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Association between hypermethylation of DNA repetitive elements in white blood cell DNA and early-onset colorectal cancer

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Abbreviations: DNA, deoxyribonucleic acid; LINE-1, long interspersed nucleotide element 1; Sat2, satellite 2; OR, odds ratio; 95% CI, 95% confidence intervals; PMR, percent of methylated reference; BMI, body mass index

Changes in the methylation levels of DNA from white blood cells (WBCs) are putatively associated with an elevated risk for several cancers. The aim of this study was to investigate the association between colorectal cancer (CRC) and the methylation status of three DNA repetitive elements in DNA from peripheral blood. WBC DNA from 539 CRC cases diagnosed before 60 years of age and 242 sex and age frequency-matched healthy controls from the Australasian Colorectal Cancer Family Registry were assessed for methylation across DNA repetitive elements Alu, LINE-1 and Sat2 using MethyLight. The percentage of methylated reference (PMR) of cases and controls was calculated for each marker. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using multivariable logistic regression adjusted for potential confounders. CRC cases demonstrated a significantly higher median PMR for LINE-1 ($p < 0.001$), Sat2 ($p < 0.001$) and Alu repeats ($p = 0.02$) when compared with controls. For each of the DNA repetitive elements, individuals with PMR values in the highest quartile were significantly more likely to have CRC compared with those in the lowest quartile (LINE-1 OR = 2.34, 95% CI = 1.48–3.70; $p < 0.001$, Alu OR = 1.83, 95% CI = 1.17–2.86; $p = 0.01$, Sat2 OR = 1.72, 95% CI = 1.10–2.71; $p = 0.02$). When comparing the OR for the PMR of each marker across subgroups of CRC, only the Alu marker showed a significant difference in the 5-fluoruracil treated and nodal involvement subgroups (both $p = 0.002$). This association between increasing methylation levels of three DNA repetitive elements in WBC DNA and early-onset CRC is novel and may represent a potential epigenetic biomarker for early CRC detection.

Introduction

Colorectal cancer (CRC) screening programs, including fecal occult blood testing and colonoscopy, have proven to be effective in reducing CRC incidence for people aged 50 years and older in the US but the protection is incomplete.[1-3] A particular cause for concern is evidence of increasing trends in CRC incidence at younger ages.[4] An increase in the younger segment of the population is unlikely to be arrested by current screening programs, as individuals younger than age 50 are not routinely tested unless they are symptomatic or have a family history of CRC. Therefore, reliable markers of CRC risk would be important to develop targeted screening and consequently lead to further reductions in CRC related morbidity and mortality.

The measurement of DNA methylation levels from white blood cells (WBCs) has recently been investigated as a method of identifying cancer-affected individuals or those who are at a higher risk of cancer.[5] Previous studies have shown that both hypo- and hyper-methylation of DNA from WBCs is associated with several different types of neoplasia, including bladder, breast, renal, and head and neck cancer.[6-9] However, the association between methylation levels of DNA from leukocytes and the risk of CRC has not been well investigated. The measurement of methylation across DNA repetitive elements has been shown to be associated

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Table 1. Characteristics of CRC-affected cases and healthy controls

Characteristic	Controls		Cases		p-value
	(n = 242)		(n = 539)		
Demographic data	Freq	%	Freq	%	
Male	128	52.9%	293	54.4%	0.70
Age (years)					
At diagnosis/ interview*	50	(43, 56)	50	(43, 55)	0.37
At blood draw*	50	(43, 56)	52	(45, 57)	0.03
Caucasian	231	95.5%	512	95%	0.78
Smoking status					
Never	110	45.5%	249	46.2%	0.70
Former	87	36%	179	33.2%	
Current	45	18.6%	111	20.6%	
Drinking status					
Never	32	13.2%	107	19.9%	0.08
Former	21	8.7%	43	8%	
Current	189	78.1%	387	72.1%	
Body mass index (kg/m^2)*	25.1	(22.4, 28.4)	26.5	(23.5, 29.7)	< 0.001
Medicines/ supplements					
2 years ago, taking					
Folic acid	8	3.3%	12	2.3%	0.39
Aspirin	14	5.8%	25	4.6%	0.50
Ibuprofen	11	4.5%	21	3.9%	0.69
Have you ever taken					
Folic acid	39	16.2%	67	12.5%	0.17
Aspirin	30	12.4%	59	11%	0.56
Ibuprofen	50	20.7%	76	14.2%	0.02
Family history					
FDR$^{\Delta}$ with CRC	45	18.6%	130	24.1%	0.09
FDR/SDR† with CRC	80	33.1%	249	46.2%	0.001
Repetitive DNA elements					
Alu PMR*	70	(46, 94)	75	(50, 110)	0.02
LINE-1 PMR*	72	(48, 107)	87	(59, 130)	< 0.001
Sat2 PMR*	66	(49, 96)	80	(57, 111)	< 0.001

