

Epigenetic-Genetic Interactions in the *APC/WNT*, *RAS/RAF*, and *P53* Pathways in Colorectal Carcinoma

Yutaka Suehiro,^{1,4} Chi Wai Wong,⁶ Lucian R. Chirieac,¹ Yutaka Kondo,² Lanlan Shen,² C. Renee Webb,¹ Yee Wai Chan,⁶ Annie S.Y. Chan,⁶ Tsun Leung Chan,⁶ Tsung-Teh Wu,¹ Asif Rashid,¹ Yuichiro Hamanaka,⁵ Yuji Hinoda,⁵ Rhonda L. Shannon,⁴ Xuemei Wang,³ Jeffrey Morris,³ Jean-Pierre J. Issa,² Siu Tsan Yuen,⁶ Suet Yi Leung,⁶ and Stanley R. Hamilton¹

Abstract **Purpose:** Early events in colorectal tumorigenesis include mutation of the *adenomatous polyposis coli* (*APC*) gene and epigenetic hypermethylation with transcriptional silencing of the *O*⁶-methylguanine DNA methyltransferase (*MGMT*), *human mut L homologue 1* (*hMLH1*), and *P16/CDKN2A* genes. Epigenetic alterations affect genetic events: Loss of *MGMT* via hypermethylation reportedly predisposes to guanine-to-adenine or cytosine-to-thymine (G:C→A:T) transition mutations in *KRAS* and *P53*, and silencing of *hMLH1* leads to high levels of microsatellite instability (MSI-H)/mutator phenotype, suggesting that epigenetic-genetic subtypes exist. **Experimental Design:** We evaluated the relationships of aberrant methylation of *APC*, *MGMT*, *hMLH1*, *P16*, *N33*, and five *MINTs* to mutations in *APC*, *KRAS*, *BRAF*, and *P53* in 208 colorectal carcinomas. **Results:** We found that *APC* hypermethylation was age related ($P = 0.04$), in contrast to the other genes, and did not cluster with CpG island methylator phenotype (CIMP) markers. Hypermethylation of *APC* concurrently with either *MGMT* or *hMLH1* was strongly associated with occurrence of G-to-A transitions in *APC* [odds ratio (OR), 26.8; $P < 0.0002$ from multivariable logistic regression model], but C-to-T transitions had no associations. There was no relationship of hypermethylation of any gene, including *MGMT*, with G-to-A or C-to-T transitions in *KRAS* or *P53*, although *APC* hypermethylation was associated with *P53* mutation ($P < 0.0002$). CIMP with MSI-H due to *hMLH1* hypermethylation, or CIMP with loss of *MGMT* expression in non-MSI-H tumors, was associated with *BRAF* mutation (OR, 4.5; $P < 0.0002$). CIMP was also associated with *BRAF* V600E T-to-A transversion (OR, 48.5; $P < 0.0002$). **Conclusions:** Our findings suggest that the heterogeneous epigenetic dysregulation of promoter methylation in various genes is interrelated with the occurrence of mutations, as manifested in epigenetic-genetic subgroups of tumors.

Authors' Affiliations: ¹Department of Pathology, Division of Pathology and Laboratory Medicine; ²Department of Leukemia, Division of Cancer Medicine; and ³Department of Biostatistics and Applied Mathematics, Division of Quantitative Sciences, The University of Texas M. D. Anderson Cancer Center; ⁴Department of Pathology, St. Luke's Episcopal Hospital, Houston, Texas; ⁵Department of Clinical Laboratory Science, Yamaguchi University School of Medicine, Ube, Yamaguchi, Japan; and ⁶Department of Pathology, The University of Hong Kong Queen Mary Hospital, Pokfulam, Hong Kong

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Requests for reprints: Stanley R. Hamilton, Division of Pathology and Laboratory Medicine, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 085, Houston, TX 77030. Phone: 713-792-2040; Fax: 713-792-4094; E-mail: shamilton@mdanderson.org.

