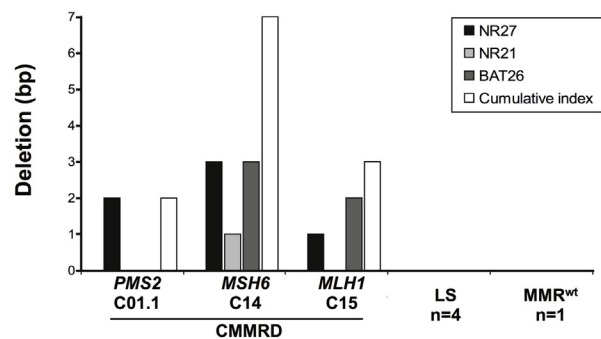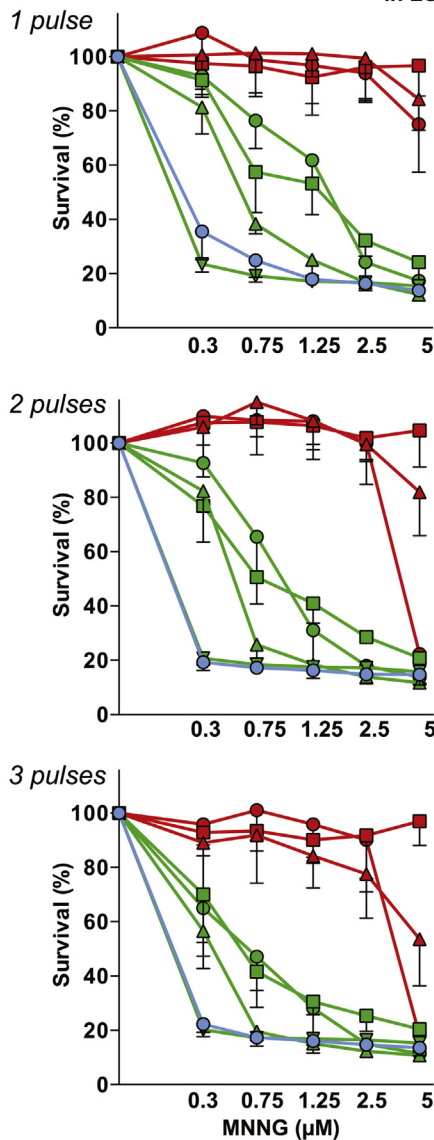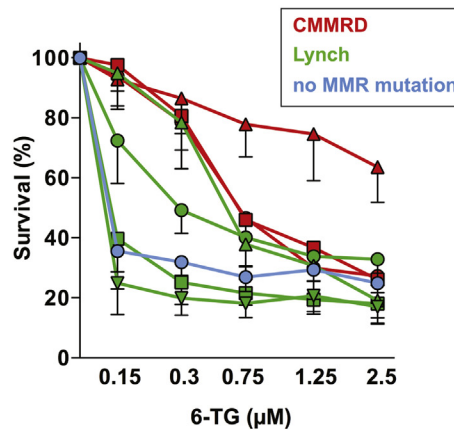
C CMMRD patients display MNNG tolerance in LCLs

B CMMRD patients but not controls display *ev*MSI

D 6-TG response in LCLs

status in tumor cells. In contrast, a clear MSI phenotype showing characteristic, aberrant alleles was observed in LCLs from all 3 CMMRD patients (Figure 1A), whereas the 5 control LCLs displayed stable allelic profiles (Figure 1B). The MSI phenotype was only demonstrated ex vivo in LCLs and was thus termed evMSI to distinguish it from the in vivo MSI phenotype detected in MMR-deficient cancer cells.

Drug tolerance assay. We first evaluated the cytotoxic effects of MNNG (methylating agent) and 6-thioguanine (6-TG, thiopurine) in 11 human colorectal cancer cell lines. Cell lines that were *MLH1-*, *MSH2-*, or *MSH6-*deficient were on average up to 10-fold more tolerant to $1 \mu M$ MNNG than MMR-proficient cell lines and 2-fold more tolerant to $15 \mu M$ 6-TG (Supplementary Figure 1). We next investigated the response of LCLs to both drugs. All 3 CMMRD-derived LCLs were phenotypically distinguishable from cells with heterozygous or wild-type MMR status. They displayed better cell survival compared to controls and there was no overlap between the two groups under several MNNG experimental conditions (Figure 1C), whereas 6-TG was less discriminant (Figure 1D).

Case Control Study

We next sought to identify experimental conditions that would best discriminate CMMRD patients from controls. Among 42 patients collected from several European cancer centers (including the 3 patients previously analyzed in the proof of concept study), 19 had been diagnosed as definite CMMRD cases by molecular analysis, ie, bi-allelic pathogenic MMR gene alterations. Of these, LCLs from 14 cases were available for the present case-control study (Table 1 and Figure 2). Pedigrees for all previously unreported patients are shown in Supplementary Figure 2. Clinical and tumor data together with detailed results of germline MMR analysis are provided in Supplementary Table 1.

evMSI. LCLs from the 14 CMMRD patients comprising carriers of bi-allelic mutations in *PMS2* (n = 10), *MSH6* (n = 3) or *MLH1* (n = 1) displayed microsatellite deletions ranging from 1 to 7 base pairs. Detection of the evMSI phenotype in the cell lines was achieved 120 days after immortalization at the latest. The median culture time for a positive evMSI phenotype was 83.6 ± 22.6 days (range 45-120) (Supplementary Table 2). In contrast, LCLs from all 23 MMR-proficient controls (12 LS patients and 11 MMR

wild-type individuals) showed no deletions. For all 23 controls except two, the cell lines were grown for at least 120 days without any evidence of deletions (median culture time = 175 days ± 62.6, range 83-304 days). Five control cell lines were grown for longer than 220 days without any evidence of deletions. Therefore, for subsequent experiments the cut-off value used to define a cell line as positive for evMSI was set as a 1 base pair deletion across all three markers and a maximum culture time of 120 days was used.

