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MGMT methylation is associated primarily with the germline C > T SNP (rs16906252) in colorectal cancer and normal colonic mucosa

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O^6-methylguanine DNA methyltransferase (MGMT) is a DNA repair protein that restores mutagenic O^6-methylguanine to guanine. *MGMT* methylation is frequently observed in sporadic colorectal cancer and was recently correlated with the C > T allele at SNP rs16906252, within the transcriptional enhancer element of the promoter. *MGMT* methylation has also been associated with *KRAS* mutations, particularly G > A transitions. We studied 1123 colorectal carcinoma to define the molecular and clinicopathological profiles associated with *MGMT* methylation. Furthermore, we assessed factors contributing to *MGMT* methylation in the development of colorectal cancer by studying the allelic pattern of *MGMT* methylation using SNP rs16906252, and the methylation status of neighbouring genes within 10q26 in selected tumours and matched normal colonic mucosa. *MGMT* methylation was detected by combined bisulphite restriction analysis in 28% of tumours and was associated with a number of characteristics, including *CDKN2A* methylation, absent lymphovascular space invasion and *KRAS* mutations (but not specifically with *KRAS* G > A transitions). In a multivariate analysis adjusted for age and sex, *MGMT* methylation was associated with the T allele of SNP rs16906252 ($P < 0.0001$, OR 5.5, 95% CI 3.8–7.9). Low-level methylation was detected by quantitative methylation-specific PCR in the normal colonic mucosa of cases, particularly those with a correspondingly methylated tumour, as well as controls without neoplasia, and this was also associated with the C > T SNP. We show that the T allele at SNP rs16906252 is a key determinant in the onset of *MGMT* methylation in colorectal cancer, whereas the association of methylation at *MGMT* and *CDKN2A* suggests that these loci may be targets of a common mechanism of epigenetic dysregulation.
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Epigenetic inactivation of tumour-suppressor and DNA repair genes through aberrant methylation of the CpG island promoter is a frequent occurrence in sporadic colorectal cancer.[1] Specific patterns of gene methylation have been reported in particular subsets of colorectal cancer. These include the CpG island methylator phenotype (CIMP), in which numerous genes scattered throughout the genome are simultaneously methylated,[2,3] and long-range epigenetic silencing, in which multiple genes within a large (>1 Mb) contiguous chromosomal region are concomitantly methylated, as described for chromosomes 2q14[4] and 3p22 encompassing the DNA mismatch repair gene *MLH1*.[5] Particular subsets of colorectal cancer are characterised by the concurrence of genetic and epigenetic alterations. This phenomenon is most clearly exemplified by the strong concordance between *MLH1* methylation, microsatellite instability (MSI), the BRAF V600E mutation and CIMP.[3,6] Furthermore, specific molecu-

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lar subtypes of colorectal cancer have been associated with distinct clinicopathological features, including differences in natural history and response rates to particular chemotherapeutic agents.[6,7] Therefore, the identification of distinct molecular profiles and associated clinicopathological features, as well as molecular susceptibility factors, is important not only in the recognition of different colorectal cancer phenotypes, but also has the potential to significantly influence targeted treatment regimes.[8]

Loss of expression of the O^6-methylguanine DNA methyltransferase (MGMT) protein is a frequent occurrence in many types of cancer, including 30–46% of sporadic colorectal cancers.[9–13] This is almost invariably associated with methylation of the *MGMT* promoter.[9] MGMT is a ubiquitously expressed DNA repair protein that protects against mutagenesis by repairing mutagenic O^6-methylguanines within DNA. By direct cleavage of the methyl adducts, the enzyme can restore the affected guanine nucleotides to normal.[14] If this fails to occur, O^6-methylguanines can pair erroneously with thymine during DNA replication, resulting in G:C>A:T transitions in the DNA, which can be important in neoplastic transformation.[14]

MGMT methylation or loss of MGMT protein expression is reportedly associated with an increased frequency of mutations in the *KRAS* oncogene and *P53* tumour-suppressor gene in colorectal cancer, particularly G>A transitions, consistent with a causative function of G>A mutagenesis in tumourigenesis.[11,12,15–18] However, other studies have found no specific associations with this sequence change,[19,20] or only a weak association with the presence of a G>A mutation within one of the four cancer-associated genes *KRAS*, *P53*, *APC* and β-catenin.[21]

Association studies between *MGMT* methylation, CIMP and microsatellite stability (MSS) status in colorectal cancer have also reported inconsistent findings. *MGMT* methylation has been correlated with MSS and low-level MSI,[17,20] though the existence of low-level MSI as an entity distinct from MSS remains controversial. Although *MGMT* methylation was initially included as one of the panel of markers to predict CIMP in some studies,[22–25] it has since been shown to be a poor marker for this epigenetic phenotype and may be independent of CIMP status altogether.[26,27] Yet in one recent study, *MGMT* methylation was associated with CIMP in combination with MSS/low-level MSI,[20] whereas in another, it correlated with the newly proposed low-level CIMP phenotype in combination with low-level MSI.[10] Thus, tumours showing *MGMT* methylation have not as yet been classified consistently into any distinct subtype of colorectal cancer with a clearly defined and universally accepted molecular profile. However, cancers in which MGMT is absent are more sensitive to treatment with alkylating agents,[28] and thus *MGMT* methylation may be a predictive marker for drug responsiveness in colorectal cancer.

MGMT methylation has been identified in early neoplastic lesions including aberrant crypt foci, indicating that it is an early event in colorectal carcinogenesis.[29,30] It is seen frequently in serrated polyps and adenomas with a villous component, suggesting an involvement in the serrated neoplasia pathway.[23–25,31,32] Low levels of *MGMT* methylation have also been detected in the normal-appearing colonic mucosae of individuals with *MGMT*-methylated colorectal cancers as well as individuals without neoplasia.[12,30,33] This led to the suggestion that *MGMT* methylation may precede and predispose to malignant transformation through Slaughter's concept of 'field cancerisation', whereby the accrual of molecular alterations in patches of preneoplastic cells underlies the development of locally recurrent epithelial cancers.[34] Recently, *MGMT* methylation was found to be closely associated with the C>T SNP (rs16906252) within the first exon of *MGMT* in colorectal cancer.[35] This SNP is located 56 bp upstream of the translation start site, within a 59-bp *cis*-acting enhancer element that spans the first exon–intron boundary and is required for efficient promoter activity (Figure 1a).[36]

At this point, therefore, the relationship between *MGMT* methylation, MGMT protein loss and particular genetic and epigenetic lesions requires further clarification. To this end, we determined the frequency of *MGMT* methylation and protein loss in a large Australian series of sporadic colorectal carcinoma and sought correlations with other molecular events and clinicopathological features. Furthermore, we aimed to identify the key determinants of *MGMT* methylation in the development of colorectal cancer. To address this, we investigated whether *MGMT* methylation status correlated with long-range epigenetic silencing of the chromosome region 10q26 surrounding *MGMT*, the CIMP phenotype or the genotype of the germline *MGMT* exon 1 SNP rs16906252. The presence of low levels of *MGMT* methylation in the normal colonic mucosa of individuals with colorectal cancer and without neoplasia was also studied in conjunction with the SNP genotype.

