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JOURNAL OF THE NATIONAL COMPREHENSIVE CANCER NETWORK

Supplemental online content for:

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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J Natl Compr Canc Netw 2023;21(7):743–752.e11

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eAppendix 1. Supplemental Information

Patients with colorectal cancer (CRC) were selected from the Columbus-area HNPCC study (Columbus) cohort and the Ohio Colorectal Cancer Prevention Initiative (OCCPI) cohort, who were prospectively recruited to assess universal screening for mismatch repair deficiency (MMRd), Lynch syndrome (LS), and other hereditary cancer conditions. The overall process of selection of cases and sample size for this study is illustrated in the CONCORD diagram in supplemental eFigure 1.

Analytical Sensitivity of the Pyrosequencing Assay for the Detection of *MLH1* Methylation

To determine the limit of detection (LoD) of the *MLH1* pyrosequencing assay, at which a methylation-positive test result would be designated, a dilution series of expected methylation levels was generated and assayed. DNA from the *MLH1*-hypermethylated RKO CRC cancer line (American Type Culture Collection) was mixed with DNA from unmethylated peripheral blood lymphocytes of a healthy subject to a total of 500 ng of DNA in proportions of RKO:PBL of 100:0 (RKO only), 75:25, 50:50, 25:75, 10:90, 5:95, 2.5:97.5, 2:98, 1:99, and 0:100 (PBL only). The average methylation level of the hypermethylated RKO was 97%, so the expected values for *MLH1* methylation in the dilution series were adjusted accordingly. This series emphasized the lower range of values (0%–5% RKO) for increased precision in the range we anticipated the methylation-positive threshold value would lie. Each 500-ng sample in the dilution series was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research). A 50-ng aliquot of bisulfite-converted dilution point was input into each PCR, purified, and pyrosequenced in triplicate in 3 separate experiments. The actual values of *MLH1* methylation measured were plotted against the expected methylation values (supplemental eFigure 2). There was strong correlation between the actual versus expected values across the full 0% to 97% methylation range ($R^2>0.95$) (supplemental eFigure 2A). The LoD of methylation distinguishable above background noise was 2.3% (actual value) for the RKO:PBL 2.5%:97.5% dilution point with an expected value of 2.4% (supplemental eFigure 2B). This was also the lowest dilution point for which each of the 5 individual CpG sites within the assay gave a value ≥1 in all tests. Therefore, for designation of a methylation-positive test result, we applied a threshold of 2.3% average methylation detected across the 5 CpG sites measured, with each of the 5 CpG sites yielding a value ≥1.

Analytical Sensitivity of the Real-Time Methylation-Specific PCR Assay for the Detection of *MLH1* Methylation

Real-time semi-quantitative methylation-specific PCR (qMSP) for the detection of *MLH1* methylation was performed from c.-188 to c.-403 using SYBR Green fluorescent intercalating dye, followed by melt curve analysis (melt peak at 76°C ± 0.5°C indicates methylation specificity of the amplicon). The *MLH1* qMSP was performed alongside *MYOD* as a control for sample input and integrity (same run, separate reactions). Results were considered valid if the *MYOD* control had a cycle threshold (Ct) value <32. Semiquantification of methylated *MLH1* was calculated against *MYOD* with reference to a commercial enzymatically (*M.Ss*sI) fully methylated reference sample (Universal Methylated DNA Standard, Zymo Research) to produce a percentage of methylated reference (PMR) value as follows: The ΔΔCt value for each sample was calculated as $\Delta Ct_{Sample(MYOD-MLH1)} - \Delta Ct_{Reference(MYOD-MLH1)}$. The PMR was calculated as $2^{(\Delta\Delta Ct)} \times 100$. A dilution series of fully methylated DNA diluted into unmethylated DNA was generated by diluting the *M.Ss*sI enzymatically methylated reference sample into PBL DNA from unmethylated healthy subjects for expected values of 75%, 50%, 25%, 10%, 5%, 1%, 0.5%, and 0.1% *MLH1* methylation, followed by bisulfite conversion. Samples were tested in triplicate on 3 separate occasions, and the PMR was measured against the 100% methylated reference sample. This qMSP assay has high analytical sensitivity, with a LoD of 0.1% *MLH1* methylation (supplemental eFigure 3), enabling the detection of low-level mosaicism.

Patient samples were considered positive for constitutional *MLH1* methylation if their blood DNA sample yielded a PMR value ≥0.1% by qMSP. Illustrative amplification curves of the fully methylated reference sample used to calculate PMR values, as well as patient samples with positive and negative test results, are shown in supplemental eFigure 4. Illustrative postamplification melt curves indicating methylation specificity at 76°C ± 0.5°C are shown in supplemental eFigure 5.