Drug tolerance assays. By varying the MNNG concentration and number of treatments, we found the optimal experimental condition that allowed CMMRD patients to be discriminated from controls was two rounds of 2.5 μM MNNG. Using this condition, LCLs from all 14 CMMRD patients displayed a cell survival rate above 60%. In contrast, 51/52 LCLs from controls displayed a cell survival rate lower than 40% (median cell survival rates of 87.5% and 20.9%, respectively; $P < .0001$; Student t test) (Figures 3 and 4A). At an arbitrary cut-off value of 50% cell survival, the methylation tolerance assay was therefore shown to be 100% sensitive (14 of 14) and 98% specific (51 of 52; the positive sample was from an LS patient with an *MSH6* defect). The thiopurine tolerance test was found to be less discriminatory and hence was not continued further (Supplementary Figure 3). This result concurs with previous findings that MMR-deficient cells are 100-fold more tolerant than MMR-proficient cells to death induced by methylating agents, but only about 10-fold more tolerant to 6-TG treatment.[23]

Overall, evMSI and methylation tolerance assays were found to be highly specific and sensitive and gave concordant results for all cases tested with both methods. In subsequent studies we therefore deemed that both assays must show abnormal results in order to conclude a definite diagnosis of CMMRD. To rule out a diagnosis of CMMRD, both assays should display normal results. Diagnosis should be considered as doubtful if results from the two functional tests are discordant.

Application of Functional Tests for Detection of CMMRD in At-Risk Individuals

In 23 of the 42 patients from our series, a diagnosis of CMMRD was suspected based on clinical presentation, but the diagnosis could not be confirmed by MMR gene mutation analysis (Table 1, Figure 2). These comprised 8 patients

Figure 1. Proof of concept study. (*A*) LCLs but not PBLs from constitutional MMR deficiency (CMMRD) patients display evMSI. Electropherograms of fluorescent amplification products for NR27, NR21, and BAT26 microsatellites. The length of the predominant allele in base pairs (bp) and the fluorescence intensity are indicated in the *box* below each profile. Deletions (*red arrows*) occurred at these loci in lymphoblastoid cell lines (LCLs) derived from CMMRD patients C01.1 (*PMS2* deficient), C14 (*MSH6* deficient), and C15 (*MLH1* deficient) compared with their respective peripheral blood lymphocytes (PBLs). In PBLs, the PCR profiles were similar in CMMRD patients C01.1 and C14 and their respective parents, demonstrating that MSI could only be demonstrated ex vivo. (*B*) CMMRD patients but not controls display evMSI. Deletions, expressed as the size of deletion for each marker and the cumulative size of deletion (ie, the sum of the deletions observed in the 3 markers) were observed in LCLs from the 3 CMMRD patients but not those from MMR-proficient controls, which included 4 LS patients and 1 individual with wild-type (wt) MMR status. (*C*) LCLs from CMMRD patients displayed methylation tolerance. One, two, or three rounds of MNNG treatment at 24-hour intervals were performed. LCLs from the 3 CMMRD patients (*red*) were phenotypically distinguishable from heterozygous (*green*) and wild-type (*blue*) LCLs, using several experimental conditions. (*D*) 6-TG response of LCLs from the 3 CMMRD patients (*red*), 4 LS patients (*green*), and 1 individual with wild-type MMR status (*blue*).

Table 1. Data Set for Known and Putative CMMRD Patients

Patient	Clinical score[a]	Germline MMR analysis				Study, reference (country)
		Gene	Mutation type	Class	Status	
CMMRD patients with confirmed molecular diagnosis (ie, with bi-allelic pathogenic MMR gene alterations)						
C01.1	7	PMS2	Frameshift / Missense	DM / DM	Compound heterozygous	Auclair et al, 2007[32]
C01.2	7	PMS2	Frameshift / Missense	DM / DM	Compound heterozygous	Auclair et al, 2007[32]
C02	3	PMS2	Frameshift / Frameshift	DM / DM	Homozygous	Ilencikova (Slovakia)
C03.1	8	PMS2	Frameshift / Frameshift	DM / DM	Homozygous	Patient 1 in Chmara et al, 2013[33]
C03.2	9	PMS2	Frameshift / Frameshift	DM / DM	Homozygous	Patient 1.2 in Chmara et al, 2013[33]
C04	7	PMS2	Large deletion / Large deletion	DM / DM	Compound heterozygous	Patient 2 in Chmara et al, 2013[33]
C05	10	PMS2	Nonsense / Nonsense / Frameshift	DM / DM / DM	Compound heterozygous	Brugières (France)
C06	8	PMS2	Splice / Splice	DM / DM	Homozygous	Brugières (France)
C07	8	PMS2	Missense / Missense	DM / DM	Homozygous	Colas (France)
C08	8	PMS2	Missense / Missense	DM / DM	Homozygous	Malka (France)
C09.1	5	PMS2	Splice / Splice	DM / DM	Homozygous	Brugières (France)
C10	4	PMS2	Splice / Splice	DM / DM	Homozygous	Brugières (France)
C11	11	PMS2	Large deletion / Large deletion	DM / DM	Homozygous	Fedhila (Tunisia) / Colas (France)
		MSH2	Missense	VUS	Heterozygous	
		MSH6	Missense	VUS	Heterozygous	
C12	5	MSH6	Frameshift / Frameshift	DM / DM	Homozygous	Patient PIV.5 in Ilencikova et al, 2011[34]
C13.1	10	MSH6	Frameshift / Frameshift	DM / DM	Compound heterozygous	Patient P6 in Gardes et al, 2012[35]
C14	8	MSH6	Frameshift / Frameshift	DM / DM	Compound heterozygous	Auclair et al, 2007[32]
C15	10	MLH1	Splice / Splice	DM / DM	Homozygous	Entz-Werle (France)
C16	9	MLH1	Missense / Missense	DM / DM	Homozygous	Raevaara et al, 2004[36]
C17	6	MSH2	Large deletion / Large deletion	DM / DM	Homozygous	Verloes (France)
Patients with clinical characteristics of CMMRD syndrome but a lack of confirmatory standard genetic defect						
C18	6	PMS2	In frame deletion / Missense	DM / VUS	Compound heterozygous	Lejeune (France)
C19	8	PMS2	Missense / Frameshift	VUS / DM	Compound heterozygous	Mathieu-Dramard (France)
C20.1	7	MSH6	Missense / Missense	VUS / VUS	Homozygous	Leis (Afghanistan)
C20.2	7	MSH6	Missense / Missense	VUS / VUS	Homozygous	Leis (Afghanistan)
C21	14	MSH6	Frameshift / In frame deletion	DM / VUS	Compound heterozygous	Bougeard et al, 2014[29]
		MSH2	Missense	VUS	Heterozygous	
C22	8	MSH6	Missense / Missense	VUS / VUS	Homozygous	Wafaa (Marocco)/ Colas (France)
		PMS2	Missense	VUS	Heterozygous	
C23	13	MSH6	In frame duplication / In frame duplication	VUS / VUS	Heterozygous	Gauthier-Villars (France)
C24	6	MSH2	Splice / Splice	DM / VUS	Compound heterozygous	Ruiz-Ponte (Spain)
C25	5	PMS2	Frameshift	DM	Heterozygous	Colas (France)
C26	4	PMS2	Large deletion	DM	Heterozygous	Kinzel (Germany)
C27	3	MLH1	Frameshift	DM	Heterozygous	Colas (France)
		MSH2	Missense	VUS	Heterozygous	
C28	1	MLH1	Splice	DM	Heterozygous	Caron (France)
C29.1	4	-	No MMR mutation identified[b]	-	-	Brugières (France)
C30	4	MSH2	Splice	VUS	Heterozygous	Brugières (France)
C31	3	-	No MMR mutation identified	-	-	Mortemousque (France)
C32	3	-	No MMR mutation identified	-	-	Brugières (France)
C33	4	-	No MMR mutation identified	-	-	Wang (France)
C34.1	4	-	No MMR mutation identified	-	-	Grandjouan (France)
C35	6	-	No MMR mutation identified	-	-	Brugières (France)

