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ONCOGENOMICS

Promoter methylation of the *mutated in colorectal cancer* gene is a frequent early event in colorectal cancer

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The *mutated in colorectal cancer* (*MCC*) gene is in close linkage with the *adenomatous polyposis coli* (*APC*) gene on chromosome 5, in a region of frequent loss of heterozygosity in colorectal cancer. The role of *MCC* in carcinogenesis, however, has not been extensively analysed, and functional studies are emerging, which implicate it as a candidate tumor suppressor gene. The aim of this study was to examine loss of *MCC* expression due to promoter hypermethylation and its clinicopathologic significance in colorectal cancer. Correspondence of *MCC* methylation with gene silencing was demonstrated using bisulfite sequencing, reverse transcription–polymerase chain reaction and Western blotting. *MCC* methylation was detected in 45–52% of 187 primary colorectal cancers. There was a striking association with *CDKN2A* methylation (*P* < 0.0001), the CpG island methylator phenotype (*P* < 0.0001) and the *BRAF* V600E mutation (*P* < 0.0001). *MCC* methylation was also more common (*P* = 0.0084) in serrated polyps than in adenomas. In contrast, there was no association with *APC* methylation or *KRAS* mutations. This study demonstrates for the first time that *MCC* methylation is a frequent change during colorectal carcinogenesis. Furthermore, *MCC* methylation is significantly associated with a distinct spectrum of precursor lesions, which are suggested to give rise to cancers via the serrated neoplasia pathway.
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Introduction

The *mutated in colorectal cancer* (*MCC*) gene was discovered in 1991 through its linkage to the region containing the susceptibility locus for familial adenomatous polyposis on chromosome 5q, which was subsequently shown to be the *adenomatous polyposis coli* (*APC*) gene (Groden *et al.*, 1991; Kinzler *et al.*, 1991; Nishisho *et al.*, 1991). The *APC* gene is altered somatically in ∼60–80% of sporadic colorectal cancers and this is considered an important early event in carcinogenesis. In contrast, little is known about the role of *MCC* in the pathogenesis of colorectal cancer. Early reports indicated that loss of heterozygosity (LOH) at the *MCC* locus is common, that is, ∼42% in colorectal cancers (Ashton-Rickardt *et al.*, 1991), whereas somatic mutation of *MCC* is relatively rare, 3–7% (Nishisho *et al.*, 1991). More recent studies have shown that the wild-type MCC protein may have a functional role in important cellular processes relevant in carcinogenesis, such as cell cycle regulation and the nuclear factor-κB (NF-κB) pathway (Matsumine *et al.*, 1996; Bouwmeester *et al.*, 2004). The activation and translocation of NF-κB to the nucleus is part of a signal-transduction pathway, which forms a basis for several physiologic and pathologic processes including cancer. These findings warrant further investigation of *MCC* as a potential tumor suppressor gene.

The molecular alterations involved in the progression from adenoma to colorectal carcinoma are now well established (Vogelstein *et al.*, 1988). LOH in chromosome 5q and mutations in the *APC* locus are considered among the earliest changes in adenoma formation. Hypermethylation of gene promoters is an important alternative mechanism by which tumor suppressor genes are silenced during colon carcinogenesis. A subset of sporadic colorectal cancers displays concordant methylation of multiple promoters and this is known as the CpG island methylator phenotype (CIMP) (Toyota *et al.*, 1999), although the definition of CIMP is controversial. CIMP comprises 20–30% of sporadic