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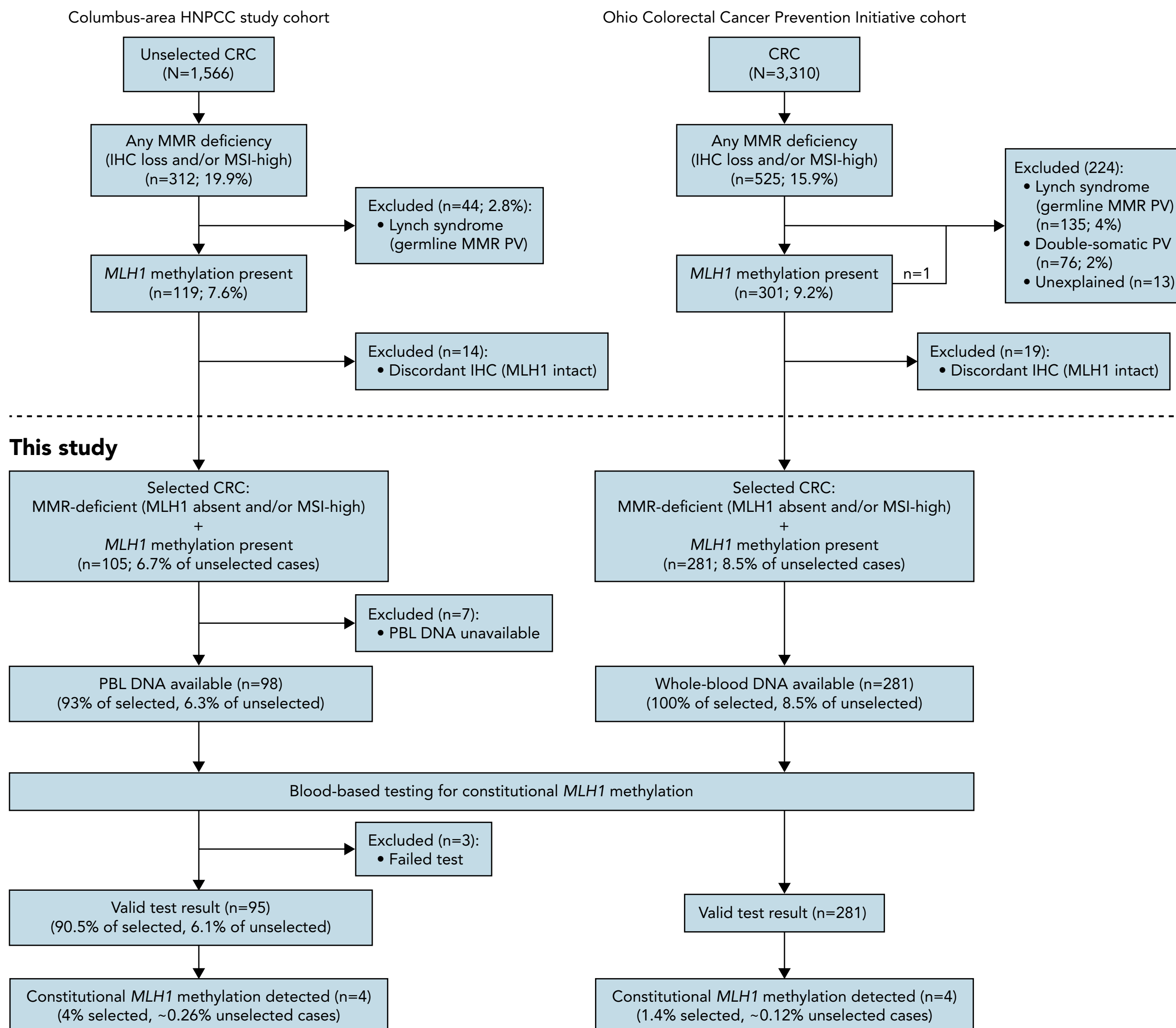
eAppendix 1. Supplemental Information (cont.)