with bi-allelic MMR mutations that included one or two VUS, 5 patients with a single MMR mutation and 10 patients in which no MMR mutation was detected. We evaluated these patients using the functional assay conditions described above (Table 2, Figure 4B). Six patients displayed positive results for both the evMSI and methylation tolerance assays, indicating a highly probable diagnosis of CMMRD. They included 5 patients with MSH6 or PMS2 bi-allelic MMR

Table 1. Continued

Patient	Clinical score[a]	Gene	Mutation type	Class	Status	Study, reference (country)
			Germline MMR analysis			
C36	3	-	No MMR mutation identified	-	-	Grandjouan (France)
C37	4	-	No MMR mutation identified	-	-	Colas (France)
C29.2	NA	-	No MMR mutation identified[b]	-	-	Brugières (France)
C34.2	4	-	No MMR mutation identified	-	-	Brugières (France)

NOTE. Detailed description of the MMR gene alterations is provided in Supplementary Table 1.
DM, deleterious mutation; NA, not applicable; VUS, variant of unknown significance.
[a]Clinical score according to Wimmer et al.[9]
[b]Extensive genetic screening was performed post-hoc in view of the abnormal functional assay results found in patient C29.1. It led to the identification of a homozygous deletion of exons 14-15 of the PMS2 gene, c.276-? (*160?) del, whereas the brother (patient C29.2) was found to be heterozygous for the PMS2 deletion.

alterations comprising VUS. Consistent with our results, in silico prediction favored a pathogenic nature for the *MSH6* and *PMS2* variants in four of these patients (C20.1, C20.2, C18, C22). In the sixth patient (C29.1), no apparent germline MMR mutations were detected. In another 15 patients, evMSI and methylation tolerance assays were both negative, indicating that a diagnosis of CMMRD was very unlikely. These included one compound heterozygote for an *MSH2* variant, 5 patients with a single *MLH1*, *PMS2*, or *MSH2* alteration, and 9 patients where no MMR alteration had been detected. In the two remaining patients (C21 and C23,

with bi-allelic *MSH6* mutations comprising one or two VUS, respectively), the data showed methylation tolerance but no evMSI phenotype. We therefore concluded a result of "doubtful" for both patients.

Comparison of Functional Assays With Other Methodological Approaches

We trialed a recently described method that evaluates dinucleotide repeats for the detection of MSI in germline DNA (gMSI).[24] In the case-control cohort, the gMSI assay
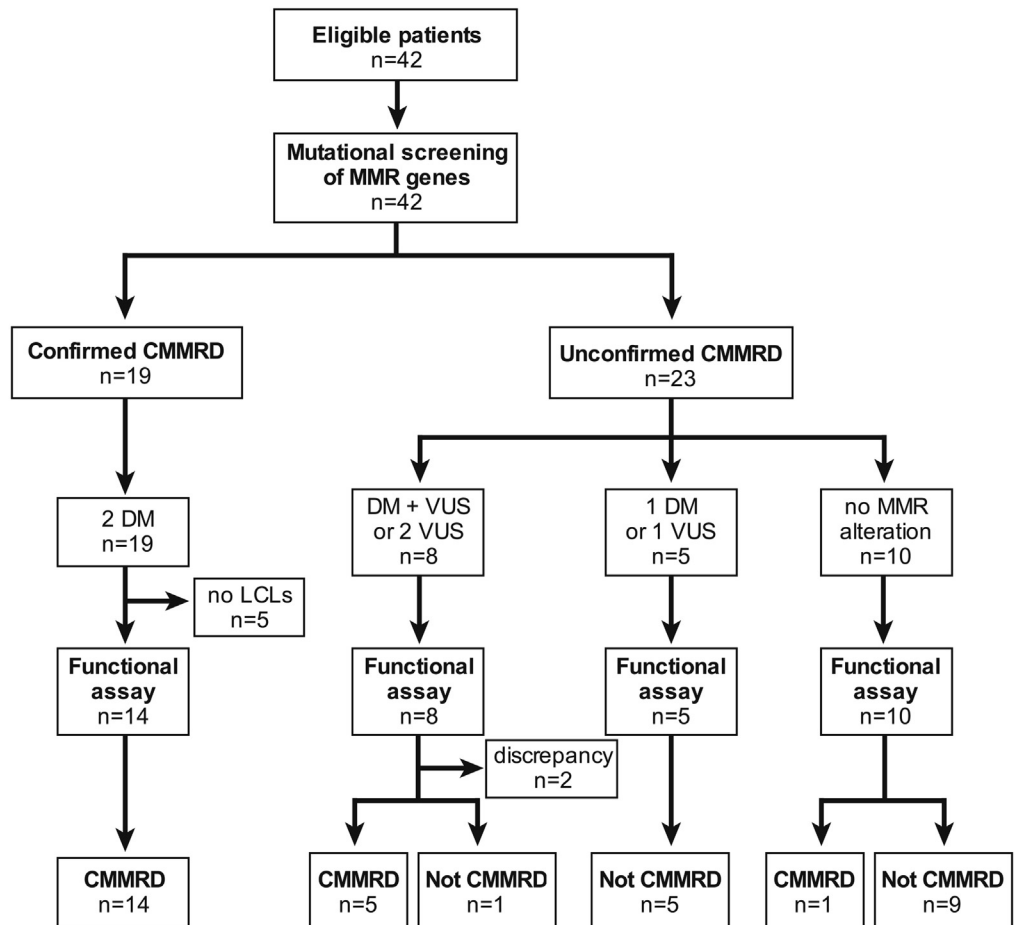

