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Characterisation of colorectal cancers showing hypermethylation at multiple CpG islands

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Background and aims: A subgroup of colorectal cancers (CRC) referred to as the CpG island methylator phenotype (CIMP+) shows simultaneous methylation of multiple CpG islands. The clinicopathological and molecular characteristics of this phenotype remain uncertain however.
Methods: We analysed methylation of CpG islands in the *p16* and *MDR1* genes and *MINT-2* clone in 275 stage II/III CRCs.
Results: Concurrent methylation of two or more CpG islands was observed in 32% of cases and was considered to represent CIMP+. These were often poorly differentiated, had less *TP53* mutations, and originated frequently in the proximal or higher stage CRC compared with CIMP– tumours ($p<0.05$ for each). CIMP+ had no prognostic significance in stage II or stage III CRC treated by surgery alone. *hMLH1* methylated tumours comprised the majority (81%) of cases with microsatellite instability, were frequently observed in older female patients, were often poorly differentiated or CIMP+, and contained wild-type K-*ras* ($p<0.05$ for each). Females who were heterozygous or homozygous for the C677T *MTHFR* polymorphism were at increased risk of developing CIMP+ CRC (odds ratio 2.17, 95% confidence interval 1.03–4.57; p=0.037).
Conclusions: These observations made in a relatively large unselected series of CRC support the notion that CIMP+ characterises a subgroup of tumours with distinctive phenotypic features.

Transcriptional silencing of tumour suppressor genes by hypermethylation of CpG islands located in the promoter region has been proposed as an important mechanism for the development of a variety of cancer types.[1 2] A CpG island methylator phenotype (CIMP+) has been described in colorectal cancer (CRC) and is characterised by simultaneous methylation of multiple genes including *p16*, *THBS1*, *IGF2*, *HIC-1*, and *COX-2*.[3–5] In a small proportion of CRCs the DNA mismatch repair gene *hMLH1* is also methylated. This specific epigenetic alteration gives rise to the majority of tumours with the microsatellite instability (MSI+) phenotype.[6–8] The precise mechanism for the aberrant DNA methylation seen in CIMP+ tumours remains to be determined but two features of this phenotype that are consistently observed in CRC are origin in the proximal colon and poorly differentiated histology.[4 9 10] Less clear cut are possible associations between CIMP+ and patient features such as age, sex, and survival.[9 11–13] Contradictory reports have also been published on the relationship between CIMP+ and the tumour specific molecular features of *TP53* mutation, K-*ras* mutation, and MSI+.[11–15]

One of the difficulties in attempts to characterise CIMP+ has been the use of selected CRC series, in particular enrichment with MSI+ cases. The majority of sporadic MSI+ tumours have a methylated *hMLH1* gene[6–8] and are therefore likely to be CIMP+ whereas familial MSI+ cases from the hereditary non-polyposis colon cancer syndrome show less *hMLH1* methylation[8] and are more likely to be CIMP–. Hence the methylation characteristics of MSI+ tumours may depend on whether they are sporadic or familial in origin. Another problem has been analysis of relatively small tumour cohorts, thereby limiting the statistical power to detect associations between CIMP+ and other molecular or clinical features. With the exception of a recent study in 119 CRC cases examined for p*16* and *p14* methylation,[16] there have been no reports to our knowledge examining methylation of multiple CpG sites in a large number of unselected CRCs. Consequently, the biological and clinical properties of CIMP+ remain largely unknown. Possible involvement of either or both the MSI+ and CIMP+ phenotypes in response of CRC to 5-fluorouracil (5-FU) based adjuvant therapy[17] highlights the need for further characterisation.

In the present study we have attempted to overcome the above limitations by examining the CIMP+ phenotype in a large (n=275) unselected CRC series. We chose to analyse hypermethylation of the *p16* and *MDR1* genes and of the *MINT-2* clone because these sites are frequently (20–40%) and specifically methylated in CRC, but not in adjacent normal epithelium.[3 4 15] Methylation of *p16*[18 19] and *MDR1*[20] genes is known to be associated with transcriptional silencing of expression and may therefore have functional significance for the development of a malignant phenotype. The CRC series investigated in this study has previously been characterised for site of tumour origin, histological grade, patient outcome,[17] *TP53* mutation,[21] K-*ras* mutation,[22] MSI+,[21] and the C677T polymorphism of the methylenetetrahydrofolate reductase (*MTHFR*) gene.[23] This has allowed us to shed additional light on the characteristics of CIMP+ CRC.