Materials and methods

Clinical Specimens

A total of 1249 colorectal carcinoma specimens and matching normal colonic mucosa were drawn from a prospective series of 1178 patients who had undergone complete surgical resection of a primary colorectal cancer at St Vincent's Hospital Sydney from March 1993 to November 2007. In the 61 individuals with two or more tumours, one cancer was selected at random for inclusion in this study. A further 50 individuals were excluded, as there was either insufficient tumour sample for methylation analysis or no result was obtained. The cohort for which *MGMT* methylation results were obtained, therefore,

included 1123 individuals (505 females and 618 males) with a mean age at diagnosis of 69 ± 12 years (range 25–99 years). The distribution of tumour TNM stages was 229 (20%) stage I, 391 (35%) stage II, 355 (32%) stage III and 148 (13%) stage IV. The normal colonic mucosa from 20 individuals (7 females and 13 males) of mean age 60 ± 18 years (range 33–91 years) who had undergone colonic resection for clinical indications other than colorectal cancer were included as controls without neoplasia. This study was approved by the St Vincent's Campus Human Research Committee (approval numbers H02/022 and H07/002) and all individuals provided their informed consent. Individuals with a known germline mutation in the mismatch repair genes, *MYH* or *APC* were excluded from this study. The clinical and pathological characteristics of the majority of cases in this patient cohort have been documented earlier.[37,38] The microsatellite status of each tumour was assessed at the Bat 25, Bat 26, Bat 40, D5S346, D2S123 and D17S250 loci as described earlier.[37] Tumours with instability at two or more markers were classified as MSI, whereas all others were designated as MSS. The identification of *KRAS* mutations within the codon 12 and 13 hotspot, as well as the BRAF V600E mutation, was determined by pyrosequencing.[39]

SNP (rs16906252) Genotyping

For germline genotyping, DNA derived from normal colonic mucosa was amplified using primers TGCAGGACCACTCGAGGCTGCCA and CCCGGA TATGCTGGGACAGCCC flanking the C>T SNP (rs16906252) with annealing at 68°C. The 167-bp amplification fragments were digested with *Hh*aI (New England Biolabs) and resolved by agarose gel electrophoresis. Presence of the G allele resulted in digestion to fragments of 97 and 70 bp, whereas the A allele remained undigested. A subset of tumours from heterozygous cases was also studied to confirm retention of both alleles before allelic bisulphite sequencing.

Methylation Analyses

Genomic DNA derived from the peripheral blood mononuclear cells of a healthy volunteer were used as an unmethylated control. The same DNA was treated with M.Sss1 methyltransferase (New England Biolabs) to generate an *in vitro* methylated control. Up to 2 μg of DNA from tumours and the methylated and unmethylated controls were converted with sodium bisulphite using the EZ Methylation-Gold kit (Zymo Research, Orange, CA, USA). Methylation analyses were performed using 100 ng bisulphite-converted DNA as PCR template. A map of the *MGMT* CpG island and assays used to assess the methylation status at the *MGMT* promoter is given in Figure 1a.

Combined Bisulphite Restriction Analysis

Combined bisulphite restriction analyses (COBRA) within the CpG island spanning the *MGMT* promoter and six other CpG-island-associated genes within chromosome 10q26 were performed using primers specific for bisulphite-converted DNA and unbiased with respect to the methylation status of the templates. Primers, amplification conditions and restriction enzymes used for the detection of methylation are listed in Supplementary Table 1. A CpG dinucleotide and restriction map for the *MGMT* COBRA assay is given in Figure 1 and for the other 10q26 genes in Supplementary Figure 1. After gel electrophoresis, the degree of methylation was estimated visually by the relative intensities of the digested and undigested fragments. On this basis, the samples were independently classified as methylated or unmethylated by two observers who were blinded with respect to the results of other analyses. For *MGMT*, methylation was detected by the presence of the digested bands of 100 and 62 bp (Figure 1a and b). There was complete concordance in the interpretation of *MGMT* methylation status for 1123 tumours between the two observers. Samples for which no reliable methylation result was obtained, or the two observers failed to agree, were excluded from further analysis.

Allelic Bisulphite Sequencing

Allelic *MGMT* methylation patterns were determined in tumours heterozygous for the C>T SNP (rs16906252) using primers GTTTGTAGGATTATT YGAGGTTGTTAT and CCCCRAATATACTAAAACA ACCC to amplify a 171-bp fragment containing the SNP and 18 CpG sites. These primers were specific to bisulphite-converted templates from the antisense strand, such that the C/T SNP was detected as the complementary bases G/A. The PCRs were conducted using cycling conditions that preferentially amplified methylated templates to reduce the number of unmethylated alleles amplified, as a proportion of the tumour specimens had relatively low levels of methylation as detected by COBRA. The first seven cycles were performed with annealing at 72°C in the first cycle, then at −1°C for each subsequent cycle, followed by 30 cycles with annealing at 65°C. PCR products were cloned using the pGEMTeasy PCR cloning system (Promega) and 12–20 colonies picked. Plasmid inserts were sequenced using the SP6 vector primer by fluorescent dideoxy-sequencing on an ABI PRISM 3700 DNA Sequencer.