Figure 2. Flow diagram of patient study cohort. The functional assay, which includes the evMSI and methylation tolerance tests, allowed either diagnosis or exclusion of CMMRD. CMMRD, constitutional MMR deficiency; DM, deleterious mutation; LCL, lymphoblastoid cell line; VUS, variant of unknown significance.
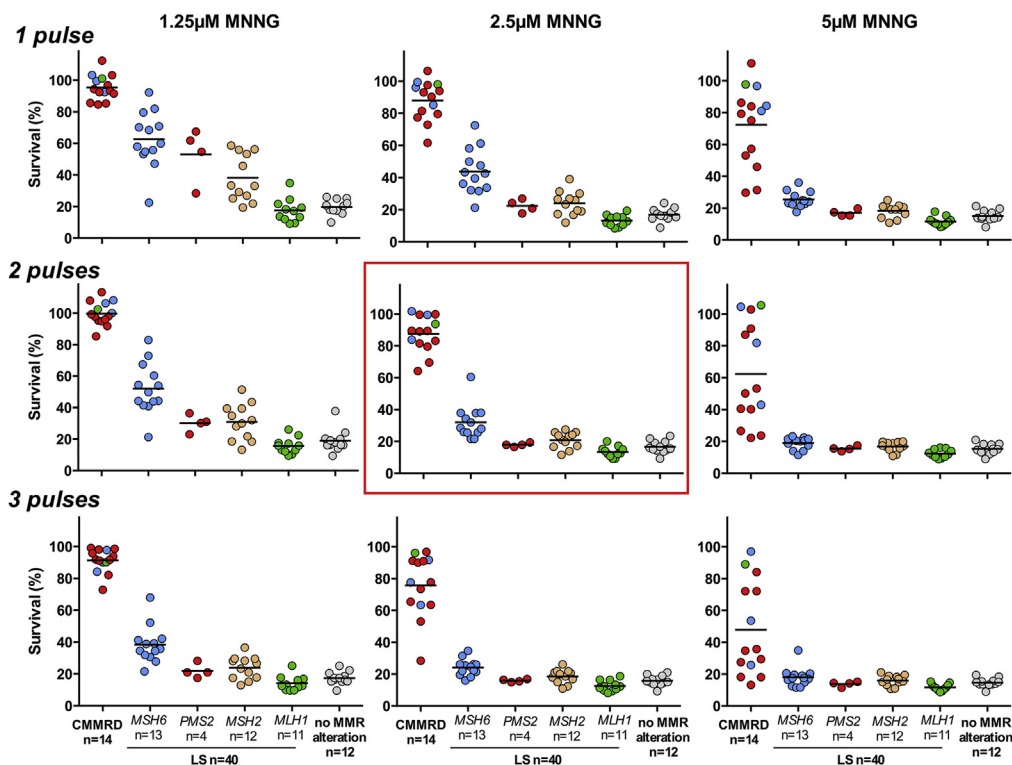
Figure 3. Tolerance of immortalized lymphoblasts derived from 14 constitutional MMR deficiency (CMMRD) patients and a series of MMR-proficient controls (including LS patients and MMR wild-type individuals) to increasing concentrations of MNNG. Because of the short half-life of MNNG in aqueous solution, 1, 2, or 3 pulses of treatment were performed. With the exception of $MLH1^{+/-}$ lymphoblastoid cell line (LCLs; n = 11) that behaved similarly to MMR wild-type lymphoblasts (n = 12) in all experimental conditions, $MSH2^{+/-}$ (n = 12), $PMS2^{+/-}$ (n = 4), and mainly $MSH6^{+/-}$ cell lines (n = 13) exhibited increased cell survival under low MNNG concentrations. At higher MNNG concentrations and/or increasing numbers of drug treatments, the survival of $MSH2^{+/-}$, $PMS2^{+/-}$, and $MSH6^{+/-}$ lines decreased towards that of $MLH1^{+/-}$ and MMR wild-type cells, whereas CMMRD LCLs remained quite tolerant to the drug. The best experimental condition to discriminate CMMRD patients from controls was 2 rounds of 2.5μM MNNG (*red box*). Patients with CMMRD or LS are represented with distinct colors depending on the MMR gene that was mutated (*red for PMS2, blue for MSH6, yellow for MSH2*, and *green for MLH1*).

yielded interpretable results in 15 of 18 CMMRD patients and in 16 of 19 controls. CMMRD patients with bi-allelic mutations involving *PMS2* (n = 11), *MLH1* (n = 1), or *MSH2* (n = 1) displayed abnormal gMSI values. In agreement with the original report,[24] we found however that CMMRD patients with bi-allelic deleterious mutations involving *MSH6* (n = 2) displayed normal gMSI ratios, thus reducing the sensitivity of this method ([Table 2] and [Supplementary Table 3] and [Supplementary Figure 4]). gMSI ratios were normal for all controls. gMSI also yielded interpretable results in 21 of 23 patients suspected of having CMMRD. The five carriers of bi-allelic *MSH6* alterations displayed normal gMSI, as expected. Moreover, gMSI corroborated the results of our functional assays in all patients with *PMS2*, *MLH1*, or *MSH2* mutations, with the exception of one (C18). This patient carried one deleterious mutation and one VUS in the *PMS2* gene. He displayed normal gMSI but abnormal evMSI and methylation tolerance results ([Table 2]). The c.2249G>A missense mutation found in the *PMS2* gene of patient C18, together with complete deletion of the other *PMS2* allele, was previously reported in a patient diagnosed with rectal cancer and a brain tumor at

22 and 23 years of age, respectively.[25] This further corroborates a pathogenic role for the VUS in patient C18 and is consistent with the results of our functional assay. The evMSI, methylation tolerance and gMSI assay results were all abnormal in patient C29.1 who lacked apparent MMR germline mutations. This prompted us to conduct additional *PMS2* screening using multiplex ligation-dependent probe amplification, which led to the identification of a homozygous deletion of exons 14-15. Normal functional test results and gMSI ratio were found in an asymptomatic brother aged 11 years (C29.2) who was later found to be heterozygous for the *PMS2* deletion.