*Figures shown are median (Q1, Q3). $^{\Delta}$ FDR, first degree relative; †SDR, second degree relative.

with global methylation levels,[10] due to the fact they are well interspersed throughout the genome, comprising approximately 45% of the total DNA sequence.[11] A number of different classes of repetitive elements including Alu [a short interspersed nucleotide element (SINE) that is the most abundant repeat in the human genome[12]], LINE-1 (a long interspersed nucleotide element (LINE) comprising approximately 17% of the human genome[13]), and Satellite 2 (Sat2) (a short tandem repeat), have all been previously shown to demonstrate significantly different methylation levels in DNA from WBCs of cancer-affected cases compared with unaffected controls.[7,14-16]

The aim of this study was to investigate associations between the DNA methylation levels of three repetitive DNA elements, LINE-1, Alu and Sat2, in peripheral blood leukocytes and having CRC.

Results

The characteristics of the participants in this study comprising 539 cases diagnosed with CRC between the ages of 18 and 59 years (54.4% male) and 242 healthy controls (52.9% male) are listed in **Table 1**. The MethyLight inter-assay coefficients of variation (CV) for the Alu, LINE-1 and Sat2 markers were 0.52, 0.37 and 0.30, respectively. There was no significant difference in the mean or median age at diagnosis of the cases [mean age at diagnosis ± standard deviation (SD) = 48.8 ± 7.6 y; median = 50.0 y], when compared with the mean and median age at interview/blood draw for controls (49.0 ± 8.5 y; p = 0.74; median = 50.0 y, p = 0.37) (**Table 1**). The median time elapsed between age at diagnosis and blood draw for cases was 1 year, ranging from blood drawn at the time of diagnosis up to 14 years after diagnosis, where 92% of the CRC cases had their blood drawn within 2 years after CRC diagnosis. The median age of blood draw for the controls was 2 years younger than the median age of blood draw for the CRC cases (p = 0.03). Cases had a higher BMI and were less likely to use ibuprofen than controls (p ≤ 0.001 and p = 0.02, respectively); cases also reported a stronger family history of CRC than controls (**Table 1**).

Cases demonstrated significantly higher median PMR values across each of the three repetitive elements compared with controls (Alu, p = 0.02; LINE-1, p ≤ 0.001; Sat2, p ≤ 0.001; **Table 1**). When individuals were grouped into quartiles based on the PMR distribution for the controls, the highest levels of methylation (Q4) for each of the repetitive elements were significantly associated with higher odds of CRC compared with the lowest quartile (**Table 2**). The association was strongest for the LINE-1 repetitive element (OR = 2.34, 95% CI = 1.48–3.70, p < 0.001) followed by Alu (OR = 1.83, 95% CI = 1.17–2.86, p = 0.01) and Sat2 (OR = 1.72, 95% CI = 1.10–2.71, p = 0.02). For the LINE-1 and Sat2 DNA repetitive elements, higher odds of being CRC-affected were also observed for PMR values in the third quartile compared with the lowest (OR = 1.71, 95% CI = 1.07–2.71, p = 0.02; OR = 1.81, 95% CI = 1.14–2.85, p = 0.01, respectively; **Table 2**). For each of the DNA repetitive elements, a statistically significant increasing linear trend in ORs was observed from Q1 to Q4 (Alu p_{trend} = 0.005, LINE-1 p_{trend} < 0.001, Sat2 p_{trend} = 0.002; **Table 2**). There was evidence for a log-linear trend for LINE-1 and Sat2 when the PMR were analyzed as a continuous variable (p < 0.001 and p = 0.001, respectively), although less so for the Alu marker (p = 0.04). Similar effects were observed for each of the DNA repetitive elements when the analysis was restricted to CRC-affected cases that had their blood taken within 1 year of their diagnosis (n = 292, 54% of cases; **Table S1**).

