

COLORECTAL CANCER

Extensive DNA methylation in normal colorectal mucosa in hyperplastic polyposis

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Background: Hyperplastic polyposis of the colorectum is a precancerous condition that has been linked with DNA methylation. The polyps in this condition have been distinguished from typical small hyperplastic polyps and renamed sessile serrated adenomas. Sessile serrated adenomas also occur sporadically and appear to be indistinguishable from their counterparts in hyperplastic polyposis.

Aims and methods: The existence of distinguishing molecular features was explored in a series of serrated polyps and matched normal mucosa from patients with and without hyperplastic polyposis by assessing mutation of *BRAF*, DNA methylation in 14 markers (*MINTs* 1, 2 and 31, *p16*, *MGMT*, *MLH1*, *RASSF1*, *RASSF2*, *NORE1* (*RASSF5*), *RKIP*, *MST1*, *DAPK*, *FAS*, and *CHFR*), and immunoprecipitation of *MLH1*.

Results: There was more extensive methylation in sessile serrated adenomas from subjects with hyperplastic polyposis ($p < 0.0001$). A more clearcut difference in patients with hyperplastic polyposis was the finding of extensive DNA methylation in normal mucosa from the proximal colon.

Conclusions: A genetic predisposition may underlie at least some forms of hyperplastic polyposis in which the earliest manifestation may be hypermethylation of multiple gene promoters in normal colorectal mucosa. Additionally, some of the heterogeneity within hyperplastic polyposis may be explained by different propensities for *MLH1* inactivation within polyps.

Colorectal hyperplastic polyps (HPs) with their characteristic serrated glandular architecture are easily distinguished on morphological grounds from adenomas and have been classified as non-neoplastic lesions that are lacking in malignant potential.¹ While an individual HP may have extremely limited potential for malignant transformation, there are reports linking the condition hyperplastic polyposis (HPP) with colorectal cancer (CRC).^{2–4} This raises the question of whether HPs occurring in the condition HPP are qualitatively different from their sporadic counterparts.

The definition of HPP allows for two major phenotypes: (1) multiple small and mainly distal polyps; and (2) small numbers of large and mainly proximal polyps.⁵ This morphological heterogeneity may explain why some studies have failed to show a convincing link with CRC.^{6–7} It is pertinent that investigations that have focused on right sided HPs have suggested that these may be the precursors of the subset of CRC with high level DNA microsatellite instability (MSI-H) whether presenting in HPP⁸ or sporadically.^{9–12} The first detailed morphological characterisation of large and proximal HPs occurred in the context of HPP.² The authors argued that the polyps in question were distinct from traditional HPs and that the condition they were describing was more aptly termed serrated adenomatous polyposis. The same authors then went on to demonstrate that identical "sessile serrated adenomas" (SSA) could occur sporadically and described in considerable detail the morphological features that distinguished these lesions from typical HPs.¹³

Parallel studies focusing on immunohistochemical and molecular changes also showed differences between proximal and distal lesions, whether these were sporadic or presented in the context of HPP. Specifically, proximal polyps and/or polyps with the features of SSA showed more aberrant proliferation,¹⁴ and high frequencies of *BRAF* mutation^{15–16} and DNA methylation.^{15–17} In contrast, small and distal HPs had frequent *KRAS* mutation^{3–15} or alterations implicating

chromosome 1p³ and low frequencies of both *BRAF* mutation¹⁶ and DNA methylation.^{3–15–17}

It is not known if the serrated polyps presenting in HPP are qualitatively different from their sporadic counterparts that are large, proximal, and show the morphological features of SSA. Based on findings with respect to *BRAF* mutation and DNA methylation, it would appear that the serrated polyps occurring in these two clinical scenarios do not differ qualitatively and the link between HPP and colorectal cancer is observed merely because the polyps are very numerous. Nevertheless, there are grounds for suspecting that the polyps in HPP may differ. A key rate limiting step in the evolution of sporadic CRC with MSI-H is methylation of the promoter region and subsequent inactivation of the DNA mismatch repair gene *MLH1*.¹⁸ Using immunohistochemistry to test for loss of expression of *MLH1* in 44 sporadic serrated polyps that were all resected from the proximal colon, none of the polyps showed even focal loss of *MLH1*.¹⁴ It would therefore appear that loss of expression of *MLH1* is an uncommon event in sporadic serrated polyps, although such loss has been described in a selected series of sporadic mixed polyps.¹⁹ In contrast, loss of *MLH1* has been observed in a high proportion of serrated polyps occurring within the condition HPP.⁸

In the present study, we have identified three patients with HPP in which the phenotype was particularly severe, as manifested by the presence of at least one CRC, serrated polyps with adenomatous dysplasia (mixed polyps or serrated adenomas), as well as very numerous polyps with the features of SSA. We compared patterns of DNA methylation

Abbreviations: HP, hyperplastic polyp; HPP, hyperplastic polyposis; SSA, sessile serrated adenoma; TSA, traditional serrated adenoma; HNPCC, hereditary non-polyposis colorectal cancer; CRC, colorectal cancer; MSI, microsatellite instability; CIMP, CpG island methylator phenotype; PCR, polymerase chain reaction; MSP, methylation specific polymerase chain reaction; MAPK, mitogen activated protein kinase

and expression of MLH1 in polyps and normal mucosa from these patients with samples obtained from patients with only small numbers of right sided serrated polyps. As inhibition of apoptosis has been linked to the aetiology of serrated polyps,^{20,21} the marker panel for DNA methylation included multiple genes with a proapoptotic function.

MATERIALS AND METHODS

Patients and tissues

Patient Nos 1–3 met one or more of the diagnostic criteria for HPP, each having multiple serrated polyps in the proximal colon of which at least two exceeded 10 mm in diameter.⁵ The numbers and types of polyps are shown in table 1. In patient No 2, over 100 polyps were counted and 54 of these were diagnosed histologically. Patient No 4 and 5 presented with proximal CRC and small numbers of proximal serrated polyps that did not meet the criteria for HPP. The serrated polyps in patient No 1–5 were classified as HPs, mixed polyps, traditional serrated adenomas (TSA), and the variant HP described as SSA¹³ (table 1, fig 1). Diffuse mucosal hyperplasia was present in the appendix in patient No 1, 4, and 5. Patient No 1 and No 3–5 were treated by right hemicolectomy and patient No 2 by proctocolectomy. Patient No 6–12 had a single SSA of the proximal colon that was removed endoscopically. Patient No 13–15 had a right sided CRC and at least one right sided HP but only DNA obtained from normal mucosa was tested.