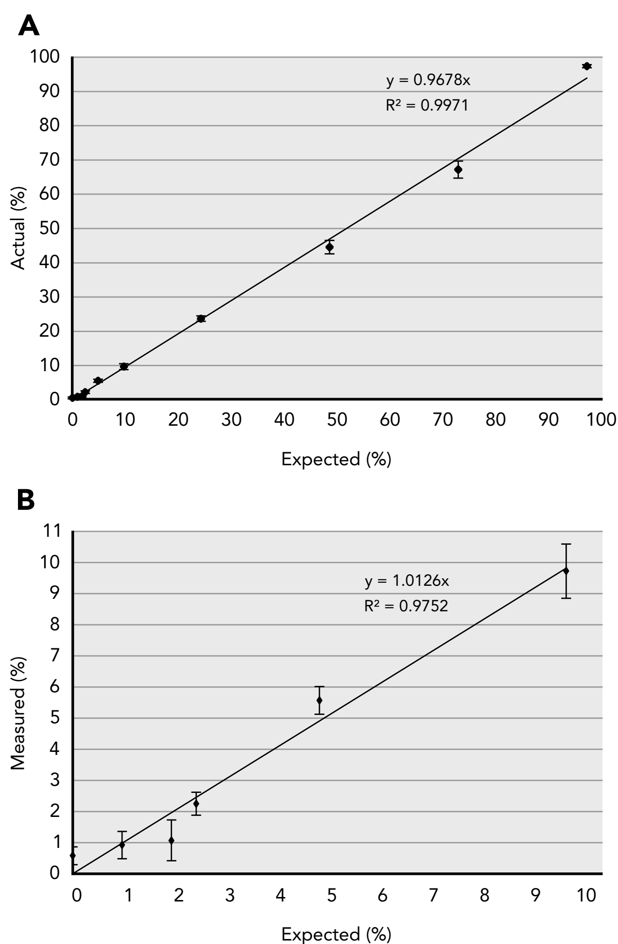
Posterior Probability of Detection of Constitutional *MLH1* Methylation at Various Rates and Ages

A Bayesian approach was used to estimate the posterior distribution for the probability of positive detection of constitutional *MLH1* methylation among the selected CRC cases tested from each cohort. The number of patients with constitutional *MLH1* methylation, Y, was modeled using a $Binomial(n,p)$ distribution, with $n =$ sample size and $p =$ probability of detection of constitutional *MLH1* methylation. A Jeffreys noninformative prior distribution, $Beta\ (1/2, 1/2)$, was assumed for p, such that posterior distribution is given by $Beta(1/2 + \mathrm{y}, 1/2 + \mathrm{n} - \mathrm{y})$. Based on the posterior distributions, the probability that positive detection of constitutional *MLH1* methylation is above a given detection threshold was calculated (supplemental eFigure 7).

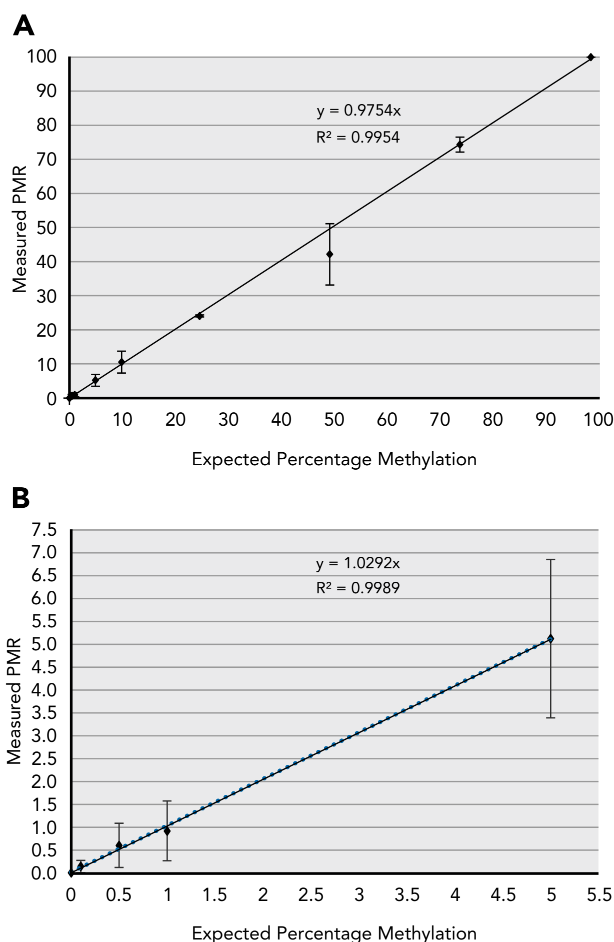
Actual values for posterior probability for detection of constitutional *MLH1* methylation in the 2 cohorts at age thresholds of particular interest and feasibility for screening are given in supplemental eTable 1.