MATERIALS AND METHODS

Patients

A series of 133 stage II or III CRCs that were surgically resected between 1991 and 1996 at the Sir Charles Gairdner Hospital, Nedlands, Australia, were prospectively included in a study designed to evaluate the prognostic significance of various molecular genetic alterations. Tumour samples were snap frozen immediately after surgery and stored at –80°C. An additional unselected series of 142 paraffin embedded stage III CRC cases that were surgically treated between 1991 and

Abbreviations: CRC, colorectal cancer; MSI, microsatellite instability; CIMP+, CpG island methylator phenotype; MTHFR, methylenetetrahydrofolate reductase; PCR, polymerase chain reaction; MSP, methylation sensitive PCR; SSCP, single strand conformation polymorphism; 5-FU, 5-fluorouracil.

1996 were also analysed. All tumours had negative resection margins. Adjuvant chemotherapy according to the Moertel regimen[24] was administered to 39 patients. These cases were excluded from survival analyses. Ethics approval for the project was obtained from the Sir Charles Gairdner Hospital Human Research Ethics Committee. Information on disease specific survival was obtained from the West Australian Health Department death register and from hospital medical records. Survival data on patients who died from causes other than CRC were censored at the time of death. The splenic flexure was used as the anatomical boundary to define proximal and distal CRC.

Methylation specific PCR (MSP)

DNA suitable for polymerase chain reaction (PCR) analysis was extracted from frozen tissue according to standard methods and from paraffin embedded samples using previously described methods.[17] Methylation specific PCR (MSP) for the detection of methylation in the promoter regions of *p16*, *hMLH1*, and the clone *MINT-2* was carried out as described previously.[9 25] This technique uses bisulphite modification to convert unmethylated, but not methylated, cytosine to uracil. MSP utilises this difference to specifically amplify either methylated or unmethylated DNA. Bisulphite (Sigma, St Louis, Missouri, USA) conversion of 1 μg of genomic DNA was carried out for 16 hours at 55°C according to a modified method[26] but without prior digestion. Primers for the promoter region of *MDR1* were designed to include six CpG dinucleotides that have been linked to regulation of *MDR1* expression.[20] Primer sequences for amplification of unmethylated *MDR1* were:

forward 5′-GGGTGTGGGTTGAGTATAGTTGTTTT-3′

reverse 5′-CCAACTTTACATACCCCTACCTCACA-3′,

and for methylated *MDR1* were:

forward 5′-GGGCGTGGGTTGAGTATAGTCGTTTC-3′

reverse 5′-CGCTCCTTAAAACAACCACCAAAACG-3′.

The annealing temperature was 56°C for unmethylated DNA and 60°C for methylated DNA, with a Mg^{2+} concentration of 1.6 mM. PCR reaction mixes contained 1×polymerisation buffer, 1×Q-solution (Qiagen, Melbourne, Australia), the optimal Mg^{2+} concentration for that primer pair, 0.4 μM of each primer, and 0.5 U *Taq* polymerase (Qiagen, Melbourne, Australia) in a total volume of 20 μl. PCR reactions were "hot started" at 94°C by addition of 100 ng of bisulphite converted DNA, followed by 35 cycles (30 seconds at 94°C, 30 seconds at the appropriate annealing temperature, 30 seconds at 72°C) and seven minutes of final extension at 72°C. PCR products were analysed on 2.5% agarose gels. Both methylated (HT-29 CRC cell line) and unmethylated DNA (peripheral blood lymphocytes) controls were included in every bisulphite conversion.