colon cancers and includes, but is not confined to, tumors with high microsatellite instability (MSI-H), which are largely caused by hypermethylation of the *MLH1* mismatch repair gene (Samowitz *et al.*, 2005a; Weisenberger *et al.*, 2006). Hypermethylation of gene promoters is seen early in colon carcinogenesis and is found in a subset of both adenomas and hyperplastic polyps (HP) (Rashid *et al.*, 2001; O'Brien *et al.*, 2004). However, the MSI-H phenotype is more common in HP than in sporadic adenomas. HP were previously considered benign lesions with no malignant potential. The finding of MSI-H in a subset of these lesions (Jass *et al.*, 2000; Hawkins and Ward, 2001) led to the hypothesis that they represent an alternative pathway of colon carcinogenesis, called 'the serrated pathway'. This pathway also features CIMP and implicates other types of serrated polyps including 'serrated adenomas (SA)', 'mixed polyps' and 'sessile serrated adenomas (SSA)' (Jass, 2005). A subset of serrated polyps are also characterized by the V600E mutation of the *BRAF* oncogene, which is found in 5–15% of colorectal cancers and is associated with MSI-H and CIMP but inversely correlated with *KRAS* mutations (Kambara *et al.*, 2004).

MCC is a highly conserved cytoplasmic protein with orthologs (> 95% identity) across many species. It is expressed in the surface epithelium of the colon and other well-differentiated cells (Senda *et al.*, 1999). However, its loss of expression in colorectal cancer has not been studied previously. Given the importance of the chromosome 5 region in colorectal cancer and the emerging functional evidence relating MCC to pathways that are relevant in carcinogenesis, we set out to establish whether the *MCC* gene could be inactivated through promoter hypermethylation in colorectal cancer. We also wanted to determine any relationships of this feature with other molecular and clinicopathologic variables.

Results

### Characterization of CpG island methylation in the MCC promoter

A 514-bp section of the *MCC* promoter was obtained from the Ensembl Genome Browser and sequenced from 11 bisulfite-treated colon cancer cell lines. The sequencing revealed extensive methylation (48 CpG sites) in the HCT-15, HT-29, KM12SM, LISP-1 and LS411N colon cancer cell lines and partial methylation in the SW480 line. Most of the CpG sites in the other cell lines (Caco-2, HCT-116, LIM1215, LoVo, SW620) appeared unmethylated. Methylation-specific PCR (MSP) and quantitative PCR were designed for the area of highest CpG site density upstream of the transcription start site (TSS) (Figure 1). A PCR product was amplified only in the methylated cell lines (Figure 2a) and the two sets of primers produced the same results. The primers specific for the unmethylated promoter were amplified only in Caco-2, HCT 116, LIM1215, LoVo, SW620 and SW480 (Figure 2a).

Reverse transcription (RT)–PCR was carried out for 10 of the 11 cell lines, to correlate *MCC* promoter methylation with loss of gene expression. The predicted 265-bp fragment was consistently amplified in the unmethylated cell lines HCT-116, LIM1215, SW620, and in the partially methylated SW480, but not in Caco-2, which may have a different MCC defect (Figure 2b). The fully methylated cell lines HCT-15, HT-29, KM12SM, LISP-1 and LS411N had no detectable *MCC* expression. Expression of *MCC* mRNA was restored with the demethylation agent 5-aza-2′-deoxycytidine (Aza) treatment in HCT-15, KM12SM and LS411N but not in the Caco-2 line (Figure 2c). Western blotting of 10 tumor biopsies revealed loss or clearly reduced MCC protein expression in at least three strongly methylated tumors compared with matching normal tissue (Figure 2d and e). Another two methylated specimens were less conclusive possibly because of significant contamination by normal cells, including stromal and inflammatory cells.

