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Constitutional *MLH1* Methylation Is a Major Contributor to Mismatch Repair–Deficient, *MLH1*-Methylated Colorectal Cancer in Patients Aged 55 Years and Younger

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Abstract

Background: Most mismatch repair–deficient (MMRd) colorectal cancer (CRC) cases arise sporadically, associated with somatic *MLH1* methylation, whereas approximately 20% have germline mismatch repair pathogenic variants causing Lynch syndrome (LS). Universal screening of incident CRC uses presence of *MLH1* methylation in MMRd tumors to exclude sporadic cases from germline testing for LS. However, this overlooks rare cases with constitutional *MLH1*

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methylation (epimutation), a poorly recognized mechanism for LS. We aimed to assess the frequency and age distribution of constitutional *MLH1* methylation among incident CRC cases with MMRd, *MLH1*-methylated tumors.

Methods: In retrospective population-based studies, we selected all CRC cases with MMRd, *MLH1*-methylated tumors, regardless of age, prior cancer, family history, or *BRAF* V600E status, from the Columbus-area HNPCC study (Columbus) and Ohio Colorectal Cancer Prevention Initiative (OCCPI) cohorts. Blood DNA was tested for constitutional *MLH1* methylation by pyrosequencing and real-time methylation-specific PCR, then confirmed with bisulfite-sequencing.

Results: Results were achieved for 95 of 98 Columbus cases and all 281 OCCPI cases. Constitutional *MLH1* methylation was identified in 4 of 95 (4%) Columbus cases, ages 34, 38, 52, and 74 years, and 4 of 281 (1.4%) OCCPI cases, ages 20, 34, 50, and 55 years, with 3 showing low-level mosaic methylation. Mosaicism in blood and normal colon, plus tumor loss of heterozygosity of the unmethylated allele, demonstrated causality in 1 case with sample availability. Age stratification showed high rates of constitutional *MLH1* methylation among younger patients. In the Columbus and OCCPI cohorts, respectively, these rates were 67% (2 of 3) and 25% (2 of 8) of patients aged <50 years but with half of the cases missed, and 75% (3 of 4) and 23.5% (4 of 17) of patients aged 55 years with most cases detected.

Conclusions: Although rare overall, a significant proportion of younger patients with *MLH1*-methylated CRC had underlying constitutional *MLH1* methylation. Routine testing for this high-risk mechanism is warranted in patients aged 55 years for a timely and accurate molecular diagnosis that will significantly alter their clinical management while minimizing additional testing.

Background

Approximately 15% of colorectal cancer (CRC) cases are mismatch repair (MMR)–deficient (MMRd), detected by immunohistochemistry (IHC),[1] microsatellite instability (MSI),[2] and/or the hypermutator phenotype.[3] Most MMRd CRC cases arise sporadically in older patients, associated with acquired (somatic in origin) aberrant methylation of the *MLH1* promoter in the tumor.[4–6] Approximately 20% of MMRd CRC cases are associated with Lynch syndrome (LS), caused by a germline pathogenic variant within an MMR gene, *MLH1, MSH2, MSH6, PMS2*, or terminal *EPCAM* deletion.[7] Constitutional *MLH1* methylation (epimutation), characterized by promoter methylation and transcriptional inactivation of a single allele throughout normal tissues, is an alternative and poorly recognized mechanism for LS.[8] Cases with constitutional *MLH1* methylation have presented with early-onset and/or multiple primary tumors consistent with *MLH1*-LS[9]; hence, clinical management according to *MLH1*-LS guidelines has been suggested for this subgroup.[10]

Universal screening of all CRC for MMRd is recommended as standard of care.[11–16] This stratifies patients by prediction of response to systemic therapies and identifies those warranting genetic testing for LS.[6,17,18] Current stepwise algorithms entail tumor testing for MMRd by IHC and/or MSI in the first tier.[12] For tumors exhibiting MLH1 loss and/or MSI, second-tier testing for *MLH1* methylation is recommended, and cases with

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MLH1 methylation are considered sporadic and hence omitted from follow-up evaluation for LS.[15,16,19–22] The *BRAF* V600E tumor mutation is sometimes used as a surrogate for *MLH1* methylation, given that it correlates, albeit imperfectly, with somatic-in-origin *MLH1* methylation and is rare in LS-associated CRC.[22–25] Cases with MMR protein loss and unmethylated *MLH1*, or *BRAF* wild-type, are eligible for LS testing. Next-generation sequencing tumor panels that detect MSI, hypermutator phenotype, and *BRAF* V600E may replace these stepwise algorithms.[26] A potential problem with these algorithms is that they may misdiagnose cases with constitutional *MLH1* methylation as common sporadic cases, thereby omitting them from appropriate follow-up testing. In these high-risk cases, the constitutional *MLH1* methylation serves as the "first hit," predisposing to tumors that exhibit MLH1 loss, MSI, and *MLH1* methylation—features that overlap with common sporadic cases.[8,27–34] Furthermore, most cases arise de novo due to a "primary epimutation" with no apparent genetic basis; consequently, carriers tend to have no remarkable family history.[8,31,35,36] Although constitutional *MLH1* methylation is potentially heritable, few familial cases have been described. These have included families with autosomal dominant inheritance linked to a genetic variant and others with non-Mendelian inheritance without an associated genetic variant.[34,35,37–46] Thus, differentiating seemingly sporadic cases with constitutional *MLH1* methylation from "true sporadic" cases with somatic *MLH1* methylation poses a clinical and molecular diagnostic challenge.