Seven of the CRCs shown in table 1 for patient No 1–5 presented in the proximal colon. Patient No 2 had synchronous rectal cancer arising in a tubulovillous adenoma that was not subjected to further analysis. CRCs in patient No 2, 4, and 5 were contiguous with residual SSA (not included in totals shown in table 1). None of the patients had inflammatory bowel disease. The study was approved by the institutional review board of McGill University.

MLH1 immunostaining

Immunohistochemical staining for the mismatch repair protein MLH1 was undertaken for 18, 54, and four serrated polyps from patient No 1–3, respectively, and seven CRCs (including any contiguous polyps) from patient No 1–5. Sections (4 µm) of formalin fixed paraffin embedded tissues were cut, mounted onto charged slides, and prepared for incubation with the primary mouse monoclonal antibody to MLH1 (1:100 clone G168-15; BD Pharmingen, Bedford, Massachusetts, USA), as described previously.¹⁴ Loss of MLH1 expression was scored only when there was complete

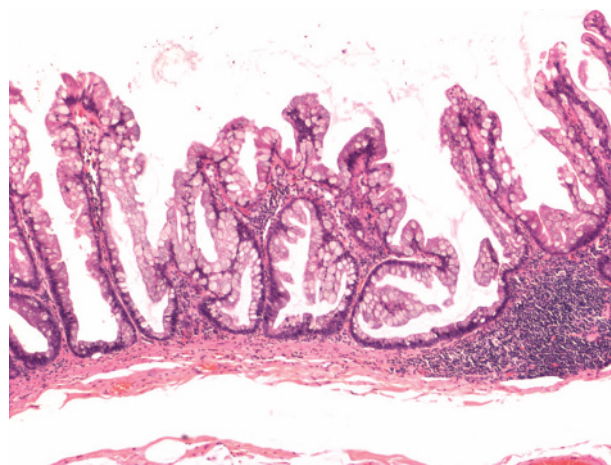


Figure 1 Typical histological appearances of a sessile serrated adenoma from patient No 2. The crypts are dilated and mucin filled, show exaggerated serration, and increased branching, and extend horizontally along the muscularis mucosae. Haematoxylin and eosin stain.

loss of nuclear expression within a distinct subclone or an entire colorectal crypt, including the crypt base cells.

DNA extraction and bisulphite modification

Samples were microdissected from paraffin embedded tissue using two 8 µm thick sections that included 23 SSAs, two mixed polyps, one TSA, two conventional adenomas, and seven CRCs. Care was taken to exclude as much normal mucosa as possible. In the case of polyps, no more than 5% of crypts would have been derived from normal mucosa. Normal mucosa was obtained from two separate samples in patient No 1, 2, 3, and 5, and one sample in patient No 4, 6, and 13–15. Meticulous care was taken to exclude any non-normal tissue. Samples of normal mucosa were all obtained from the proximal colon. Cell lysis and DNA extraction were performed using a QIAamp DNA mini kit (QIAGEN, Mississauga, Ontario, Canada) according to the manufacturer's protocol. Extracted genomic DNA was diluted in 40 µl of distilled water and denatured by adding 6 µl of 2N NaOH and incubation at 75°C for 20 minutes. Freshly prepared 4.8 M sodium bisulphite (500 µl) and 28 µl of 10 mM hydroquinone were added to the denatured genomic DNA and the reaction was carried out overnight in the dark at 55°C. DNA was then purified using a Wizard DNA clean-up (Promega, Madison, Wisconsin, USA) and then ethanol precipitated after five minutes of alkali treatment with 8.8 µl of 2 N NaOH at room temperature.

Methylation specific polymerase chain reaction (PCR)

Methylation of promoter region of 11 genes and three MINT loci (see below) was examined by methylation specific polymerase chain reaction (MSP) using AmpliTaq Gold kit (Roche, Branchburg, New Jersey, USA). MINT1, MINT2, MINT31, *p16* (*CDKN2A*), *MLH1*, and *O-6-methylguanine DNA methyltransferase* (*MGMT*) have been employed in several previous studies defining CpG island methylator phenotype (CIMP) in colorectal polyps and cancers.^{22–26} RASSF1, RASSF2, and NORE1 (RASSF5) are members of the RAS association domain family (RASSF) of RAS effectors, known to link RAS activation to cell cycle arrest and apoptosis.^{27–31} Mammalian sterile20-like 1 (MST1) is a ubiquitously expressed proapoptotic protein kinase that mediates the apoptotic effect of RAS by forming a complex with RASSF1/NORE1 and induction of the caspase cascade.^{31–33} The cell surface receptor FAS and death associated protein kinase

Table 1 Clinical features and polyp diagnoses in subjects with colorectal cancer (CRC)†

Patient No	Age (y)	Sex	HP	SSA	MP	TSA	AD	CRC
1	64	F	3	10	4	1	4	1
2	63	M	32	21	1	0	3	2*
3	70	F	0	8	3	1	4	3
4	28	F	0	3	0	0	0	1
5	75	M	0	3	0	0	1	1

*A synchronous rectal cancer was not subjected to molecular analysis. HP, hyperplastic polyp; SSA, sessile serrated adenoma; MP, mixed polyp (all were part SSA, part AD); TSA, traditional serrated adenoma; AD, adenoma; CRC, colorectal cancer.

SSAs contiguous with CRCs from patient No 2, 5, and 6 are not included in the polyp count but the contiguous SSA from patient No 4 was analysed for DNA methylation.

†Respective age and sex for patient No 6–15 with diagnoses presented under methods are: 75 M; 63 M; 65 F; 72 M; 74 M; 64 F; 48 F; 73 F; 74 F; 72 F.

