COLORECTAL CANCER

Molecular correlates with *MGMT* promoter methylation and silencing support CpG island methylator phenotype-low (CIMP-low) in colorectal cancer

Shuji Ogino, Takako Kawasaki, Gregory J Kirkner, Yuko Suemoto, Jeffrey A Meyerhardt, Charles S Fuchs

Gut 2007;**56**:1564–1571. doi: 10.1136/gut.2007.119750

See end of article for authors' affiliations

Correspondence to: Dr Shuji Ogino, Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, 75 Francis St, BWH Pathology, Boston, MA 02115, USA; shuji_ogino@dfci.harvard.edu

Revised 18 February 2007
Accepted 28 February 2007
Published Online First 2 March 2007

Background: The CpG island methylator phenotype (CIMP or CIMP-high) with widespread promoter methylation is a distinct epigenetic phenotype in colorectal cancer. In contrast, a phenotype with less widespread promoter methylation (CIMP-low) has not been well characterised. O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation and silencing have been associated with G>A mutations and microsatellite instability-low (MSI-low).
Aim: To examine molecular correlates with *MGMT* methylation/silencing in colorectal cancer.
Methods: Utilising MethyLight technology, we quantified DNA methylation in *MGMT* and eight other markers (a CIMP-diagnostic panel; *CACNA1G*, *CDKN2A (p16)*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*) in 920 population-based colorectal cancers.
Results: Tumours with both *MGMT* methylation and loss were correlated positively with MSI-low (p = 0.02), CIMP-high (≥6/8 methylated CIMP markers, p = 0.005), CIMP-low (1/8–5/8 methylated CIMP markers, p = 0.002, compared to CIMP-0 with 0/8 methylated markers), *KRAS* G>A mutation (p = 0.02), and inversely with 18q loss of heterozygosity (p = 0.0002). Tumours were classified into nine MSI/CIMP subtypes. Among the CIMP-low group, tumours with both *MGMT* methylation and loss were far more frequent in MSI-low tumours (67%, 12/18) than MSI-high tumours (5.6%, 1/18; p = 0.0003) and microsatellite stable (MSS) tumours (33%, 52/160; p = 0.008). However, no such relationship was observed among the CIMP-high or CIMP-0 groups.
Conclusion: The relationship between *MGMT* methylation/silencing and MSI-low is limited to only CIMP-low tumours, supporting the suggestion that CIMP-low in colorectal cancer may be a different molecular phenotype from CIMP-high and CIMP-0. Our data support a molecular difference between MSI-low and MSS in colorectal cancer, and a possible link between CIMP-low, MSI-low, *MGMT* methylation/loss and *KRAS* mutation.

Transcriptional inactivation by cytosine methylation at the promoter CpG islands of tumour suppressor genes is an important carcinogenic mechanism.[1] A number of tumour suppressor genes have been shown to be silenced by promoter methylation in colorectal cancers.[1–3] In fact, a subset of colorectal cancers exhibit promoter methylation in multiple genes, which is referred to as the CpG island methylator phenotype (CIMP).[2 4] CIMP-positive colorectal tumours have a distinct clinical, pathologic and molecular profile, such as associations with proximal tumour location, female sex, poor differentiation, microsatellite instability (MSI), and high *BRAF* and low *TP53* mutation rates.[5–12] Promoter CpG island methylation has been shown to occur early in colorectal carcinogenesis.[13 14]

Although CIMP (which we designate as ''CIMP-high'', to be distinguished from ''CIMP-low'') appears to be a distinct biological phenotype in colorectal cancer, the existence of CIMP-low (with less extensive CIMP-specific promoter methylation) is still controversial. We have previously shown that CIMP-low is associated with male sex and *KRAS* mutations compared to CIMP-high and CIMP-0 (absence of methylation in five CIMP-specific promoters).[15] However, differences between CIMP-low and CIMP-0 are not as clear-cut as those between CIMP-low and CIMP-high,[15] and additional evidence is necessary to establish CIMP-low as a different phenotype from CIMP-high and CIMP-0.

O-6-methylguanine-DNA methyltransferase (*MGMT*) acts to repair inappropriately methylated guanine residues in DNA. Chronic exposure to alkylating/methylating agents can lead to increased MGMT activity,[16] and MGMT expression protects from spontaneous G:C to A:T transition mutations.[17] Promoter methylation and silencing of *MGMT* are commonly present in colorectal cancer, and associated with G>A mutations in the *KRAS* and *TP53* genes.[18 19] *MGMT* promoter methylation in normal-appearing colonic mucosa (perhaps as a field effect) may be a predisposing factor for the development of colorectal neoplasia.[20 21] *MGMT* methylation in colorectal cancer has been suggested to predict non-recurrence after chemotherapy, while it does not predict non-recurrence among colorectal cancer patients who have not received chemotherapy.[22] In agreement

Abbreviations and official gene symbols: CACNA1G, calcium channel, voltage-dependent, T type alpha-1G subunit; CDKN2A, cyclin-dependent kinase inhibitor 2A (p16/INK4A); CIMP, CpG island methylator phenotype; CRABP1, cellular retinoic acid binding protein 1; DAB, diaminobenzidine; HNPCC, hereditary non-polyposis colorectal cancer; IGF2, insulin-like growth factor 2; LOH, loss of heterozygosity; MGMT, O-6-methylguanine-DNA methyltransferase; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite stable; NCI, National Cancer Institute; NEUROG1, neurogenin 1; PMR, percentage of methylated reference (degree of methylation); RUNX3, runt-related transcription factor 3; SOCS1, suppressor of cytokine signaling 1; TGFBR2, transforming growth factor-beta receptor type 2; WGA, whole genome amplification

with that report, another study has shown that MGMT loss of expression does not affect survival among stage C colon cancer patients who have not received chemotherapy.[23] Compared to the high performance characteristics of CIMP-specific promoters (*CACNA1G*, *CDKN2A (p16)*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*), the sensitivity and specificity of *MGMT* methylation for CIMP-high are low (62% and 66%, respectively),[10 24] raising the possibility that *MGMT* methylation may be a marker for less extensive promoter methylation (ie, CIMP-low). In addition, *MGMT* methylation and loss have been associated with MSI-low in colorectal cancer.[23 25] However, no study to date has examined *MGMT* methylation and silencing in relation to combined MSI and CIMP status. Previous studies on molecular correlates with *MGMT* methylation in colorectal cancer have been based on small numbers of cases and/or non-quantitative methylation-specific PCR.