Quantitative Real-Time Methylation-Specific PCR with Temperature Dissociation

Quantitative real-time methylation-specific PCR was carried out for *MGMT* using primers (5′-3′)

TCGTTCGGTTTGTATTGGTC and TCTACGCATCCT CGCTAAAC. *MyoD* was used as a control for DNA input and integrity.[40] Amplifications were conducted in triplicate in 20 μl volumes using 0.3 μM primers and 1 $\times$ iQ SYBR-Green Supermix (BioRad) on the MyiQ single-colour PCR detection system (BioRad). Cycle threshold (C_T) values were obtained using MyiQ software version 5.0 (BioRad). Annealing was at 62°C and a fourth step at 81°C was included in each cycle during which the fluorescence output was measured to avoid any non-specific signal from primer dimers. The number of methylated fragments was calculated at the C_T against a standard curve of serially diluted plasmids containing 25, 50 and 10^2–10^6 copies of the target sequence for *MGMT* and *MyoD*. Percentage methylation reference (PMR) values were calculated with reference to the Human CpGenome Universal Methylated DNA (Chemicon) control, as described earlier.[41] After amplification, a melt curve was performed from 72 to 95°C with fluorescence measurements at 0.5°C intervals to determine the melting temperature of the amplicons. Uniform dissociation of amplification products at the correct temperature ensured product specificity. This assay was capable of detecting methylation at PMR levels of 0.01 in the presence of $>2.5 \times 10^4$ copies of the *MyoD* control gene (approximately 200 ng input DNA), which was achieved for each sample tested.

CpG Island Methylator Phenotype

The CIMP status of tumours was assessed by MethyLight at the *CACNA1G*, *RUNX3*, *IGF2*, *NEUROG1* and *SOCS1* loci, as described earlier.[3] Tumours were classified as CIMP positive (CIMP+) in which ≥ 3 loci showed methylation levels at a PMR value >4.[27]

MGMT Expression Analyses

Semi-quantitative real-time RT–PCR
Total RNA was extracted from fresh-frozen colorectal cancer and matched normal colonic mucosa specimens using the Midi-Mini RNA extraction kit (Invitrogen). RNA samples were treated with DNase I and cDNAs were prepared from 2 μg total RNA using the First Strand Superscript III cDNA synthesis kit with oligo-dT_{20} primers (Invitrogen). A control with reverse transcriptase omitted was performed for each sample. PCR amplification was conducted across the final two exons of *MGMT* using primers (5′-3′) GAGGAGCAATGAGAGGCA ATCCT and CATCCGATGCAGTGTTACACGT, and the *HPRT* housekeeping gene using primers AATTA TGGACAGGACTGAACGTC and GGCGATGTCAAT AGGACTCCAGATG. Amplifications were performed in triplicate using 200 ng cDNA or RT minus control as template with annealing at 59°C on the MyiQ. Quantitation of the relative levels of *MGMT* expression in tumour *versus* paired normal colonic mucosa was performed using the C_T values according to the Pfaffl method.[42]

Immunohistochemistry
Tissue microarrays were constructed using duplicate cores from formalin-fixed paraffin-embedded tumour tissues. A measure of 4 μM sections were dewaxed and rehydrated on silane-coated slides before antigen retrieval and blocking with 3% hydrogen peroxidise and 2% skimmed milk. Sections were incubated with a 1:50 dilution of monoclonal mouse anti-human MGMT antibody (Clone MT3.1, Santa Cruz) for 1 h at room temperature and bound antibody was detected with horseradish peroxidise conjugated polymer antibody (Novocastra). Sections were counterstained with haematoxylin. A negative staining control with the primary antibody omitted was included. Slides were visualised under white light at $\times 10$ magnification and interpreted by a pathologist (NJH) blinded to methylation status. Staining was considered assessable in which nuclear staining of MGMT was visible in either stromal or germinal follicle lymphocyte cells, or in normal colonic epithelial cells at the margins of the tumour. Tumours were considered negative for MGMT expression in which nuclear staining for MGMT in tumour cells was either entirely absent or significantly reduced in comparison to adjacent normal cells.

Statistical Analyses

The methylation status of the *MGMT* promoter was analysed as a categorical variable. Analyses to detect any differences in frequency between categorical variables were performed using the χ^2 test. An independent *t*-test was used to test whether *MGMT* methylation correlated with age. The Mann–Whitney *U*-test was used to assess whether non-normally distributed values (methylation, mRNA expression levels) differed between groups. Multivariate analysis using the binary logistic regression model was performed to determine independent factors among covariates that had shown significant associations. The Spearman and Pearson tests were used to determine whether the levels of methylation in normal colonic mucosa were associated with age, and if this was linear. All reported probability (*P*) values were two sided and a value of ≤ 0.05 was considered significant. The SPSS v17.0 statistical package (SPSS Chicago, IL, USA) was used for all statistical analyses.

Results

Frequency and Allelic Pattern of *MGMT* Promoter Methylation in Colorectal Cancer

The frequency of *MGMT* promoter methylation in this series of colorectal carcinoma was 28% (312 of

1123 tumours), as detected by COBRA. However, it was notable that there was considerable variability in the degree of methylation between tumours (Figure 1b). Clearly, some tumours displayed quite low levels of *MGMT* methylation, whereas in others, high levels of methylation were detected consistent with biallelic methylation. To determine whether indeed both alleles were affected, allelic bisulphite sequence analysis was performed across a promoter fragment containing the exon 1 C>T SNP (rs16906252) site and a number of flanking CpG dinucleotides in heterozygous tumours with variable levels of methylation as assessed by COBRA (Figure 1a). Allelic bisulphite sequencing was performed from the antisense strand of *MGMT* on which the polymorphic content of the SNP was preserved as G/A after sodium bisulphite conversion (Figure 1a). Of the 18 *MGMT*-methylated tumours studied, eight (with methylation levels estimated by COBRA to be between 10 and 80%) showed biallelic methylation (Figure 1c; Supplementary Figure 2). However, 10 (methylated at levels of 10–50% by COBRA) showed monoallelic methylation. Interestingly, methylation in each of these 10 tumours was specific to the T:A allele (Figure 1c; Supplementary Figure 3). One heterozygous tumour that was unmethylated by COBRA was confirmed to be unmethylated by allelic bisulphite sequencing (Supplementary Figure 2).

Correlation with Transcriptional Repression and Loss of Protein Expression

The levels of *MGMT* transcription were compared between 14 primary colorectal carcinoma with either confirmed biallelic methylation or high levels of promoter methylation ($\geq$50% by COBRA) and 16 unmethylated tumours, with respect to their matched normal colonic mucosa, using semi-quantitative real-time RT–PCR. *MGMT* expression was significantly reduced in the methylated tumours compared with unmethylated tumours, confirming that methylation results in transcriptional repression (Figure 1d).