Table 2 Primer sequences and methylation specific polymerase chain reaction conditions

Primer name	Sense primer (5'-3')	Antisense primer (5'-3')	Annealing temp (°C)	Reference
MINT1				
U	AATTTTTTATATATATTTTGAAGT	AACAAAAACCTCAACCCCAACA	54	Park ²³
M	AATTTTTTATATATATTTTGAAGC	AAAAACCTCAACCCCGCG	54	Park ²³
MINT2				
U	GATTTGTAAAGTGTGAGTTTGT	CAAATAATAACAACAATCCATACA	54	Park ²³
M	TTGTTAAAGTGTGAGTTTCGTC	AATAACGACGATTCGCTACG	54	Park ²³
MINT31				
U	TAGATGTGGGGAAGTGTTTTGGT	TAAATACCCAAAAACAAACACCACA	59	Park ²³
M	TGTTGGGGAAGTGTTTTTCGGC	CGAAAACGAAACGCCGCG	59	Park ²³
P16				
U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCCACAACCCATAA	59	Park ²³
M	TTATTAGAGGGTGGGGCGGATCGC	GACCCGAAACCCGACCGTAA	59	Park ²³
MLH1				
U	AATGAATTAATGGAAGAGTGGATAGT	TCTCTTACCTCCCTAAACA	54	Petko ⁴¹
M	CGGATAGCGATTTTAAACGC	CCTAAACGACTACTACCCG	54	Petko ⁴¹
MGMT				
U	TTTGTGTTTGTATGTTGTAGGTTTTGT	AACTCCACACTCTTCCAAAACAAAACA	59	Esteller ⁴²
M	TTTCGACGTCGTAGGTTTTCGC	GCACTCTCCGAAAACGAAACG	59	Esteller ⁴²
RASSF1				
U	TTTAGGTTTTTATTGTGTGTT	CCAATTAACCCATACTTCACTAA	52	–
M	GTATTAGGTTTTTATTGCGCG	GATTAACCCGTACTTCGCTAA	52	–
RASFF2				
U	AGTTGTTGTTGTTTTAGGTGG	AAAAAACCAACACCCCCACA	54	Hesson ⁴³
M	GTTGTCGTCGTTTTTATAGGCG	AAAAACCAACGACCCCGCG	58	Hesson ⁴³
NORE1				
U	AAGGAAGGGGAAATTAATTAGAGT	CCCCTCTAAAACAAAACCTCAACA	56	–
M	AGGAAGGGGAAATTAATTAGAGC	CCTCTAAAACGAAACTCGACC	56	–
MST1				
U	TTTTGGTAATGTAGGAAGATAGTGT	AAACCACAACCTAATCACATA	54	–
M	GTTTCGTAACGTAGGAAGATAGC	AACCACGACCCTAATCACGTA	54	–
FAS				
U	AATTAATGGAGTTTTTTAATTGG	AACACCTATATACACTTTACACAAA	54	–
M	ATTAATGGAGTTTTTTAATTCGG	CACCTATATACACTTTACGCGAA	54	–
DAPK				
U	GGTAAGGAGTGTGAGAGGTGTTTT	ACCCTACACTACAAATTACCAAA	54	–
M	GTAAGGAGTCGAGAGGTGTTTC	CCTACCGTACGAATTACCG	54	–
CHFR				
U	TTGGTTAGGATTAAGATGGTTGA	CCCACAATTAACCAACAACACT	54	–
M	TTGGTTAGGATTAAGATGGTGC	GCGATTAACCAACGACGACG	54	–
RKIP				
U	TTTAGTGATATTTTTGAGATATGA	CACTCCCTAACCTCTAATTAACCAA	52	–
M	TTTAGCGATATTTTTGAGATACGA	GCTCCCTAACCTCTAATTAACCG	52	–

(DAPK) are known mediators of the extrinsic (death receptor initiated) apoptotic pathway.^{34–35} Checkpoint gene with fork-head and RING finger domains (CHFR) functions as a mitotic checkpoint to delay entry into metaphase.³⁶ Raf

kinase inhibitor protein (RKIP) negatively regulates mitogen activated protein kinase (MAPK) by direct binding and inhibition of Raf kinase.^{37–38} The primer sequences of these

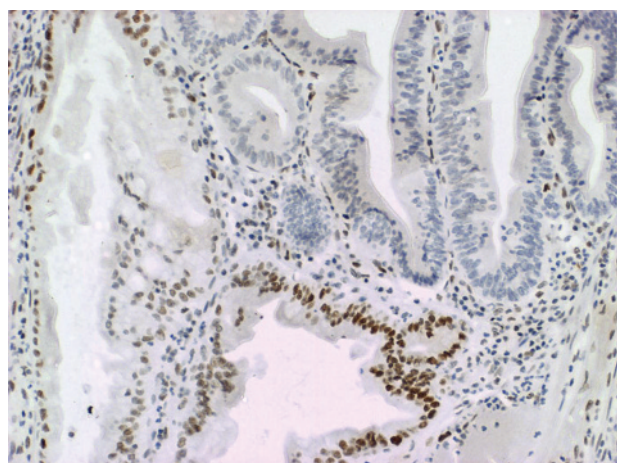


Figure 2 Loss of nuclear expression of MLH1 in adenomatous or dysplastic clone (upper right) within a sessile serrated adenoma (giving a mixed polyp) from patient No 1. ABC technique.

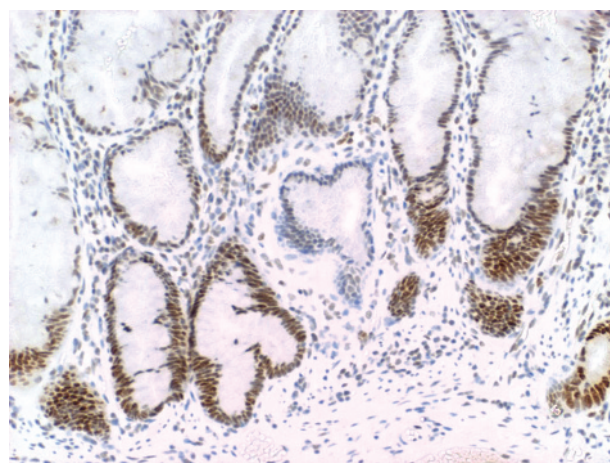


Figure 3 Loss of nuclear expression of MLH1 (possible mild residual staining) in a single crypt non-dysplastic crypt in a sessile serrated adenoma from patient No 2. The crypt is not longitudinally sectioned but loss is clearly occurring near the crypt base. This was the only evidence of loss of expression of MLH1 in a total of 54 polyps that were tested in this patient (the colorectal cancer also showed loss of MLH1). ABC technique.

genes are listed in table 2. Conditions for amplification were 10 minutes at 95°C followed by 39 cycles of denaturing at 95°C for 30 seconds, annealing at certain temperatures (table 2) for 30 seconds, and 30 seconds of extension at 72°C. PCR products were subjected to electrophoresis on 8% acrylamide gels and visualised by SYBR gold nucleic acid gel stain (Molecular Probes, Eugene, USA). CpGenome Universal Methylated DNA (Chemicon, Temecula, California, USA) was used as a positive control for methylation. Gene promoters were considered methylated if the intensity of methylated bands was more than 10% of their respective unmethylated bands.