In this study, using MethyLight technology and a large number of population-based colorectal cancer samples, we examined *MGMT* methylation and silencing in relation to various molecular features, particularly combined MSI and CIMP status. MethyLight assays have been shown to be robust and reproducible in quantifying methylation in DNA from paraffin-embedded tumour tissue.[26] Discovering molecular correlates is important in cancer research because this may: (1) provide clues to carcinogenic mechanisms; (2) propose or support a new molecular subtype; (3) alert investigators to be aware of potential confounding in association studies; and (4) suggest surrogate markers in clinical or research settings.[15 27]

METHODS

Study group

We utilised the databases of two large prospective cohort studies: the Nurses' Health Study (n = 121 700 women followed since 1976),[28] and the Health Professional Follow-up Study (n = 51 500 men followed since 1986).[29] Informed consent was obtained from all participants prior to inclusion in the cohorts. A subset of the cohort participants developed colorectal cancers during prospective follow-up. Thus, these colorectal cancers represented population-based, relatively unbiased samples (compared to retrospective or single-hospital-based samples). Previous studies on the Nurses' Health Study and the Health Professionals Follow-up Study have described baseline characteristics of cohort participants and incident colorectal cancer cases, and confirmed that our colorectal cancer cases were well representative as a population-based sample.[28 29] We collected information from cohort participants through questionnaire although our questionnaire was not designed for the identification of hereditary non-polyposis colorectal cancer (HNPCC). We collected paraffin-embedded tissue blocks from hospitals where cohort participants with colorectal cancers had undergone resections of primary tumours. We excluded cases if adequate paraffin-embedded tumour tissue was not available or if tumours were previously treated by chemotherapy or radiation. As a result, a total of 920 colorectal cancer cases (410 from the men's cohort and 510 from the women's cohort) were included. Besides the 920 tumours analysed in this study, we excluded 25 cancers in which there was no tissue available other than tumour tissue at metastatic sites, and also excluded 19 patients who had received chemotherapy prior to tumour resection. Among our cohort studies, there was no significant difference in demographic features between cases with tissue analysed and those without tissue data.[30] Many of the cases have been previously characterised for status of CIMP, MSI, *KRAS* and *BRAF*.[15 24] However, we have not examined *MGMT* methylation and silencing in relation to MSI/CIMP status or various other molecular variables. Follow-up of these cohorts was still ongoing and analysis on patient outcomes was not possible at the time of the study. Tissue collection and analyses were approved by the Dana-Farber Cancer Institute and Brigham and Women's Hospital Institutional Review Boards.

Histopathologic evaluations

Haematoxylin and eosin (H&E) stained tissue sections were examined under a light microscope by a pathologist (SO) in a blinded fashion without knowledge of clinical and other laboratory data. Tumours were classified into well/moderately differentiated (<50% solid areas) and poorly differentiated tumours (≥50% solid areas).[12] In addition, the extent and type of mucinous component in each tumour were evaluated, and tumours were classified into five categories: (1) tumours with no mucinous or signet ring cell component (non-mucinous tumours); (2) tumours with 1–49% mucinous component but no signet ring cells; (3) tumours with ≥50% mucinous component but no signet ring cells; (4) tumours with 1–49% signet ring cell component; and (5) tumours with ≥50% signet ring cell component.

Genomic DNA extraction and whole genome amplification

Genomic DNA was extracted from dissected tumour tissue sections using the QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA) as previously described.[31] Normal DNA was obtained from colonic tissue at resection margins. Whole genome amplification (WGA) of genomic DNA was performed by PCR using random 15-mer primers[31] for subsequent MSI analysis and *KRAS* and *BRAF* sequencing. Previous studies by us and others showed that WGA did not significantly affect *KRAS* mutation detection or microsatellite analysis.[31 32]

Microsatellite instability and 18q loss of heterozygosity analyses

Methods for analysing for MSI status have been previously described.[33] In addition to the recommended NCI (National Cancer Institute) panel consisting of D2S123, D5S346, D17S250, BAT25 and BAT26,[34] we also used BAT40, D18S55, D18S56, D18S67 and D18S487 (ie, a 10-marker panel).[33] A high degree of MSI (MSI-H) was defined as the presence of instability in ≥30% of the markers. A low degree of MSI (MSI-L) was defined as the presence of instability in <30% of the markers, and microsatellite stable (MSS) tumours were defined as tumours without an unstable marker. We defined MSI-L cases very strictly. We repeated PCR every time there was an altered peak inconclusive for instability, to confirm instability and exclude sporadic PCR artifact. There were a total of 131 (15%) MSI-H among 889 tumours with MSI status determined. Among the MSI-H tumours, the frequencies of *MLH1* methylation, *TGFBR2* mutation and *BRAF* mutation were 73% (96/131), 73% (96/131) and 46% (59/128), respectively. Methods for analysis of *TGFBR2* mutation have been previously described,[35] and the presence of a peak at an altered size in tumour DNA relative to normal DNA was interpreted as positivity for a mutation in the *TGFBR2* mononucleotide repeat.

Methods for 18q loss of heterozygosity (LOH) analysis (on microsatellite markers D18S55, D18S56, D18S67 and D18S487) have been previously described.[33] The presence of LOH at each locus was defined as 40% or greater reduction of one of two allele peaks in tumour DNA relative to normal DNA, in two duplicated runs to exclude allele dropout and sporadic PCR bias. Overall 18q LOH positivity was strictly defined as the presence of at least two informative markers with LOH, and 18q LOH negativity as the absence of LOH in all (at least two) informative markers. These stringent criteria enabled us to select biologically homogeneous groups of tumours. When we

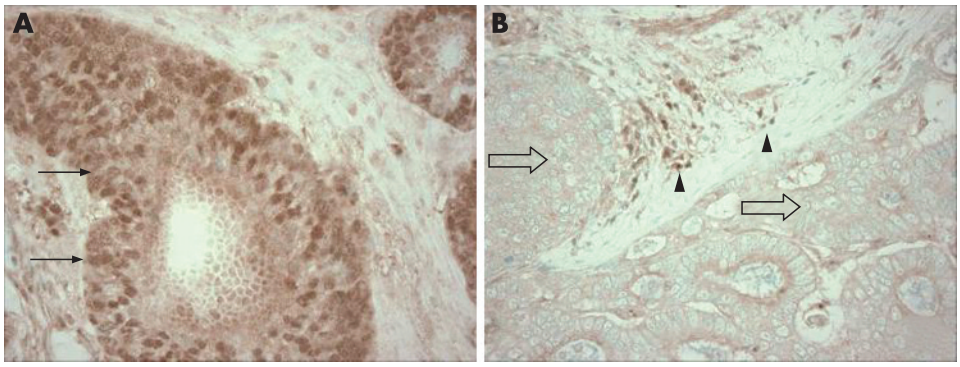

Figure 1 Evaluation of MGMT expression by immunohistochemistry. (A) MGMT expression is present in colorectal cancer cells (arrows). (B) Colorectal cancer cells show loss of MGMT expression (empty arrows). Inflammatory cells serve as an internal positive control (solid arrowheads).

used less stringent criteria in which 18q LOH positivity was defined as ⩾1 informative markers with LOH and 18q LOH negativity as ⩾1 informative markers without evidence of LOH, we could include a larger number of cases; however, the relationship between 18q LOH and *MGMT* became weaker (data not shown).