Screening for MSI+, TP53 mutation, K-ras mutation, and MTHFR genotype

The MSI status of each tumour was evaluated by fluorescent single strand conformation polymorphism (SSCP) analysis of deletions in the BAT-26 mononucleotide repeat, as described previously.[27] Deletions in the quasimonomorphic BAT-26 allele establish the MSI status of tumours with more than 99% accuracy in Caucasian populations.[28] Tumour samples were also screened for mutations in exons 4–10 of the *TP53* gene[21] and in codons 12 and 13 of the K-*ras* gene using fluorescent-SSCP,[22] as described previously by our laboratory. All suspected *TP53* and K-*ras* mutations were confirmed at least once by separate PCR-SSCP analyses as well as by DNA sequencing.[29] Genotyping for the C677T polymorphism in the *MTHFR* gene was carried out using silver stain and fluorescent SSCP methods, as described recently by our laboratory, and confirmed by *HinfI* restriction enzyme digestion.[23] The incidence of CT and TT *MTHFR* genotypes in CRC cases was compared to that observed in an age matched series of 467 healthy Australian individuals (range 60–92 years).[23]

Statistical analysis

Data were analysed using the SPSS 10.0 (Chicago, Illinois, USA) software package. Contingency tables were analysed using the χ^2 test (Pearson statistic) or Fisher's exact test when expected frequencies were lower than five. Kaplan-Meier analysis was used to assess five year cumulative survival probability and differences were evaluated using the log rank test. Cox's proportional hazards univariate and multivariate analyses were used to calculate hazard ratios and 95% confidence intervals. Wald's test was used to calculate 95% confidence intervals for odds ratios. All p values are derived from two tailed statistical tests.

RESULTS

Methylation of *p16*, *MDR1*, and *MINT-2* was analysed in 275 stage II/III CRC. To assess the sensitivity of MSP, DNA obtained from methylated and unmethylated control samples was mixed at different ratios prior to bisulphite treatment. MSP was able to detect as little as 3% methylated *p16* allele content. The ratio of methylated/non-methylated allele for each sample was not evaluated because microdissection had not been carried out to remove contaminating normal tissue. Comparison of MSP results for *p16*, *MDR1*, and *MINT-2* revealed perfect concordance between DNA obtained from 23 frozen tumours and from the corresponding paraffin embedded archival tissue blocks. This confirms previous observations on the suitability of DNA obtained from paraffin embedded tissue for methylation studies.[30] Although the success rate for the MSP technique using archival DNA was approximately 70% in the current study, the intensity of bands was similar to that observed from frozen tissue DNA. Only samples showing clear results for all three CpG sites were included in the analysis. An example of MSP results obtained from archival DNA for the *p16* gene are shown in fig 1.

Methylation and clinicopathological features

Methylation of *p16*, *MDR1*, and *MINT-2* was detected in 36%, 24%, and 37% of tumours, respectively. The characteristics of tumours with individual or multiple methylated sites are summarised in table 1. When methylation of individual sites was analysed, no sex or age differences were apparent. Methylation of at least one or two CpG islands was more frequent in higher stage tumours. All three sites showed approximately twofold more frequent methylation in proximal compared with distal colon tumours, with even less methylation seen in rectal tumours ($p<0.001$ for each). Poorly differentiated tumours showed 2–3-fold higher frequency of *p16*, *MDR1*, and *MINT-2* methylation compared with well/moderately differentiated tumours ($p<0.005$ for each). Methylation of CpG islands occurred in a non-random manner, with concordant methylation at two or more sites observed in 32% of cases (table 1, fig 2). These were defined arbitrarily in the present study as being CIMP+.

Figure 1 Methylation sensitive polymerase chain reaction (MSP) results for the *p16* gene. DNA for MSP amplification was obtained from paraffin embedded tissue (lanes 1–4) and from frozen tissue (lanes 5–7). Samples 1, 2, 3, 5, and 6 were scored as unmethylated for the *p16* gene whereas samples 4 and 7 were scored as methylated.