Correlation of MCC methylation with clinicopathologic variables

MCC promoter methylation was first assessed using the MSP in 103 clinicopathologic stage C colon cancers. *MCC* methylation was detected in 52% of the cancers but was not found in the matched normal tissue from the same patients. Frequency of *CDKN2A* methylation was 33% and there was a highly significant correlation between the two methylated genes ($P = 0.0001$) (Table 1). *MCC* methylation was also associated with high tumor grade ($P = 0.0135$) and metastasis to four or more lymph
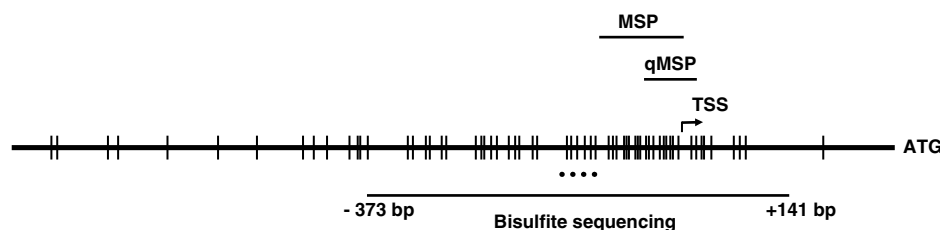
Figure 1 Schematic representation of the *MCC* promoter region and CpG island with the TSS 220 bp upstream from codon 1. The vertical bars indicate the position of each CpG site. Six CpG sites located in the middle of the CpG island were resistant to bisulfite modification (indicated by a dotted line). MSP and qMSP tests were designed upstream from the TSS in the region of highest CpG density.
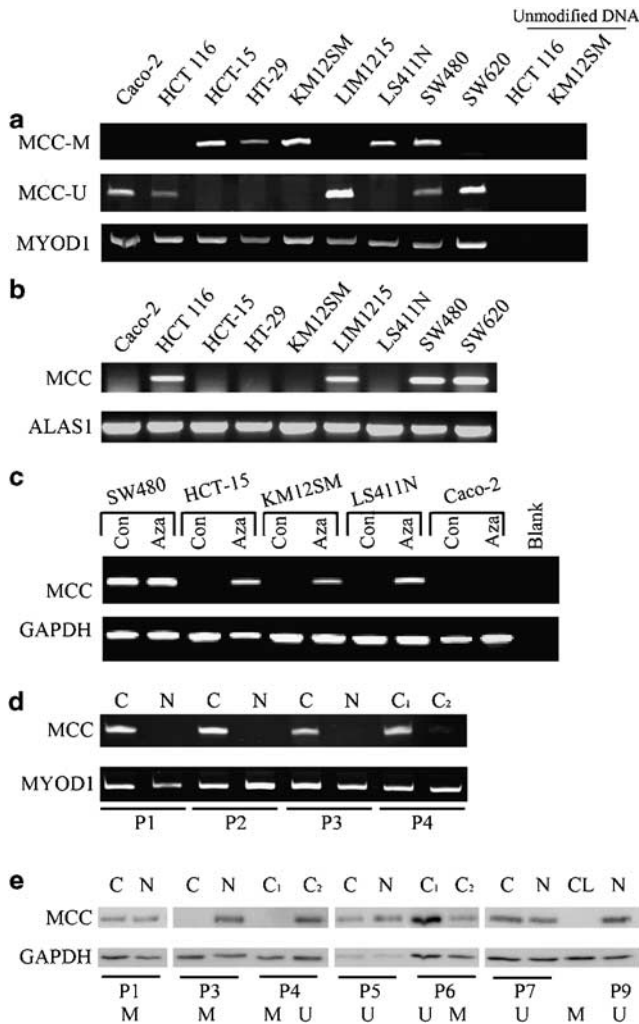
Figure 2 Correspondence of *MCC* gene promoter hypermethylation with loss of RNA or protein expression in cell lines (**a** and **b**) and primary colorectal cancers (**d** and **e**). (**a** and **d**) MSP using primers specific for the methylated (M, 99 bp) and unmethylated (U, 94 bp) *MCC* promoter and control PCR using *MYOD1* primers in bisulfite-treated DNA samples. (**b** and **c**) cDNA amplification from colorectal cancer cell lines with primers for *MCC* and housekeeping genes *ALAS1* and *GAPDH*. Addition of Aza to the control (Con) medium reversed *MCC* expression in HCT-15, KM12SM and LS411N cell lines, but not in Caco-2 cells, which are unmethylated. (**e**) Western blotting of primary colorectal cancer (C) and matching normal (N) tissue specimens. Loss or reduced MCC protein expression is evident in patients (P) 3, 4, 6 and in the cell line (CL) HCT-15.

nodes ($P = 0.0260$). *MCC* methylation was not associated with differential overall survival (hazard ratio (HR) 1.2, confidence interval (CI) 0.7–1.8) nor with survival to death due to colon cancer (HR 1.7, CI 0.9–3.2). There was no association with gender, tumor size, apical node or vein involvement. *MCC* methylation was more common in proximal than in distal colon cancer ($P = 0.0011$) (Table 1). There was a trend for more frequent *MCC* methylation in tumors that showed loss of MLH1 protein expression ($P = 0.0642$) or MSI-H ($P = 0.0930$) but this was not statistically significant.