Sequencing of *KRAS* and *BRAF*

Methods of PCR and sequencing targeted for *KRAS* codons 12 and 13, and *BRAF* codon 600 have been previously described.[15 31] Pyrosequencing was performed using the PSQ96 HS System (Biotage AB and Biosystems, Uppsala, Sweden) according to the manufacturer's instructions.

Real-time PCR (MethyLight) for quantitative DNA methylation analysis

Sodium bisulfite treatment on genomic DNA was performed as previously described.[26] Real-time PCR to measure DNA methylation (MethyLight) was performed as previously described.[36 37] Utilising ABI 7300 (Applied Biosystems, Foster City, CA, USA) for quantitative real-time PCR, we amplified *MGMT* and eight other promoters (*CACNA1G*, *CDKN2A (p16)*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*); methylation in the latter eight promoters has been shown to be sensitive and specific for CIMP,[24] and thus we used these eight markers as a CIMP diagnostic panel. *COL2A1* (the collagen 2A1 gene) was used to normalise for the amount of input bisulfite-converted DNA.[26 37] Primers and probes were previously described as follows: *CACNA1G*, *CRABP1* and *NEUROG1*[10 11]; *MGMT*, *CDKN2A* and *COL2A1*[37]; *MLH1*[26]; and *IGF2*, *RUNX3* and *SOCS1*.[11] The percentage of methylated reference (PMR; ie, degree of methylation) at a specific locus was calculated by dividing the *GENE:COL2A1* ratio of template amounts in a sample by the *GENE:COL2A1* ratio of template amounts in *Sss*I-treated human genomic DNA (presumably fully methylated) and multiplying this value by 100.[36] A PMR cut-off value of 4 was based on previously validated data.[10 26 36 37] We set a PMR cut-off value of 6 for *CRABP1* and *IGF2*, based on PMR distribution. Precision and performance characteristics of bisulfite conversion and subsequent MethyLight assays have been previously evaluated and the assays have been validated.[26]

In particular, we validated the *MGMT* PMR cutoff of 4 by examining PMR values in relation to loss of expression. Among 567 tumours with valid *MGMT* methylation and expression data, the frequencies of MGMT loss were as follows: 14% (46/331) in tumours with PMR = 0; 18% (5/28) in tumours with PMR of 0–1; 33% (3/9) in tumours with PMR of 1–4; 60% (6/10) in tumours with PMR of 4–10; and 79% (150/189) in tumours with PMR>10.

CIMP-high was defined as the presence of ⩾6/8 methylated CIMP markers (excluding *MGMT*), CIMP-low as the presence of 1/8 to 5/8 methylated markers, and CIMP-0 as the absence (0/8) of methylated markers, based on the data that CIMP-high and CIMP-low are associated with *BRAF* mutations and *KRAS* mutations, respectively.[15 24]

Immunohistochemistry for MGMT

For MGMT immunohistochemistry, antigen retrieval was performed; deparaffinised tissue sections were treated in 1 mM EDTA buffer (pH 8) in a pressure cooker by a microwave for 20 min. Tissue sections were incubated with 3% H_2O_2 (20 min) to block endogenous peroxidase for 20 min, and then

Table 1 Frequency of *MGMT* promoter methylation and loss of expression in colorectal cancer

	MGMT promoter methylation		MGMT expression		
	Total examined, n	Methylated (%)	Total examined, n	Loss (%)	p Value
All cases	920	354 (38%)	599	224 (37%)	
Sex					
Men	410	150 (37%)	250	91 (36%)	
Women	510	204 (40%)	349	133 (38%)	
Tumour differentiation					
Well/moderate	815	311 (38%)	519	190 (37%)	
Poor	84	34 (40%)	54	22 (41%)	
Non-mucinous tumours	593	211 (36%)	393	133 (34%)	Referent
Mucinous 1–49% (no signet ring cell)	185	85 (46%)	123	59 (48%)	0.005
Mucinous ⩾50% (no signet ring cell)	81	41 (51%)	44	16 (36%)	
Signet ring cells 1–49%	46	13 (28%)	35	16 (46%)	
Signet ring cells ⩾50%	15	4 (27%)	4	0	
Location					
Right	255	103 (40%)	176	70 (40%)	
Left (excluding rectum)	175	62 (35%)	121	50 (41%)	
Rectum	107	37 (35%)	65	18 (28%)	

Only a statistically significant p value is described.

incubated with 2% horse serum (Vector Laboratories, Burlingame, California, USA) in phosphate-buffered saline (20 min). Primary antibody against MGMT (clone MT3.1, Lab Vision, Fremont, CA; dilution 1:25) was applied for 1 h at room temperature. Secondary antibody (Vector Laboratories) (30 min), and then avidin biotin complex conjugate (Vector Laboratories) (30 min) were applied. Sections were visualised by diaminobenzidine (DAB) (2 min) and methyl-green counterstain. Normal colonic epithelial cells and inflammatory cells served as internal positive controls when a tumour lost MGMT expression (fig 1). Appropriate positive and negative controls were included in each run of immunohistochemistry. All immunohistochemically-stained slides were interpreted by a pathologist (SO) blinded from clinical and other molecular data.

Statistical analysis

In statistical analysis, χ^2 test (or Fisher's exact test when the number in any category was less than 10) was performed for categorical data, using the SAS program (Version 9.1, SAS Institute, Cary, NC). All p values were two-sided, and statistical significance was set at $p \leqslant 0.05$.