To examine the relationship between MGMT methylation and protein expression, immunoperoxidase staining was performed on a subset of 402 tumours of which 120 (30%) displayed methylation of the *MGMT* promoter. As expected, MGMT protein loss was highly concordant with promoter methylation (χ^2 $P<0.0001$). In tumours showing immunohistochemical loss of MGMT expression ($n=62$), 48 (77%) were methylated, consistent with earlier findings that methylation of this gene represents a major cause of protein loss in colorectal cancer.[9] However, methylation was also found in 72 (21%) of the 340 tumours with normal staining. Immunohistochemistry results were available for nine of the tumours that were monoallelically methylated (Supplementary Figure 3), and protein expression was retained in six of these. Expression of MGMT was presumably derived from the unmethylated allele in these six tumours. This contrasted to the complete loss of MGMT protein in the five biallelically methylated tumours for which immunohistochemistry data was available (Supplementary Figure 3).

MGMT Methylation Occurs as a Localised Event within Chromosome Region 10q26

To determine whether methylation of *MGMT* occurred as an isolated event or was subject to long-range epigenetic silencing in tumourigenesis, we sought evidence of concomitant promoter methylation of *MGMT* and neighbouring genes within 10q26. The methylation status of six gene-associated CpG islands flanking *MGMT* and spanning a 2.3 Mb region of 10q26 was examined by COBRA in 40 pairs of primary colorectal carcinoma and their matched normal colonic mucosae, of which 20 tumours had $\geq$50% *MGMT* methylation and 20 were unmethylated at *MGMT* (Figure 2). The *FOXI2* gene, over 1.5 Mb upstream of *MGMT*, was methylated at high levels in all tumours irrespective of the *MGMT* methylation status, and at lower levels in the majority of normal colonic mucosae. *FOXI2* methylation is thus independent of *MGMT* and may occur in a tissue-dependent manner, consistent with the lack of expression of the encoded Foxi2 transcription factor in the colon.[43] The *EBF3* gene, situated approximately 500 kb downstream of *MGMT* and expressed from the opposite strand, was more frequently methylated in tumours that were also methylated at *MGMT* (Mann–Whitney *U*: $P=0.00015$, Figure 2). No other genes in the 2.3 Mb region studied were found by COBRA to be methylated in tumours or normal colonic mucosa. Although *MGMT* and the neighbouring *EBF3* gene may frequently be concomitantly methylated in colorectal cancer, this is distinct from earlier observations of long-range epigenetic silencing, in which multiple genes within large contiguous regions spanning >1 Mb were co-ordinately methylated.[4,5]

Correlations of *MGMT* Methylation with Clinicopathological and Molecular Features of Colorectal Cancer

MGMT methylation correlated with increased age, female gender, mucinous histology, conspicuous intraepithelial lymphocytes and absence of lymphovascular space invasion (Table 1). In addition, a number of molecular factors including MSI and *MLH1* methylation, CIMP+, *KRAS* and BRAF V600E mutations, and methylation of *CDKN2A* also correlated with *MGMT* methylation (Table 1). Tumour stage, grade and location did not show a relationship with *MGMT* methylation (data not shown). In univariate analysis, the strongest asso-