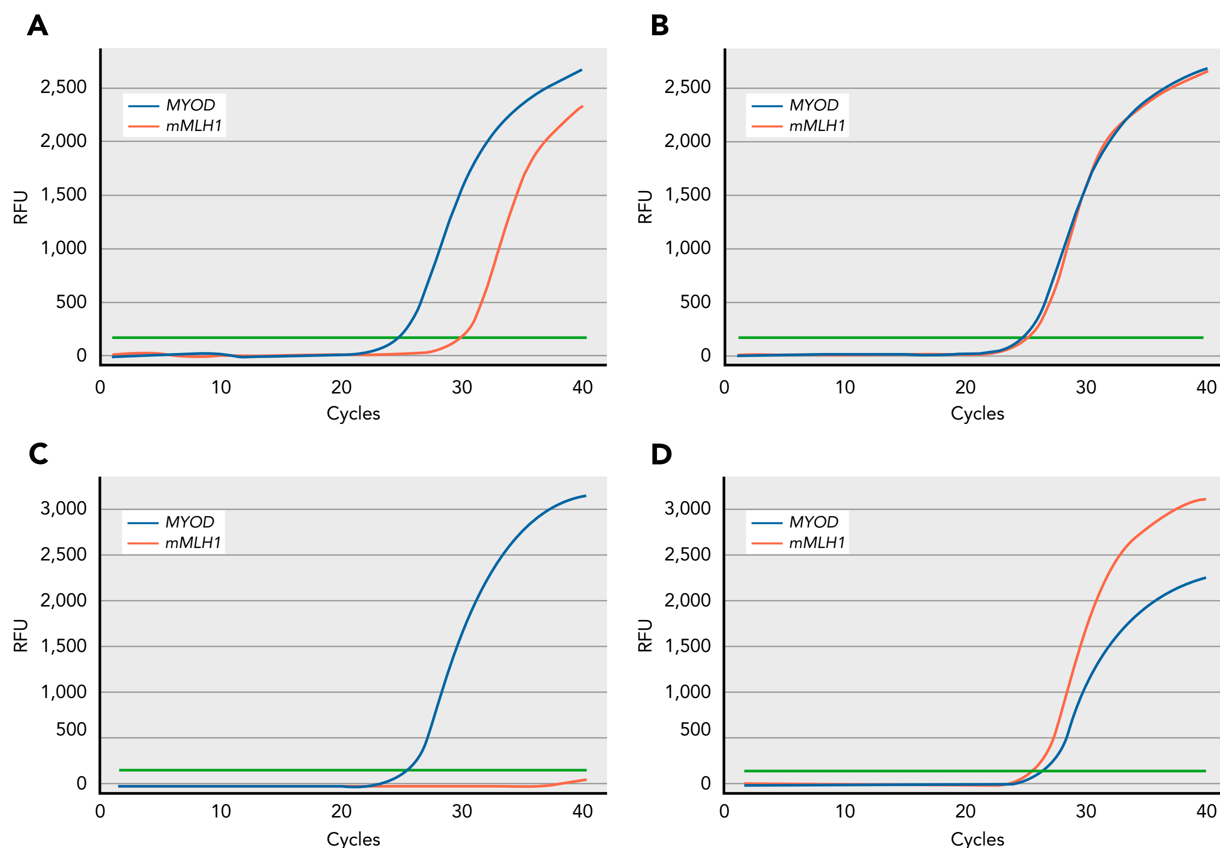
eFigure 1. Overall strategy for patient selection and numbers for constitutional *MLH1* methylation screening in 2 population-based cohorts of incident CRC in the state of Ohio.
Abbreviations: CRC, colorectal cancer; IHC, immunohistochemistry; MMR, mismatch repair; MMRd, mismatch repair–deficient; MSI, microsatellite instability; PBL, peripheral blood leukocyte; PV, pathogenic variant.