RESULTS

MGMT promoter methylation and loss of expression

Utilising MethyLight technology, we quantified DNA methylation in *MGMT* and a panel of eight promoters (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*); the latter eight markers have been shown to be sensitive and specific for CIMP[10 11 24] and were thus used as a CIMP diagnostic panel. Among the 920 tumours, 354 (38%) showed *MGMT* promoter methylation. We also assessed MGMT expression by immunohistochemistry (fig 1). Among the 599 tumours with valid expression data, 224 (37%) showed loss of expression, most consistent with gene silencing or deletion. Table 1 summarises the relationship between clinicopathologic variables and *MGMT* methylation (or loss of expression).

Molecular correlates with *MGMT* methylation or loss of expression

Table 2 shows the relationship between various molecular features and *MGMT* promoter methylation (or loss of expression) in colorectal cancer. *MGMT* promoter methylation was highly correlated with MGMT loss of expression ($p<0.0001$). *MGMT* methylation was positively correlated with *MLH1* methylation ($p = 0.02$), unlike in a previous study.[38] MSI-L showed slightly higher frequencies of *MGMT* methylation (45%) and loss (49%) than MSS tumours (37% showing *MGMT* methylation and 36% showing MGMT loss), although differences were not statistically significant. Both *MGMT* methylation and loss were significantly more common in CIMP-high and CIMP-low tumours than in CIMP-0 tumours. 18q LOH-positive tumours showed a significantly lower frequency of *MGMT* methylation (30%, $p = 0.0008$) and loss (27%, $p = 0.004$) than 18q LOH-negative tumours (45% showing *MGMT* methylation and 42% showing MGMT loss).

Tumours with *KRAS* G>A mutation showed a higher frequency of MGMT loss (47%, $p = 0.003$) than tumours with wild-type *KRAS* (32% showing MGMT loss). We also examined the frequencies of *KRAS* G>A mutation in tumours with or without *MGMT* methylation (or expression). The frequencies of *KRAS* G>A mutation were not significantly different between

Table 2 Frequency of *MGMT* methylation and loss of expression in colorectal cancer

	MGMT methylation			MGMT expression		
	Total examined, n	Methylated (%)	p Value	Total examined, n	Loss (%)	p Value
MGMT expression						
Loss	210	156 (74%)	<0.0001			
Intact	357	43 (12%)	Referent			
MLH1 methylation						
(+)	115	56 (49%)	0.02			
(−)	805	298 (37%)	Referent			
MSI status						
MSI-high	131	57 (44%)		94	37 (39%)	
MSI-low	73	33 (45%)		47	23 (49%)	
MSS	685	255 (37%)		444	158 (36%)	
TGFBR2 (only MSI-H tumours)						
Mutated	93	41 (44%)		70	31 (44%)	
Wild-type	38	16 (42%)		21	6 (29%)	
CIMP status						
CIMP-high	136	68 (50%)	<0.0001	101	42 (42%)	0.04
CIMP-low	353	154 (44%)	0.0002	199	93 (44%)	0.003
CIMP-0	431	132 (31%)	Referent	267	161 (30%)	Referent
KRAS						
Any mutation	321	135 (42%)		206	92 (45%)	0.003
G>A mutation	195	84 (43%)		132	62 (47%)	0.003
Non-G>A mutation	126	51 (40%)		74	30 (41%)	
Wild-type	553	205 (37%)		368	119 (32%)	Referent
BRAF						
Mutated	116	46 (39%)		82	23 (28%)	
Wild-type	758	294 (38%)		492	188 (38%)	
KRAS/BRAF						
K(+)B(+)	6	3 (50%)		3	2 (67%)	
K(+)B(−)	315	132 (42%)		203	90 (44%)	0.02
K(−)B(+)	110	43 (39%)		79	21 (27%)	
K(−)B(−)	443	162 (37%)		289	98 (34%)	Referent
18q LOH						
(+)	237	71 (30%)	0.0008	169	45 (27%)	0.004
(−)	217	98 (45%)	Referent	145	61 (42%)	Referent

Only statistically significant p values are described. CIMP, CpG island methylator phenotype; LOH, loss of heterozygosity; MSI, microsatellite instability; MSS, microsatellite stable.

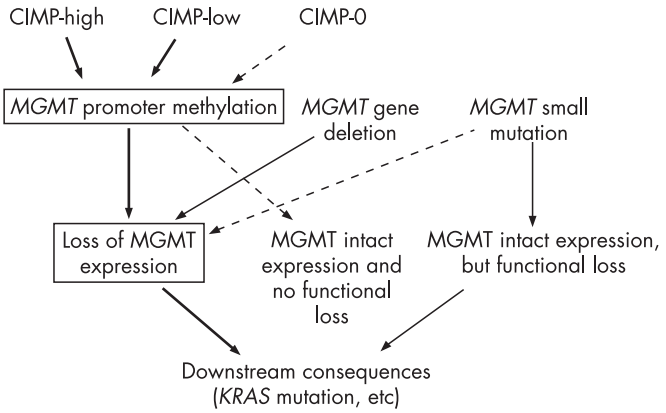

Figure 2 Various molecular aberrations surrounding *MGMT* promoter methylation and loss of expression in colorectal cancer. Promoter methylation and gene deletion (or small mutation) can occur in two different *MGMT* alleles in the same tumour. Thus, this figure is a simplified illustration. Tumours with both *MGMT* methylation and loss of expression represent a more homogeneous group of tumours than tumours with *MGMT* methylation alone or tumours with MGMT loss alone.

MGMT-methylated tumours (25%, 84/340) and *MGMT*-unmethylated tumours (21%, 111/534). However, the frequency of *KRAS* G>A mutation was significantly higher in MGMT-lost tumours (29%, 62/211; p = 0.006) than MGMT-expressing tumours (19%, 70/363).

Tumours with both *MGMT* methylation and loss of expression

As shown in table 2, the correlation between *MGMT* methylation and loss of expression was tight but not perfect. Figure 2 illustrates various molecular aberrations surrounding *MGMT* promoter methylation and loss of expression. About 26% of MGMT-lost tumours exhibited no significant *MGMT* methylation (maybe due to gene deletion or mutation that impaired gene expression), and about 12% of MGMT-expressing tumours did show *MGMT* methylation (maybe partial or monoallelic methylation that did not considerably affect expression). We calculated the frequency of tumours with both *MGMT* methylation and loss of expression among colorectal cancers with various molecular features. Tumours with both *MGMT* methylation and loss likely represented a more homogeneous group of tumours than tumours with *MGMT* methylation alone, or tumours with MGMT loss alone. Table 3 summarises the molecular correlates with simultaneous *MGMT* methylation and loss of expression. MSI-L tumours showed a significantly higher frequency of *MGMT* methylation/loss (41%, p = 0.02) than MSS tumours (25%).