Another tool proposed for CMMRD screening is IHC analysis to detect loss of MMR protein expression in normal tissues. IHC was recently reported to be 100% sensitive when performed on normal colonic or skin tissues from 5 CMMRD patients.[26] However, based on previous observations in LS patients, IHC may lack sensitivity, especially for the detection of some missense and truncating MMR gene mutations,[27,28] resulting in false negative diagnosis for CMMRD. This was demonstrated in the present study where positive MSH6 staining was observed in
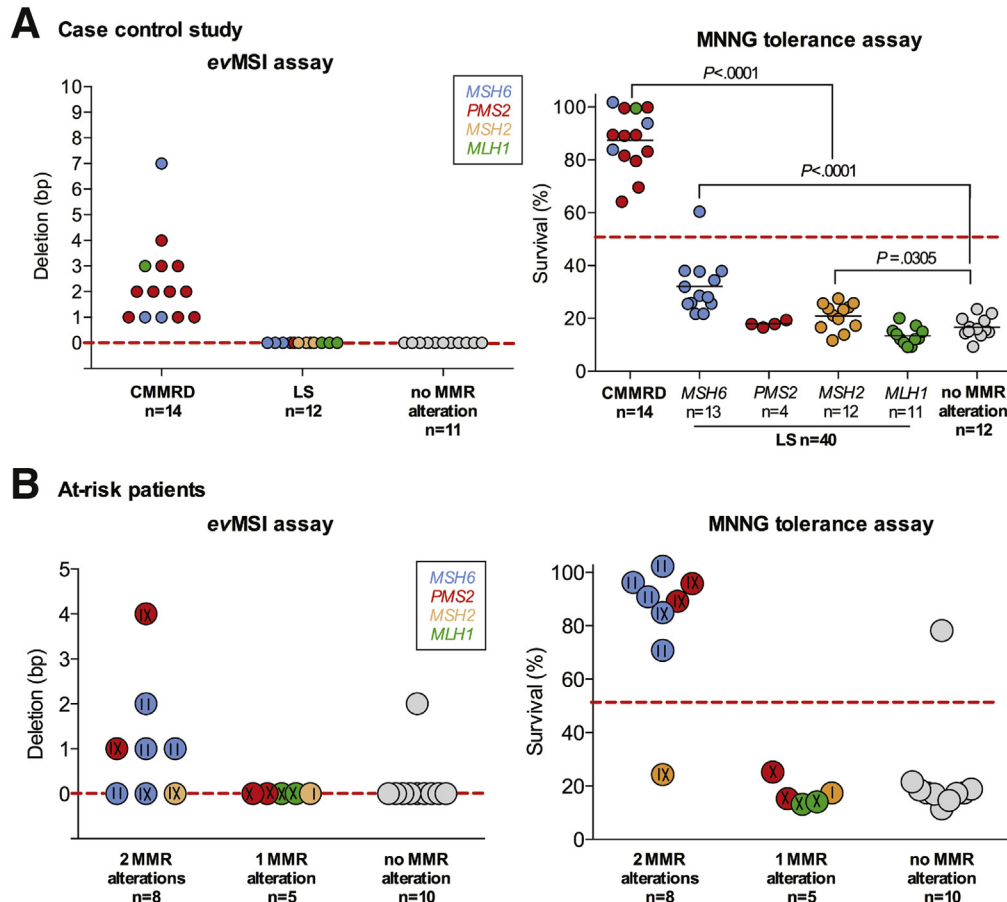
Figure 4. evMSI and methylation tolerance assays in a case-control study (*A*) and in patients considered at-risk for constitutional MMR deficiency (CMMRD) syndrome (*B*). (*A*) Case-control study involving 14 CMMRD patients and 23 MMR-proficient controls consisting of 12 LS patients with heterozygous mutations affecting the MMR genes and 11 patients with no detected germline MMR mutation. EvMSI assay (*left*): deletion sizes (in base pairs [bp]) are expressed as the sum of the deletions for the 3 markers (NR27, NR21, and BAT26). The cut-off value used to define a cell line as positive for evMSI was set at 1 bp deletion for all 3 markers (*red dotted line*). Lymphoblastoid cell line (LCLs) from all 14 CMMRD patients showed decreased allele size, regardless of which MMR gene was mutated, whereas no deletions were detected in the 23 MMR-proficient controls tested. Methylation tolerance assay (*right*): survival (%) of immortalized lymphoid cells derived from the same 14 CMMRD patients and from controls after 2 rounds of 2.5 μM MNNG treatment. Because some LS patients displayed increased tolerance to MNNG compared to MMR wild-type controls, a larger series of LS patients was used for the drug assay. Whereas $MLH1^{+/-}$ LCLs behaved similarly to MMR wild-type lymphoblasts, $MSH2^{+/-}$ and especially $MSH6^{+/-}$ cell lines exhibited significantly increased median cell survival. Overall, all CMMRD-derived LCLs displayed cell survival higher than 60%, whereas cell survival of all MMR-proficient LCLs was lower than 40%, with the exception of 1 case. The cut-off value was arbitrarily set at 50% cell survival (*red dotted line*). Student *t* test. (*B*) evMSI (*left*) and methylation tolerance (*right*) tests were applied for the detection of CMMRD syndrome in 23 patients with a clinical presentation suggestive of CMMRD but for whom the diagnosis could not be confirmed (or excluded) by sequencing of MMR genes. These consisted of 8 patients with biallelic MMR alterations involving 1 or 2 VUS, 5 patients with a single MMR alteration, and 10 patients without germline MMR mutation. One of these last patients (C29.1) showed abnormal functional assay results for both tests, which prompted us to perform additional *PMS2* genetic screening that led to the identification of a homozygous deletion. Vertical line = VUS; cross = deleterious mutation. CMMRD and LS patients are represented using distinct colors depending on the MMR gene that was mutated.

two patients (C20.2 and C22) with homozygous *MSH6* missense mutations and who are likely to be CMMRD according to the functional assays and in silico predictions. Conversely, PMS2 protein was not expressed in the normal colonic mucosa of patient C25, the carrier of a single deleterious *PMS2* mutation in which a diagnosis of CMMRD was ruled out based on normal results for the evMSI, methylation tolerance, and gMSI tests (Table 2 and Supplementary Table 1).