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The development of most colorectal carcinomas (CRC) is thought to be initiated by inactivation of the *APC/β-catenin/Wnt* signaling pathway, usually by mutation of one copy of the *APC* gene followed by a second event that inactivates the other allele (1–4). The second inactivating event is usually described as allelic deletion or mutation but can be epigenetic methylation of cytosines in CpG islands of the promoter region of *APC* that results in transcriptional silencing (5, 6). Altered *APC* has wide-ranging downstream effects on cell-cell adhesion, transcriptional regulation, chromosomal instability, cell migration, proliferation and cell cycle control, differentiation, and apoptosis (1–4).

Somatic *APC* mutation is associated with the development of intraepithelial neoplasia (dysplasia) in aberrant crypt foci and early adenomas (7). The initiating alterations in *APC* in these early lesions persist whereas additional genetic and epigenetic events accumulate and drive tumor progression. Somatic mutation in the *APC* gene is present in as many as 80% of sporadic colorectal adenomas and carcinomas (7–9), and a mutation cluster region in exon 15 (9) accounts for the majority of mutations. Most *APC* mutations alter function of the gene product via

point mutation or frameshift mutation that results in a truncated protein lacking all of the axin-binding sites and all but one or two of the 20-amino-acid β -catenin binding sites (10). Mutations of the *KRAS* or *BRAF* gene in the *RAS/RAF* pathway and of the *P53* gene are later genetic events in colorectal tumorigenesis (3).

Well-known mechanisms of spontaneous generation of point mutations in genes include deamination of cytosine and 5-methylcytosine to uracil and thymine, respectively; depurination; DNA polymerase infidelity; and oxidative damage from endogenously produced free radicals (11, 12). Epigenetic events have been hypothesized to influence specific genetic changes and to have a complementary role with genetic alterations in colorectal tumorigenesis (13), albeit with transient controversy about the existence of the hypermethylation pathway, termed the CpG island methylator phenotype (CIMP; refs. 14, 15). A few reports have addressed the influence of epigenetics on specific gene mutations. Inactivation via cytosine methylation of CpG islands in the promoter of the DNA repair gene *O*⁶-methylguanine DNA methyltransferase (*MGMT*) has been linked to subsequent G:C→A:T transition mutations in other genes, representing G-to-A mutations on the sense strand or C-to-T mutations if the G-to-A is on the antisense strand (16). An explanatory mechanism of failure to repair adducts has been proposed (17–19). The *MGMT* gene product removes mutagenic and cytotoxic methyl adducts from the *O*⁶ position of guanine. Hypermethylation of the promoter of *MGMT* has been associated with transcriptional silencing of the gene and loss of protein expression. Failure of removal of *O*⁶-methylguanine adducts results from absent *MGMT* enzyme activity, and mispairing with thymines occurs during replication so that the guanine-cytosine pair is converted to an adenine-thymine pair (16, 20). *MGMT* hypermethylation has been associated in some studies with G:C→A:T mutations in the *KRAS* proto-oncogene and the *P53* suppressor gene that are frequently mutated during progression of colorectal neoplasms (18, 19). Other studies, however, have not identified this association nor a relationship of these specific mutations to reduced or absent expression of *MGMT* protein (21, 22).

Hypermethylation of the promoter of the *hMLH1* mismatch repair gene leads to another important molecular pathway in colorectal tumorigenesis, that is, the microsatellite instability (MSI) pathway (23–25). Loss of functional *hMLH1* protein results in inability of tumor cells to repair nucleotide mismatches. This deficiency is manifested as slippage mutations in repeated nucleotide sequences (microsatellites), resulting in high levels of MSI (MSI-H), as well as instability in both homopolymeric runs and base substitutions in coding sequences called mutator phenotype (25). Hypermethylation of the *P16* gene can silence expression of this suppressor gene and is an early event in colorectal neoplasia that can occur in adenomas (26–28) and aberrant crypt foci (29).