Table 3 Frequency of tumours with both *MGMT* methylation and loss in colorectal cancer

	Total examined, n	*MGMT* methylated and lost (%)	p Value
MSI status			
MSI-high	91	29 (32%)	
MSI-low	46	19 (41%)	0.02
MSS	421	106 (25%)	Referent
CIMP status			
CIMP-high	101	35 (35%)	0.005
CIMP-low	199	66 (33%)	0.002
CIMP-0	267	55 (21%)	Referent
KRAS			
Any mutation	197	65 (33%)	0.03
G>A mutation	127	45 (35%)	0.02
Non-G>A mutation	70	20 (29%)	
Wild-type	349	85 (24%)	Referent
KRAS/BRAF			
K(+)B(+)	3	2 (67%)	
K(+)B(−)	194	63 (32%)	0.04
K(−)B(+)	76	15 (20%)	Referent
K(−)B(−)	273	70 (26%)	
18q LOH			
(+)	163	28 (17%)	0.0002
(−)	142	51 (36%)	Referent

Only statistically significant p values are described. CIMP, CpG island methylator phenotype; LOH, loss of heterozygosity; MSI, microsatellite instability; MSS, microsatellite stable.

MGMT methylation and loss in nine MSI/CIMP subtypes of colorectal cancer

Molecular classification of colorectal cancer based on MSI and CIMP status is increasingly important because MSI status and CIMP status reflect global genomic and epigenomic aberrations in tumour cells. Thus, we classified tumours into nine subtypes according to both MSI and CIMP status as follows: MSI-H CIMP-high (n = 93), MSI-L CIMP-high (n = 6), MSS CIMP-high (n = 35), MSI-H CIMP-low (n = 24), MSI-L CIMP-low (n = 33), MSS CIMP-low (n = 282), MSI-H CIMP-0 (n = 14), MSI-L CIMP-0 (n = 34) and MSS CIMP-0 (n = 368). By virtue of the large sample size, we were able to evaluate the frequencies of *MGMT* methylation and/or loss in all of the nine subtypes, even in rare subtypes such as MSI-L CIMP-high and MSI-H CIMP-0 (fig 3).

In contrast to the CIMP-high and CIMP-0 groups, the CIMP-low groups exhibited a striking difference in the frequencies of *MGMT* methylation and/or loss between MSI-H and MSI-L tumours (fig 3). In the CIMP-low groups, MSI-H tumours showed much lower frequencies of *MGMT* methylation (8.3%), loss of expression (22%) and methylation/loss (5.6%) than MSI-L tumours (61% showing methylation, p<0.0001; 67% showing loss of expression, p = 0.002; and 67% showing methylation/loss, p = 0.0003). These data indicate that MSI-L is not a mixture of misdiagnosed MSI-H and MSS tumors. These relationships between *MGMT* and MSI status were present only among CIMP-low tumours, suggesting that CIMP-low tumours constituted a different subset of colorectal cancers from CIMP-high and CIMP-0 tumours. Our data also suggest that MSI-L tumours were different from MSS tumours at least among the CIMP-low tumour group.

Within the CIMP-high group, there was no significant difference in the frequencies of *MGMT* methylation or loss between MSI-H, MSI-L and MSS tumours. Similarly, within the CIMP-0 group, there was no significant difference in the frequencies of *MGMT* methylation or loss between MSI-H, MSI-L and MSS tumours.

DISCUSSION

We conducted this study to examine the molecular correlates with *MGMT* promoter methylation and gene silencing in colorectal cancer, using a large number of samples and robust methylation detection methods. Discovering molecular correlates is important, because it may: (1) provide clues to pathogenesis; (2) propose or support a new molecular subtype; (3) alert investigators to be aware of potential confounding in association studies; and (4) suggest surrogate markers in clinical or research settings.[27 35] We used quantitative PCR assays (MethyLight)[36] to determine the degree of DNA methylation, which is robust enough to reproducibly differentiate low-level methylation from high-level methylation.[26] Our resource of a large number of samples from two large prospective cohorts (relatively unbiased samples compared to retrospective or single-hospital-based samples) has enabled us to precisely estimate the frequency of colorectal cancers with specific molecular features (eg, *MGMT* methylation, CIMP-high, MSI-H, etc).

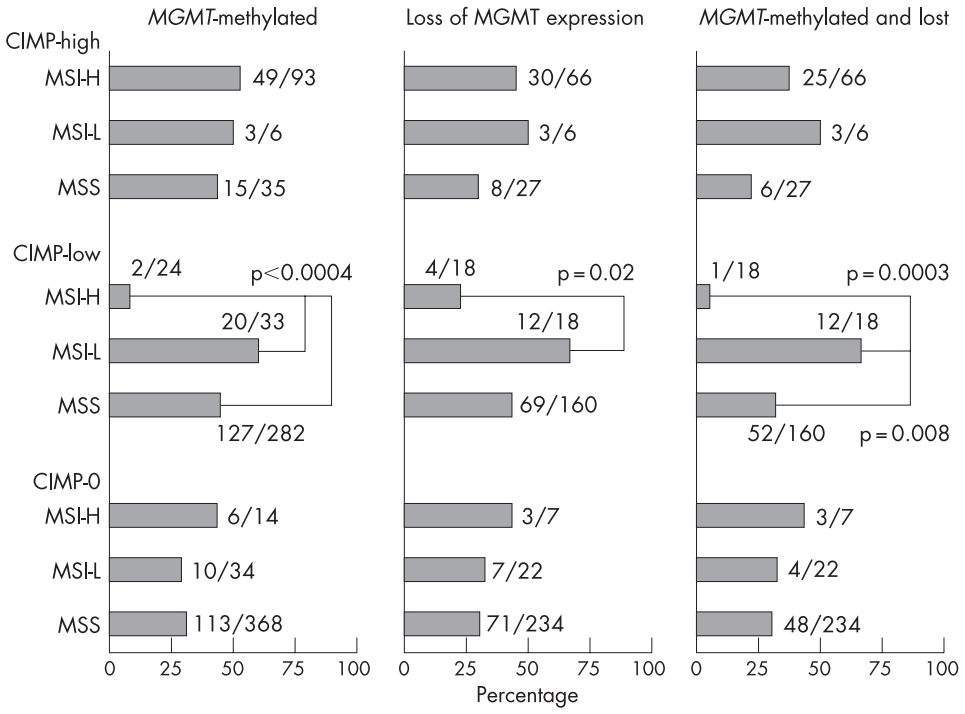

Figure 3 Frequencies of *MGMT* methylation and/or loss of expression in nine MSI/CIMP subtypes of colorectal cancer. A significant relationship between *MGMT* and MSI status is present only among the CIMP-low tumour group.