The number of DNA repetitive elements with methylation levels (PMR values) in the highest quartile was also associated with a higher prevalence of CRC (**Table 3**). Results are presented for

Table 2. PMR values of CRC-affected cases analyzed as quartiles* (Q1 lowest, Q4 highest) and as a continuous variable* (OR per unit increase of logged PMR value) for each repetitive DNA element

	PMR quartile	Q1	Q2	Q3	Q4	Test for trend	PMR continuous
Sat2	P		0.31	0.01	0.02	0.002	0.001
	95% CI		(0.48, 1.26)	(1.14, 2.85)	(1.10, 2.71)		(1.22, 2.25)
	OR	Ref	0.78	1.81	1.72		1.66
LINE-1	P		0.34	0.02	< 0.001	< 0.001	< 0.001
	95% CI		(0.78, 2.06)	(1.07, 2.71)	(1.48, 3.70)		(1.25, 2.05)
	OR	Ref	1.27	1.71	2.34		1.6
Alu	P		0.59	0.84	0.01	0.005	0.04
	95% CI		(0.72, 1.79)	(0.66, 1.66)	(1.17, 2.86)		(1.01, 1.70)
	OR	Ref	1.13	1.05	1.83		1.31

*Models adjusted for age at blood draw (continuous), sex, body mass index (quartiles), smoking status, drinking status, ethnicity (Caucasian/not), and binary use of folate, aspirin and ibuprofen two years ago, and family history (FDR or SDR) of CRC.

each category, since the likelihood ratio test provided some evidence against a linear trend across categories (p = 0.06). Having three DNA repetitive elements with PMR values in the highest quartile nearly tripled the odds of being diagnosed with CRC compared with individuals with no DNA repetitive elements with methylation levels (PMR values) in the highest quartile (OR = 2.96, 95% CI = 1.56–5.59, p = 0.001).

When cases were placed into subgroups according to age at diagnosis, tumor characteristics]site, node status, T-stage, *BRAF* p.V600E mutation, mismatch repair (MMR)-status], chemotherapy treatment, germline mutation status (MMR gene or *MUTYH* mutations), and family history of CRC, we observed no significant difference in the association between PMR levels and CRC across subgroups (**Table 4**), other than for lymph node involvement and 5FU treatment subgroups, where the ORs were significantly different for the Alu repetitive element only (p = 0.002 for both).

Table 3. Combined effect of hypermethylation over multiple repetitive elements

Number of markers in highest quartile:	OR	95% CI	P
0	Ref		
1	1.99	(1.35, 2.94)	< 0.001
2	1.63	(1.04, 2.56)	0.03
3	2.96	(1.56, 5.59)	0.001
LRT test*			0.06

*LRT, likelihood ratio test: compares the categorical PMR variable with the continuous. Model fully adjusted as per **Table 2**.

Discussion

In this study, we observed a strong association between increased methylation levels of DNA repetitive elements Alu, LINE-1 and Sat2 in peripheral blood WBC DNA and CRC. This association did not depend on age, sex, BMI, family history, tumor stage, smoking status, alcohol intake, ethnicity, medication and supplement usage, or chemotherapeutic treatment. These findings suggest that increased DNA methylation in WBCs may represent a biomarker of CRC predisposition. Assessing WBC DNA methylation has the potential to be a highly cost-effective and tissue accessible approach to population screening in order to identify individuals at greater risk of developing CRC, improve early detection rates and ultimately reduce the incidence of CRC within the population, particularly for those individuals under age 50 who would normally not be screened for CRC and a group in which the incidence of CRC may be increasing.