BRAF mutation

BRAF mutation analysis at codon 600 (V600E; formerly V599E)³⁹ was performed by a real time PCR based allelic discrimination method, as previously described.⁴⁰ Briefly, primers were designed to selectively amplify the wild-type (T1796) and mutant (A1796) *BRAF* alleles. PCR amplification and melting curve analysis was performed on a Rotor-gene 3000 (Corbett Research, NSW, Australia). Cycling conditions were as follows: 50°C for two minutes, 95°C for two minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. After amplification, samples were subjected to a temperature ramp from 60°C to 99°C, rising 1°C each step. For wild-type

Patient No	Specimen	BRAF mutation	Methylation status										
			MINT1	MINT2	MINT31	P16	RASSF1	RASSF2	NORE1	MST1	CHFR	RKIP	
1	SSA1	+											
	SSA2	+											
	SSA3	+											
	SSA4	+											
	SSA5	+											
	SSA6	+											
	SSA7	+											
	SSA8	+											
	MP1	+											
	MP2	-											
CRC	+												
2	SSA1												
	SSA2												
	CRC												
3	SSA1	+											
	SSA2	+											
	AD	+											
	TSA	+											
	CRC1												
	CRC2	+											
CRC3	+												
4	SSA												
	CRC	+											
5	SSA1												
	SSA2												
	SSA3												
	AD												
	CRC												
6	SSA	-											
7	SSA	+											
8	SSA	+											
9	SSA	+											
10	SSA	+											
11	SSA	+											
12	SSA	+											

Methylated locus	Not amplified
Unmethylated locus	Not assessed

Figure 4 Methylation patterns in gene promoters in polyps and cancers. SSA, sessile serrated adenoma; TSA, traditional serrated adenoma; CRC, colorectal cancer.

samples, single peaks were observed at 80°C and samples containing mutant alleles produced additional peaks at 85°C.

Statistical analysis

The χ^2 test was used to compare the frequency of locus methylation in polyps of patients with or without hyperplastic polyposis. Loci that did not show amplification with primers for both methylated and unmethylated sequences were excluded from analysis. A p value of less than 0.05 was considered statistically significant.

RESULTS

Immunoexpression of MLH1

There was complete loss of expression of MLH1 in the six proximal CRCs from patient No 1, 2, 3, and 5, and these CRCs also showed high level DNA MSI (data not shown). Of 18 serrated polyps from patient No 1, seven showed loss of expression of MLH1. This included the unequivocally dysplastic subclones in four of four mixed polyps (fig 2) and clusters of non-dysplastic crypts in three SSAs. Among 54 serrated polyps from patient No 2, only one showed convincing loss of expression of MLH1 and this was limited to a single non-dysplastic crypt (fig 3). Of four serrated polyps from patient No 3, a mixed polyp showed loss of MLH1 within the adenomatous subclone and multifocal loss of MLH1 was present in an SSA. A large SSA contiguous with the CRC from patient No 4 showed normal expression of MLH1.

CpG island methylation

The methylation status of markers was examined in 23 SSAs, two mixed polyps, one TSA, two conventional adenomas, and seven CRCs from 12 patients (fig 4). Markers *MGMT*, *MLH1*, *FAS*, and *DAPK* were less specific than the others in showing relatively high frequencies of methylation in normal mucosa of three subjects (No 4, 5 and 6) without HPP (data not shown). The range of locus methylation in the polyps of patient No 1–3 with HPP was 70–100% (7–10 of 10 markers) versus 30–100% (3–10 of 10 markers) in the polyps of patient No 4–12 with sporadic SSAs. The 93% methylated loci (112 of 120) demonstrated in DNA samples derived from 12 SSAs

from patient No 1–3 with HPP was significantly more frequent than 73% of methylated loci (79 of 108) in 11 sporadic SSAs (patient No 4–12) ($p < 0.0001$) (fig 4).

Although there was more methylation in the polyps of patients with HPP, there was considerable overlap between the patient groups (fig 4). A more striking difference was found with respect to the frequency of methylation in normal mucosa in patients with and without HPP. While patient No 1–3 with HPP showed methylation rates of 85%, 75%, and 90% in normal colonic mucosa (17, 15, and 18 of 20 loci, respectively), we detected methylation in only 13% of loci when testing normal mucosal samples derived from patient No 4–6 and 13–15 with sporadic serrated polyps of the proximal colon (fig 5). Examples of MSP on DNA derived from normal mucosa as well as polyps in patients with and without HPP are shown in fig 6.

BRAF mutation

Mutation of *BRAF* was found in 100% of SSAs (10 of 10) in patients with HPP and in 87% (7 of 8) of sporadic SSAs (fig 4). Mutation of *KRAS* was found in a single mixed polyp from patient No 1 (data not shown). SSAs from patients with and without HPP were therefore well matched in terms of anatomical location and *BRAF* mutation.

DISCUSSION

The role of DNA methylation or the CIMP^{25, 44} in the evolution of CRC has been a matter of some controversy. It has been argued by some that CRCs with CIMP do not have distinct clinical, pathological, and molecular features and that DNA methylation is largely an epiphenomenon.⁴⁵ However, this interpretation ignores three important groups of observations. Firstly, CIMP positive CRCs do in fact share a number of features regardless of the presence or absence of DNA MSI-H status. These include proximal location, female predilection, mucinous and poor differentiation, and a high frequency of the serrated pathway specific *BRAF* mutation.^{46–49} Secondly, CIMP (as well as mutation of *BRAF*) is fully established in the putative precursor lesions of CIMP positive CRCs, namely large and proximal HPs or SSAs (see above). Thirdly, the observations that: (a) there is extensive