In particular, we sought to decipher the relationship between *MGMT* and MSI/CIMP status. Molecular classification based on MSI and CIMP status is increasingly important[39] because MSI and CIMP status reflect global genomic and epigenomic aberrations in cancer cells. We have found that, among CIMP-low tumours, *MGMT* methylation and silencing are correlated positively with MSI-L and inversely with MSI-H. However, no such relationship was present among CIMP-high and CIMP-0 tumours. A previous study demonstrated an insignificant trend towards an inverse relationship between *MGMT* methylation and MSI-H MINT++ (ie, CIMP)[40] (this MINT++ could represent both CIMP-high and CIMP-low, because MINT markers are not specific for CIMP-high but are also frequently methylated in CIMP-low tumours[11]). Our data indicate unique molecular features of CIMP-low, which differ from CIMP-high and CIMP-0. Our data also support a possible link between CIMP-low, MSI-L, *MGMT* methylation/loss and *KRAS* mutation.

We have previously shown that CIMP-low in colorectal cancer is associated with male sex and *KRAS* mutations, while CIMP-high is associated with female sex and *BRAF* mutations, and CIMP-0 is associated with wild-type *KRAS*/*BRAF* genes and shows no sex predilection.[15] However, the difference between CIMP-low and CIMP-0 was not as clear-cut as the difference between CIMP-high and CIMP-low.[15] Our data from the current study provide additional supporting evidence for a molecular difference between CIMP-low and CIMP-0. Further studies are necessary to identify the best set of markers for the diagnosis of CIMP-low, because the eight marker panel we used is sensitive and specific for CIMP-high[10 24] but perhaps not the best markers to separate CIMP-low from CIMP-0.

We have shown an association between *MGMT* methylation/loss and MSI-L, which is in agreement with the previous studies.[23 25] In addition, we have shown that the association between *MGMT* methylation/loss and MSI-L is only present in CIMP-low tumours but not in CIMP-high or CIMP-0 tumours. As shown in fig 3, MSI-L is not a mixture of under-diagnosed MSI-H and MSS. MSI-L has been associated with shorter survival in stage C colon cancer compared to MSS tumours.[23] A cDNA microarray expression study has also supported MSI-L as a distinct phenotype from MSS and MSI-H.[41] These data collectively support molecular and biological differences between MSI-L and MSS in colorectal cancer. Additional studies are necessary to find underlining molecular defects for MSI-L and better biomarkers for MSI-L (other than microsatellites), since there has been a controversy whether MSI-low exists as a distinct molecular phenotype from MSS.[42 43] It also remains to be elucidated why the significant relationship between *MGMT* and MSI-L is limited to CIMP-low tumours.

We have shown the positive correlation between *MGMT* methylation and *MLH1* methylation, in contrast to a previous study.[38] We have shown that *MLH1* methylation is very specific for CIMP-high,[10] and that *MGMT* methylation is also correlated with CIMP-high (in this study). Thus, in our large scale study, the observed correlation between *MGMT* methylation and *MLH1* methylation is logical, and likely mediated by CIMP-high.

We have detected other interesting molecular correlates between *MGMT* methylation/loss and various molecular features, including *KRAS* mutation, *KRAS* G>A mutation and 18q LOH (inverse correlation). Esteller *et al*[18] have previously described a relationship between *KRAS* G>A mutation and *MGMT* methylation, and the frequency of *MGMT* methylation was more common in colorectal cancers with *KRAS* G>A mutation (71%, 36/51), compared to tumours with *KRAS* non-G>A mutation (32%, 12/37 showing *MGMT* methylation) and tumours with wild-type *KRAS* (35%, 55/156 showing *MGMT* methylation). In the current study, we could show the significant relationship between MGMT loss of expression and *KRAS* G>A mutation ($p = 0.003$) (or any *KRAS* mutation, $p = 0.003$). Halford *et al*[44] demonstrated that MGMT loss of expression in colorectal cancer was significantly more common in tumours with G>A mutation in *APC*, *CTNNB1* (the β-catenin gene) or *KRAS*.

MGMT methylation and/or CIMP have been related to the serrated pathway of colorectal carcinogenesis.[25 39 45 46] *MGMT* methylation has been detected in 22% of hyperplastic polyps, in 25% of sessile serrated adenomas,[47] in 16–22% of serrated adenomas with a variable degree of dysplasia, and in 50% of serrated adenocarcinomas.[48] However, since *MGMT* methylation and CIMP are positively correlated, it is also possible that serrated neoplasias may be correlated with CIMP and only indirectly with

MGMT methylation through CIMP. Nonetheless, MSI-L occurs more commonly (~30%) in serrated adenocarcinomas while MSI-L is less common (~14%) in non-serrated adenocarcinomas,[49] favouring the suggestion that factors inducing MSI-L are important for the development of serrated neoplasias.[46]

In conclusion, *MGMT* methylation and loss of expression are correlated with MSI-L, CIMP-high and CIMP-low in colorectal cancer. In particular, among CIMP-low tumours, *MGMT* methylation and loss are correlated positively with MSI-L and inversely with MSI-H. No such relationship is present among CIMP-high or CIMP-0 tumours, supporting the suggestion that CIMP-low may be a different phenotype from CIMP-high and CIMP-0. Our data also support a molecular difference between MSI-L and MSS in colorectal cancer, as well as a possible pathogenetic link between CIMP-low, MSI-L, *MGMT* methylation/loss and *KRAS* mutation.

Note added in proofs: As additional supporting evidence for molecular differences between CIMP-low and CIMP-0, we have recently shown that 18q LOH in non-MSI-high tumour is correlated positively with CIMP-0 and inversely with CIMP-low and CIMP-high.[50] With regard to the frequency of 18q LOH, CIMP-low is similar to CIMP-high, but different from CIMP-0.

ACKNOWLEDGEMENTS

We deeply thank the Nurses' Health Study and Health Professionals Follow-up Study cohort participants who have generously agreed to provide us with biological specimens and information through responses to questionnaires. We thank Graham Colditz, Walter Willett and many other staff members who implemented and have maintained the cohort studies. We deeply thank Peter Laird, Daniel Weisenberger and Mihaela Campan for assisting in the development of the MethyLight assay.