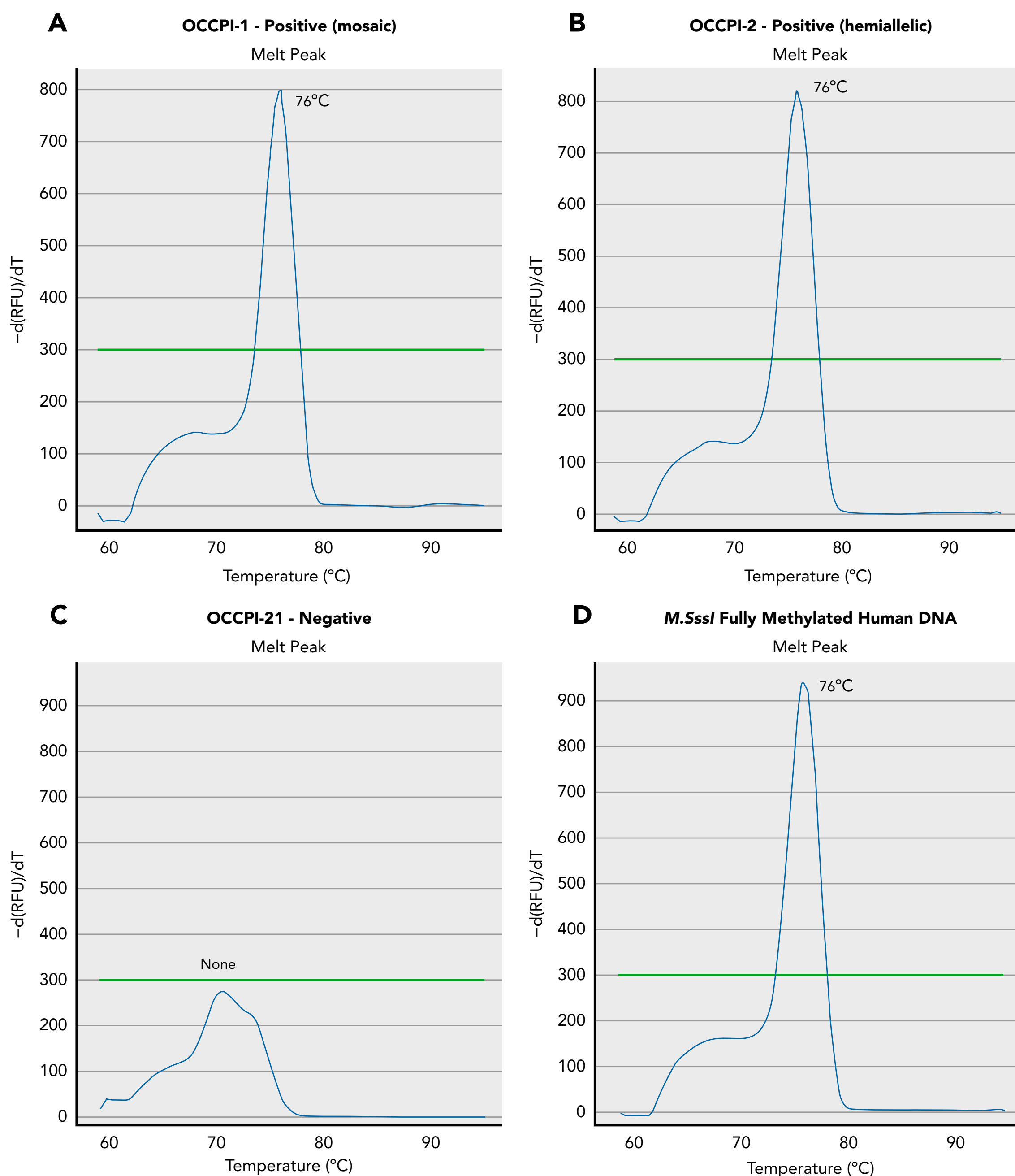
eFigure 2. Analytical sensitivity and linearity of *MLH1* methylation to measure expected percentage methylation plotted against expected percentage methylation levels in a dilution series of the hypermethylated colorectal cancer cell line, RKO, mixed with unmethylated healthy control peripheral blood lymphocytes in designated ratios. Plotted are the means of the mean methylation values across 3 experiments, where each dilution point was assayed in triplicate. Error bars show the SD of the means across the 3 experiments. **(A)** Full range of measured values versus expected values from 0% to 97%, showing a strong linear correlation between the actual values measured and the expected values ($R^2 > 0.95$). **(B)** Expanded view of the range of values between 0% and 10%, also showing a strong correlation between actual and expected values ($R^2 > 0.95$). Some background noise was consistently observed, even in the unmethylated sample (0% expected). The lowest actual value of methylation measured that was distinguishable above background noise and with nonoverlapping standard error bars was 2.3% (expected value 2.4%).



eFigure 3. Analytical sensitivity and linearity of the semiquantitative real-time methylation-specific PCR assay used to screen for constitutional *MLH1* methylation, particularly low-level mosaic methylation. **(A)** Full range of measured values versus expected values from 0% to 100%, showing a strong linear correlation between the measured values and the expected values ($R^2 > 0.99$), when calculated as a PMR. **(B)** Expanded view of the range of values between 0% and 5%, also show a strong correlation between measured and expected values ($R^2 > 0.99$). The limit of detection was a PMR of 0.1%.
Abbreviation: PMR, percentage of methylated reference.