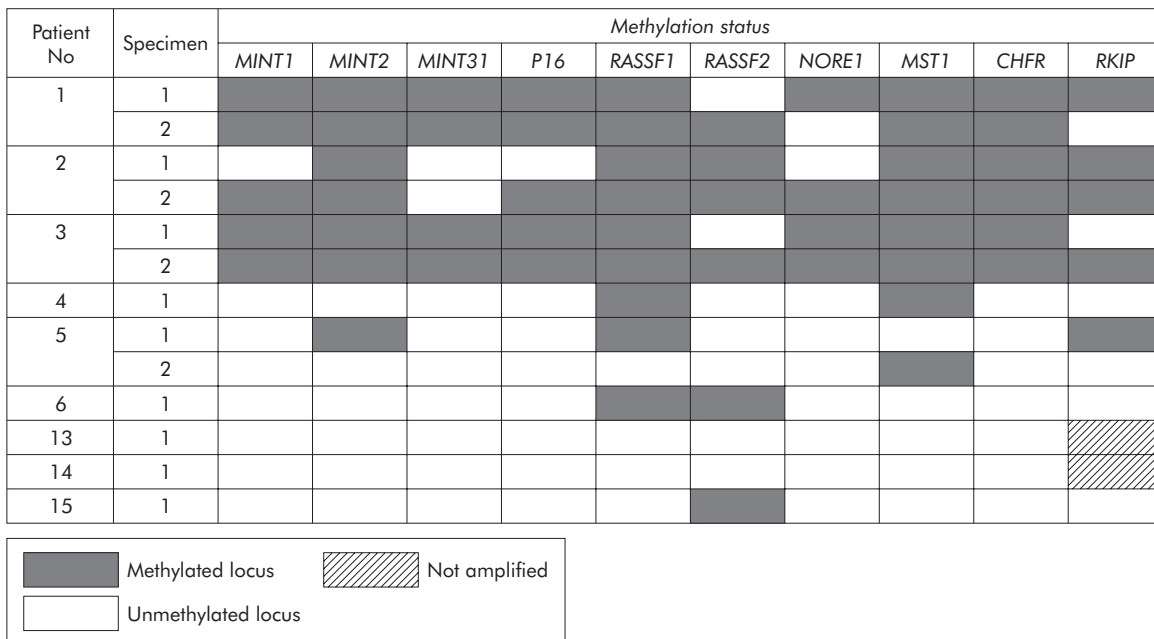


Figure 5 Methylation patterns in gene promoters in normal colonic mucosa.

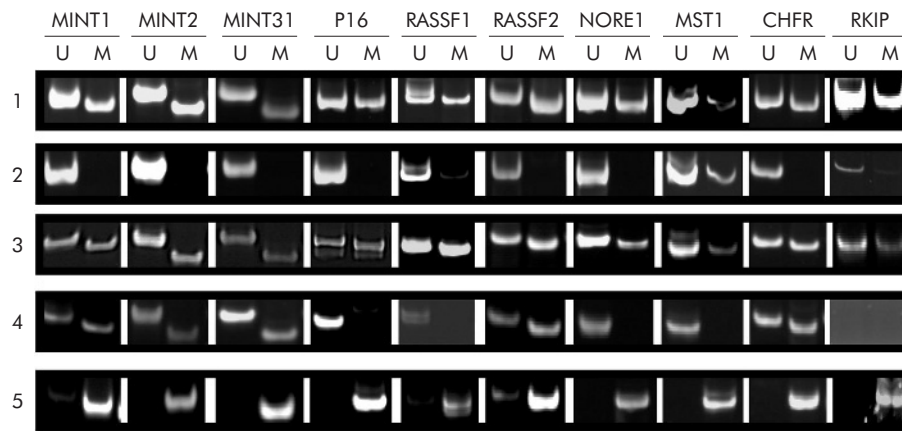


Figure 6 Representative examples of methylation specific polymerase chain reaction at different loci in normal mucosa and polyps from patients with or without hyperplastic polyposis. Methylation of MINT1, 2, 31 markers, and *p16*, *RASSF1*, *RASSF2*, *NORE1*, *MST1*, *CHFR*, and *RKIP* was examined by methylation specific polymerase chain reaction using primers for unmethylated (U) or methylated (M) loci. (1) Normal colonic mucosa from patient No 3 (sample 2) with hyperplastic polyposis; (2) normal colon mucosa from patient No 5 (sample 2) with sporadic sessile serrated adenoma (SSA); (3) SSA from patient No 1 (sample 1) with hyperplastic polyposis; (4) SSA from patient No 9 with sporadic SSA; and (5) control methylated DNA (see materials and methods).

and concordant DNA methylation across all polyps in HPP,²² (b) the condition HPP segregates within families,^{3 50} and (c) CRCs with *BRAF* mutation and/or DNA methylation occur within families,^{40 49 51} serve as evidence for a genetic mechanism underlying CIMP although this remains controversial.^{52 53} We have therefore adopted the premise that CIMP is an early pathogenic change that serves to mould colorectal tumorigenesis whether this culminates in MSI-H or non-MSI-H CRCs with CIMP.

The range of DNA methylation markers used in studies of serrated polyps of the colorectum has been limited to date. Assuming that a subset of serrated polyps is characterised by a state known as the CpG island methylator phenotype (CIMP) then it is desirable to identify an optimum panel that is both sensitive and specific for CIMP. This study employed markers that have been used extensively in studies of colorectal cancers and polyps (MINT1, MINT2, MINT31, *p16*, *MLH1*, and *MGMT*) as well as less widely used markers. Inhibition of apoptosis has been regarded as a pathogenic mechanism in serrated polyps^{20 21} but is regulated by multiple signalling pathways. We therefore reasoned that more advanced serrated polyps may be characterised by a more comprehensive inactivation of apoptosis signalling pathways through methylation of proapoptotic genes. Oncogenic activation of *BRAF* appears to be both proapoptotic and antiapoptotic.⁵⁴ *RASSF1*, *RASSF2*, and *NORE1* (*RASSF5*) are proapoptotic genes downstream of *KRAS* that are known to be methylated as a component of CIMP.^{43 55–61} Silencing of these genes by methylation could therefore direct MAP kinase activation towards antiapoptotic signalling. *MST1* proapoptotic kinase is activated by *RASSF1/NORE1* complex^{31 32} although it has not been shown to be methylated previously. Augmentation of MAPK signalling may occur through inactivation of *RKIP*.^{37 38} Two further proapoptotic genes, cell surface receptor *FAS* and *DAPK*, and the mitotic checkpoint gene *CHFR* are all known to be methylated in colon cancer.^{62–65} It is possible that *BRAF* mutation initiates a tissue alteration (that is, a serrated polyp) when antiapoptotic signalling becomes dominant through silencing of proapoptotic genes. This could explain the close association between *BRAF* mutation and CIMP. However, the present study has not shown that methylation of proapoptotic genes has functional consequences with respect to gene silencing.