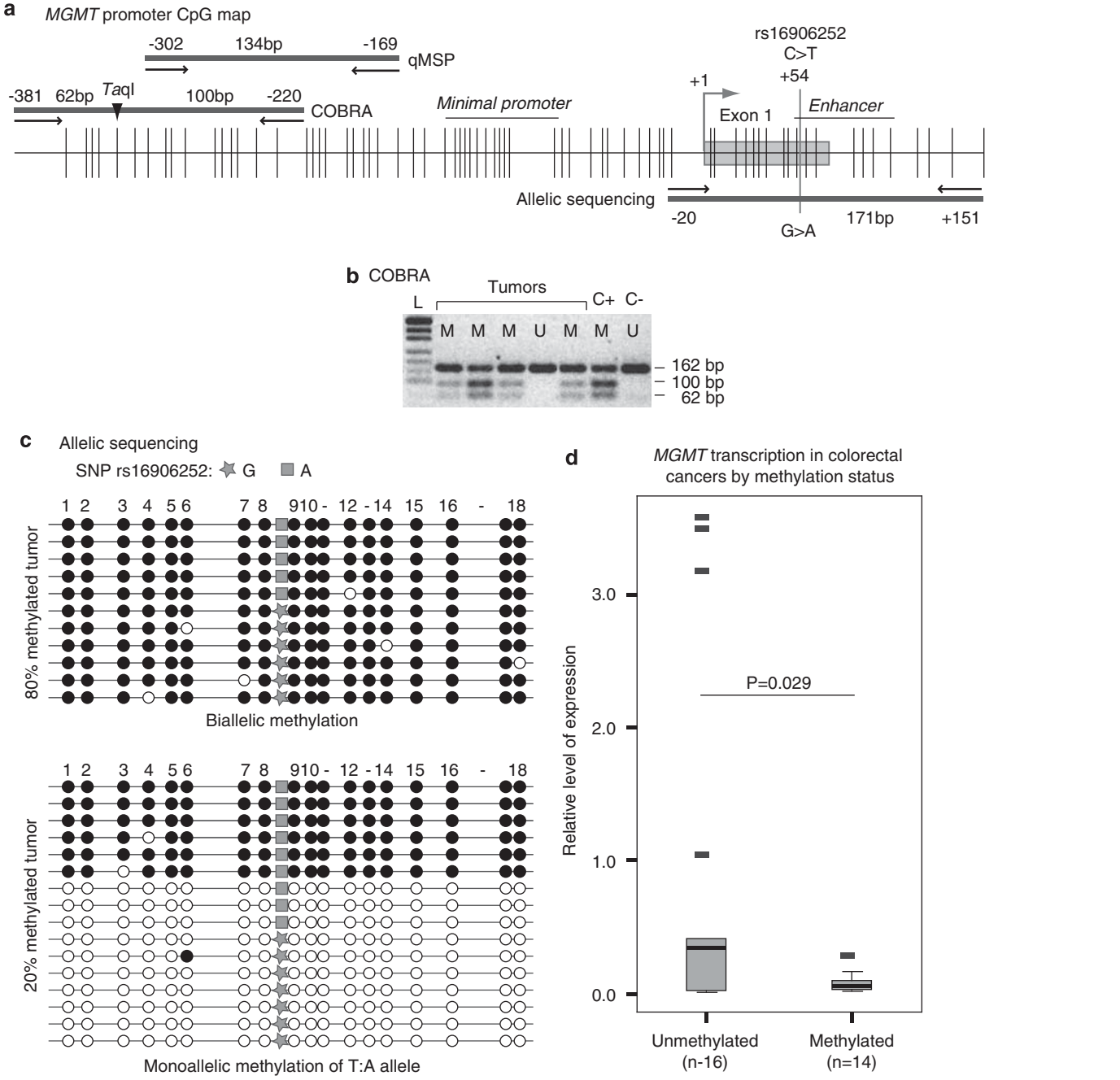

Figure 1 Methylation of the CpG island spanning the *MGMT* promoter in colorectal carcinoma. (**a**) CpG map of the *MGMT* promoter region and assays used to determine methylation status in bisulphite-converted DNA. The region shown consists of 507 bp of the CpG island affected by methylation in colorectal cancer, spanning the minimal promoter region, first exon and 59 bp enhancer element, as labelled. The *MGMT* transcription start site, denoted by the grey arrow, is at position +1. The C>T SNP (rs16906252) is located at +54 bp within exon 1 and the enhancer. Vertical lines show the positions of CpG dinucleotides. Horizontal bars indicate PCR amplification fragments for methylation assays, with horizontal arrows showing primer positions, with fragment sizes in base pairs shown, and numbers (+/−) their position with respect to the transcription start site. COBRA was used to determine the methylation status and levels in colorectal carcinoma, with *Taq*I digestion indicating the presence of methylation at the CpG site indicated by a downward arrow. qMSP assay was used to detect and measure methylation levels in normal colonic mucosa. Allelic sequencing was performed from the antisense strand on which the C>T SNP was detected as G>A. (**b**) Representative examples of COBRA in tumours to determine methylation status. L, pUC19/MSpI DNA ladder, C+, M.SssI *in vitro* methylated control DNA, C−, unmethylated control DNA from peripheral blood, M, methylated tumour, U, unmethylated tumour. (**c**) Representative patterns of allelic bisulphite sequencing of two colorectal carcinoma. Horizontal lines indicate individual alleles, circles denote individual CpG dinucleotides with black indicating a methylated CpG and white indicating an unmethylated CpG. Above, a tumour that is methylated on both genetic alleles of the *MGMT* promoter. Below, a tumour that is methylated specifically on the A:T allele of SNP rs16906252. (**d**) Box plot showing the pooled relative levels of *MGMT* transcription between unmethylated and methylated tumours with respect to their matched normal colonic mucosa and normalised to *HPRT*. The level of *MGMT* transcription was significantly reduced in the methylated tumours (median 0.047) compared with the unmethylated tumours (median 0.34). Low levels of *MGMT* expression detected in the methylated tumours may be derived from normal cells within the tumour tissue.

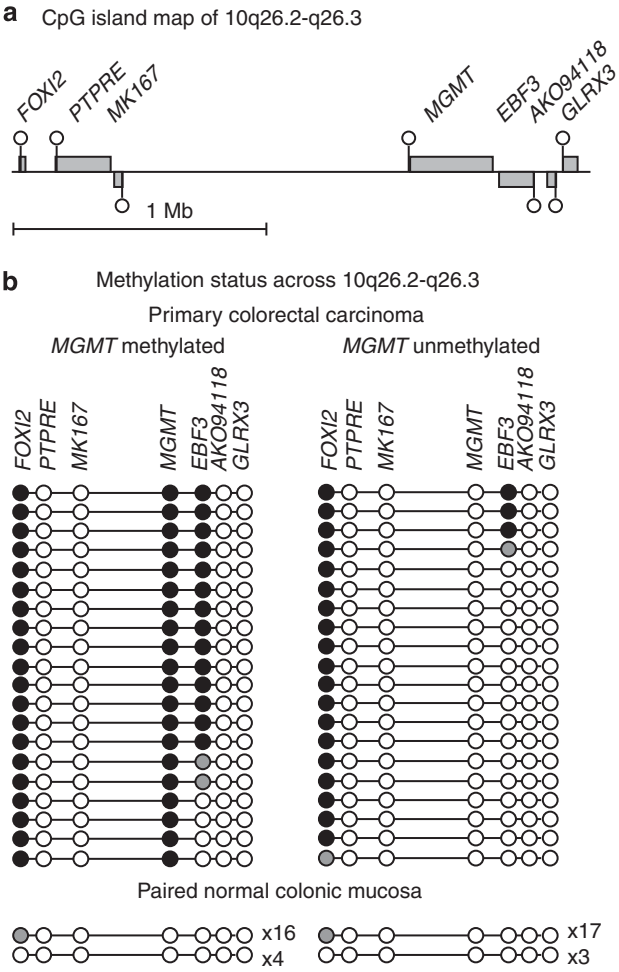

Figure 2 Chromosome region 10q26 methylation status in colorectal carcinomas. (**a**) Map of genes associated with CpG islands within chromosome region 10q26.2–q26.3 flanking the *MGMT* gene. Genes are depicted as grey boxes, with those above the horizontal line expressed from the sense strand and those below the line transcribed from the antisense strand. CpG islands spanning the promoters of the genes are indicated by lollipops. (**b**) Methylation status of the CpG islands spanning the 10q26 genes as determined by COBRA in 20 primary colorectal carcinoma with *MGMT* promoter methylation at $\geq$50% and 20 tumours unmethylated at *MGMT* and their paired normal colonic mucosa. Each circle represents the CpG island of a gene, as labelled above. Black circles indicate methylation at levels of >10%, grey circles indicate methylation at $\leq$10% and white circles indicate absence of methylation. Each line linking the circles represents an individual specimen, or multiples thereof showing the same methylation profile, as indicated.

ciations with *MGMT* methylation were found with *CDKN2A* methylation (OR 2.3, 95% CI 1.7–3.0, $P<0.0001$), *KRAS* mutations (OR 2.1, 95% CI 1.6–2.7, $P<0.0001$), CIMP+ (OR 1.8, 95% CI 1.2–2.5, $P=0.002$), absent lymphovascular space invasion (OR 1.7, 95% CI 1.3–2.2, $P<0.0001$) and female gender (OR 1.6, 95% CI 1.2–2.0, $P<0.0001$). When variables significant in univariate analysis were included in a multivariate analysis, only female gender (OR 1.5, 95% CI 1.2–2.0, $P=0.001$), *CDKN2A* methylation (OR 2.0, 95% CI 1.5–2.7, $P<0.0001$), absent lymphovascular space invasion (OR 1.6, 95% CI 1.2–2.1, $P=0.003$) and presence of *KRAS* mutations (OR 1.9, 95% CI 1.5–2.6, $P<0.0001$) remained significant. As reported earlier,[26,44] there was a strong association between *CDKN2A* methylation, CIMP+ (OR 9.4, 95% CI 5.9–15.1, $P<0.0001$) and *MLH1* methylation (OR 5.7, 95% CI 3.6–8.9, $P<0.0001$), respectively.