Several previous studies have investigated the association between WBC global DNA methylation levels and neoplasia in the colorectum. Pufulete and colleagues[17] reported an association between hypomethylation in both colonic mucosa and leukocyte DNA and an increased risk for colorectal adenomas and a non-significant increased risk for CRC in 35 and 28 patients, respectively. Lim et al.[18] also described an association between global DNA hypomethylation and an increased risk for colorectal adenomas in the peripheral blood DNA of 115 women. In contrast, Nan et al.,[19] found no association between overall WBC DNA methylation level and CRC risk among 358 female CRC cases where blood samples had been collected prior to CRC diagnosis. More recent studies have focused on measuring DNA repetitive elements as surrogate markers for global DNA methylation levels. Kitkumthorn et al.[20] demonstrated significantly lower LINE-1 DNA methylation in the WBCs of 36 CRC-affected cases compared with controls. In a prospective study of WBC DNA methylation and cancer risk, including CRC, Zhu et al.[14] demonstrated a 3-fold increased incidence of all cancers in individuals with low LINE-1 methylation levels. No significant association however was observed between WBC DNA methylation and CRC, either at baseline or longitudinal CRC development, based on only 23 and 3 CRC cases, respectively.

A recent review by Brennan and Flanagan[21] has discussed some of the limitations of these previous studies regarding the reported associations between WBC DNA methylation and cancer risk. Differences in study design (retrospective vs. prospective), methylation testing methodology as well as the case and control

Table 4. Comparison of the association between PMR values (on the logarithm scale) and CRC risk between subgroups

Subgroup	Cases	Alu			LINE-1			Sat2		
	n	OR	95% CI	P*	OR	95% CI	P*	OR	95% CI	P*
5FU**										
No 5FU	173	2.01	(1.41, 2.84)	0.002	1.83	(1.33, 2.53)	0.33	1.91	(1.28, 2.85)	0.68
5FU	196	1.14	(0.81, 1.60)		1.56	(1.15, 2.11)		1.76	(1.20, 2.58)	
BRAF										
Negative	452	1.38	(1.05, 1.80)	0.41	1.64	(1.27, 2.12)	0.59	1.78	(1.29, 2.44)	0.09
Positive	31	1.08	(0.59, 1.97)		1.4	(0.79, 2.50)		0.98	(0.48, 2.00)	
MMR†										
Proficient	451	1.41	(1.08, 1.85)	0.23	1.66	(1.29, 2.15)	0.3	1.73	(1.26, 2.38)	0.78
Deficient	39	1.01	(0.57, 1.78)		1.27	(0.75, 2.15)		1.59	(0.84, 3.00)	
Nodes										
No positive	224	1.68	(1.25, 2.28)	0.002	1.73	(1.30, 2.30)	0.44	1.87	(1.32, 2.66)	0.24
Positive	204	1.05	(0.76, 1.45)		1.54	(1.14, 2.08)		1.52	(1.05, 2.20)	
T-stage										
T1/T2	219	1.32	(0.97, 1.79)	0.96	1.47	(1.09, 1.97)	0.29	1.66	(1.16, 2.38)	0.99
T3/T4	320	1.31	(0.98, 1.73)		1.7	(1.29, 2.24)		1.66	(1.19, 2.31)	
Age at diagnosis										
< 40 y	76	1.37	(0.89, 2.10)	0.35	1.98	(1.30, 3.00)	0.31	1.66	(1.01, 2.73)	0.75
40–< 50 y	170	1.12	(0.80, 1.56)		1.42	(1.03, 1.95)		1.81	(1.23, 2.67)	
50–59 y	293	1.41	(1.06, 1.88)		1.62	(1.23, 2.14)		1.58	(1.13, 2.20)	
Site										
Rectum	189	1.35	(0.97, 1.86)	0.08	1.58	(1.16, 2.15)	0.35	1.88	(1.29, 2.76)	0.81
Left colon	148	1.71	(1.20, 2.42)		1.9	(1.36, 2.66)		1.66	(1.10, 2.49)	
Right colon	126	1.09	(0.76, 1.57)		1.46	(1.04, 2.05)		1.7	(1.11, 2.60)	
Germline Mutation										
None Found	492	1.37	(1.05, 1.78)	0.07	1.63	(1.26, 2.10)	0.69	1.66	(1.22, 2.26)	0.96
MMR†	37	1.04	(0.58, 1.87)		1.46	(0.84, 2.53)		1.78	(0.94, 3.38)	
MUTYH	10	0.46	(0.17, 1.26)		1.09	(0.41, 2.90)		1.51	(0.46, 4.97)	
Family History										
None	40	1.18	(0.68, 2.04)	0.83	1.53	(0.91, 2.59)	0.97	2.01	(1.07, 3.76)	0.57
FDR$^{\Delta}$ CRC	130	1.26	(0.88, 1.80)		1.64	(1.16, 2.32)		1.74	(1.14, 2.65)	
FDR$^{\Delta}$ polypectomy	356	1.35	(1.02, 1.78)		1.6	(1.23, 2.08)		1.52	(1.10, 2.10)	