Constitutional *MLH1* methylation is rare in CRC overall.[47] However, its frequency and age distribution among incident MMRd, *MLH1*-methylated CRC cases remains unclear because prior screens have been conducted primarily in single-center studies on small sample sizes. Ascertainment biases in estimations of frequency are likely, given the use of different inclusion criteria with respect to age, clinicopathologic characteristics including *BRAF* V600E status, and prior genetic testing. The frequency of constitutional *MLH1* methylation was 3% to 9%[8,30,35,41,47–50] in patients ascertained via cancer clinics as fulfilling at least one of the clinical criteria in the revised Bethesda guidelines for LS evaluation,[51] with MSI and MLH1 loss in their tumor and negative germline test results. Marginally higher rates of constitutional *MLH1* methylation were found with inclusion of *MLH1* methylation as a tumor feature, at 3.5% to 15.6%.[33,47,52]

In this study, we leveraged the biospecimen resources and prior clinicopathologic and molecular results from the population-based Columbus-area HNPCC study (Columbus)[53,54] and Ohio Colorectal Cancer Prevention Initiative (OCCPI)[55] cohorts, designed for prospective studies on universal screening for hereditary cancer, to determine the frequency and age distribution of constitutional *MLH1* methylation among incident CRC cases selected solely by the tumor features of MMRd and presence of *MLH1* methylation.

Methods

Patients

The patient selection strategy is shown in supplemental eFigure 1 (available with this article at www.JNCCN.org). The first case series was derived from 1,566 unselected CRC cases in the Columbus cohort (recruited 1999–2004).[53,54] The second series was derived from 3,310 CRC cases in the OCCPI cohort (recruited 2013–2016).[55] Eligibility criteria

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for inclusion in this study were patients whose CRC showed (1) MLH1 absence and/or MSI-high if IHC was missing/uninterpretable, (2) *MLH1* methylation, and (3) availability of peripheral blood leukocyte (PBL) DNA (Columbus) or whole-blood DNA (OCCPI). We leveraged the existing tumor molecular pathology data from prior universal screening for MMRd and LS to select cases for inclusion, including MMR IHC, MSI status (Bethesda 5-marker panel),[51] and *MLH1* methylation status previously determined using methylation-specific PCR (Columbus)[5,56,57] or CpG pyrosequencing[58] (OCCPI) assays. All patients were deidentified before study initiation.

Screen for Constitutional *MLH1* Methylation and Promoter Variants

Bisulfite conversion was performed on 1 μg of DNA using the EZ DNA Methylation-Gold Kit (Zymo Research), with approximately 50 ng input into each assay. Screening for constitutional *MLH1* methylation was performed using 2 assays previously described for this purpose: (1) quantitative CpG pyrosequencing using the Pyro-Mark Q96 system (Qiagen)[31,36,59] and (2) high-sensitivity real-time semi-quantitative methylation-specific PCR (qMSP) followed by melt curve analysis (methylation-specific peak at 76.5°C ± 0.5°C) using the CFX96 Thermal Cycler system (Bio-Rad Laboratories), enabling the detection of low-level mosaicism.[34–36,42,60] Samples were considered methylation-positive if either assay yielded a signal above the respective limit of detection, at 2.3% for pyrosequencing and percentage of methylated reference (PMR) value at 0.1% for qMSP (supplemental eFigures 2 and 3).[42,61] Presence of methylation was confirmed by clonal bisulfite sequencing (24 clones) across a promoter fragment encompassing 16 CpGs and single-nucleotide polymorphism (SNP) c.-93G>A (rs1800734).[34–36,61] This also allowed allele-specific methylation patterns to be determined in patients heterozygous for this SNP. The *MLH1* CpG island was Sanger sequenced in methylation-positive cases to identify potential genetic variants associated with constitutional *MLH1* methylation.[34,42]

Statistics

Descriptive statistics were performed using SPSS Statistics, Version 27. Based on a Bayesian approach, posterior probabilities and 95% high-density probability confidence intervals for various rates of detection were calculated as described in supplemental eAppendix 1.

Results

Detection of Constitutional *MLH1* Methylation

Columbus Cohort—Among 105 of 1,566 (6.7%) eligible cases, 98 cases with MLH1 loss (n=95; 3 had missing data) and/or MSI-high (n=98) plus *MLH1* methylation in their tumor had PBL DNA available (supplemental eFigure 1). Median age at diagnosis was 72 years (range, 34–98 years). Methylation testing was successful for 95 cases, with complete concordance between pyrosequencing and qMSP results. Constitutional *MLH1* methylation was detected in 4 of 95 (4%) of the selected cases, aged 34, 36, 52, and 74 years at diagnosis (Figure 1). Clonal bisulfite sequencing confirmed the presence of constitutional *MLH1* methylation affecting a proportion of alleles in PBL DNA from all 4 cases (supplemental eFigure 6). The levels and allelic patterns of constitutional *MLH1*

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methylation and clinical, demographic, and molecular pathology features are summarized in Table 1. Patient Columbus-6 had a classic pattern of hemiallelic methylation (approximately half of alleles methylated), consistent with an epimutation affecting a single parental allele. Notably, the other 3 patients (Columbus-1, -2, and -65) had low-level methylation consistent with mosaicism affecting a proportion of cells (Table 1, supplemental eFigure 6). CRC was the first presentation of cancer in all 4 cases. No genetic alterations were identified within the *MLH1* CpG island in the 4 carriers. Only Columbus-65 had a positive family history recorded, which included CRC and/or endometrial cancer in a first-degree relative, but precise details are unknown.