Relationship of *KRAS* G>A Transitions and *MGMT* Methylation

Activating mutations within codons 12 and 13 of *KRAS* were found in 32% of cancers in this series and 60% of the mutations were G>A transitions (Table 2).[39] As stated above, *MGMT* methylation had a close and independent correlation with the presence of a *KRAS* mutation. However, on classification of the *KRAS* mutant cancers by mutation type, no association was found between *MGMT* methylation and G>A mutations compared with non-G>A mutations, and in fact frequency of methylated and unmethylated tumours was approximately equal for each mutation category (Table 2). Furthermore, no association was found between loss of MGMT protein expression and presence of a *KRAS* mutation ($P=0.09$), nor G>A transitions ($P=0.2$).

Close Association Between *MGMT* Methylation and Germline C>T SNP (rs16906252)

Of the cohort of 1123 cases, constitutional DNA was available for 1039 cases and these were genotyped for the germline C>T SNP (rs16906252). The frequency of the T allele in this cohort was 8% (two cases homozygous T/T, 151 heterozygous C/T, 886 homozygous C/C, conforming to Hardy–Weinberg equilibrium) (Table 1). In a univariate analysis, the presence of the T allele (C/T and T/T genotypes combined) was strongly associated with *MGMT* methylation (OR 5.5, 95% CI 3.8–7.9, $P<0.0001$) and these results were unaltered in a multivariate analysis, which adjusted for age and sex.

Low-Level *MGMT* Methylation in Normal-Appearing Colonic Mucosa

The possibility that methylation of *MGMT* might precede and predispose to the development of colorectal cancer was assessed by screening the normal colonic mucosa from 100 cases with colorectal cancer and 20 individuals without neoplasia for low levels of promoter methylation using a sensitive real-time methylation-specific PCR assay followed by a temperature dissociation curve to verify any positive signals (qMSP) (Figure 1a). The results were obtained for 50 cases with a methylated tumour selected at random from the cohort and 47

Table 1 Statistical comparisons of the clinicopathological and molecular features of patients by methylation status of the *MGMT* promoter

	Valid cases, tumours (%)	*MGMT Meth (%)*	*MGMT Unmeth (%)*	P-*value*
Clinicopathological tumour features				
Mean age, years (±1 s.d.)	68.9 ± 12.2	70.4 (±12.6)	68.3 (±12.0)	0.012*
Gender	1123 (100)			
Female	505 (45.0)	167 (33.1)	338 (66.9)	0.000
Male	618 (55.0)	145 (23.5)	473 (76.5)	
Mucinous histology	1119 (99.6)			
Mucinous	230 (20.5)	78 (33.9)	152 (66.1)	0.033
Non-mucinous	889 (79.2)	234 (26.3)	655 (73.7)	
IELs	1122 (99.9)			
Conspicuous	176 (15.7)	64 (36.4)	112 (63.6)	0.018
Inconspicuous	946 (84.2)	248 (26.2)	698 (73.8)	
Lymphovascular space invasion	1113 (99.1)			
Absent	669 (59.6)	213 (31.8)	456 (68.2)	0.001
Present	444 (39.5)	96 (21.6)	348 (78.4)	
Molecular features of tumour				
Microsatellite status	1121 (99.8)			
MSI	146 (13.0)	53 (36.3)	93 (63.7)	0.037
MSS	975 (86.8)	258 (26.5)	717 (73.5)	
CIMP status	1120 (99.8)			
Positive	156 (13.9)	60 (38.5)	96 (61.5)	0.003
Negative	964 (85.8)	252 (26.1)	712 (73.9)	
BRAF mutation status	1119 (99.6)			
V600E Mutant	138 (12.3)	49 (35.5)	89 (64.5)	0.047
Wild type	981 (87.4)	263 (26.8)	718 (73.2)	
KRAS mutation status	1121 (99.9)			
Mutant	358 (31.9)	137 (38.3)	221 (61.7)	0.000
Wild type	763 (68.0)	175 (22.9)	588 (77.1)	
MLH1 methylation	1101 (98.1)			
Methylated	127(11.3)	49 (38.6)	78 (61.4)	0.003
Unmethylated	974 (86.8)	261 (26.8)	713 (73.2)	
CDKN2A methylation	1075 (95.7)			
Methylated	481 (42.8)	182 (37.8)	299 (62.2)	0.000
Unmethylated	594 (52.9)	126 (21.2)	468 (78.8)	
Patient genotype				
SNP rs16906252	1039 (92.5)			
CT	151 (13.4)	91 (60.3)	60 (39.7)	
TT	2 (0.2)	2 (100)	0 (0)	0.000
CC	886 (78.9)	195 (22.0)	691 (78.0)	

The number of cases (tumours) for which data was available is listed for each parameter tested. The percentages provided in the third and fourth columns are with respect to the group against which *MGMT* methylation status is being tested. All *P*-values were performed using χ^2 test, except for a comparison of age in which a Student's *t*-test was used. Meth, methylated; Unmeth, unmethylated; IELs, intraepithelial lymphocytes.