*P, likelihood ratio test p-value, comparing the OR for the PMR marker across subgroups. **Excluding T-stages 1 and 4. †MMR, mismatch repair; $^{\Delta}$FDR, first degree relative.

characteristics, including ethnicity, gender and mean age at diagnosis/blood draw, are likely to explain some of the differences in findings between previous studies of colorectal neoplasia and the present study. For example, Kitkumthorn et al.,[20] studied an Asian population of CRC-affected cases and controls compared with our study of predominantly Caucasian CRC-affected cases and controls. Across the previous studies described above, the mean age at diagnosis of colorectal neoplasia ranged from 60 to 74 years, which contrasts the median age at CRC diagnosis of the cases in this study (50 years). Furthermore, a potential confounder of the findings of two previous studies[17,20] was the difference in the age of the controls, which on average, were more than 10 years younger than the CRC-affected cases without mention of statistical adjustment for this age difference. The age at which blood is drawn from cases and controls is an important consideration since previous studies have shown that global de-methylation can occur with increasing age.[22,23] Therefore, we studied cases and controls matched for ethnicity, age and sex frequency in order to minimize any age-related effects on methylation levels. As a result, our findings may be more relevant for early-onset CRC risk rather than related to CRC risk overall, where the majority of CRC cases in the population are diagnosed over the age of 60 years.

Another potential explanation for the discrepancy between study findings is the different methodologies used for testing

methylation levels, each with their own limitations (discussed in Brennan and Flanagan, 2012).[21] Currently, sequencing of bisulphite converted DNA is considered the "gold standard" for DNA methylation analysis, however, this is a low-throughput method. More recent studies have utilized pyrosequencing to measure DNA repetitive element methylation; however, this approach only captures a small region of an otherwise large repeat, measuring only 4 CpGs between nucleotide 318–331 within the LINE-1 5' UTR.[24] In this study, we have utilized MethyLight, a methylation specific PCR-based assay that captures methylation across 10 CpGs between nucleotide 251 and 331 of the LINE-1 5' UTR.[10] When considering our findings we have shown hypermethylation of not just the LINE-1 repetitive element but for all three of the repetitive DNA elements tested, adding support to the concept that methylation levels throughout the genome may be altered. Replication of these and other study findings using the same methodology is needed.

Consistent with our findings of increased WBC DNA methylation being associated with CRC, a recent study demonstrated a significant increase in WBC DNA methylation levels for breast cancer cases compared with controls using the LUMA assay, however, no specific association with LINE-1 methylation was observed.[25] In addition, a study by Liao et al.,[7] on 328 renal cell carcinoma patients demonstrated an association between LINE-1 hypermethylation of WBC DNA and an increased risk of renal cell carcinoma. This suggests that peripheral blood DNA hypermethylation may be a marker of predisposition not only for CRC, but also for other cancers, including breast and renal cell carcinoma. However, these results differ from the majority of previous studies that have shown WBC DNA hypomethylation to be associated with cancer.[6,15,20,25-27]