Table 1 Comparison of *MCC* promoter methylation with *CDKN2A* promoter methylation, MLH1 protein expression, MSI and other clinicopathologic variables in 103 clinicopathologic stage C colon cancer patients

	MCC Methylated	*MCC* Unmethylated	Total number of patients	P
CDKN2A				
Methylated	27 (79.4)	7 (20.6)	34	
Unmethylated	27 (39.1)	42 (60.9)	69	**0.0001**
MLH1 expression				
Negative	13 (72.2)	5 (27.8)	18	
Positive	41 (48.2)	44 (51.8)	85	0.0642
MSI-H	17 (68.0)	8 (32.0)	25	
MSI-L/MSS	37 (48.7)	39 (51.3)	76	0.0934
Female	21 (52.5)	19 (47.5)	40	
Male	33 (52.4)	30 (47.6)	63	0.9906
Proximal colon	48 (61.5)	30 (38.5)	78	
Splenic flexure and descending colon	6 (24.0)	19 (76.0)	25	**0.0011**
Low–average grade	21 (40.4)	31 (59.6)	52	
High grade	33 (64.7)	18 (35.3)	51	**0.0135**
1–3 nodes	32 (45.1)	39 (54.9)	71	
⩾4 nodes	22 (68.8)	10 (31.2)	32	**0.026**
Apical node involved				
Yes	7 (70.0)	3 (30.0)	10	0.2416
No	47 (50.5)	46 (49.5)	93	
Veins involved				
Yes	13 (61.9)	8 (38.1)	21	
No	41 (50.0)	41 (50.0)	82	0.3297
Tumor size				
<5 cm	23 (46.9)	26 (53.1)	49	0.288
⩾5 cm	31 (57.4)	23 (42.6)	54	

Abbreviations: *MCC, mutated in colorectal cancer*; MSI-H, high microsatellite instability; MSI-L, low microsatellite instability; MSS, microsatellite stable.

Correlation of MCC methylation with the BRAF mutation and CIMP

In a separate cohort of colorectal cancer patients of known CIMP and *BRAF/KRAS* mutation status, *MCC* methylation was detected in 38 out of 84 sporadic cancer specimens (45%) and one out of seven hereditary non-polyposis colon cancer (HNPCC) tumors using quantitative MSP. *MCC* methylation was detected in all CIMP + tumors ($P < 0.0001$) and in all 17 tumors with the *BRAF* V600E mutation ($P < 0.0001$; Table 2). There was no correlation with the presence of *KRAS* mutation. MSI-H cancers showed *MCC* methylation more often ($P = 0.0444$). There was no correlation with the stage of cancer but there was a trend for more frequent methylation in proximal compared with distal cancers. *APC* methylation was detected in 25% of the specimens and did not correlate with *MCC* methylation ($P = 0.3191$), CIMP ($P = 0.0858$) or *BRAF* V600E ($P = 0.3388$). Only two out of 20 CIMP + tumors and two out of 16 tumors with the *BRAF* mutation showed *APC* methylation.