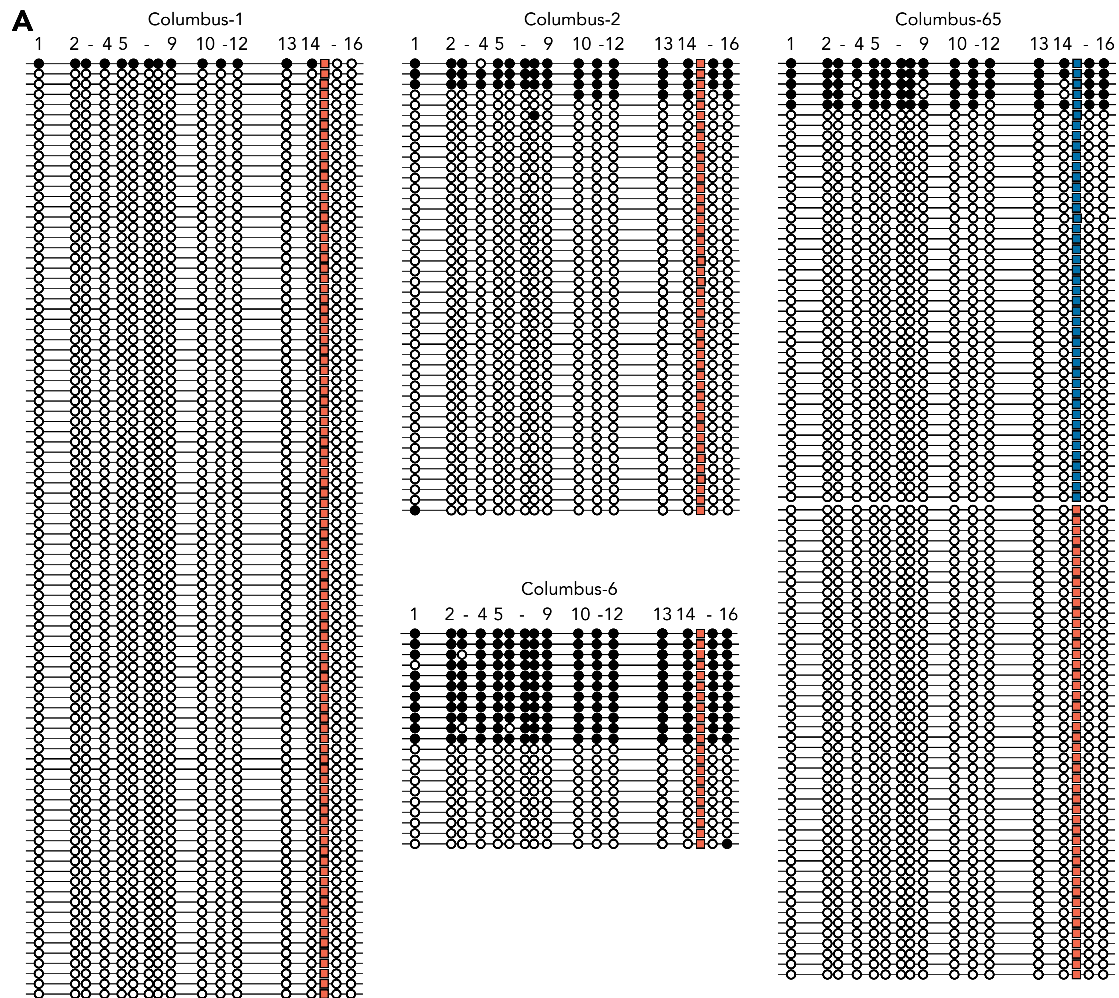


eFigure 4. Illustrative amplification profiles from real-time methylation-specific PCR assay for the detection and semiquantification of constitutional *MLH1* methylation. Blue curve shows amplification of the *MYOD* locus, which amplified regardless of methylation status and serves as the input control for sample sufficiently and integrity. Orange curve shows amplification of the *MLH1* locus only if it is methylated (*mMLH1*), because this assay is methylation-specific. Green horizontal line shows the cycle threshold at which levels of *MYOD* and *mMLH1* are measured. **(A, B)** Samples from CRC cases OCCPI-1 and OCCPI-2, which were positive for constitutional *MLH1* methylation at different levels (pyrosequencing and clonal bisulfite sequencing showed OCCPI-1 was mosaic and OCCPI-2 was hemiallelic). **(C)** OCCPI-21, a case that tested negative for constitutional *MLH1* methylation, illustrating the *mMLH1* line lies beneath the green cycle threshold. **(D)** *M.Sss*I fully methylated human DNA, which served as the reference sample for calculating percentage of methylated reference.
Abbreviations: CRC, colorectal cancer; OCCPI, Ohio Colorectal Cancer Prevention Initiative; RFU, relative fluorescence units.