DNA hypomethylation is believed to lead to chromosomal instability[28] by allowing silenced areas of the genome, such as retrotransposons, to become active. In contrast, our results showed that DNA hypermethylation in WBCs is associated with CRC risk. Whether it is DNA hypomethylation or hypermethylation that has been associated with cancer risk, the mechanism underlying this association between aberrant DNA methylation and cancer is unknown. When considering an underlying mechanism in the context of our findings, a previous study has observed in the DNA from WBCs of healthy controls and from cell lines that LINE-1 sequences containing double stranded DNA breaks have increased methylation levels around the area of the break when compared with samples without double strand breaks.[29] These findings demonstrate an association between DNA damage and hypermethylation in WBC DNA. The association we have observed between WBC DNA hypermethylation and CRC risk may be related to an increased frequency of double stranded DNA breaks in cancer-affected individuals, however, further studies are required to test this hypothesis.

Chemotherapy is an important factor to consider when investigating DNA methylation changes, as some studies suggest that chemotherapy can result in increased DNA methylation.[30-32] Apart from the Alu repetitive element, our results show no significant difference in the methylation levels of cases who underwent treatment with 5FU and those who did not, suggesting that 5FU treatment is unlikely to be the reason for the association with DNA hypermethylation and CRC risk we observed. In addition to chemotherapy, other exogenous or environmental factors may influence DNA methylation.[5] In this study, we adjusted for and observed no evidence of association with potential confounders of DNA methylation including cigarette smoking, drinking alcohol, BMI, use of NSAIDs and folate intake. Despite this, we cannot exclude the possibility that other unmeasured factors have contributed to the differences in methylation levels between cases and controls, as has been previously reported including *MTHFR* polymorphism[33] (altering folate metabolism), phytoestrogen intake,[34] arsenic exposure[35] and amount of physical activity.[36]

The strengths of this study include the large sample size of cases from a population-based resource, use of sex and age matched controls, and statistical analysis adjusting for multiple potential confounding factors including 5FU chemotherapy. Protocols for blood collection, processing and DNA extraction were consistent between cases and controls. When considering the limitations of this study, the time of blood draw for cases was after the diagnosis of CRC, which makes it unclear as to whether the DNA hypermethylation predisposes to CRC development (cause), or is related to metabolic changes caused by cancer growth or treatment (consequence). To further address this question of cause or consequence, prospective cohort studies will be needed to identify and quantify the risk, if any, between differences in peripheral blood methylation and cancer risk. Another consideration is that the DNA extracted from our participants was from whole blood. There can be differences in the cell count of a particular type of circulating WBC, with a recent study showing different DNA methylation levels between different WBC cell types.[37] Therefore, an individual's methylation profile may be dependent upon their composition of WBCs.[5] Inflammation is one biological response which can cause a shift in the distribution of particular WBC types, for example after surgery or during an infection.[38]

In summary, this study is the first to identify an association between the association of CRC and increased levels of methylation in three repetitive DNA elements in WBC DNA. Further studies are needed using prospective cohort designs in order to confirm this finding, further elucidate a cause or consequence association and to provide further evidence for the utility of measuring methylation levels within WBC DNA as a potential biomarker of CRC. Assessing the methylation levels of repetitive DNA elements from a routine blood sample is an attractive prospect for identifying individuals with early stage CRC, or possibly those at a higher risk of developing CRC, and therefore, has important health and economic benefits. Furthermore, an individual's WBC methylation level could be used in conjunction with other known CRC risk factors in risk prediction modeling to ultimately determine an accurate personalized CRC risk prediction.