The relationships among epigenetic alterations of various individual genes, concurrent hypermethylation of multiple genes in CIMP, and specific genetic mutations are incompletely described and discordant among different studies. Comprehensive characterization of these multiple pathways that are important in CRC will contribute to understanding the spectrum of intertumoral heterogeneity. We therefore examined the interrelationships among these genomic pathways in 208 CRCs. Our findings provide additional evidence for the occurrence of epigenetic-genetic interactions in tumorigenesis

and have important implications for understanding the molecular pathogenesis of subgroups of CRC.

Materials and Methods

Specimens. We evaluated CRC specimens from 208 patients who were included in an ongoing study that compares the molecular pathology of CRC in Hong Kong and the United States. (The results of the international comparison of molecular characteristics are the subject of another report.) The patients had their resection between 1991 and 2003 at Queen Mary Hospital in Hong Kong ($n = 90$) or the Texas Medical Center in Houston at The University of Texas M. D. Anderson Cancer Center ($n = 70$) or St. Luke's Episcopal Hospital ($n = 48$). The mean age of the patients was 61.6 years; 56.3% were male; 73.6% of the tumors were in the left colon or rectum; and 9.1% were stage I, 37.5% were stage II, 36.1% were stage III, and 17.3% were stage IV (30). No patients received preoperative neoadjuvant therapy before the surgical or biopsy procedure that produced the specimen used for our evaluation. The institutional review board at each collaborating institution approved the study.

Microdissection and DNA extraction. Routine formalin-fixed, paraffin-embedded or frozen archived resection or biopsy specimens were analyzed. DNA was prepared from tumor and from nonneoplastic mucosa after manual microdissection of 5- μ m histopathologic sections, as described previously (31).

Hypermethylation of *APC*, *MGMT*, *hMLH1*, *P16*, *N33*, and *MINTs*. The methylation status of the *APC* promoter 1A; the promoters of the *MGMT*, *hMLH1*, *P16/CDKN2A*, and *N33* genes; and methylated in tumor (*MINT*) loci 1, 2, 25, 27, and 31 were determined by bisulfite treatment of DNA followed by methylation-specific PCR, as shown in Fig. 1. The methods were those described previously (5, 26–29, 32, 33) except for *N33* and *MINT27*. The primers for the *N33* were 5'-CGGAG-GGTTAGTTAGCGGTTTTTC-3' and 5'-GACAAAACAATATCTCCTC-CACGCG-3' for the methylated allele, and 5'-TGGAGGGTTAGTTAG-TGGGTTTTT-3' and 5'-CAACAAAACAATATCTCCTCCACACA-3' for the unmethylated allele. The primers used for *MINT27* were 5'-GGAGT-TTGTGTTAGACGCGGC-3' and 5'-AAAACGCCAAAACTCCCTACG-3' for the methylated allele, and 5'-GTGTGGAGTTTGTGTAGATGTGGT-3' and 5'-CAAAAACACCAAAAACCTCCCTACA-3' for the unmethylated allele. DNA from normal lymphocytes was used as the control for unmethylated genes and loci, except for *N33*, whereas placental DNA treated with *SssI* (CpG) methylase (New England Biolabs) was used as

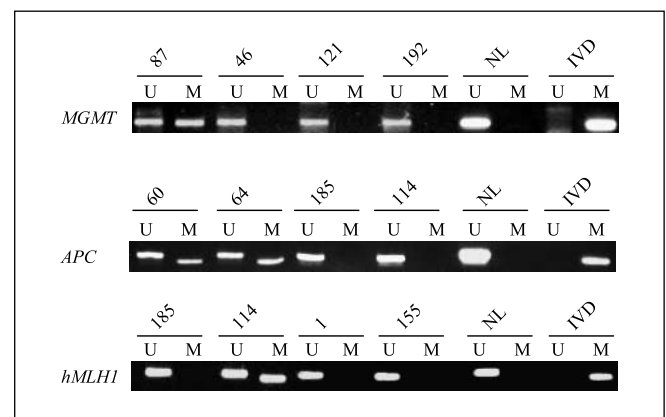


Fig. 1. Examples of methylation assays of *MGMT*, *APC*, and *hMLH1* gene promoters in CRCs. The presence of a visible PCR product from methylation-specific PCR in lanes labeled U indicates the presence of an unmethylated allele, and in lanes labeled M, the presence of a methylated allele. *MGMT* hypermethylation is evident in case 87; *APC* hypermethylation is evident in cases 60 and 64; and *hMLH1* hypermethylation is evident in case 114. DNA from normal lymphocytes (NL) was used as a control for unmethylated *MGMT* and *APC*, and *in vitro* methylated DNA (IVD) from placenta was used as a control for the hypermethylated genes.