Table 1 Associations between methylation and clinicopathological features of colorectal cancer

Feature (n)	Methylation (%)					
	p16	*MDR1*	*MINT-2*	One	Two	Three
Total (275)	100 (36)	67 (24)	102 (37)	141 (51)	88 (32)	40 (15)
Sex						
Female (137)	51 (37)	35 (26)	56 (41)	70 (51)	47 (34)	25 (18)
Male (138)	49 (36)	32 (23)	46 (33)	71 (51)	41 (30)	15 (11)†
Age						
<71 y (144)	50 (35)	35 (24)	49 (34)	69 (48)	47 (33)	18 (13)
⩾71 y (131)	50 (38)	32 (24)	53 (40)	72 (55)	41 (31)	22 (17)
Stage						
II (72)	21 (29)	13 (18)	20 (28)	27 (38)	16 (22)	11 (15)
III (203)	79 (39)*	54 (27)*	82 (40)†	114 (56)‡	72 (36)‡	29 (14)
Site						
Rectum (42)	9 (21)	2 (5)	9 (21)	15 (36)	5 (12)	0 (0)
Distal colon (77)	20 (26)	11 (14)	18 (23)	29 (38)	16 (21)	4 (5)
Proximal colon (152)	71 (47)§	54 (36)§	75 (49)§	114 (64)§	67 (44)§	36 (24)§
Grade						
Well/moderate (211)	62 (29)	39 (19)	65 (31)	93 (44)	55 (26)	18 (9)
Poor (61)	36 (59)§	27 (44)§	36 (59)§	46 (75)§	32 (53)§	21 (34)§

*p<0.2; †p<0.1; ‡p<0.05; §p<0.005.

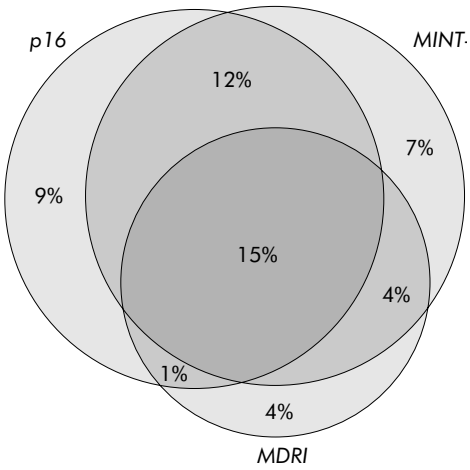

Figure 2 Venn diagram showing overlap in the methylation of *p16*, *MDR1*, and *MINT-2* observed in 275 colorectal cancer cases. CpG island methylator phenotype tumours were defined as having two or more sites methylated. Percentages shown are absolute values.

Because methylation was more frequent in stage III cases (table 1), the prognostic significance of CpG island methylation was examined separately for patients with stage II or III disease. Only patients treated by surgery alone were included in these analyses because of a possible interaction between CIMP+ and chemotherapy.[17] Kaplan-Meier and Cox regression analyses revealed no associations between methylation at individual or multiple sites and overall patient survival (results not shown).

Methylation and somatic alterations

Associations between CpG island methylation and several other molecular features that were previously characterised in this tumour series[21 22 29] are shown in table 2. Tumours with wild-type *TP53* showed more frequent methylation compared with those with mutant *TP53*, especially for *MINT-2* and for multiple sites. Similarly, methylation was more frequent in tumours with wild-type K-*ras*, reaching significance for tumours with methylated *MDR1*, two or more, and with all three sites methylated. No differences between the type of *TP53* or K-*ras* mutation and methylation were observed (results not shown). As expected, methylation was 2–3-fold more frequent in MSI+ compared with MSI– tumours.

Differences between CIMP+ and MSI+ phenotypes

Recent studies suggest that the CIMP+ and MSI+ phenotypes are closely related.[4 31] In order to characterise CIMP+ independently of MSI+, all 41 MSI+ cases were removed from the present series. The remaining 58 tumours with two or more methylated sites (CIMP+/MSI–) still showed significant associations with the features of proximal tumour origin,

Table 2 Associations between methylation and molecular features of colorectal cancer