Methods

Study participants. Incident CRC cases (n = 618) diagnosed before 60 years of age (45.6% diagnosed less than 50 years of

age) between 1997 and 2001 were ascertained from the Victorian Cancer Registry and recruited to the Australasian Colorectal Cancer Family Registry (ACCFR).[39] Recruitment of CRC affected individuals included all cases diagnosed between 18 and 44 years of age and 50% of cases with CRC diagnosed between 45 and 59 years of age, irrespective of family history. Cases diagnosed with Familial Adenomatous Polyposis were excluded from the study. WBC DNA was available from 588 of the 618 probands diagnosed with CRC (95%). Of these 588 cases, 547 had sufficient DNA available for bisulphite conversion and methylation analysis, of which, 8 samples failed testing. The remaining 539 cases with methylation data were included in the study. Healthy controls sex and age frequency matched to cases that were living in Victoria were randomly selected from the population using the Victorian Electoral Roll.

Interviews were conducted to obtain epidemiological information, personal and family history of cancer and dietary information using three questionnaires. Cigarette smoking status, alcohol consumption, body mass index (BMI) and use of medication (folic acid, aspirin and ibuprofen) used in this study were derived from the period two years before date of diagnosis for cases, or date of interview for controls. Cigarette smoking and alcohol consumption were categorized as current (within two years of date of diagnosis/interview), former (stopped before two years of date of diagnosis/interview) or never smokers/drinkers. Blood was taken at time of recruitment and is described here as the age at blood draw. Written informed consent was obtained from all cases and controls to collect a blood sample and tumor pathology materials (tumor blocks and diagnostic slides). This study was approved by the Human Research Ethics Committees of all participating institutions.

Molecular and pathological characterization. Colorectal tumors were characterized for mismatch repair deficiency using a ten-marker panel to assess microsatellite instability (MSI) and/or by immunohistochemistry for the four mismatch repair proteins as has been previously described.[40-42] Tumors were described as (1) mismatch repair deficient if they showed microsatellite instability and/or loss of expression of one or more of the mismatch repair proteins by immunohistochemistry or (2) mismatch repair proficient if tumors were microsatellite stable (or low level of instability) or showed stable expression of all four mismatch repair proteins by immunohistochemistry. In addition, tumors demonstrating loss of the MLH1 and PMS2 proteins by immunohistochemistry were characterized for methylation of the *MLH1* promoter using the MethyLight assay as previously described.[43,44] The *BRAF* V600E mutation status was tested for in all CRCs using a fluorescent allele-specific PCR assay to detect the somatic t: a mutation at nucleotide 1799 in exon 15 of the *BRAF* gene as has been previously described.[45]

Cases whose CRCs demonstrated mismatch repair deficiency through loss of expression of one or more of the mismatch repair proteins by immunohistochemistry and/or showed instability in 30% or more of the microsatellite markers underwent germline mutation testing (Sanger sequencing and MLPA) irrespective of the tumor *BRAF* V600E mutation status or methylation of the *MLH1* gene promoter.[39,44,46-48] All cases were tested for mutations in the *MUTYH* gene as previously described.[49] Those cases carrying a pathogenic mutation in a mismatch repair gene (Lynch Syndrome) or biallelic or compound heterozygous mutations in *MUTYH* were identified for the stratified analysis.

Primary CRC tissue from the Australasian Colorectal Cancer Family Registry Jeremy Jass Memorial Tissue Bank were reviewed by specialist GI pathologists for site, tumor grade, tumor stage, tumor margin, nodal involvement, presence of mucinous component, peritumoral lymphocytes, Crohn's-like lymphocytic reaction, tumor-infiltrating lymphocytes and synchronous CRC. Tumors from the ileo-cecal junction through the cecum, ascending colon, hepatic flexure, and transverse colon were grouped as right-sided (proximal) colon cancers (ICD-O-3 codes C180, C182, C183 and C184). Tumors in the splenic flexure (C185), descending (C186), sigmoid colon (C187) and recto-sigmoid junction (C199) were classified as left-sided (distal) colon cancers, with tumors in the rectum (C209) considered as a third distinct group.

LINE-1, Alu and Sat2 methylation detection. For each participant, WBCs were isolated from 18 mL of whole blood treated with the anti-coagulant EDTA. The DNA was extracted using a proteinase-K digestion and a salt-ethanol precipitation method which has been previously described.[50] DNA was suspended in 1× TE buffer (0.01 M Tris, pH 7.4; 0.1 mM EDTA, pH 8.0) for quantification, then an aliquot was diluted in water to a concentration of 50 ng/μL and stored at 4°C to limit freeze thawing.