OCCPI Cohort—Eligible CRC cases included 281 of 3,310 (8.5%) with MLH1 loss (n=280, 1 equivocal was MSI-high) plus *MLH1* methylation in their tumor and had whole-blood DNA available (supplemental eFigure 1).[55] Median age at diagnosis was 71.7 years (range, 20–93 years). Methylation testing was successful for all 281 cases, with complete concordance between pyrosequencing and qMSP results. Constitutional *MLH1* methylation was detected in 4 of 281 (1.4%) selected cases, aged 20, 34, 50, and 55 years (Figure 1). These were confirmed by clonal bisulfite sequencing (supplemental eFigure 6), as summarized in Table 1. CRC was the first presentation of cancer in all 4 cases. All 4 cases had an unremarkable family history, and no genetic alterations were identified within the *MLH1* CpG island. Patients OCCPI-2, -10, and -15 had classic hemiallelic constitutional *MLH1* methylation, with methylation levels of approximately 50% measured by pyrosequencing (Figure 1B) and about half of alleles found to be methylated by clonal bisulfite sequencing (supplemental eFigure 6), consistent with epimutation of one parental allele. OCCPI-1 had low-level mosaic (4.2%) constitutional *MLH1* methylation in blood (Figure 1B); he was heterozygous for the c.-93G>A promoter SNP, with methylation affecting about 20% of "G" alleles (supplemental eFigure 6). Together, these data are consistent with monoallelic, mosaic constitutional *MLH1* methylation affecting approximately 8% to 10% of leukocytes. Tumor and paired normal colorectal mucosa (NCM) DNA samples were retrieved for OCCPI-1. Pyrosequencing detected similarly low-level mosaic *MLH1* methylation (2.6%) in NCM, therefore affecting approximately 5% NCM cells (Figure 2A). In the tumor, a high level of methylation (61%) was detected (Figure 2A), and clonal bisulfite sequencing found only "G" alleles at c.-93G>A, which were predominantly methylated (Figure 2B). Sequencing across the *MLH1* promoter showed loss of heterozygosity (LoH) of the unmethylated "A" allele at c.-93G>A in the tumor (Figure 2C). These findings are consistent with constitutional *MLH1* methylation of the "G" allele in a small proportion of cells as the "first hit" predisposing to tumorigenesis, accompanied by somatic LoH of the unmethylated "A" allele as the "second hit."

Stratification by Age

To determine the optimal age threshold at which screening yielded the highest rate of detection of constitutional *MLH1* methylation with minimal cases missed while also minimizing the number of cases screened overall, we calculated the frequency of constitutional *MLH1* methylation by age and within 5-year age bins for each cohort (Figure 3, Table 2).

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Columbus Cohort—Of the 4 cases with constitutional *MLH1* methylation detected, 2 were aged <50 years, an age bin comprising only 3 of 95 (3%) of the cohort, yielding a positive detection rate of 2 of 3 (67%), but with 2 older cases missed. The only patient with unmethylated PBL in the <50 years bin carried the 5′ untranslated region promoter variant c.-11C>T (rs776898290).[54] This variant has been identified in additional suspected LS cases and showed significantly diminished transcriptional activity in promoter reporter assays,[42] but it remains a variant of uncertain significance. The highest positive detection rate for constitutional *MLH1* methylation at 3 of 4 (75%) was <55 years, an age threshold that comprised only 4 of 95 (4%) of the cohort, but with 1 older case missed. To detect all 4 cases, screening of 58 of 95 (61%) cases <75 years of age yielded a detection rate of 4 of 58 (7%).

OCCPI Cohort—Constitutional *MLH1* methylation was detected in 2 of 8 (25%) patients aged <50 years, an age bin comprising just 8 of 281 (2.8%) of the cohort, but with 2 older cases missed. The detection rate was 3 of 14 (21%) patients aged <55 years, an age threshold that comprised 14 of 281 (5%) of the cohort, but with 1 case (aged 55 years) missed. Increasing the age threshold to <60 years entailed screening 31 of 281 (11%) patients in the cohort, yielding a detection rate of 4 of 31 (13%), with all 4 cases detected. The youngest age threshold at which all 4 cases were detected was 55 years, which entailed screening of 17 of 281 (6%) of the cohort, yielding a positive detection rate of 4 of 17 (23.5%).

Posterior probabilities for various rates of detection were calculated for each cohort to assess the age limit at which an acceptable detection rate was yielded (supplemental eFigure 7 and eTable 1). Assuming testing for constitutional *MLH1* methylation is considered acceptable when it yields a detection rate >10% with very high probability, the age threshold for testing implementation would be <60 years for the Columbus-area cohort (probability >0.99) and 55 years for the OCCPI cohort (probability 0.96).

Discussion

Current universal testing algorithms may result in patients with constitutional *MLH1* methylation going underdiagnosed and underserved as the opportunity for a clinical and molecular diagnosis at first presentation of cancer is missed. For routine implementation of additional blood-based methylation testing into the existing universal screening algorithm for MMRd and LS, multiple factors would be taken into consideration, including the rate/probability of detection among those selected for screening and the number (and cost) of additional tests needed to identify each new case. Our study aimed to address the frequency of constitutional *MLH1* methylation among incident MMRd/*MLH1*-methylated CRC cases and whether age at diagnosis could be incorporated into existing algorithms to select patients warranting referral for additional methylation testing to detect this rare high-risk defect.