the positive control. DNA obtained from the cell line UMUC3 was used as the control for unmethylated *N33*. PCR products from methylated and unmethylated reactions were electrophoresed on 3% agarose gels and visualized by ethidium bromide staining. Each sample was analyzed in triplicate. The criterion for presence of hypermethylation was detection of a methylated band in all three independent assays.

Hierarchical cluster analysis (34) of all tumors based on CpG island methylation in the 10 evaluated markers (Fig. 2) was used to identify the marker panel that defined CIMP status. The five markers identified in this fashion (*MINT1*, *MINT2*, *MINT31*, *p16*, and *hMLH1*) were found to be the same as those used in our previous studies (26, 32, 35). CRC were classified as having CIMP if three or more ($\geq 60\%$) of the evaluated markers were methylated. Classification of CIMP on the basis of two or more methylated markers in the panel ($\geq 40\%$) yielded similar results.

Mutation of *APC*, *KRAS*, *BRAF*, and *P53* genes. DNA sequencing was used to evaluate mutations in the *APC*, *KRAS*, *BRAF*, and *P53* genes. For the *APC* gene, four sets of oligonucleotide primers were used to amplify the mutation cluster region in exon 15 from codons 1260 to 1592 as described previously (36), except for redesigned primers C1 and C2. For codons 1416 to 1510, C1 was 5'-AAGCCCCAGT-GATCTTCCA-3' and C2 was 5'-CAGAGCACTCAGGCTGGAT-3'. The primers for exon 11 and exon 15 of *BRAF* and exon 2 of *KRAS* used in the current study were described previously (37).

For the *P53* gene, five sets of oligonucleotide primers were used to amplify exons 5 to 8 (exon 5 was amplified in two overlapping segments). Exon 5a-forward was 5'-CTTCAACTCTGTCTCCTCCTC-3' and exon 5a-reverse was 5'-GCTGTGACTGCTTGTAGATGG-3'. Exon 5b-forward was 5'-GCCAAGACCTGCCCTGTG-3' and exon 5b-reverse was 5'-CAACCAGCCCTGCTCTCT-3'. Exon 6-forward was 5'-GGCC-TCTGATTCTCACTGA-3' and exon 6-reverse was 5'-CTCCTCCCAGAG-ACCCAGT-3'. Exon 7-forward was 5'-CCTCATCTGGCCCTGTGTT-3' and exon 7-reverse was 5'-GCAGGGTGGCAAGTGGCTCC-3'. Exon 8-forward was 5'-CCTTACTGCCTTGTCTCTCT-3' and exon 8-reverse was 5'-CTCCACCGCTTCTGTCTG-3'.

For each primer pair, PCR products were generated from two independent PCR reactions for sequencing of the forward and reverse products. The PCR products were purified by use of shrimp alkaline phosphatase and exonuclease I (Amersham) in accordance with the manufacturer's instructions. The purified PCR products were sequenced on an ABI Prism 3730 DNA Analyzer with the ABI Prism Big Dye Terminator Cycle Sequencing Kit version 1.1 (Applied Biosystems). The primers used for amplification were also used for sequencing. The sequencing results were analyzed using DNA Sequencing Analysis Software Version 5.0 (Applied Biosystems). All mutations were confirmed by analysis in both the forward and reverse directions.