Sodium bisulphite modification was performed on 1 μg of this DNA using the EZ-96 Methylation Gold Kit (Zymo Research, catalog number D5007), according to manufacturer's instructions. Following bisulphite conversion, 15 ng of DNA was used in the MethyLight real-time polymerase chain reaction for each DNA repetitive element region tested as previously described by Weisenberger et al.[10] and labeled as Alu-M2, LINE-1-M1 and Sat2-M1, with a separate Alu region (Alu-C4) being utilized as the reference gene.[10] The primer and probe sequences, mastermix and cycling conditions have also been previously described,[10] with amplification performed on the Corbett Rotorgene 6000 qPCR platform (Qiagen).

A standard curve was run in triplicate for each marker using four 1:10 serial dilutions of Universal Methylated DNA Standard (Zymo Research, catalog number D5011) with 1 μg bisulphite converted on each 96 well plate of samples undergoing conversion. The Universal Methylated DNA standard was also used as a calibrator (run in duplicate) for each MethyLight marker per run performed. A random subset of 56 DNA samples was tested in triplicate to assess the variation of the assay. The level of DNA methylation was expressed as the percentage of methylated reference (PMR), using the following formula as previously described:[30]

$$\mathrm{PMR}=100\times\frac{(\text{target gene})_{\text{sample}}/(\text{reference gene})_{\text{sample}}}{(\text{target gene})_{\text{calibrator}}/(\text{reference gene})_{\text{calibrator}}}$$

Statistical analysis. All statistical analyses were performed using Stata version 11.2 (StataCorp). The PMR values of each sample were obtained by calculating the above formula using

each calibrator replicate from that run, then taking the average of those two PMR values. Baseline characteristics of case and control subjects were compared using chi-square tests or Wilcoxon rank-sum tests, as appropriate.

Unconditional logistic regression models were used to assess the association between PMR values and CRC. Models were adjusted for all measured potentially confounding factors: age at blood draw (continuous), sex, BMI (categorized into quartiles), smoking status (current, former and never), alcohol drinking status (current, former and never), self-reported ethnicity (Caucasian/not), use of folic acid, aspirin and ibuprofen two years before recruitment (yes, no) and family history (first-degree relative (FDR) or second-degree relative (SDR)) of CRC. PMR values of cases were categorized into four ordered groups based on the quartiles among the control individuals for each repetitive DNA element. The association between PMR quartile and CRC was assessed using three separate logistic regression models. Tests for trend were conducted by assigning each PMR quartile its median value and fitting this as a linear effect. Models were also fit using the natural logarithm of each PMR variable as a linear effect, with odds ratios representing the association per unit increase of the logged PMR variable. Odds ratios (ORs) were calculated with 95% confidence intervals (CIs) and two-tailed Wald test p-values.

A combined measure of hypermethylation across the three repetitive elements was assessed by counting the number of repetitive elements with PMR levels in the highest quartile (Q4) for each individual. This variable was included in a logistic regression model similar to those described previously. Linearity of the association between the combined hypermethylation variable and CRC was assessed using the likelihood ratio test.

Associations between PMR values and CRC risk were compared between different subgroups of CRC using multinomial regression adjusted for the confounding variables given above, allowing the effects of age, sex and PMR to differ between subgroups, while restricting the effects of other confounders to be the same across subgroups. The natural logarithms of the PMR variables were fitted as linear effects. Models using grouped PMR values gave similar results. The likelihood ratio test was used to assess whether the associations between PMR and CRC differed by subgroup. For analyses comparing subgroups defined by receipt of 5-fluoruracil (5FU) treatment, cases with T-stage 1 or 4 were excluded from the analysis as T-stage 1 cases rarely had chemotherapy and T-stage 4 cases almost always had chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Ethical Statement

Written informed consent was obtained from all study participants. The study protocol was approved by the QIMR HREC under protocol P628.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/25178

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