Authors' affiliations

S Ogino, Department of Pathology, Brigham and Women's Hospital, Boston, MA, USA; Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA; Department of Pathology, Harvard Medical School, Boston, MA, USA
T Kawasaki, Y Suemoto, Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA
G J Kirkner, Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA
J A Meyerhardt, C S Fuchs, Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA; Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA; Department of Medicine, Harvard Medical School, Boston, MA, USA

Funding: This work was supported by the US National Institute of Health (NIH) grants P01 CA87969 and P01 CA55075.

Competing interests: None.

REFERENCES

1 **Laird PW**. Cancer epigenetics. *Hum Mol Genet* 2005;**14**(Spec No 1):R65–76.
2 **Issa JP**. CpG island methylator phenotype in cancer. *Nat Rev Cancer* 2004;**4**:988–93.
3 **Wong JJ**, Hawkins NJ, Ward RL. Colorectal cancer: a model for epigenetic tumorigenesis. *Gut* 2007;**56**:140–8.
4 **Toyota M**, Ahuja N, Ohe-Toyota M, *et al*. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A* 1999;**96**:8681–6.
5 **van Rijnsoever M**, Grieu F, Elsaleh H, *et al*. Characterisation of colorectal cancers showing hypermethylation at multiple CpG islands. *Gut* 2002;**51**:797–802.
6 **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**:710–5.
7 **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.
8 **Samowitz W**, 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.
9 **Kambara T**, Simms LA, Whitehall VLJ, *et al*. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. *Gut* 2004;**53**:1137–44.
10 **Ogino S**, Cantor M, Kawasaki T, *et al*. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut* 2006;**55**:1000–6.
11 **Weisenberger DJ**, Siegmund KD, Campan M, *et al*. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006;**38**:787–93.
12 **Ogino S**, Odze RD, Kawasaki T, *et al*. Correlation of pathologic features with CpG island methylator phenotype (CIMP) by quantitative DNA methylation analysis in colorectal carcinoma. *Am J Surg Pathol* 2006;**30**:1175–83.
13 **Minoo P**, Jass J. Senescence and serration: a new twist to an old tale. *J Pathol* 2006;**210**:137–40.
14 **Jass JR**. Serrated adenoma of the colorectum and the DNA-methylator phenotype. *Nat Clin Pract Oncol* 2005;**2**:398–405.
15 **Ogino S**, Kawasaki T, Kirkner GJ, *et al*. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. *J Mol Diagn* 2006;**8**:582–8.
16 **Lees NP**, Harrison KL, Hall CN, *et al*. Human colorectal mucosal O6-alkylguanine DNA-alkyltransferase activity and DNA-N7-methylguanine levels in colorectal adenoma cases and matched referents. *Gut*. Published Online First 4 Aug 2006. doi: 10, 1136/gut.2006.097899.
17 **Aquilina G**, Biondo R, Dogliotti E, *et al*. Expression of the endogenous O6-methylguanine-DNA-methyltransferase protects Chinese hamster ovary cells from spontaneous G:C to A:T transitions. *Cancer Res* 1992;**52**:6471–5.
18 **Esteller M**, Toyota M, Sanchez-Cespedes M, *et al*. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. *Cancer Res* 2000;**60**:2368–71.
19 **Esteller M**, Risques RA, Toyota M, *et al*. Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. *Cancer Res* 2001;**61**:4689–92.
20 **Shen L**, Kondo Y, Rosner GL, *et al*. MGMT promoter methylation and the field defect in sporadic colorectal cancer. *J Natl Cancer Inst* 2005;**97**:1330–8.
21 **Giovannucci E**, Ogino S. DNA methylation, field effects, and colorectal cancer. *J Natl Cancer Inst* 2005;**97**:1317–9.
22 **Nagasaka T**, Sharp GB, Notohara K, *et al*. Hypermethylation of O6-methylguanine-DNA methyltransferase promoter may predict nonrecurrence after chemotherapy in colorectal cancer cases. *Clin Cancer Res* 2003;**9**:5306–12.
23 **Kohonen-Corish MR**, Daniel JJ, Chan C, *et al*. Low microsatellite instability is associated with poor prognosis in stage C colon cancer. *J Clin Oncol* 2005;**23**:2318–24.
24 **Ogino S**, Kawasaki T, Kirkner GJ, *et al*. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer using a large population-based sample. *J Mol Diagn* 2007;**9**:305–14.
25 **Whitehall VL**, Walsh MD, Young J, *et al*. Methylation of O-6-methylguanine DNA methyltransferase characterizes a subset of colorectal cancer with low-level DNA microsatellite instability. *Cancer Res* 2001;**61**:827–30.
26 **Ogino S**, Kawasaki T, Brahmandam M, *et al*. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. *J Mol Diagn* 2006;**8**:209–17.
27 **Ogino S**, Kawasaki T, Kirkner GJ, *et al*. Down-regulation of p21 (CDKN1A/CIP1) is inversely associated with microsatellite instability and CpG island methylator phenotype (CIMP) in colorectal cancer. *J Pathol* 2006;**210**:147–54.
28 **Colditz GA**, Hankinson SE. The Nurses' Health Study: lifestyle and health among women. *Nat Rev Cancer* 2005;**5**:388–96.
29 **Giovannucci E**, Liu Y, Rimm EB, *et al*. Prospective study of predictors of vitamin D status and cancer incidence and mortality in men. *J Natl Cancer Inst* 2006;**98**:451–9.
30 **Chan AT**, Ogino S, Fuchs CS. Aspirin use and risk of colorectal cancer according to cyclooxygenase-2 expression. *New Engl J Med* 2007;**356**:2131–42.
31 **Ogino S**, Kawasaki T, Brahmandam M, *et al*. Sensitive sequencing method for KRAS mutation detection by pyrosequencing. *J Mol Diagn* 2005;**7**:413–21.
32 **Dietmaier W**, Hartmann A, Wallinger S, *et al*. Multiple mutation analyses in single tumor cells with improved whole genome amplification. *Am J Pathol* 1999;**154**:83–95.
33 **Ogino S**, Brahmandam M, Cantor M, *et al*. Distinct molecular features of colorectal carcinoma with signet ring cell component and colorectal carcinoma with mucinous component. *Mod Pathol* 2006;**19**:59–68.
34 **Boland CR**, Thibodeau SN, Hamilton SR, *et al*. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res* 1998;**58**:5248–57.
35 **Ogino S**, Kawasaki T, Ogawa A, *et al*. TGFBR2 mutation is correlated with CpG island methylator phenotype (CIMP) in microsatellite instability-high colorectal cancer. *Hum Pathol* 2007;**38**(4):614–20.
36 **Eads CA**, Danenberg KD, Kawakami K, *et al*. MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res* 2000;**28**:E32.
37 **Widschwendter M**, Siegmund KD, Muller HM, *et al*. Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. *Cancer Res* 2004;**64**:3807–13.
38 **Fox EJ**, Leahy DT, Geraghty R, *et al*. Mutually exclusive promoter hypermethylation patterns of hMLH1 and O6-methylguanine DNA methyltransferase in colorectal cancer. *J Mol Diagn* 2006;**8**:68–75.
39 **Jass JR**. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 2007;**50**:113–30.
40 **Whitehall VL**, Wynter CV, Walsh MD, *et al*. Morphological and molecular heterogeneity within nonmicrosatellite instability-high colorectal cancer. *Cancer Res* 2002;**62**:6011–4.