Our retrospective nested study of the population-based Columbus and OCCPI cohorts selected CRC cases based solely on tumor molecular features (MLH1 loss/MSI-high and *MLH1* methylation) used in universal screening algorithms; therefore, it was unbiased with respect to age at diagnosis, tumor *BRAF* V600E status, personal or family history

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of cancer, or whether prior germline genetic testing had been undertaken. With selection by these key tumor features, constitutional *MLH1* methylation was detected in just 4% (4/95) of Columbus and 1.4% (4/281) of OCCPI cases. This low frequency across all ages is unsurprising, given the rarity of this defect and because *MLH1* methylation is typically somatic in origin in CRC and associated with advanced age. Although the full cohorts of unselected cases were not screened, by extrapolation this makes constitutional *MLH1* methylation extremely rare among unselected CRC series, at 4 of 1,566 (~0.26%) of Columbus cases and 4 of 3,310 (~0.12%) of OCCPI cases. This mechanism also represents a minor fraction (<10%) of LS cases, because MMR germline pathogenic variants were previously identified in 2.8% of Columbus cases and 4% of OCCPI cases.[53–55] Nevertheless, a key finding of this study was that after stratification by age at diagnosis, constitutional *MLH1* methylation was a major contributor to incident MMRd, *MLH1*-methylated CRC among younger cases, which, in turn, represented a small fraction of cases overall. This shows that selection by key tumor features with age limitation would incur screening of small numbers. The highest rates of detection of patients with constitutional *MLH1* methylation were 75% (3/4 tested) of those aged <55 years in the Columbus cohort and 25% (2/8 tested) of those aged <50 years in the OCCPI cohort; however, these age thresholds resulted in missed cases. In the OCCPI cohort, increasing the age of testing to 55 years maintained a high detection rate at 23.5% (4/17 tested), with all 4 cases with constitutional *MLH1* methylation detected and minimal additional screening. The 2022 NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) for Genetic/Familial High-Risk Assessment: Colorectal recommend that testing for constitutional *MLH1* methylation be considered in patients with CRC whose tumor exhibited MLH1 loss and *MLH1* methylation if it is early onset (occurs at <50 years of age) or they have a family history.[16] However, in the 2 cohorts we studied, restricting testing to patients aged <50 years would have resulted in half (4/8) of cases being missed. Furthermore, probands with constitutional *MLH1* methylation often have no family history, as was found for 7 of 8 cases herein, given that epimutations tend to arise de novo. Only the oldest patient had a family history but no apparent underlying genetic variant. The finding of this 74-year-old patient with constitutional *MLH1* methylation illustrates that age-limited screening will result in occasional missed cases. However, given the rarity of this defect, triaging is needed to increase detection rates for routine implementation of screening. By using age 55 years at diagnosis as the threshold for referral of incident cases of CRC with MMRd, *MLH1*-methylated tumors for blood-based methylation testing, most cases with constitutional *MLH1* methylation herein would have been identified with high detection rates (23.5% Columbus, 75% OCCPI) and with minimal cases screened (4% Columbus, 6% OCCPI with MMRd/*MLH1*-methylated tumors). This would provide a timely and correct molecular diagnosis of this defect at first presentation of CRC at an age when intensive surveillance for *MLH1*-LS cancers could result in the prevention or earlier diagnosis of metachronous cancer and potential life-years saved.

The role of low-level mosaic constitutional *MLH1* methylation in cancer predisposition has remained in question. An interesting observation herein was that 3 patients had low-level mosaic constitutional *MLH1* methylation. Although it is plausible that this may confer lower risks for cancer than hemiallelic methylation, mosaicism was found in both

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the oldest (age 74 years) and the youngest (age 20 years) patients. Prior cases with low-level mosaicism have been reported with early-onset and/or multiple primary cancers.[42,62] Although additional samples were unavailable from Columbus cases, for patient OCCPI-1 (age 20 years), we were able to demonstrate that methylation was present in NCM (tumor tissue of origin), albeit also low level. Furthermore, by tracing allelic representation and methylation at the c.-93G>A SNP in tumor and NCM, we found high levels of monoallelic methylation in the tumor combined with LoH of the unmethylated allele. These findings are consistent with a causal role for mosaic constitutional *MLH1* methylation in early-onset tumorigenesis, whereby monoallelic methylation in the cell(s) of origin is predisposing, followed by somatic LoH of the remaining functional allele.

A limitation of this study was the lack of systematic *BRAF* V600E tumor testing within the cohorts. For 4 of 8 constitutional *MLH1* methylation carriers with available data, all 4 were *BRAF* wild-type (Table 1). This is consistent with prior case reports suggesting a higher frequency of *BRAF* wild-type, although a few cases with *BRAF* V600E CRC have been reported.[31,45] Nevertheless, in the absence of tumor *MLH1* methylation results or in universal screening scenarios where tumor *BRAF* V600E testing is used in place of *MLH1* methylation testing, carriers of constitutional *MLH1* methylation may undergo germline genetic testing for LS (on account of MLH1 loss plus *BRAF* wild-type in their tumor). This will likely yield a negative/uninformative germline test result. Therefore, testing for constitutional *MLH1* methylation should also be considered in patients with CRC aged 55 years whose tumor showed MLH1 loss and *BRAF* wild-type (in the absence of tumor *MLH1* methylation information) if germline genetic testing returns a nonactionable result to correctly diagnose or rule out constitutional *MLH1* methylation. Finally, referral for constitutional *MLH1* methylation testing should also be considered in patients with a clinical history of synchronous or metachronous LS-associated cancers displaying MMRd and *MLH1* methylation at any age.