Feature (n)	Methylation (%)					
	p16	*MDR1*	*MINT-2*	One	Two	Three
Total (275)	100 (36)	67 (24)	102 (37)	141 (51)	88 (32)	40 (15)
TP53						
Wild-type (152)	59 (39)	40 (26)	62 (41)	79 (52)	54 (36)	28 (18)
Mutant (103)	30 (29)*	18 (18)†	26 (25)‡	46 (44)	21 (20)‡	7 (7)‡
K-*ras*						
Wild-type (138)	51 (37)	41 (30)	50 (36)	67 (46)	49 (36)	26 (19)
Mutant (65)	17 (26)*	7 (11)§	18 (28)	28 (44)	11 (17)‡	3 (5)‡
MSI						
– (228)	70 (31)	40 (18)	70 (31)	106 (45)	58 (25)	16 (7)
+ (41)	28 (68)§	27 (66)§	30 (73)§	32 (77)§	29 (71)§	24 (59)§

*p<0.2; †p<0.1; ‡p<0.05; §p<0.005.
MSI, microsatelite instability.

Table 3 Characteristics of microsatellite instability (MSI+) colorectal cancer with or without *hMLH1* methylation

Feature (n)	*hMLH1* (%)		
	Unmethylated	Methylated	p Value
Total (54)	10 (19)	44 (81)	
Sex			
Female (33)	3 (9)	30 (91)	
Male (21)	7 (33)	14 (67)	0.035†
Age			
<68 y (27)	9 (33)	18 (67)	
⩾68 y (27)	1 (4)	26 (96)	0.005‡
Site			
Distal (4)	2 (50)	2 (50)	
Proximal (49)	7 (14)	42 (86)	0.13†
Grade			
Well/moderate (31)	9 (29)	22 (71)	
Poor (22)	1 (5)	21 (95)	0.033†
TP53			
Wild-type (42)	9 (21)	33 (79)	
Mutant (8)	1 (13)	7 (87)	NS
K-*ras*			
Wild-type (41)	5 (12)	36 (88)	
Mutant (8)	4 (50)	4 (50)	0.028†
CpG methylation*			
<2 sites (15)	10 (67)	5 (33)	
⩾2 sites (34)	0 (0)	34 (100)	<0.001†

**p16*, *MDR1*, and *MINT-2*.
†Fisher's exact test.
‡χ^2 test.

higher tumour stage, poor differentiation, and wild-type *TP53* ($p<0.05$ for each), but not with the presence of wild-type K-*ras*. These features appear therefore to be characteristic of CIMP+ tumours independently of the MSI+ phenotype.

The majority of sporadic MSI+ tumours show methylation of the *hMLH1* promoter[6–8] and these are presumably CIMP+/MSI+. The remaining MSI+ cases are more likely to have germline or somatic mutations, or loss of heterozygosity of one or more of the DNA mismatch repair genes[8] and might therefore be CIMP–/MSI+. In the present study, we examined *hMLH1* methylation in the 41 MSI+ cases, as well as an additional 14 MSI+ cases from an earlier series.[21] This allowed us to compare the features of MSI+ tumours with or without *hMLH1* methylation (table 3). MSI+ tumours with methylated *hMLH1* frequently were CIMP+ or poorly differentiated and tended to be more common in older (mean age 70 *v* 49 years for non-methylated cases) female patients. They also showed less frequent K-*ras*, but not *TP53*, mutations. Although the number of unmethylated MSI+ cases in this study was small (n=10), our findings suggest that in contrast with methylated tumours, they were mostly found in younger (9/10) male (7/10) patients and did not show a tendency to be poorly differentiated (1/10).

Methylation and *MTHFR* genotype

The C677T polymorphism in the *MTHFR* gene has only 30% the level of enzymatic activity when present as the homozygote (TT) compared with the wild-type.[32] Compared with age matched controls, we recently found the TT genotype to be twice as frequent in CRC patients with MSI+ tumours.[23] We hypothesised that aberrant methyl group metabolism in these individuals could predispose them to CIMP+ CRC. In the present study we investigated possible associations between *MTHFR* genotype and tumour specific methylation at CpG islands. Because of the relatively small numbers, individuals with the TT genotype were combined with CT genotype individuals. No associations between CIMP+ and the CT/TT genotype were observed for the entire group (table 4). However, a twofold increased risk of CIMP+ CRC was observed for CT/TT females compared with age matched controls. The CT/TT *MTHFR* genotype was also significantly associated with methylation of *p16* (odds ratio 2.40 (95% confidence interval 1.19–4.85); p=0.01) and *MINT-2* (1.97 (1.01–3.84); p=0.04) in females. When all MSI+ cases were excluded, significant associations between the *MTHFR* genotype and CIMP+, *p16*, or *MINT-2* methylation were still observed. These results suggest that a CT/TT *MTHFR* genotype can predispose females to the development of CIMP+ tumours independently of MSI status.