Estimation of Sensitivity, Specificity, Positive Predictive Value, and Negative Predictive Value for Functional Assays and for Other Methodological Approaches

In our case-control cohort, the functional assay (ie, evMSI and methylation tolerance) was 100% sensitive and 100% specific whereas gMSI testing was 86.7% (13/15) sensitive and 100% (16/16) specific. It is worth noting that the sensitivity of gMSI is likely to be an overestimate, since

Table 2. evMSI, Methylation Tolerance, gMSI, and IHC Data in the Series of 14 CMMRD Patients With Bi-Allelic Pathogenic MMR Gene Alterations and in 23 At-Risk Patients for Whom Diagnosis Could Not Be Confirmed by MMR Sequencing

Patient	MMR sequencing		Functional assays		Diagnosis according to functional assays	Comparison with other tests	
	Status	MMR gene	evMSI	Methylation tolerance		gMSI	MMR protein expression in normal tissue (IHC)
CMMRD patients n=14							
C15	hmz DM	MLH1			CMMRD		Lost
C06, C07, C08, C09.1	hmz DM	PMS2					Lost
C10	hmz DM	PMS2	+	+		+	NA
C04, C05	cpd htz DM	PMS2					Lost
C01.1	cpd htz DM	PMS2					NA
C01.2	cpd htz DM	PMS2	+	+		NA	Lost
C02	hmz DM	PMS2	+	+		NI	NA
C14	cpd htz DM	MSH6					Lost
C12	hmz DM	MSH6	+	+		-	NA
C13.1	cpd htz DM	MSH6					NA
Patients at-risk for CMMRD n=23							
C29.1	No mutation[a]		+	+	CMMRD	+	Lost
C20.1	hmz VUS	MSH6	+	+		-	Lost
C20.2, C22	hmz VUS	MSH6					Conserved
C18	DM + VUS	PMS2					Lost
C19	DM + VUS	PMS2	+	+		NI	Lost
C24	DM + VUS	MSH2	-	-	Not CMMRD	-	Conserved
C30	htz VUS	MSH2					NA
C25	htz DM	PMS2					Lost
C26	htz DM	PMS2					Conserved
C27	htz DM	MLH1					NA
C28	htz DM	MLH1					Conserved
C34.2	No mutation						Conserved
C29.2, C31, C32, C34.1, C35, C36, C37	No mutation[a]						NA
C33	No mutation		-	-		NI	NA
C21	DM + VUS	MSH6	-	+	Doubtful	-	Lost
C23	hmz VUS	MSH6					Lost

NOTE. Detailed data on MMR gene alterations, on the expression of MMR proteins in normal tissue, and on gMSI test are provided in Supplementary tables 1 to 3.
−, negative/normal; +, positive/abnormal; cpd, compound; DM, deleterious mutation; hmz, homozygous; htz, heterozygous; NA, not available; NI, not interpretable; VUS, variant of unknown significance
[a]Extensive genetic screening that was performed post-hoc led to the identification of a deletion of exons 14-15 in the *PMS2* gene that was found at an homozygous or heterozygous status in patients C29.1 and C29.2, respectively.

this depends on the proportion of CMMRD patients with *MSH6* alterations. In our series this was only 13% (2/15), however *MSH6* alterations are thought to be responsible for a higher proportion (∼20%) of all CMMRD patients.[9] The performance of IHC could not be evaluated properly due to the lack of a standardized method for the analysis of MMR gene expression in the normal tissue of controls (ie, MMR-proficient cases).

Next, we estimated the performance of functional testing compared to the standard method of MMR gene sequencing. This was done for the entire cohort, including patients deemed to be at-risk. Using a Metropolis-Hastings algorithm, the functional assay revealed higher sensitivity (94.2% (95% CI: 79.4%-99.9%) vs 80.1% (54.1%-99.0%)), higher NPV (97.2% (89.8%-99.9%) vs 91.2% (76.6%-99.6%)), but lower specificity (90.1% [range, 76.1%-99.5%] vs 97.6% [range, 91.2%-99.9%]) and lower PPV (80.5% [range, 53.9%-99%] vs 93.6% [range, 77.9%-99.8%]) for CMMRD diagnoses. In order to include gMSI in the comparison, an estimation of the sensitivity and specificity was made in the smaller series of patients and controls for which results from all tests were available. Functional testing still offered the highest sensitivity (93.3% [range, 76.7%-99.8%]) and a lower specificity (Supplementary Table 4), however these differences did not reach statistical significance because of small cohort sizes. As stated above, the lack of a standardized method for IHC analysis of normal tissues meant we were unable to properly evaluate the sensitivity and specificity of this method.

Discussion

In this article, we propose a new approach for the diagnosis of CMMRD that involves the common and specific functional characteristic of all CMMRD patients (ie, MMR deficiency). Our approach was based on the

exploitation of this feature through the evaluation of MSI and methylation tolerance in MMR-deficient, immortalized lymphoblastoid cells. This method gave unequivocal results in CMMRD patients with known bi-allelic deleterious mutations. If one assumes that abnormal results for both assays indicate a diagnosis of CMMRD, whereas normal results for both assays rule this out, our method was 100% sensitive and 100% specific in this case-control study. When applied to additional patients suspected of having CMMRD syndrome because of evocative clinical criteria but who lacked the confirmatory standard genetic defects, a clear discrimination into two groups was obtained. In the first group showing abnormal results for both tests, we considered that CMMRD was highly probable. In contrast, a diagnosis of CMMRD was highly unlikely in the second group of patients showing normal results for both tests. Our novel functional approach may therefore be especially useful for the confirmation or rejection of CMMRD diagnosis in patients with VUS by providing an assessment of the pathogenicity of MMR variants. It is also useful in cases where the diagnostic method failed to detect bi-allelic MMR mutations despite an evocative CMMRD clinical phenotype (eg, patient C29.1). Furthermore, our approach can rule out that a second mutation has been missed in patients with heterozygous, pathogenic PMS2 or MSH2 mutations who nevertheless show an unusually early onset of cancer (eg, colon tumors at 12, 17, and 25 years of age in patients C24, C26, and C25, respectively). The results from our functional approach support the existence of a clinical continuum that spans the less severe CMMRD phenotypes that mimic LS (eg, patient C18), to more severe and early onset LS phenotypes that mimic CMMRD.[29] Overall, our findings highlight that functional tests capable of assessing constitutional MMR-deficiency are highly desirable for the accurate diagnosis of CMMRD patients.