Table 2 Relationship of KRAS G>A transitions and *MGMT* promoter methylation

Molecular feature	*Valid cases (%)*	*MGMT Meth (%)*	*MGMT Unmeth (%)*	P-*value*, χ^2
KRAS mutation type	358 (100)			
G>A transitions	213 (59.5)	81 (59.1)	132 (59.7)	$P=0.91$
Other mutations	145 (40.5)	56 (40.9)	89 (40.3)	

age-matched (±5 years) cases with an unmethylated tumour. The age (mean 68 ± 12 years) and gender (44 females and 53 males) of these cases was representative of the cohort. *MGMT* methylation was detected in the normal colonic mucosa of 53/97 (55%) patients with colorectal cancer (PMR range 0–9.7) and 9/20 (45%) individuals without neoplasia (PMR range 0–1.8), but there was no significant difference between the two groups (Figure 3a), indicating that presence of *MGMT* methylation in normal colonic mucosa was not associated with colorectal cancer *per se*. However, when the cases were stratified by the *MGMT* methylation status of their matched tumours, methylation was significantly increased in the normal colonic mucosa of patients whose tumours were correspondingly methylated (Figure 3b). When the normal colonic mucosa samples of colorectal cancer cases were classified according to the genotype of the *MGMT* C>T SNP (rs16906252), irrespective of the methylation status of the matched tumour, *MGMT* methylation was significantly increased in those harbouring the T allele (Figure 3c).

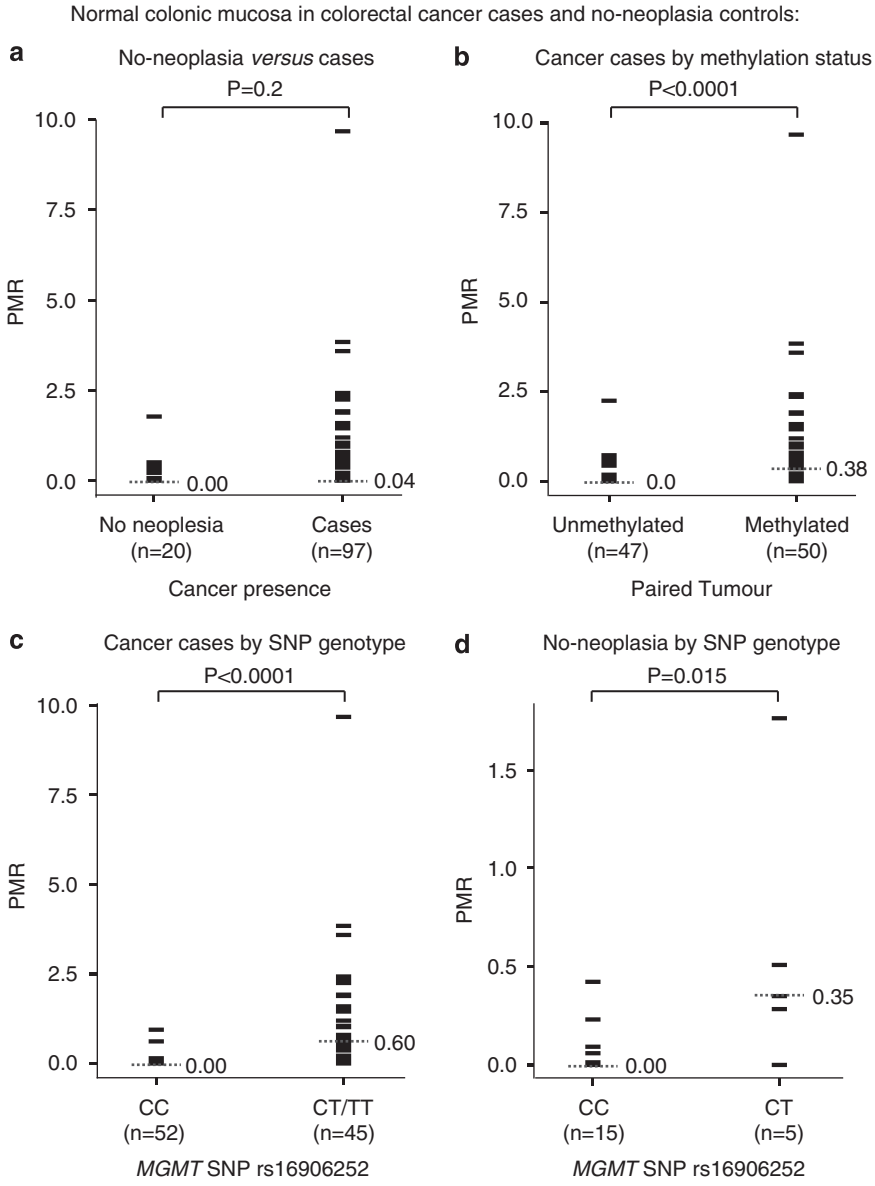

Figure 3 *MGMT* promoter methylation in normal colonic mucosa. Plots showing the degree of *MGMT* methylation expressed as the PMR detected by quantitative real-time methylation-specific PCR in normal colonic mucosa. Methylation levels were compared between groups of normal colonic mucosa using the Mann–Whitney *U*-test and the *P*-values are indicated for each of the groups tested. (**a**) *MGMT* methylation levels in the normal colonic mucosa of individuals without neoplasia and paired normal colonic mucosa of colorectal cancer cases were not significantly different. (**b**) Paired normal colonic mucosa from colorectal cancer cases as divided by the *MGMT* methylation status of the paired tumour showed that methylation levels were higher in the normal colonic mucosa of cases with a correspondingly methylated tumour. (**c**) Paired normal colonic mucosa of cases with colorectal cancer and (**d**) normal colonic mucosa from individuals without neoplasia, each divided according to genotype at the germline C/T SNP (rs16906252) within the *MGMT* transcriptional enhancer. *MGMT* methylation levels were significantly higher in the normal colonic mucosa of both cases and no-neoplasia controls harbouring the T allele of the SNP.

In the individuals without neoplasia, of whom 15 were homozygous CC and five were heterozygous C/T (conforming to Hardy–Weinberg equilibrium), methylation was also associated with the T allele (Figure 3d). There was a non-linear association between age and the level of *MGMT* methylation detected in normal colonic mucosa, both for colorectal cancer cases (Spearman correlation $r=0.36$, $P=0.0004$) and for individuals without neoplasia (Spearman correlation $r=0.3$, $P=0.036$). There was no association between gender and methylation levels in normal colonic mucosa.

Discussion

This investigation used a large colorectal cancer cohort to determine the frequency of *MGMT* promoter methylation in sporadic colorectal cancer, as well as associated clinicopathological and molecu-

lar features. *MGMT* methylation was identified in 28% of tumours. This is a slightly lower prevalence than the generally reported range, perhaps reflecting the analytical sensitivities of the various methylation assays.[9–13] The strong but incomplete concordance between *MGMT* methylation, transcriptional repression and protein loss is well established,[9–11] and our findings confirm that promoter methylation is the predominant cause of MGMT loss in sporadic colorectal cancer.