DISCUSSION

In the present study, we examined methylation of *p16*, *MDR1*, and *MINT-2* in a large unselected series of CRC. These cases were well characterised for clinicopathological features, including patient survival, as well as for various molecular alterations. The major findings were: (i) CIMP+ tumours were frequently poorly differentiated, often occurred in proximal or higher stage tumours, and had a lower frequency of *TP53* mutations compared with CIMP– tumours; (ii) methylation of the CpG sites investigated had no prognostic value for patients treated by surgery alone; (iii) *hMLH1* methylation status defined two groups of MSI+ tumours with different features; and (iv) females with the CT or TT *MTHFR* genotype were at increased risk of developing CIMP+ CRC.

Although CIMP+ was originally described using six CpG islands,[4] no consensus panel currently exists for defining this phenotype. In a later publication the same authors stated that investigation of 2–4 CpG loci may be sufficient for identification of CIMP+ CRC.[11] Previous studies have shown that CIMP+ tumours are characterised by concordant methylation of multiple type C CpG islands such as *p16* and *MINT-2*.[3–5] Our earlier work suggested that *MDR1* was also a type C gene[15] and hence in the current study we arbitrarily defined tumours with methylation at two or more of *p16*, *MINT-2*, and *MDR1* as being CIMP+. Taking into account that most previous studies have been carried out on selected CRC series, the frequencies observed here for *p16*, *MDR1*, and *MINT-2* methylation (table 1) are similar to those reported previously.[4 9 13 15] These tumours

Table 4 Risk of CIMP+ and CIMP– colorectal cancer (CRC) according to the methylenetetrahydrofolate reductase (*MTHFR*) genotype

	Females* (n=118)	Males* (n=109)
Controls *v* all CRC	1.13 (0.75–1.71)	0.92 (0.60–1.39)
Controls *v* CIMP+†	2.17 (1.03–4.57)‡	0.68 (0.32–1.42)
Controls *v* CIMP–	0.84 (0.52–1.35)†	1.03 (0.60–1.39)

*Odds ratio and 95% confidence interval for frequency of the CT or TT genotype in CRC patients compared with age matched control group.
†Defined as having two or more CpG sites methylated.
‡p=0.037.
CIMP+, CpG island methylator phenotype.

comprised 32% of the current unselected stage II/III CRC series and were characterised by the features of poor histological grade and proximal tumour location. Previous studies on *p16*[4 9 10] and *MINT-2*[4] methylation have reported the same positive associations with tumour grade and site of origin. In agreement with a previous study,[11] the present results show a lower frequency of *TP53* mutation in CIMP+ tumours (table 2). The incidence of K-*ras* mutations in methylated tumours is less clear, with some studies reporting a higher incidence in CIMP+[11] or *p16* methylated[14] tumours, but not others for *p16* methylation alone.[12 13] The present results show a trend for lower frequency of K-*ras* mutations in CIMP+ but this was due mainly to the low frequency of K-*ras* mutations in the closely related MSI+ group.[22]

To date, little is known of the prognostic significance of CpG island methylation in CRC. A study in 84 stage III CRC patients found an association between *p16* methylation and shortened survival.[12] However, the current work and that of Esteller and colleagues[13] found no prognostic significance for *p16* methylation alone. Methylation of *MDR1*, *MINT-2*, or multiple sites was also found to have no prognostic value for patients treated by surgery alone in the present study. We previously reported evidence for a greater extent of survival benefit from 5-FU based chemotherapy for CRC patients with proximal tumours compared with those with distal tumours, with the benefit appearing to be most pronounced for patients with MSI+ tumours.[17] Interestingly, in the present study we found 44% of proximal (table 1) and 71% of MSI+ tumours (table 2) to be CIMP+. The degree of survival benefit from 5-FU based therapy appears therefore to correlate with the frequency of CIMP+. This phenotype is characterised by aberrant DNA methylation and we hypothesise that CIMP+ may be an important predictive factor for the response of CRC to antifolate therapies.