eFigure 5. Postamplification melt curves indicating specificity for *MLH1* methylation after qMSP. **(A, B)** Samples for which qMSP amplification products were generated from methylated *MLH1* templates, and melt curves of the amplification products showed peak melt temperature of 76°C. **(C)** Sample that did not generate a qMSP amplification product, because it was negative for *MLH1* methylation, but low levels of primer dimer may have been present. The melt curve was not measured, because this was below the threshold level. **(D)** The fully methylated reference sample provided the reference melt peak temperature at which methylated products were measured. The bump to the left of the peak is produced by primer dimers or other nonspecific binding.

Abbreviations: OCCPI, Ohio Colorectal Cancer Prevention Initiative; qMSP, real-time methylation-specific PCR; RFU, relative fluorescence units.



eFigure 6. Clonal bisulfite sequencing confirms the presence of constitutional *MLH1* methylation across individual alleles in blood DNA. The methylation status at 16 consecutive CpG sites (circles) across a fragment of the *MLH1* CpG island promoter region from c.-48 to c.-370 (GenBank accession no. NG_007109.2) is shown within individual bisulfite-sequenced alleles (horizontal lines) in the blood DNA from CRC cases (as labeled) who tested positive for methylation by the CpG pyrosequencing and real-time methylation-specific PCR screening assays. The *MLH1* promoter fragment encompassed the common promoter SNP c.-93G>A (rs1800734), enabling the demonstration of allele-specific methylation in heterozygotes. **(A)** Columbus cohort cases. Columbus-1 had just ~1% alleles methylated, indicating low-level mosaicism. Columbus-2 had ~9% methylated alleles sequenced, whereas pyrosequencing measured higher methylation levels at 27%, indicating mosaicism. Columbus-6 had hemiallelic methylation (~50% alleles methylated), consistent with pyrosequencing (47.6% methylation) and epimutation of a single parental allele. Columbus-65 was heterozygous at SNP c.-93G>A, and methylation was specific to ~11% of alleles bearing the "A" genotype, indicating monoallelic mosaic methylation in up to 11% of leukocytes.

Abbreviations: Columbus, Columbus-area HNPCC study; CRC, colorectal cancer; SNP, single-nucleotide polymorphism.

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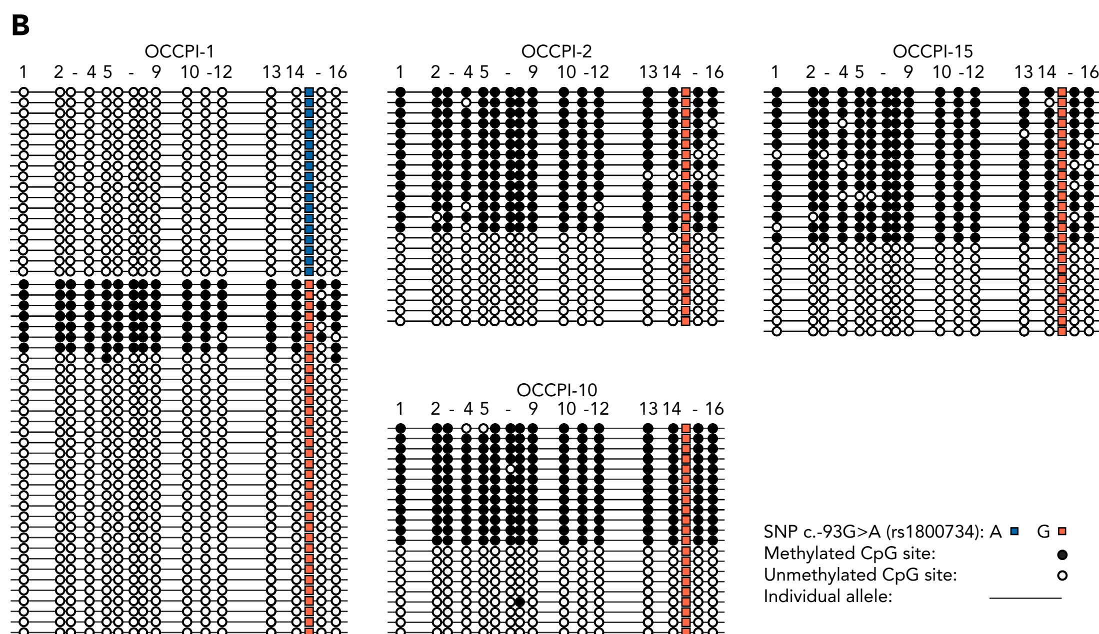
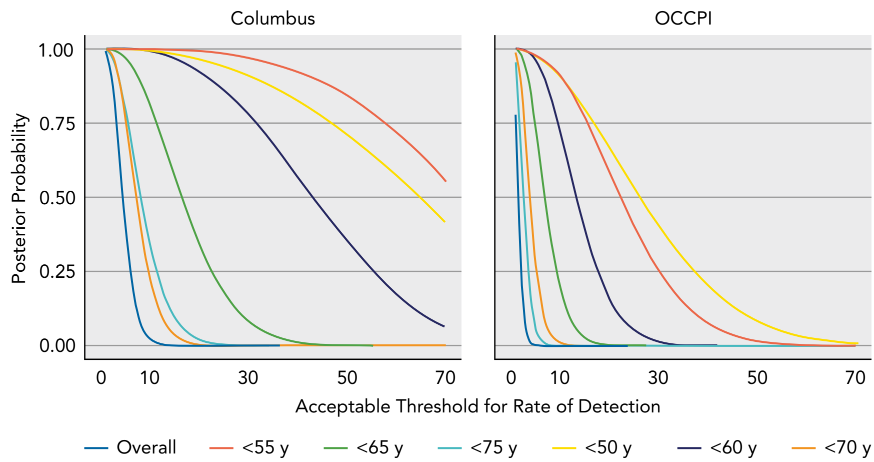