41 **Mori Y**, Selaru FM, Sato F, *et al*. The impact of microsatellite instability on the molecular phenotype of colorectal tumors. *Cancer Res* 2003;**63**:4577–82.
42 **Tomlinson I**, Halford S, Aaltonen L, *et al*. Does MSI-low exist? *J Pathol* 2002;**197**:6–13.
43 **Jass JR**. Re: Tomlinson, *et al*. Does MSI-low exist. *J Pathol* 2002;**197**:6–13. *J Pathol* 2003;**199**:267–9; author reply 9–70.
44 **Halford S**, Rowan A, Sawyer E, *et al*. O6-methylguanine methyltransferase in colorectal cancers: detection of mutations, loss of expression, and weak association with G:C>A:T transitions. *Gut* 2005;**54**:797–802.
45 **Jass JR**, Biden KG, Cummings MC, *et al*. Characterisation of a subtype of colorectal cancer combining features of the suppressor and mild mutator pathways. *J Clin Pathol* 1999;**52**:455–60.
46 **Makinen MJ**. Colorectal serrated adenocarcinoma. *Histopathology* 2007;**50**:131–50.
47 **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.
48 **Dong SM**, Lee EJ, Jeon ES, *et al*. Progressive methylation during the serrated neoplasia pathway of the colorectum. *Mod Pathol* 2005;**18**:170–8.
49 **Tuppurainen K**, Makinen JM, Junttila O, *et al*. Morphology and microsatellite instability in sporadic serrated and non-serrated colorectal cancer. *J Pathol* 2005;**207**:285–94.
50 **Ogino S**, Kawasaki T, Kirkner GJ, *et al*. 18q loss of heterozygosity in microsatellite stable colorectal cancer is correlated positively with CpG island methylator phenotype-negative (CIMP-negative) and inversely with CIMP-low and CIMP-high. *BMC Cancer* 2007;**7**:7272 (electronic full-length article).

EDITOR'S QUIZ: GI SNAPSHOT

Answer

From questions on page 1542

Endoscopic sphincterotomy was performed on the fifth hospital day. A fluke was found and was removed from the common bile duct by balloon extraction (fig 1). The fluke which measured 1.9×0.9 mm was identified as *Fasciola hepatica* (fig 2). On the sixth hospital day, the patient started to eat again and had no recurrence of epigastric pain, fever, or nausea. The IgE level was 273 U/ml.

He was treated for fascioliasis with a single 625 mg dose of triclabendazole (Egaten). On the 14th hospital day, liver function tests showed that the aspartate aminotransferase level was 22 U/l, while alanine aminotransferase was 32 U/l, alkaline phosphatase was 296 U/l, γ-glutamyltransferase was 274 U/l and total bilirubin was 22 μmol/l. He was discharged on the 15th hospital day. Two months later, the IgE level had fallen to 117 U/ml.

Cattle, sheep and other domesticated herbivores are the definitive hosts and major reservoirs of *Fasciola hepatica*, with human infection usually occurring owing to consumption of aquatic plants contaminated with metacercariae (encysted larval parasites). After infection occurs, the larvae migrate through the wall of the small intestine into the peritoneal cavity and then penetrate the liver capsule within a few days. Next, the larvae invade the bile ducts and grow to maturity there after 2–4 months, after which the flukes start laying eggs, which may continue for years.

doi: 10.1136/gut.2006.107912a

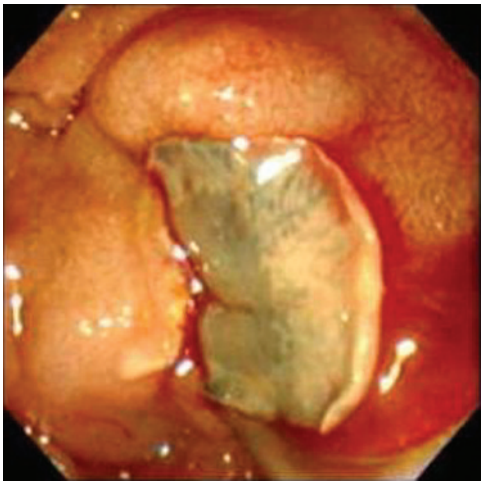

Figure 1 Endoscopic images of a *Fasciola hepatica* fluke in the biliary tract.

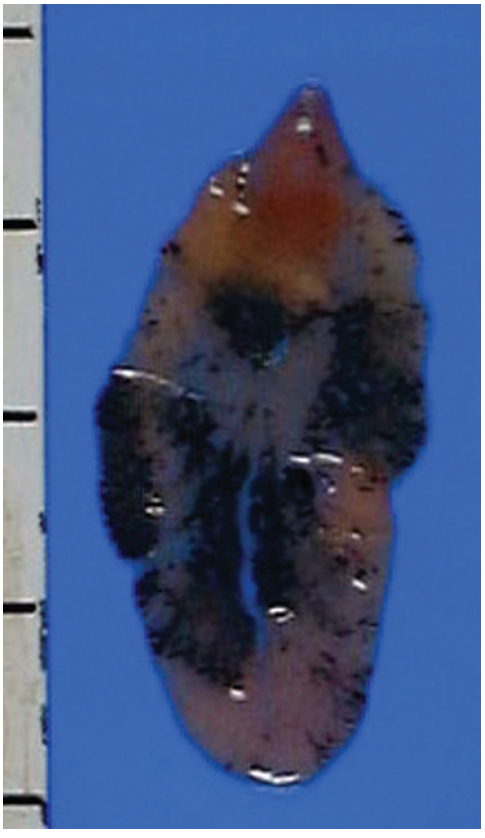

Figure 2 *Fasciola hepatica* fluke recovered from the bile duct by endoscopic retrograde cholangiopancreatography and endoscopic sphincterotomy.