In order to highlight pictorially any differences among the serrated polyps with respect to DNA methylation in the

preceding panel of 14 markers, we elected to exclude all markers that were methylated in normal mucosa in patients with sporadic serrated polyps. On this basis, we excluded *MLH1*, *MGMT*, *FAS*, and *DAPK*, leaving a panel of 10 markers. We and others have shown previously that some of the preceding genes, notably *MGMT* and *MLH1*, may show non-specific methylation in normal colorectal samples.^{17 66} There was considerable overlap with respect to methylation in SSAs from subjects with and without HPP (fig 4). Nevertheless, there were more instances of marker methylation in the 12 SSAs in HPP patient No 1–3 compared with 11 sporadic SSAs from patient No 4–12 ($p < 0.0001$). This difference is not surprising given that the polyps in subjects with HPP arose within an environment of normal colonic mucosa that showed very extensive DNA methylation. However, there was no significant difference between the sets of polyps from the two groups of patients when the markers *RASSF1*, *p16*, and *RKIP* were excluded ($p = 0.1$) (fig 4). It is possible that more time is required for some markers to become methylated than others. Very extensive DNA methylation was seen in all three relatively large sporadic polyps from elderly patient No 5 and in the largest (15 mm) sporadic SSA from patient No 11 (fig 4).

The normal mucosa in patients with HPP showed very extensive DNA methylation (fig 5). Two previous reports have noted the finding of high level CIMP in the normal mucosa of subjects with hyperplastic polyposis.^{17 22} The present study extends these observations by showing concordant methylation across a large marker panel. CIMP markers generally show little or no methylation in normal colorectal mucosa and this was confirmed in the DNA samples derived from the normal colorectal mucosa of six subjects with small numbers of proximally located serrated polyps. The finding of extensive DNA methylation in normal colorectal mucosa may serve as a useful diagnostic biomarker for high cancer risk forms of HPP. However, there is a need to confirm these findings in additional subjects with HPP and this should include the detailed mapping of the extent of methylation in different regions of the colon. Given the predilection for CIMP positive CRC to occur in the proximal colon, it is possible that DNA hypermethylation would be more extensive proximally. The present findings should be distinguished from the increased methylation restricted to *MGMT* that has been demonstrated as a field change in normal colorectal mucosa from subjects in whom CRCs show *MGMT* methylation.⁶⁷

An explanation for the finding of extensive DNA methylation in normal mucosa in HPP can only be speculative but nevertheless warrants consideration. As noted above, CRCs with CIMP have been linked with family history in two cancer family clinic based studies^{40,51} and one large population based study.⁴⁹ The findings in these three studies support the possibility of genetic predisposition to aberrant DNA methylation. The population based study was large (911 subjects) and bias free. Patients were stratified on the basis of *BRAF* mutation but the authors demonstrated a very strong correlation between *BRAF* mutation and CIMP, regardless of whether CRCs were DNA microsatellite stable or MSI-H.⁴⁹ In subjects with microsatellite stable CRCs, the odds ratio for a positive family history when comparing *BRAF* mutation positive and *BRAF* wild-type groups was 4.23 (95% confidence interval (CI) 1.65–10.84). In the case of MSI-H CRCs, the odds ratio for a positive family history was only 0.64 (95% CI 0.18–2.19) for subjects with *BRAF* mutation positive versus *BRAF* wild-type CRCs. However, one third of subjects with *BRAF* wild-type/MSI-H CRCs were aged less than 55 years and a proportion would be expected to have Lynch syndrome.⁴⁹

In a separate study using the same population, the authors demonstrated a weaker association between CIMP and family history.⁵³ However, they employed a very broad definition of CIMP (two or more of five markers methylated) to the extent that less than one third of CIMP positive cancers had *BRAF* mutation. Using a similarly broad definition of CIMP, a hospital based study found no link between CIMP positive CRC and family history.⁵² However, this study is difficult to evaluate because it excluded families with “hereditary non-polyposis colorectal cancer (HNPCC)” without stating how HNPCC was defined. Families may meet the Amsterdam criteria without having HNPCC/Lynch syndrome. While there is no consensus in the literature, there is good evidence for the heritability of CIMP and the contrary evidence can be explained by limitations in study design.

HPP is associated with CIMP positive polyps and CRCs but is an extremely uncommon condition. Nevertheless, instances of HPP have been documented within the setting of CRC families in which cancers show *BRAF* mutation and/or DNA methylation⁴⁰ and in siblings.⁵⁰ It is conceivable that the usual CIMP positive CRCs develop through a “two hit” mechanism whereby one mutant copy of a gene that is implicated in the regulation of DNA methylation is inherited, while the wild-type allele is inactivated at the somatic level. The widespread methylation of normal mucosa found in HPP might then occur in subjects who have inherited two copies of such a mutated gene. On the basis of the above, HPP may serve as an important genetic model for CIMP. These suggestions remain highly speculative and do not preclude a role for environmental factors in the aetiology of both DNA methylation and HPP. Age and inflammatory bowel disease are known to influence DNA methylation in colorectal mucosa.⁶⁸

The phenotypes in patient No 1 and 3 are very similar. Both patients had only moderate numbers of serrated polyps but a high proportion of these showed dysplasia (mixed polyps or traditional serrated adenomas) and/or loss of expression of MLH1. In contrast, patient No 2 had very large numbers of serrated polyps, including many typical hyperplastic polyps. Only one polyp (a mixed polyp) showed mild dysplasia. In addition, loss of MLH1 expression was confined to a single non-dysplastic crypt in a sessile serrated adenoma. While all three subjects showed extensive DNA methylation in normal colorectal mucosa, it is possible that methylation leading to loss of expression of MLH1 is influenced by an additional partly independent mechanism. This would explain why some patients with hyperplastic polyposis, such as patient No

3 and others reported in the literature,^{8,69} develop multiple synchronous CRCs that may be MSI-H.

In summary, we have documented the finding of extensive DNA methylation in samples of normal mucosa from the proximal colon of subjects with HPP and suggest that there may be a genetic basis for this observation. Some of the observed heterogeneity within HPP may be explained by differing propensities for *MLH1* inactivation that may be due to mechanisms that are partly independent of a general predisposition to DNA methylation. Finally, observations relating to methylation of multiple proapoptotic genes provide an explanation for the synergy between DNA methylation and mutation of *BRAF* although this will need to be confirmed by functional studies.