Our study has provided new insight into the mechanisms underpinning *MGMT* methylation in colorectal cancer. We found no evidence for a mechanism of long-range epigenetic silencing operating within the vicinity of *MGMT*. Rather, the localised methylation of *MGMT* and the adjacent *EBF3* gene are most likely attributable to epigenetic dysregulation confined to one or both closely linked genes. Consistent with the involvement of a *cis*-acting factor, presence of the T allele of the linked C/T germline SNP within the transcriptional enhancer element of *MGMT* was a key predictor of *MGMT* promoter methylation in colorectal cancer. This finding confirms an earlier report in another colorectal cancer population.[35] Furthermore, we showed that the T allele is preferentially methylated in colorectal cancer through the observation of monoallelic methylation of this allele in a subset of tumours from heterozygous patients. Some of these cancers retained MGMT protein expression (data not shown), presumably through translation of transcripts from the unmethylated allele. Certainly, this provides a plausible explanation for the occurrence of methylation in tumours with normal MGMT expression, as assessed by immunohistochemistry. In other cancers with monoallelic methylation, protein expression was lost, possibly because of disruption of the unmethylated allele by genetic mechanisms.[21] In addition, we found that the T allele was associated with detectable levels of *MGMT* methylation in the paired normal colonic mucosa of cases with colorectal cancer, particularly those cases in which the tumour was correspondingly methylated, indicating that methylation may indeed be a precursor to neoplasic development. Other studies have also shown that *MGMT* methylation occurs in the normal colonic mucosa in a proportion of colorectal cancer cases, and it has been proposed that this may serve as a field defect predisposing to the development of cancer.[12,30,33] However, others and we found similar levels of *MGMT* methylation in the normal colonic mucosa of individuals without neoplasia as well,[12,33] which we additionally show is associated with the T allele of SNP rs16906252. This provides strong evidence that the T allele predisposes to *MGMT* methylation in normal colonic mucosa. The relatively low frequency (28%) of *MGMT* methylation identified may reflect the reduced frequency (8%) of the C>T allele of the SNP in this colorectal cancer cohort (compared with 35% methylation in colorectal cancers with a C>T allele frequency of 9.2% in the study cohort by Ogino *et al*),[35] as opposed to any technical idiosyncrasy in the method used for methylation detection.

The precise mechanism by which the C>T polymorphism renders the promoter susceptible to methylation remains to be clarified. Functional studies have shown that deletion of the 59-bp enhancer element, within which this SNP is located, reduces transcriptional activity of the *MGMT* promoter by 95%.[36] A minimal protein binding motif of 9 bp located just 24–33 bp downstream of the SNP site has been shown to bind a 45 kDa transcriptional activator termed as the MGMT enhancer-binding protein.[45] Yet, enhancer activity increased with the incorporation of additional sequences flanking this motif, suggesting that nearby sequences also contribute to transcriptional activity.[45] Thus, it is possible that the C>T change results in downregulation of transcription with resultant accrual of methylation. Definitive evidence to show that the T allele incurs methylation of the *MGMT* promoter and that this in turn directly precedes and predisposes to neoplastic development, would require further functional assessment, as well as prospective and population-based cohort studies. If proven to be the case, this germline SNP may serve as a genetic risk marker for colorectal cancer, as well as other types of cancer in which *MGMT* methylation is frequently observed.

We found a strong association between *MGMT* methylation and methylation of *CDKN2A* and *MLH1*, as well as CIMP+ in our cohort, consistent with earlier studies.[26,44] The independent association we identified between *MGMT* and *CDKN2A* methylation on multivariate analysis suggests that these two loci may be targets of a common mechanism of epigenetic dysregulation that also underlies the interrelated features of *MLH1* methylation and CIMP+. Thus, while the C>T SNP is a key feature in *MGMT* methylation, generalised epigenetic disruption that underpins the methylation of additional genes may also be a contributing factor.

The presence of *MGMT* methylation within tumours also correlated with female gender and age, consistent with some, but not other studies of this gene.[10–12] There is a general trend evident at other loci, for both women and the elderly, to show more frequent aberrant methylation in tumours.[46,47] The frequency and level of *MGMT* methylation in normal colonic mucosa was also age related, suggesting that additional factors influence the accumulation of methylation at the *MGMT* promoter.

Our study confirmed the earlier reported link between *MGMT* methylation and presence of a *KRAS* mutation, but found no specific preference for G>A transitions, and no association with MGMT protein loss and the presence or nature of *KRAS* mutations. Our findings suggest that *MGMT* methylation occurs in the context of a *KRAS* mutation, but do not support a direct causal model

between *MGMT* inactivation and accrual of G>A mutations. In a recent study of comparable size, an increased rate of *KRAS* G>A mutations was found in colorectal cancers with loss of the MGMT protein, but not with promoter methylation of *MGMT*.[10] Thus, it is plausible that loss of MGMT precedes and induces G>A transitions in a subset of colorectal cancers, irrespective of the mechanism of MGMT loss. However, the results of our study coupled with the lack of consistency in the findings of other studies[11,12,15,17,19–21] argue against this direct sequence of events as a generalised phenomenon in the development of colorectal cancer. The concurrence of these epigenetic and genetic lesions in a subset of colorectal cancers suggests a more complex relationship between these events, perhaps akin to the close association between *MLH1* methylation and the BRAF V600E mutation.

The various molecular and clinicopathological associations with *MGMT* methylation have been inconsistent between studies in different colorectal cancer populations. This study represents the largest and most comprehensive study undertaken of this kind in a single colorectal cancer cohort, and thus may help reconcile these outstanding controversies. The frequency of the various features we tested for associations were typical of other sporadic colorectal cancer populations,[37–39] and so it is unlikely that our study of a single consecutive series of colorectal cancers incurred any significant selection bias. In summary, our results suggest that the germline C>T genotype represents a strong determinant of *MGMT* methylation, and additional factors including female gender and generalised epigenetic dysfunction are also contributory factors. *MGMT* methylation is strongly associated with *KRAS* mutation, but as opposed to a causal link, this may represent an interrelated occurrence of epigenetic and genetic aberrations in this subset of colorectal cancers.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)