Conclusions

Our findings provide evidence in support of referral for blood-based testing for constitutional *MLH1* methylation among patients with first presentation of CRC at age 55 years whose tumor exhibits MMRd and *MLH1* methylation. Given the low prevalence of CRC cases with these tumor features at younger ages, additional methylation testing will be minimal yet likely to yield high rates of detection of constitutional *MLH1* methylation. Currently, CLIA-certified testing for constitutional *MLH1* methylation in the United States is available at the Mayo Clinic, Rochester, Minnesota, which uses a qMSP test similar to one of the screening tests used in this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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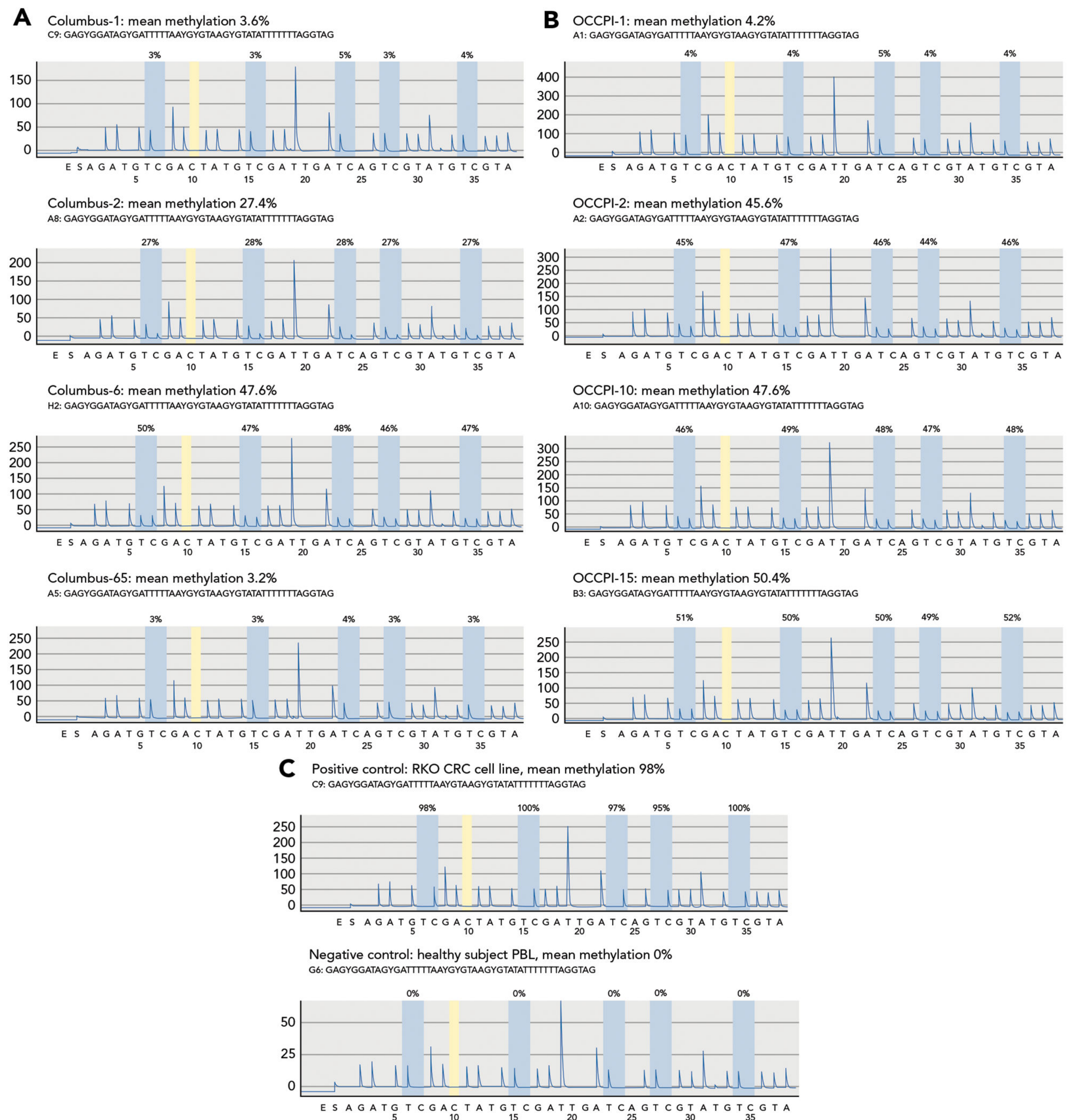

Figure 1.

Blood-based detection of constitutional *MLH1* methylation by CpG pyrosequencing in CRC cases with MMRd, *MLH1*-methylated tumors. Pyrogram traces are shown for patients with CRC in whom constitutional *MLH1* methylation was detected across 5 CpG sites within the c.-241 to c.-272 sequence (GenBank accession no. NG_007109.2) of the *MLH1* promoter in bisulfite-converted germline DNA. **(A)** *MLH1* methylation was detected in PBL DNA of 4 cases from the Columbus cohort. **(B)** *MLH1* methylation was detected in whole-blood DNA from 4 cases from the OCCPI cohort. **(C)** Illustrative examples of pyrograms from

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the hypermethylated RKO CRC cell line as positive control and an unmethylated healthy control PBL as a negative control. Methylation is detected by the presence of a peak at the cytosine (C) within each CpG site interrogated (blue bars), whereas unmethylated cytosines are detected as thymine (T) peaks within the same CpG sites, due to the conversion of unmethylated cytosines to uracils using bisulfite treatment. The percentage methylation value at each CpG site appears above each bar. The mean percentage of methylation across all 5 CpG sites is calculated and shown above each pyrogram. The yellow bar indicates a non-CpG cytosine used as a quality control measure to ensure complete bisulfite conversion to T, whereupon this yields a valid test result.