eFigure 6 (cont.). Clonal bisulfite sequencing confirms the presence of constitutional MLH1 methylation across individual alleles in blood DNA. The methylation status at 16 consecutive CpG sites (circles) across a fragment of the MLH1 CpG island promoter region from c.-48 to c.-370 (Gen-Bank accession no. NG_007109.2) is shown within individual bisulfite-sequenced alleles (horizontal lines) in the blood DNA from CRC cases (as labeled) who tested positive for methylation by the CpG pyrosequencing and real-time methylation-specific PCR screening assays. The MLH1 promoter fragment encompassed the common promoter SNP c.-93G.A (rs1800734), enabling the demonstration of allele-specific methylation in heterozygotes. **(B)** OCCPI cohort cases. OCCPI-1 was heterozygous for SNP c.-93G>A with ~20% of "G" alleles methylated. This was consistent with 4.2% methylation measured by pyrosequencing and together indicate mosaic monoallelic methylation present in ~8%–10% of leukocytes. OCCPI-2, -10, and -15 had hemiallelic methylation (~50% alleles methylated), consistent with 46%–50% methylation levels measured by pyrosequencing and epimutation affecting a single parental allele.

Abbreviations: CRC, colorectal cancer; OCCPI, Ohio Colorectal Cancer Prevention



eFigure 7. Posterior probability that the rate of detection of constitutional *MLH1* methylation will exceed the acceptable threshold for various detection rates under selected ages. Posterior probabilities are very high (*y* axis) that acceptable detection rates of 10% (*x* axis) will be exceeded by screening in patients aged <50, <55, and <60 years in the Columbus cohort and patients aged <50 and <55 years in the OCCPI cohort.
Abbreviations: Columbus, Columbus-area HNPCC study; OCCPI, Ohio Colorectal Cancer Prevention Initiative.

eTable 1. Posterior Probability That Positive Detection of Constitutional *MLH1* Methylation Is >X% in Patients With MMRd, *MLH1*-Methylated CRC

	Columbus Cohort	OCCPI Cohort
Overall (all ages)		
1%	0.993	0.778
5%	0.387	0.001
10%	0.020	0.000
Age <60 y		
1%	1.00	1.000
5%	0.999	0.964
10%	0.991	0.724
25%	0.862	0.052
Age ≤55 y[a]		
1%	1.000	1.000
5%	1.000	0.997
10%	0.999	0.956
25%	0.984	0.464
Age <55 y[a]		
1%	1.000	1.000
5%	1.000	0.988
10%	0.999	0.913
25%	0.984	0.399
Age <50 y		
1%	1.000	1.000
5%	0.999	0.981
10%	0.994	0.911
25%	0.942	0.529

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

[a]There were no cases aged 55 years in the Columbus cohort; therefore, posterior probabilities were the same for <55 years and ≤55 years in this cohort.