Although we have investigated by far the largest CMMRD series reported to date in the literature, our method requires further confirmation in additional cohorts of CMMRD patients. This will help to refine the criteria for the functional assays in cases with ambiguous results, such as the two patients who harbored VUS in the MSH6 gene and showed methylation tolerance but not evMSI (C21, C23). One possible explanation for this observation is that certain MMR gene mutations might uncouple the DNA mismatch repair and DNA damage-induced apoptosis functions, as reported in mice.[30,31] Overall, we found that functional testing showed better sensitivity than either MMR gene sequencing or gMSI, although it may have a lower specificity. Bearing this in mind, we propose a flow chart for the use of our assay alone or in combination with other tests in routine clinics in the next future (Figure 5). IHC could not be evaluated properly in this study due to the lack of a standardized method for assessment of MMR gene expression in the normal tissues of MMR-proficient subjects. The results with IHC are likely to be highly dependent on the type of tissue being studied (eg, colon, brain, skin, lymphoid cells). Moreover, it is well known that IHC can give rise to false negative results for MMR deficiency in cases where
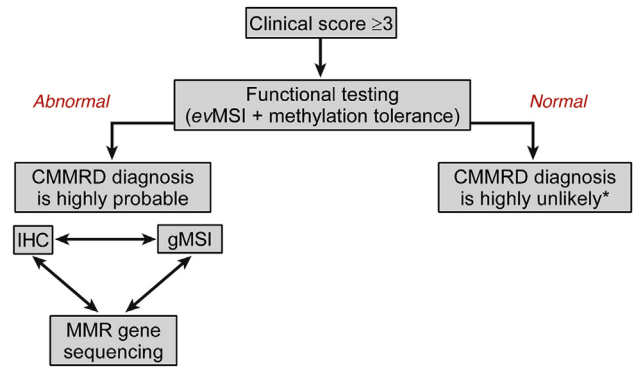

Figure 5. Proposed algorithm for the evaluation of patients suspected of having constitutional MMR deficiency (CMMRD). In the future, individuals with a clinical score of ≥3 according to Wimmer et al[9] should be initially tested by functional assays. Because this approach has a high negative predictive value, a normal result obtained with the functional assays would confidently allow diagnosis of CMMRD to be excluded without the need for additional tests. Alternatively, an abnormal result would be highly suggestive of CMMRD. However, due to the relatively low positive predictive value (80.5%) associated with this assay, we recommend that medical geneticists and pediatricians further investigate these "at-very-high-risk" cases by using other approaches (immunohistochemistry [IHC], germline microsatellite instability [gMSI], or sequencing of MMR genes) in order to confirm the diagnosis. It is worth noting that IHC results can be used to guide germline mutation analysis to a specific MMR gene, whereas in this context, the finding of a normal gMSI ratio would direct genetic analysis to the MSH6 gene. *LS should be sought in cases with evocative criteria.

inactivating missense mutations nevertheless result in expression of the mutant protein.[27,28] Further studies should evaluate MMR protein expression using standardized methods in normal and tumor tissues from large cohorts of CMMRD patients, MMR-proficient controls and Lynch syndrome patients, in the same manner as performed here to assess our functional assay.

In summary, the novel functional approach proposed here showed higher sensitivity for CMMRD diagnosis compared to MMR sequencing or gMSI, the 2 other methods used so far. This approach can be used to determine whether MMR variants of uncertain pathogenicity are responsible for functional inactivation of the MMR system. The ability to classify variants as pathogenic or neutral is a major challenge in clinical genetics, particularly with the advent of next-generation sequencing. Moreover, the diagnosis of CMMRD syndrome based solely on clinical and genetic data is presently inadequate. As an overall diagnostic strategy, we therefore recommend the implementation of our functional assays in combination with IHC and gMSI analysis (Figure 5). These tests can be performed in any order upon suggestion of CMMRD syndrome based on an evocative clinical score. This strategy has already been introduced at the Saint-Antoine Hospital in Paris with the aim of further validating our assay in an independent cohort of CMMRD patients. The service is available upon request. We are confident this assay will provide a functional

definition, or "signature," for CMMRD, similar to the chromosomal breakage test for diagnosis of Fanconi anemia. In the near future, we believe that individuals who are at-risk of CMMRD will be tested solely using functional assays as the initial test.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.06.013.

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Supplementary Material and Methods

Patients

All 19 genetically confirmed and 19 of 23 patients suspected of having constitutional mismatch repair (CMMRD) included in this study had a score ≥ 3 points according to the recently published clinical criteria for suspected diagnosis of CMMRD. Additionally, 4 patients were included in this study. One patient (C28) was included because he displayed a very severe clinical history with 4 Lynch syndrome (LS)-related tumors from 32 to 36 years of age and osteosarcoma at 11 years of age (osteosarcoma was found in CMMRD patient C05 at 24 years of age). Equally, 1 patient (C27) was included because, compared to other members of this LS family, he had a very early onset (30 years of age) of 2 synchronous colon cancers with an adenoma and a brother who had a malignant brain tumor at the age of 18 years old. Another patient (C33) had a cerebral tumor at 27 years of age as well as café-au-lait macules (CALMs), and her sister displayed a cerebral tumor (22 years of age). Finally, 1 patient was an asymptomatic sibling with CALMs of a possible CMMRD patient (C29.2). Altogether, the study included 42 patients (37 families) from several European cancer centers. Control subjects considered free of MMR germline defects included 5 familial adenomatous polyposis (FAP) and 2 neurofibromatosis type 1 (NF1) individuals with identified germline APC and NF1 mutations, respectively (FAP and NF1 were chosen because they represented cancer predisposition syndromes showing clinical overlap with CMMRD), and 8 control patients had diagnoses of sporadic colorectal cancer without familial cancer history. These patients had developed microsatellite stable tumors (6 cases) or microsatellite instability (MSI) tumors due to epigenetic silencing of MLH1 because of somatic methylation (2 cases) and thus were not suspected of having CMMRD syndrome.

Colorectal Cell Lines

Human colorectal cancer cell lines were grown in Dulbecco's modified Eagle medium (DMEM) with stable glutamine (Glutamax) supplemented with 10% fetal calf serum, 100 IU/mL penicillin and 100 mg/mL streptomycin (PAA). They included 6 microsatellite unstable (HCT116, LIM2405, LS174T, KM12 [all MLH1-deficient], HCT15 [MSH6 mutated], and LoVo [homozygous deletion of exons 2-8 of MSH2]) and 5 microsatellite stable (LS513, SW620, Caco-2, FET, and HCT116 mlh1-2 [HCT116 transfected with an MLH1-expression vector][1]) cell lines.