It was recently shown that *hMLH1* methylation is more common in females compared with males.[7] In both sexes, patients with *hMLH1* methylated MSI+ tumours were on average 20 years older compared with those with no *hMLH1* methylation.[7] In the present study of 54 MSI+ tumours, similar sex and age differences were also noted between the two subgroups defined by *hMLH1* methylation status (table 3). Tumours showing *hMLH1* methylation were also more likely to be poorly differentiated, CIMP+, and to have wild-type K-*ras* in comparison with MSI+ tumours with unmethylated *hMLH1*. Kuismanen and colleagues[8] reported similar tumour site differences between sporadic MSI+ cases depending on their *hMLH1* methylation status. As shown in table 3, the large majority (34/39, 87%) of *hMLH1* methylated tumours are CIMP+. Hence the above results suggest the existence of two groups of MSI+ tumours that can be distinguished by CIMP+ status and that are likely to evolve through different pathways. CIMP–/MSI+ tumours result from mutations and/or LOH of mismatch repair genes, whereas CIMP+/MSI+ tumours arise following widespread methylation of tumour DNA including *hMLH1*. The low number of CIMP–/MSI+ cases (n=10) prevents us from carrying out further characterisation of this subgroup. Our results (table 3) confirm those of previous studies[6–8] showing that approximately 50–90% of sporadic MSI+ CRCs have methylated *hMLH1*. Since the frequency of MSI+ in population based CRC is approximately 10–15%, we therefore estimate the incidence of *hMLH1* methylation to be about 7–12%. This is 2–3-fold lower than the frequencies of methylation observed for *p16*, *MDR1*, and *MINT-2*. The factors responsible for this difference have not been identified but may be gene, tumour, or host related.

Work by Slattery and colleagues[33 34] has implicated lifestyle factors such as diet, smoking, oestrogen exposure, and alcohol consumption in the risk of developing MSI+ CRC. We recently reported that the TT *MTHFR* genotype could also predispose to MSI+ CRC.[23] As the majority of sporadic MSI+ cases are CIMP+, it could be expected that these same lifestyle and genotypic factors are involved in the development of tumours with aberrant DNA methylation. Individuals with the TT *MTHFR* genotype have impaired remethylation of homocysteine to methionine,[32] hence we speculated this could predispose them to the development of CIMP+ CRC. In the current study, we present evidence for a twofold increased risk of CIMP+ CRC in females, but not males, with the CT/TT *MTHFR* genotype (table 4). Although several previous studies have investigated associations between *MTHFR* genotype and the overall risk of CRC,[35–37] this is the first study that has specifically examined the association between this genotype and a CRC subgroup characterised by DNA hypermethylation. Prospective studies that incorporate genotypic, dietary, and lifestyle factors are required to determine the relative contribution that each makes to the risk of developing CIMP+ tumours and may also shed light on the apparent sex difference observed here.

The site related distribution of CIMP+ observed in the current and previous studies[4 9 10] add to the growing list of differences between proximal and distal CRC.[38–40] We speculate that the higher incidence of CIMP+ tumours in the proximal compared with the distal colon may account for our previously reported site difference in the degree of survival benefit from antifolate based chemotherapy.[17] Our results and those of others[7 8] also reveal important differences between MSI+ tumours with or without *hMLH1* methylation. The present work is the first to identify a genotypic risk factor for the development of CIMP+ CRC. Dietary and lifestyle factors are no doubt also involved in the aetiology of this CRC subgroup. A recent report suggesting that hyperplastic polyps and serrated adenomas may be the precursors of MSI+ CRCs with methylated *hMLH1*[41] should facilitate further investigations into the development of MSI+ and the closely related CIMP+ tumours.

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