Hyperplastic polyps and adenomas

In a well-characterized series of 42 precursor lesions, *MCC* methylation was significantly more common in serrated polyps (20/25; 80%) than in traditional adenomas (AD) (6/17; 35%, $P = 0.0084$), including eight

Table 2 Comparison of *MCC* promoter methylation with other molecular variables

	MCC Methylated	*MCC* Unmethylated	Total number of patients	P
CIMP+	21 (100)	0 (0)	21	**<0.0001**
CIMP−	18 (25.7)	52 (74.3)	70	
CDKN2A				
Methylated	23 (88.5)	3 (11.5)	26	**<0.0001**
Unmethylated	16 (24.6)	49 (75.4)	65	
APC				
Methylated	11 (52.4)	10 (47.6)	21	0.3191
Unmethylated	26 (40.0)	39 (60.0)	65	
MSI-H	12 (63.2)	7 (36.8)	19	
MSI-L/MSS	27 (37.5)	45 (62.5)	72	**0.0444**
BRAF V600E mutation				
Yes	17 (100)	0 (0)	17	**<0.0001**
No	22 (29.7)	52 (70.3)	74	
KRAS codon 12/13 mut.				
Yes	9 (34.6)	17 (65.4)	26	0.356
No	30 (46.2)	35 (53.8)	65	
Proximal colon	20 (55.6)	16 (44.4)	36	
Distal colon	17 (34.7)	32 (65.3)	49	0.0553
Stage A	1 (50.0)	1 (50.0)	2	
Stage B	15 (34.9)	28 (65.1)	43	
Stage C	10 (45.5)	12 (54.5)	22	
Stage D	7 (50.0)	7 (50.0)	14	0.7098

Abbreviations: *APC*: *adenomatous polyposis coli*; CIMP: CpG island methylator phenotype; *MCC*: *mutated in colorectal cancer*; MSI-H: high microsatellite instability; MSI-L, low microsatellite instability; MSS, microsatellite stable.

out of nine SSA (Figure 3). *APC* methylation was less common in serrated polyps (4/18; 22%) than in AD (9/16; 56%) but this was not statistically significant ($P = 0.0764$). There was no association between *APC* and *MCC* methylation ($P = 0.2376$).

Discussion

This study has shown for the first time that the *MCC* gene is frequently methylated in colorectal cancer, which leads to loss of RNA and protein expression of this gene. This raises questions about the biological significance of the *MCC* defect and the molecular pathways involved. Here, we also provide new evidence that *MCC* methylation is an early event in colon carcinogenesis and correlates with CIMP but not with *APC* methylation. This suggests that the two linked chromosome 5 genes are methylated independently and exert independent effects on colorectal cancer phenotype.

APC mutations, methylation and LOH are common in adenomas and are considered important early events in colorectal carcinogenesis. In this study, we detected a clear difference in the spectrum of *MCC* methylation in early lesions. *MCC* methylation was more common in serrated polyps than in AD. The series analysed here included nine SSA, three SA and one mixed polyp, of which all but one showed *MCC* methylation. Advanced serrated polyps have recently emerged as a precursor lesion for the MSI-H subset of colorectal cancers that
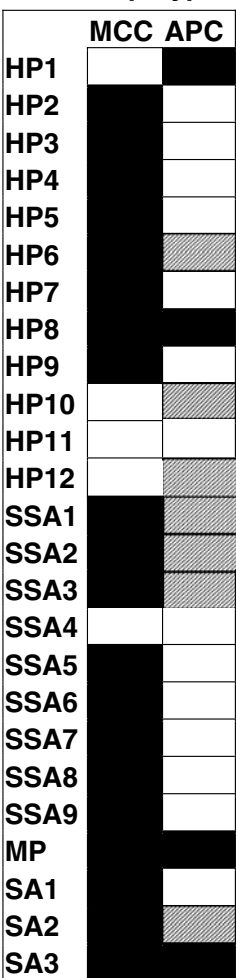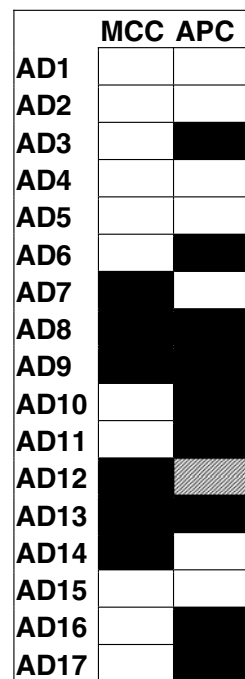
Figure 3 *MCC* and *APC* methylation in a series of hyperplastic polyps (HP), serrated adenomas (SA), sessile SA (SSA), a mixed polyp (MP) and traditional adenomas (AD). Black cells indicate presence of methylation, white cells lack of methylation and shaded cells indicate those specimens where no data are available. HP2-3, HP5-6, HP9, SSA1-SSA9 and SA3 are from hyperplastic polyposis patients, the other serrated polyps are sporadic.