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REFERENCES

- 1 **Hamilton SR**, Aaltonen LA. *World Health Organization Classification of Tumors, Pathology and Genetics*. Lyon: IARC Press, 2000.
- 2 **Torlakovic E**, Snover DC. Serrated adenomatous polyposis in humans. *Gastroenterology* 1996;**110**:748–55.
- 3 **Rashid A**, Houlihan PS, Booker S, *et al*. Phenotypic and molecular characteristics of hyperplastic polyposis. *Gastroenterology* 2000;**119**:323–32.
- 4 **Leggett BA**, Devereaux B, Biden K, *et al*. Hyperplastic polyposis: association with colorectal cancer. *Am J Surg Pathol* 2001;**25**:177–84.
- 5 **Burt RW**, Jass JR. Hyperplastic polyposis. In: Hamilton SR, Aaltonen LA, eds. *World Health Organization Classification of Tumors. Pathology and Genetics. Tumors of the Digestive System*. Berlin: Springer-Verlag, 2000:135–6.
- 6 **Williams GT**, Arthur JF, Bussey HJ, *et al*. Metaplastic polyps and polyposis of the colorectum. *Histopathology* 1980;**4**:155–70.
- 7 **Ferrandez A**, Samowitz W, DiSario JA, *et al*. Phenotypic characteristics and risk of cancer development in hyperplastic polyposis: case series and literature review. *Am J Gastroenterol* 2004;**99**:2012–8.
- 8 **Jass JR**, Iino H, Ruzkiewicz A, *et al*. Neoplastic progression occurs through mutator pathways in hyperplastic polyposis of the colorectum. *Gut* 2000;**47**:43–9.
- 9 **Jass JR**, Young J, Leggett BA. Hyperplastic polyps and DNA microsatellite unstable cancers of the colorectum. *Histopathology* 2000;**37**:295–301.
- 10 **Biemer-Huttmann AE**, Walsh MD, McGuckin MA, *et al*. Mucin core protein expression in colorectal cancers with high levels of microsatellite instability indicates a novel pathway of morphogenesis. *Clin Cancer Res* 2000;**6**:1909–16.
- 11 **Hawkins NJ**, Ward RL. Sporadic colorectal cancers with microsatellite instability and their possible origin in hyperplastic polyps and serrated adenomas. *J Natl Cancer Inst* 2001;**93**:1307–13.
- 12 **Goldstein NS**, Bhanot P, Odish E, *et al*. Hyperplastic-like colon polyps that preceded microsatellite-unstable adenocarcinomas. *Am J Clin Pathol* 2003;**119**:778–96.
- 13 **Torlakovic E**, Skovlund E, Snover DC, *et al*. Morphologic reappraisal of serrated colorectal polyps. *Am J Surg Pathol* 2003;**27**:65–81.
- 14 **Higuchi T**, Sugihara K, Jass JR. Demographic and pathological characteristics of serrated polyps of colorectum. *Histopathology* 2005;**47**:32–40.
- 15 **O'Brien MJ**, Yang S, Clebanoff JL, *et al*. Hyperplastic (serrated) polyps of the colorectum: relationship of CpG island methylator phenotype and K-ras mutation to location and histologic subtype. *Am J Surg Pathol* 2004;**28**:423–34.