Microsatellite instability. MSI was evaluated in genomic DNA extracted from microdissected tumor tissue and nonneoplastic mucosa as described previously (38). A panel of seven microsatellites was evaluated: four mononucleotide repeats (BAT25, BAT26, BAT40, and *TGF β R2*) and three dinucleotide repeats (D2S123, D5S346, and D17S250). Tumors were categorized according to criteria from the National Cancer Institute conference on MSI (39) as microsatellite-stable (MSS) if no marker had altered size, MSI-low (MSI-L) if at least one marker but $<40\%$ of total markers were altered, and MSI-high (MSI-H) if at least 40% of markers were altered.

MGMT and hMLH1 gene product expression. Histologic sections of formalin-fixed paraffin-embedded tissue cut 5 μ m thick were evaluated for immunohistochemical staining of tumor cell nuclei with commercially available mouse anti-MGMT monoclonal antibody (Chemicon) or anti-hMLH1 monoclonal antibody (PharMingen) and routine methods, as described previously (40, 41). Nuclear expression was classified as present or absent relative to staining of nuclei of nonneoplastic cells that provided a positive internal control for each slide.

Statistical analysis. Each assay was done and interpreted independently of all other assays, and the results were then entered into a database for statistical analysis. Hierarchical cluster analysis was done to assess the association among the 10 evaluated CpG methylation sites

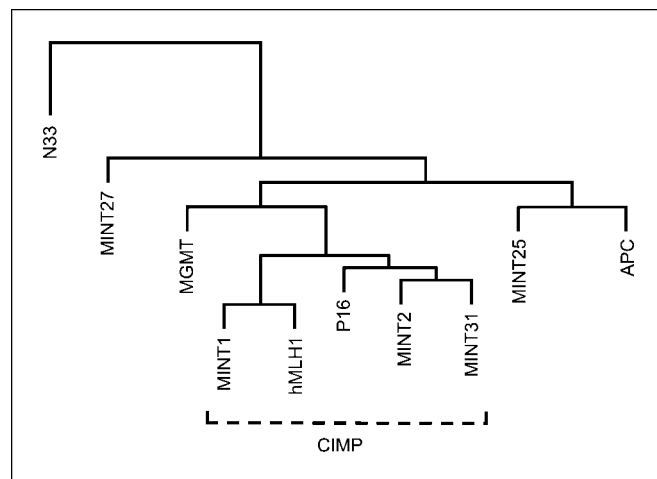


Fig. 2. Hierarchical cluster analysis of the 10 methylation makers used. *P16*, *hMLH1*, and *MINTs 1, 2, and 31* clustered together and were used to define CIMP, as in our previous studies (26, 32, 35). Hypermethylation of the *APC*, *MGMT*, and *N33* genes and of *MINTs 25* and *27* did not cluster with the CIMP markers.

(34). Descriptive statistics were calculated. Associations between categorical variables were evaluated with two-tailed Fisher's exact tests. The odds ratios (OR) and 95% confidence intervals were estimated for each association. *P* values <0.05 were considered statistically significant.

In addition, a multivariable logistic regression model (42) was fit for the binary outcome of presence or absence of mutation of the *APC*, *Ki-ras*, *BRAF*, and *P53* genes and for the subsets of G-to-A, C-to-T, and G:C→A:T transition mutations, and of *BRAF* V600E nucleotide 1799 T-to-A transversion. Methylation, gene product expression, and microsatellite instability were used as predictors. Logistic regression is one type of logistic regression model that uses the logic or Boolean combinations of binary covariates as potential predictors. A subset of cases with all available results was created. Through implementing a "greedy search" algorithm (42), we then found the optimal logic regression model that minimized the score function. The greedy search algorithm first identified a best single predictor among all candidate single predictors. Then, it searched among the remaining predictors to find the best combination that lowered the score function. This process continued until the score function could no longer be reduced. To account for multiple testing inherent in the logic regression procedure, permutation tests were carried out using 5,000 permutations to compute the *P* values. The ORs were adjusted by tumor stage. All statistical analyses were carried out in Splus.

Results

The epigenetic, genetic, and associated phenotypic alterations in the 208 CRCs we studied are summarized in Table 1 and Fig. 3.