also display high-frequency methylation of tumor suppressor genes (Kambara *et al*., 2004; Wynter *et al*., 2004). This has been extensively studied in the setting of hyperplastic polyposis, where patients present with multiple, large, mostly proximal SSA. However, not all HP show extensive methylation, in particular the common small polyps that are typically present in the distal colon and rectum. Our data support the view that CIMP could be a marker for those HP that have malignant potential (Wynter *et al*., 2004).

MCC methylation was further analysed in two independent cancer patient cohorts. The stage C cohort included extensive clinicopathologic follow-up data, whereas the second cohort was more suitable for correlation with other gene alterations. The latter cohort was a subset of the patients recently analysed for 195 methylated gene markers, which did not include the *MCC* gene (Weisenberger *et al*., 2006). Both cohorts had

been analysed for MSI and *CDKN2A* methylation. Striking positive associations were found with the *BRAF* V600E mutation, *CDKN2A* methylation and CIMP. There was no association with *APC* methylation or with *KRAS* mutations. *MCC* methylation was found in all but one specimen with the *BRAF* mutation, out of a total of 29 cancers and serrated polyps. *BRAF* and *KRAS* oncogenes both upregulate the RAS/RAF/ERK pathway with wide-ranging effects on gene expression, cell proliferation and differentiation, cytoskeletal rearrangements and chromatin remodeling. The *BRAF* V600E mutation also increases NF-κB signaling (Ikenoue *et al.*, 2003). This mutation is found in serrated polyps but not in adenomas (Kambara *et al.*, 2004) and displays a striking correlation with MSI-H and CIMP (Nagasaka *et al.*, 2004). *KRAS* mutations are found in a wider spectrum of early lesions, but never together with *BRAF* V600E, and are possibly caused by a defect in the *MGMT* repair gene (Esteller *et al.*, 2000). Therefore, our findings add to the speculation that there is a causal relationship between the *BRAF* mutation and one or more genes methylated in CIMP.

There are strong indications that *MCC* is involved in important cellular functions. The MCC protein becomes highly phosphorylated during the G1- to S-phase transition *in vitro* and is a potential negative regulator of cell cycle progression (Matsumine *et al.*, 1996). Overexpression of wild-type MCC protein *in vitro* resulted in a decrease in the number of cells entering the cell cycle S phase. This cell cycle inhibitory effect was completely abolished using mutant forms of the MCC protein (Matsumine *et al.*, 1996). One mutation tested was the same as that initially identified in a tumor from a colorectal cancer patient (Kinzler *et al.*, 1991). Particularly interesting is the finding that MCC appears to stabilize the IκBβ protein that is an essential inhibitor of NF-κB transcription factor activation (Bouwmeester *et al.*, 2004). There is evidence that NF-κB activation is involved in colorectal carcinogenesis (Karin *et al.*, 2002) and that it can be caused by the *BRAF* V600E mutation *in vitro* (Ikenoue *et al.*, 2003). The more precise role of an *MCC* defect in this process can now be tested experimentally.

One novel clinicopathologic association detected here was that *MCC* methylation was more commonly found in clinicopathologic stage C tumors with four or more involved lymph nodes. This may suggest an effect on tumor metastasis. However, a survival analysis did not reveal any difference between patient groups with or without *MCC* methylation. One study has demonstrated that CIMP is associated with adverse prognosis, but only in those patients who do not have MSI-H tumors (Ward *et al.*, 2003). Similarly, the *BRAF* V600E mutation is associated with poor survival but only in MSS colon cancers (Samowitz *et al.*, 2005b). Therefore, it is possible that an adverse effect of gene methylation on survival is difficult to demonstrate because of the survival benefit attributed to the MSI-H phenotype, which comprises a subgroup of CIMP. Detailed molecular analysis of much larger cohorts of patients will be required to further define the outcome of these patient subgroups.