- 16 Yang S, Farraye FA, Mack C, et al. BRAF and KRAS mutations in hyperplastic polyps and serrated adenomas of the colorectum: relationship to histology and CpG island methylation status. *Am J Surg Pathol* 2004;**28**:1452-9.
- 17 Wynter CV, Walsh MD, Higuchi T, et al. Methylation patterns define two types of hyperplastic polyp associated with colorectal cancer. *Gut* 2004;**53**:573-80.
- 18 Kane MF, Loda M, Gaida GM, et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res* 1997;**57**:808-11.
- 19 Oh K, Redston M, Odze RD. Support for hMLH1 and MGMT silencing as a mechanism of tumorigenesis in the hyperplastic-adenoma-carcinoma (serrated) carcinogenic pathway in the colon. *Hum Pathol* 2005;**36**:101-11.
- 20 Tateyama H, Li W, Takahashi E, et al. Apoptosis index and apoptosis-related antigen expression in serrated adenoma of the colorectum: the saw-toothed structure may be related to inhibition of apoptosis. *Am J Surg Pathol* 2002;**26**:249-56.
- 21 Jass JR, Whitehall VL, Young J, et al. Emerging concepts in colorectal neoplasia. *Gastroenterology* 2002;**123**:862-76.
- 22 Chan AO, Issa JP, Morris JS, et al. Concordant CpG island methylation in hyperplastic polyposis. *Am J Pathol* 2002;**160**:529-36.
- 23 Park SJ, Rashid A, Lee JH, et al. Frequent CpG island methylation in serrated adenomas of the colorectum. *Am J Pathol* 2003;**162**:815-22.
- 24 Chan AO, Broadus RR, Houlihan PS, et al. CpG island methylation in aberrant crypt foci of the colorectum. *Am J Pathol* 2002;**160**:1823-30.
- 25 Toyota M, Ho C, Ahuja N, et al. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. *Cancer Res* 1999;**59**:2307-12.
- 26 Nagasaka T, Sasamoto H, Notohara K, et al. Colorectal cancer with mutation in BRAF, KRAS, and wild-type with respect to both oncogenes showing different patterns of DNA methylation. *J Clin Oncol* 2004;**22**:4584-94.
- 27 Vos MD, Ellis CA, Bell A, et al. Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. *J Biol Chem* 2000;**275**:35669-72.
- 28 Vos MD, Martinez A, Ellis CA, et al. The pro-apoptotic Ras effector Nore1 may serve as a Ras-regulated tumor suppressor in the lung. *J Biol Chem* 2003;**278**:21938-43.
- 29 Vos MD, Ellis CA, Elam C, et al. RASSF2 is a novel K-Ras-specific effector and potential tumor suppressor. *J Biol Chem* 2003;**278**:28045-51.
- 30 Shivakumar L, Minna J, Sakamaki T, et al. The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol Cell Biol* 2002;**22**:4309-18.
- 31 Khokhlatchev A, Rabizadeh S, Xavier R, et al. Identification of a novel Ras-regulated proapoptotic pathway. *Curr Biol* 2002;**12**:253-65.
- 32 Praskova M, Khokhlatchev A, Ortiz-Vega S, et al. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NRE1, and by Ras. *Biochem J* 2004;**381**:453-62.
- 33 Graves JD, Gotoh Y, Draves KE, et al. Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. *Embo J* 1998;**17**:2224-34.
- 34 Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998;**281**:1305-8.
- 35 Kogel D, Prehn JH, Scheidtmann KH. The DAP kinase family of pro-apoptotic proteins: novel players in the apoptotic game. *Bioessays* 2001;**23**:352-8.
- 36 Scolnick DM, Halazonetis TD. Chfr defines a mitotic stress checkpoint that delays entry into metaphase. *Nature* 2000;**406**:430-5.
- 37 Park S, Yeung ML, Beach S, et al. RKIP downregulates B-Raf kinase activity in melanoma cancer cells. *Oncogene* 2005;**24**:3535-40.
- 38 Yeung K, Seitz T, Li S, et al. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. *Nature* 1999;**401**:173-7.
- 39 Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. *Nat Rev Mol Cell Biol* 2004;**5**:875-85.
- 40 Young J, Barker MA, Simms LA, et al. Evidence for BRAF mutation and variable levels of microsatellite instability in a syndrome of familial colorectal cancer. *Clin Gastroenterol Hepatol* 2005;**3**:254-63.
- 41 Petko Z, Ghiassi M, Shuber A, et al. Aberrantly methylated CDKN2A, MGMT, and MLH1 in colon polyps and in fecal DNA from patients with colorectal polyps. *Clin Cancer Res* 2005;**11**:1203-9.
- 42 Esteller M, Hamilton SR, Burger PC, et al. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res* 1999;**59**:793-7.
- 43 Hesson LB, Wilson R, Morton D, et al. CpG island promoter hypermethylation of a novel Ras-effector gene RASSF2A is an early event in colon carcinogenesis and correlates inversely with K-ras mutations. *Oncogene* 2005;**24**:3987-94.
- 44 Toyota M, Ohe-Toyota M, Ahuja N, et al. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. *Proc Natl Acad Sci U S A* 2000;**97**:710-15.
- 45 Yamashita K, Dai T, Dai Y, et al. Genetics supersedes epigenetics in colon cancer phenotype. *Cancer Cell* 2003;**4**:121-31.
- 46 Kambara T, Simms LA, Whitehall VL, et al. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. *Gut* 2004;**53**:1137-44.
- 47 Hawkins N, Norrie M, Cheong K, et al. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. *Gastroenterology* 2002;**122**:1376-87.
- 48 Whitehall VL, Wynter CV, Walsh MD, et al. Morphological and molecular heterogeneity within nonmicrosatellite instability-high colorectal cancer. *Cancer Res* 2002;**62**:6011-4.
- 49 Samowitz WS, Sweeney C, Herrick J, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. *Cancer Res* 2005;**65**:6063-9.
- 50 Jeevaratnam P, Cottier DS, Browett PJ, et al. Familial giant hyperplastic polyposis predisposing to colorectal cancer: a new hereditary bowel cancer syndrome. *J Pathol* 1996;**179**:20-5.
- 51 Frazier ML, Xi L, Zong J, et al. Association of the CpG island methylator phenotype with family history of cancer in patients with colorectal cancer. *Cancer Res* 2003;**63**:4805-8.
- 52 Ward RL, Williams R, Law M, et al. The CpG island methylator phenotype is not associated with a personal or family history of cancer. *Cancer Res* 2004;**64**:7618-21.
- 53 Samowitz WS, Albertsen H, Herrick J, et al. Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. *Gastroenterology* 2005;**129**:837-45.
- 54 Cox AD, Der CJ. The dark side of Ras: regulation of apoptosis. *Oncogene* 2003;**22**:8999-9006.
- 55 Dammann R, Yang G, Pfeifer GP. Hypermethylation of the CpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. *Cancer Res* 2001;**61**:3105-9.
- 56 Agathangelou A, Honorio S, Macartney DP, et al. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. *Oncogene* 2001;**20**:1509-18.
- 57 van Engeland M, Roemen GM, Brink M, et al. K-ras mutations and RASSF1A promoter methylation in colorectal cancer. *Oncogene* 2002;**21**:3792-5.
- 58 Sakamoto N, Terai T, Ajioka Y, et al. Frequent hypermethylation of RASSF1A in early flat-type colorectal tumors. *Oncogene* 2004;**23**:8900-7.
- 59 Hesson L, Dallol A, Minna JD, et al. NRE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. *Oncogene* 2003;**22**:947-54.
- 60 Akino K, Toyota M, Suzuki H, et al. The Ras effector RASSF2 is a novel tumor-suppressor gene in human colorectal cancer. *Gastroenterology* 2005;**129**:156-69.
- 61 Irimia M, Fraga MF, Sanchez-Cespedes M, et al. CpG island promoter hypermethylation of the Ras-effector gene NRE1A occurs in the context of a wild-type K-ras in lung cancer. *Oncogene* 2004;**23**:8695-9.
- 62 Petak I, Danam RP, Tillman DM, et al. Hypermethylation of the gene promoter and enhancer region can regulate Fas expression and sensitivity in colon carcinoma. *Cell Death Differ* 2003;**10**:211-17.
- 63 Yamaguchi S, Asao T, Nakamura J, et al. High frequency of DAP-kinase gene promoter methylation in colorectal cancer specimens and its identification in serum. *Cancer Lett* 2003;**194**:99-105.
- 64 Toyota M, Sasaki Y, Satoh A, et al. Epigenetic inactivation of CHFR in human tumors. *Proc Natl Acad Sci U S A* 2003;**100**:7818-23.
- 65 Brandes JC, van Engeland M, Wouters KA, et al. CHFR promoter hypermethylation in colon cancer correlates with the microsatellite instability phenotype. *Carcinogenesis* 2005;**26**:1152-6.
- 66 Nakagawa H, Nuovo GJ, Zervos EE, et al. Age-related hypermethylation of the 5' region of MLH1 in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. *Cancer Res* 2001;**61**:6991-5.
- 67 Shen L, Kondo Y, Rosner GL, et al. MGMT promoter methylation and field defect in sporadic colorectal cancer. *J Natl Cancer Inst* 2005;**97**:1330-8.
- 68 Issa JP, Ahuja N, Toyota M, et al. Accelerated age-related CpG island methylation in ulcerative colitis. *Cancer Res* 2001;**61**:3573-7.
- 69 Yao T, Nishiyama K, Oya M, et al. Multiple 'serrated adenocarcinomas' of the colon with a cell lineage common to metaplastic polyp and serrated adenoma. Case report of a new subtype of colonic adenocarcinoma with gastric differentiation. *J Pathol* 2000;**190**:444-9.