Abbreviations: Columbus, Columbus-area HNPCC study; CRC, colorectal cancer; MMRd, mismatch repair–deficient; OCCPI, Ohio Colorectal Cancer Prevention Initiative; PBL, peripheral blood leukocyte.

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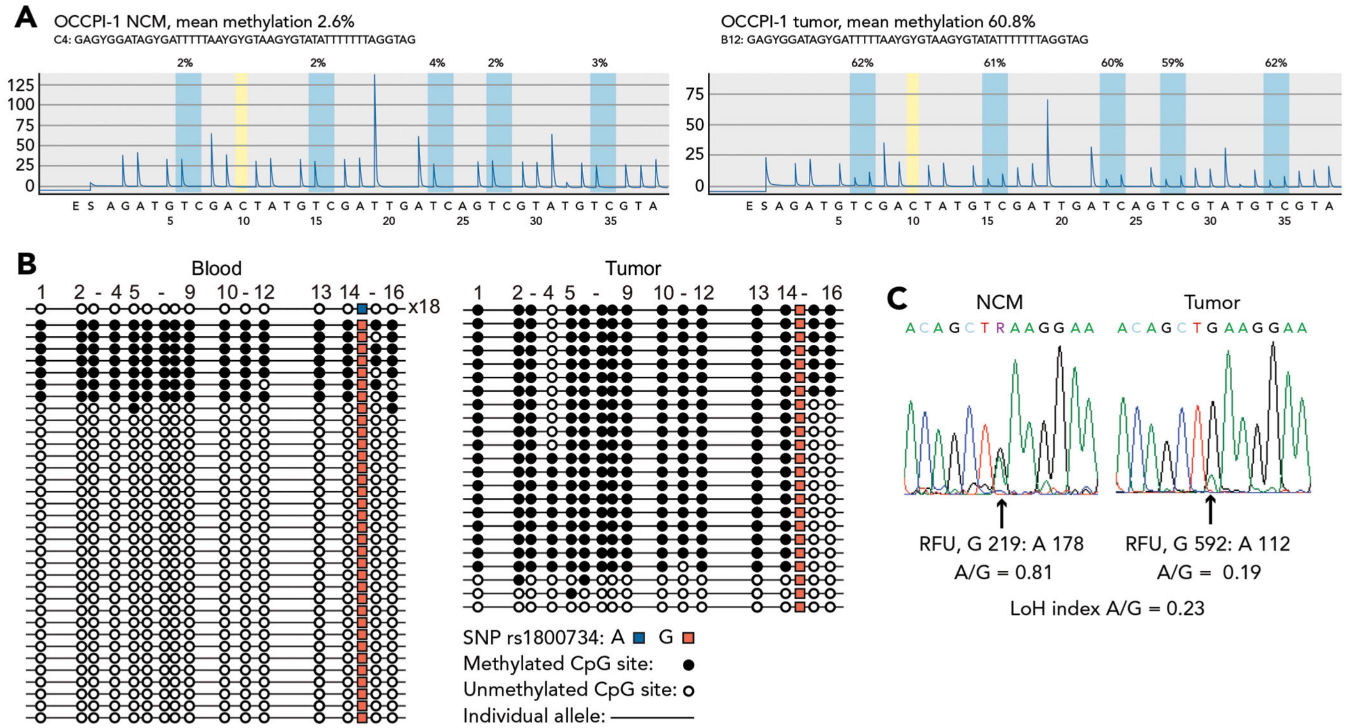

Figure 2.

Identification of LoH of the unmethylated allele in the tumor of patient OCCPI-1 indicates low-level mosaic constitutional *MLH1* methylation predisposed to CRC development. **(A)** Pyrosequencing in NCM shows low-level methylation at 2.6% in the tissue of tumor origin and confirms high-level methylation in the tumor (60.8%). Legend according to Figure 1. **(B)** Clonal bisulfite sequencing in the tumor. Legend according to supplemental eFigure 6. Although patient OCCPI-1 was heterozygous at the c.-93G>A SNP (rs1800734), only detected "G" alleles were sequenced in the tumor, most of which were methylated. The methylation patterns on individual alleles were repetitive, suggesting clonal expansion from a small number of cells that contained methylated G alleles. **(C)** Sanger sequencing electropherogram across the c.-93G>A SNP site within the *MLH1* promoter is shown in the tumor and paired NCM samples, with the RFU measured at the A and G peaks at the c.-93G>A SNP (indicated by arrows), shown for each allele below. The LoH index in the tumor was calculated with reference to the paired NCM sample as $(A/G_{Tumor})/(A/G_{NCM})$, whereby values <0.6 or >1.7 indicate LoH of the A or G allele, respectively. NCM has approximately equal representation of the G and A alleles, consistent with heterozygosity. The tumor shows reduced representation of the A allele (LoH index 0.23), consistent with LoH of the A allele as the "second hit" in the tumor. See supplemental eFigure 6, available with this article at JNCCN.org.