In conclusion, we have characterized a new methylated gene marker for early colorectal carcinogenesis. The finding of a higher frequency of *MCC* methylation in serrated polyps than in adenomas provides an interesting contrast with the *APC* gene that is closely linked with *MCC* but is methylated in a different spectrum of early lesions. Further study is required to define the role of *MCC* in colorectal carcinogenesis.

Materials and methods

Patients and tumor specimens

Paraffin-embedded tumor and matched normal tissue specimens were analysed from 103 clinicopathologic stage C colon cancer patients from the Concord Hospital. These were previously examined for microsatellite instability, *CDKN2A* methylation, MGMT and mismatch repair protein expression (Kohonen-Corish *et al.*, 2005). Frozen biopsies of tumor and matching normal tissue were available for protein analysis from an additional 10 cancers from eight patients. We also examined a further cohort of patients from the Royal Brisbane Hospital who were previously analysed for MSI, *BRAF* and *KRAS* mutations and CIMP (Kambara *et al.*, 2004; Wynter *et al.*, 2004; Weisenberger *et al.*, 2006), This cohort consisted of 84 sporadic and seven hereditary non-polyposis colon cancers and 42 adenomas and HP. The early lesions were divided into two groups for statistical analysis: group 1 included nine SSA, three SA, one mixed polyp and 12 HP; group 2 consisted of 10 adenomas, two tubular adenomas and five tubulovillous adenomas. The Royal Brisbane Hospital cohort of 91 cancer patients was a subset of the patients analysed in a recent study that redefined the classification of CIMP by using five new methylated gene markers (Weisenberger *et al.*, 2006). If at least three out of five of these genes showed methylation, the cancer was classified as CIMP + and methylation in 0–2 genes was classified as CIMP−. The study was approved by the South-Western Sydney Area Health Service Human Research Ethics Committee and the Human Research Ethics Committee of the Queensland Institute of Medical Research.

Bisulfite sequencing of the MCC promoter

A 360-kb genomic sequence containing the MCC gene was obtained from the Ensembl Genome Browser (ENSG00000171444; http://www.ensembl.org). This includes a 90.7-kb region upstream of the translation initiation codon. A 522-bp CpG island was identified using the EMBOSS-CpGPlot program (EMBL-European Bioinformatics Institute; http://www.ebi.ac.uk). A TSS is located in this CpG island 220 bp upstream of codon 1 (Figure 1) (Ensembl Transcript ID ENST00000302475).

DNA from cancer cell lines was bisulfite-treated using standard methods (Herman *et al.*, 1996). The *MCC* promoter region was amplified using primers *MCC*-promF 5′-GG TTAGTAGTTAGATAGTTGT-3′, and *MCC*-promR 5′-TAC TTAATCCCTTCTACCAC-3′ (Figure 1). PCR conditions were 95°C (30 s), 53°C (45 s) and 72°C (90 s) for 40 cycles. The 514-bp fragment was sequenced using BigDye on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Methylation-specific PCR

The *MCC* promoter was amplified from bisulfite-treated tumor and cell line DNA specimens using methylation-specific primers *MCC*-metF1 5′-TATTGTTTCGGAACGGG

GCGT-3′ and *MCC*-metR1 5′-CAAAAAACTCGATAACGC GACG-3′ (Figure 1). Primers specific for the unmethylated promoter were *MCC*-unmetF1 5′-GGTATTGTTTTGGAAT GGGGTG-3′ and *MCC*-unmetR1 5′-CTCAATAACACAAC ACACTCAC-3′. PCR conditions were 95°C (30 s), 61°C (45 s) and 72°C (30 s) for 35 cycles. *CDKN2A* promoter methylation was analysed as previously described (Herman *et al.*, 1996; Kohonen-Corish *et al.*, 2005). The *MYOD1* primers, which do not contain any CpG nucleotide sequences, were used as an internal reference PCR to ensure integrity of each DNA specimen (Eads *et al.*, 1999, 2000). PCR conditions were 95°C (45 s), 57°C (45 s) and 72°C (60 s) for 38 cycles. All PCR reactions in this study were carried out using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) in a DNA engine DYAD (MJ Research, Waltham, MA, USA). The cycling conditions included an initial denaturation step at 95°C for 12 min and a final elongation at 72°C for 7 min.