Treatment of Colorectal Cell Lines

Cells in the exponential growth phase were counted by Trypan blue exclusion and seeded into 24-well plates (Falcon) at a density of 0.2 to 5×10^5 cells/well in complete medium. After 24-hour incubation, 6-TG (1, 5, 15, and 20 μM final concentrations) or extemporaneously reconstituted MNNG (0.1, 1, 5, 20 μM final concentrations) was added. Medium was removed and replaced with fresh medium after 24-hour or 1-hour incubation, respectively. Cell growth was evaluated after a total incubation period of 7 to 9 days. To exclude differences in MNNG cytotoxicity due to variations in O^6-methylguanine methyltransferase enzyme activity, the latter was abrogated by exposure to O^6-benzylguanine (20 μmol/L final concentration) during the entire experiment. All samples were tested in quadruplicate.

6-TG Treatment of Lymphoblastoid Cell Lines

Cells suspended in complete medium (3×10^5 cell/mL) were distributed into 6 microtubes with increasing concentrations of 6-TG (0.15, 0.3, 0.6, 1.25, and 2.5 μmol/L final concentrations) into 5 of them. After 24-hour incubation, all microtubes were centrifuged, and cells were rinsed with fresh medium and then seeded in 100-μL aliquots into 96-well round-bottom plates (0.6×10^4 cells/well). Cell growth was evaluated after a total incubation time of 7 days, and all samples were tested in sextuplicate.

DNA Extraction for evMSI and gMSI Assays

Ficoll-Plaque PLUS (GE Healthcare) was used to isolate human lymphocytes from blood patients, according to the supplier's recommendations. DNA extraction from lymphocytes or lymphoblastoid cell line (LCL) was performed using QIAmp DNA kit according to the supplier's recommendations (Qiagen, Venlo, The Netherlands).

Determining the gMSI Ratio

Multiplex PCR amplification in triplicate (denaturation of 95°C for 2 min, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds, with a final extension at 72°C for 10 minutes) of the dinucleotide microsatellite markers D17S791, D2S123, and D17S250 was developed using the primers previously described,[2] and using 10 ng of patient germinal DNA. PCR products were separated by capillary electrophoresis on an ABI3100 genetic analyzer and quantified using Gene Mapper version 3.7 software. Briefly, the germline microsatellite instability (gMSI) ratio was determined by dividing the height of an allele's trailing "stutter" peak (n+1) by the height of the allele's major peak (n). Interpretation required that the size difference between alleles in heterozygous individuals was ≥ 6 bp.[2]

Statistical Analysis

We developed a Bayesian approach to conduct inference for the unknown prevalence, sensitivity, and specificity of the 3 diagnostic methods as performed in Joseph et al.[3] Our setting, however, was different from theirs, in particular, we knew the true disease status for controls and genetically confirmed CMMRD patients, which removed the lack of identifiability of in the approach used by Joseph et al, which was pointed out in Johnson et al.[4]

In the saturated model, the joint distribution of the tests or combination of tests was assumed to be multinomial with 16 categories, corresponding to all possible

observations. The multinomial parameters were expressed as the true proportion of confirmed CMMRD patients, sensitivity, and specificity of the tests. We assumed conditional independence of the tests to ensure identifiability in the unsaturated model. A Metropolis-Hastings algorithm was run on the data to estimate the 7 parameters and 2-sided confidence intervals.[3,5] Let D be the true CMMRD status $(+/-)$, T_1 $(+/-)$, T_2 $(+/-)$ and T_3 $(+/-)$ be the result of MMR gene sequencing, functional testing and gMSI testing, respectively. The true proportion of CMMRD patients, sensitivity, and specificity of the 3 tests or combination of tests are defined as:

$$\pi = \mathbb{P}(D = +)$$
$$s_1 = \mathbb{P}(T_1 = +|D = +) \text{ and } c_1 = \mathbb{P}(T_1 = -|D = -)$$
$$s_2 = \mathbb{P}(T_2 = +|D = +) \text{ and } c_2 = \mathbb{P}(T_2 = -|D = -)$$
$$s_3 = \mathbb{P}(T_3 = +|D = +) \text{ and } c_3 = \mathbb{P}(T_3 = -|D = -)$$

The observed data are summarized in Supplementary Table 5 (rows with no observations are not reported); the usual latent variables are denoted by X, Y, and Z.

We particularized *MSH6* cases, as gMSI is not relevant to identify CMMRD patients with *MSH6* defects. As a consequence, we rewrite

$$Y = Y_{\text{MSH6}} + Y_{\text{noMSH6}}$$

with $Y_{\text{MSH6}} \sim \mathcal{B}\left(2, \dfrac{\pi(1 - s_1)(1 - s_2)}{\pi(1 - s_1)(1 - s_2) + (1 - \pi)c_1 c_2}\right)$

and

$$Y_{\text{noMSH6}} \sim \mathcal{B}\left(13, \dfrac{\pi(1 - s_1)(1 - s_2)(1 - s_3)}{\pi(1 - s_1)(1 - s_2)(1 - s_3) + (1 - \pi)c_1 c_2 c_3}\right)$$

$$Z = Z_{\text{MSH6}} + Z_{\text{noMSH6}}$$

with $Z_{\text{MSH6}} \sim \mathcal{B}\left(1, \dfrac{\pi(1 - s_1)s_2}{\pi(1 - s_1)s_2 + (1 - \pi)c_1(1 - c_2)}\right)$

and

$$Z_{\text{noMSH6}} \sim \mathcal{B}\left(3, \dfrac{\pi(1 - s_1)s_2(1 - s_3)}{\pi(1 - s_1)s_2(1 - s_3) + (1 - \pi)c_1(1 - c_2)c_3}\right)$$

conditionally to the parameters.

This formulation allowed us to fit the model through the Metropolis-Hastings algorithm.[6] In this algorithm, we considered a Dirichlet prior for the joint distribution of the seven parameters. Parameters of the marginal previous distributions were chosen as (1,1) for the true proportion of CMMRD patients, the sensitivities and the specificities. The Metropolis-Hastings algorithm was run on 50,000 iterations, and the last 25,000 iterations were used to derive estimations and confidence intervals for the sensitivities, specificities, positive and negative predictive values of MMR gene sequencing, functional testing and gMSI testing. The same procedure has been applied for the comparison between MMR gene sequencing and functional testing.

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Supplementary Table 5. Data for the Hastings-Metropolis algorithm

Genetic testing	Methylation tolerance and *ev*MSI	gMSI	True status	Data
+	+	+	CMMRD+	9
+	+	-	CMMRD+	2
-	-	-	CMMRD+	Y
-	-	-	CMMRD-	9+15-Y
-	+	+	CMMRD+	X
-	+	+	CMMRD-	1-X
-	+	-	CMMRD+	Z
-	+	-	CMMRD-	4-Z