Abbreviations: CRC, colorectal cancer; LoH, loss of heterozygosity; NCM, normal colorectal mucosa; OCCPI, Ohio Colorectal Cancer Prevention Initiative; RFU, relative fluorescence units; SNP, single-nucleotide polymorphism.

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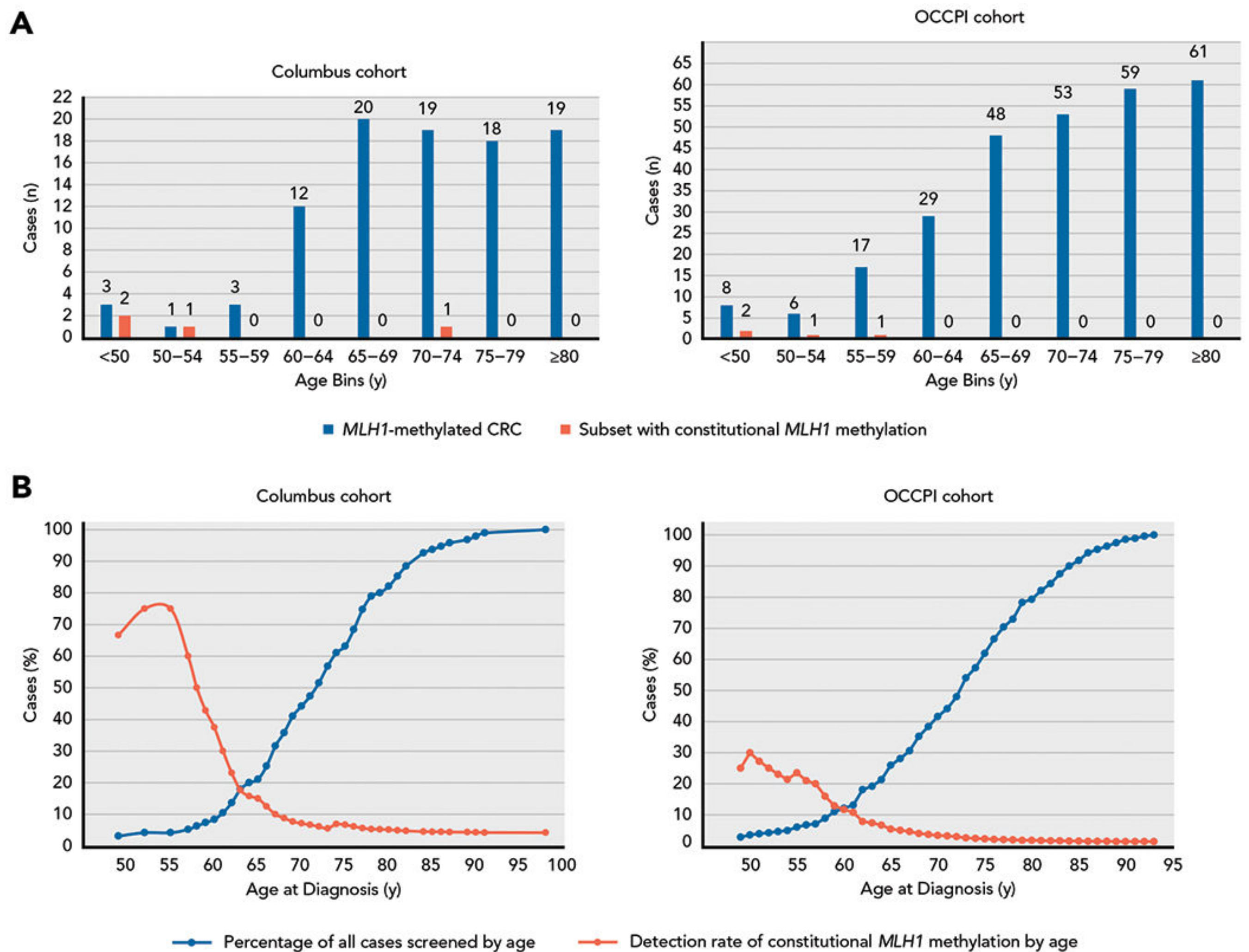

Figure 3.

Rate of detection of constitutional *MLH1* methylation by age among the Columbus and OCCPI cohorts. **(A)** Histograms showing the number of cases with constitutional *MLH1* methylation detected (orange) among CRC cases with an MMRd, *MLH1*-methylated tumor (blue) tested within 5-year age bins beginning at age <50 years for each cohort. **(B)** Line graphs showing the cumulative percentage of CRC cases tested by age (blue), beginning at age <50 years pooled, with 100% of cases being n=95 for the Columbus cohort and n=281 for the OCCPI cohort. The percentage of cases with constitutional *MLH1* methylation detected among cases tested of the same age, beginning at age <50 years, are shown in orange. In the Columbus cohort the highest detection rate was 75% (3 of 4) at age threshold <55 years, with 1 older case missed. There were no cases aged 55 years at diagnosis in the Columbus cohort; therefore, the same detection rate was observed at 55 years. In the OCCPI cohort, the highest detection rate was 25% (2 of 8) cases <50 years, but with 2 cases missed. The youngest age threshold at which all cases were detected was age 55 years (<56 years), yielding a detection rate of 23.5% (4 of 17 cases). In both cohorts, screening at age 55 years would entail screening of a small fraction of total cases: 4% for the Columbus cohort and 6% of the OCCPI cohort.