Methylation-specific quantitative PCR
Methylation-specific primers for the real-time PCR assay were *MCC*-metF2 5′-TTTCGTCGTTGTCGTAGTTG-3′ and *MCC*-metR2 5′-TTCACGAACGAACCATTACTA-3′ (Figure 1). The methylated amplicon-specific fluorogenic hybridization probe was 6FAM5′-CGCGTCGCGTTATC GAGTT-3′BHQ-1. The PCR was carried out in duplicate for each tumor specimen using the RealMasterMix Probe ROX (Eppendorf, Hamburg, Germany) with 4 mM Mg^{2+} in a Rotorgene 3000 (Corbett Research, Sydney, Australia). PCR conditions were 95°C (15 s) and 60°C (60 s) for 50 cycles, with an initial denaturation at 95°C for 2 min. *APC* methylation and the internal reference gene *MYOD1* were analysed following the previously described MethyLight protocol (Eads *et al.*, 1999, 2000). Following this method, the threshold cycle $C_{(T)}$ value for each test and reference PCR is compared to a standard curve to obtain the quantitation value for each reaction. The standard curve is obtained using serial dilutions of a fully methylated control sample with known concentration. The relative quantitation of methylation in a tumor specimen is then determined by calculating the ratio of the test gene value (*MCC* or *APC*) to the reference gene value (*MYOD1*) for the same specimen (Eads *et al.*, 1999, 2000). Limit of detection of each assay was 1.2 ng/μl.

cDNA and protein analysis
For the Aza treatment colon cancer cells were freshly seeded and allowed to grow overnight. The culture medium was then replaced with fresh medium containing 1 μM of Aza (Sigma-Aldrich Corporation, St Louis, MO, USA). Cells were allowed to grow for 72 h, with changing of Aza-containing medium every 24 h, and then harvested. A cell viability of >70% was retained after 72 h of treatment. RNA was extracted from cell lines using an RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was prepared with the Expand Reverse Transcriptase kit (Roche, Mannheim, Germany). A 265-bp fragment was amplified from exons 1 and 2 using primers *MCC*-cDNAF1 5′-CTGGGTGAAAATGGCTGTCT-3′ and *MCC*-cDNAR1 5′-GTTCCCTCTCTGTTTGCTGG-3′. PCR conditions were 95°C (30 s), 60°C (30 s) and 72°C (30 s) for 35 cycles.

Whole biopsies of 10 primary tumors were frozen after surgery for protein extraction. Protein (20 μg) from total tissue lysate was separated using a 10% SDS–polyacrylamide gel at 100 V for 1.5 h, and transferred to PVDF membrane for 1 h at 120 V. Membranes were blocked in 5% skim milk powder/ Tris-buffered-saline-Triton (0.05%), and incubated with primary antibody, 1:2000 α-MCC (Pharmingen, San Diego, CA, USA) or 1:48 000 α-GAPDH (Ambion, Austin, TX, USA), overnight at 4°C. Horseradish peroxidase-conjugated anti-mouse IgG (Amersham, Piscataway, NJ, USA) was used as the secondary antibody. Bands were visualized using ECL.

Survival analysis and statistics
The χ^2 test was used to evaluate the significance of differences in contingency tables. Patients were followed annually until death or December 2004. Overall survival was measured from the date of resection to the date of death owing to any cause, censored patients being those alive at the close of the study or lost to follow-up. For death owing to colon cancer, censored patients were those alive at the close of the study, lost to follow-up or deceased owing to other causes. Proportional hazards regression was used to determine HR and their 95% CI. The level for statistical significance was set at $\leqslant 0.05$.

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