Abbreviations: Columbus, Columbus-area HNPCC study; CRC, colorectal cancer; MMRd, mismatch repair–deficient; OCCPI, Ohio Colorectal Cancer Prevention Initiative.

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Table 1.

Demographic and Clinicopathologic Features of CRC Cases With Constitutional *MLH1* Methylation Identified as Likely Cause for Their MMRd, *MLH1*-Methylated Tumor

Identifier	Sex	Race	Cancer History	Age at Diagnosis	FH	MLH1 IHC	MSI-H	*BRAF* V600E	Constitutional *MLH1* Methylation Levels: Pyrosequencing, qMSP (PMR), Clonal bis-seq
Columbus-area HNPCC study cohort									
Columbus-1	M	Black	Ascending colon	34 y	Neg	Absent	Pos	N/T	3.6%, 1.5%, low-level mosaicism (~1% alleles methylated)
Columbus-2	F	White	Splenic flexure	38 y	Neg	Absent	Pos	WT	27.4%, 13.8%, mosaic (~9% alleles methylated)
Columbus-6	M	White	Cecum, synchronous	52 y	Neg	Absent	Pos	N/T	47.6%, 42.9%, hemiallelic (~50% alleles methylated)
Columbus-65	M	White	Ascending colon	74 y	Pos	Absent	Pos	N/T	3.2%, 1.2%, monoallelic and mosaic (methylation on ~11% of “A” alleles at heterozygous c.-93G>A SNP)
OCCPI study cohort									
OCCPI-1	M	White	CRC unspecified	20 y	Neg	Absent	Pos	WT	4.2%, 1.6%, monoallelic and mosaic (methylation on ~20% “G” alleles at heterozygous c.-93G>A SNP)
OCCPI-2	F	White	Ascending colon	34 y	Neg	Absent	Pos	WT	45.6%, 39.5%, hemiallelic (~50% alleles methylated)
OCCPI-10	F	Black	Cecum	50 y	Neg	Absent	Pos	N/T	47.6%, 35.6%, hemiallelic (~50% alleles methylated)
OCCPI-15	M	White	Transverse colon	55 y	Neg	Absent	Pos	WT	50.4%, 50.4%, hemiallelic (~50% alleles methylated)

Monoallelic methylation indicates methylation is linked to a single genetic allele at a heterozygous promoter SNP. Hemiallelic methylation indicates methylation of approximately half of alleles (~50% methylation measured).

Abbreviations: bis-seq, bisulfite sequencing; Columbus, Columbus-area HNPCC study; CRC, colorectal cancer; Dx, diagnosis; F, female; FH, family history; IHC, immunohistochemistry; M, male; MMRd, mismatch repair–deficient; MSI-H, microsatellite instability-high; N/T, not tested; Neg, negative; OCCPI, Ohio Colorectal Cancer Prevention Initiative; PMR, percentage of methylated reference; Pos, positive; qMSP, real-time semiquantitative methylation-specific PCR followed by melt analysis; SNP, single-nucleotide polymorphism; WT, wild-type.

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Table 2.

Detection Rate of Constitutional *MLH1* Methylation as a Function of Age Group Among Patients With MMRd, *MLH1*-Methylated CRC

Age Bin	Epimutation Cases Detected	Sample Size n (% of Cohort)	Detection Rate (95% CI)
Columbus cohort			
<50 y	2 of 4	3 (3%)	66.7% (22.9–99.0)
<55 y[a]	**3 of 4**	**4 (4%)**	**75.0% (34.7–99.7)**
<60 y	3 of 4	7 (7%)	42.9% (12.9–75.4)
<65 y	3 of 4	19 (20%)	15.8% (3.2–33.8)
<70 y	3 of 4	39 (41%)	7.7% (1.4–17.4)
<75 y	4 of 4	58 (61%)	6.9% (1.8–14.4)
<80 y	4 of 4	76 (80%)	5.3% (1.3–11.1)
<All ages	4 of 4	95 (100%)	4.2% (1.0–8.9)
OCCPI cohort			
<50 y	2 of 4	8 (2.8%)	25.0% (3.3–55.1)
<55 y	3 of 4	14 (5.0%)	21.4% (4.7–44.0)
55 y	**4 of 4**	**17 (6.0%)**	**23.5% (7.0–44.5)**
<60 y	4 of 4	31 (11.0%)	12.9% (3.5–26.0)
<65 y	4 of 4	60 (21.4%)	6.7% (1.7–13.9)
<70 y	4 of 4	108 (38.4%)	3.7% (0.9–7.9)
<75 y	4 of 4	161 (57.3%)	2.5% (0.6–5.3)
<80 y	4 of 4	220 (78.3%)	1.8% (0.4–3.9)
All ages	4 of 4	281 (100%)	1.4% (0.3–3.1)

The detection rate shows the percentage of cases with 95% CI in which constitutional *MLH1* methylation (epimutation) was detected under/at a designated age threshold at diagnosis of CRC exhibiting MMRd and *MLH1* methylation. The optimal age threshold at which minimal screening of cases selected by these tumor features resulted in a high rate of detection, and most carriers of constitutional *MLH1* methylation identified were aged 55 years (shown in bold).

Abbreviations: Columbus, Columbus-area HNPCC study; CRC, colorectal cancer; MMRd, mismatch repair–deficient; OCCPI, Ohio Colorectal Cancer Prevention Initiative.

[a]There were no cases in the Columbus cohort aged 55 years; therefore, the rate of detection at age 55 years was the same as for age <55 years.