Promoter hypermethylation and mutation of *APC* gene. An alteration of *APC* was found in 70.2% of the CRC; 31.7% had *APC* hypermethylation, and this alteration was age-related (43), occurring more frequently in older patients (mean age 64.6 years as contrasted with 60.2 years, $P = 0.04$; 45.8% of CRC with *APC* hypermethylation in patients in the oldest quartile >79 years of age but only 23.5% in the youngest quartile <51 years old). No other hypermethylation site we evaluated had an association with age. By contrast with the tumors, nonneoplastic colorectal mucosa rarely had *APC* hypermethylation (0.8%).

Sixty-two percent of the tumors had mutation of *APC* in the mutation cluster region between codons 1260 and 1596. Point mutation was the most common sequence alteration observed. Twenty-five G-to-A transition point mutations were found in

19 tumors (Table 2). Two tumors had two G-to-A transitions (cases 7 and 18), and two tumors had three (cases 3 and 16). Only one of the G-to-A mutations occurred in a CpG site (case 3). Twenty-four percent of the G-to-A mutations were silent, and the remainder resulted in amino acid substitutions. A C-to-T transition was found in 30 tumors. Thus, there was no evidence of strand bias in G:C→A:T mutations (25 G-to-A and 30 C-to-T on sense strand).

Promoter hypermethylation of MGMT gene and loss of MGMT protein expression. Of the CRCs, 27.4% had hypermethylation of MGMT, and 24.5% had loss of MGMT protein by immunohistochemistry. Methylation status of the MGMT gene and MGMT gene product expression status were highly concordant, with the expected inverse relationship in 82.7% of the carcinomas ($P < 0.001$).

Promoter hypermethylation of hMLH1 gene, loss of hMLH1 protein expression, and microsatellite instability. Hypermethylation

of the *hMLH1* gene was found in 9.2% of the CRC, and loss of hMLH1 protein expression was found in 9.8%, with the expected inverse relationship in 94.6% the carcinomas. Hypermethylation of *hMLH1* also had the expected strong association with high levels of MSI (MSI-H), with methylation status of *hMLH1* and MSI status concordant in 92.7% of tumors ($P < 0.0001$).

Concurrent CpG island hypermethylation. Hierarchical cluster analysis showed that the methylation status of the 10 markers had distinct relationships (Fig. 2). *MINT1*, *MINT2*, *MINT31*, *P16*, and *hMLH1* clustered together, as we found in our previous studies (26, 32, 35). These five markers therefore were used to define CIMP based on hypermethylation of three or more markers ($\geq 60\%$) and identified 10.1% of the CRC as having CIMP. The methylation status of the *APC*, *MGMT*, and *hMLH1* genes were not significantly associated (OR, 0.5-1.1; 95% confidence interval, 0.1-1.7 to 0.6-2.1), as reported in previous studies (5, 44).

Table 1. Summary of epigenetic, genetic, and associated phenotypic alterations

Site and type of alteration	Percentage of tumors	No. tumors	Total tumors evaluated
<i>APC</i> gene			
<i>APC</i> gene alteration identified	70.2	146	208
<i>APC</i> promoter hypermethylation	31.7	64	202
Mutation in <i>APC</i> mutation cluster region	62.0	129	208
Point mutation	—	87 (136)*	—
G-to-A transition	—	19 (25)*	—
C-to-T transition	—	30 (32)*	—
Frameshift mutation	—	57 (59)*	—
<i>MGMT</i> gene and MGMT expression			
<i>MGMT</i> promoter hypermethylation	27.4	57	208
Loss of MGMT protein expression	24.5	51	208
Concordance of <i>MGMT</i> methylation status with MGMT protein expression status	82.7	172	208
<i>hMLH1</i> gene and microsatellite instability			
<i>hMLH1</i> promoter hypermethylation †	9.2	19	207
Loss of hMLH1 protein expression	9.8	20	204
Concordance of <i>hMLH1</i> methylation status with hMLH1 protein expression status	94.6	192	203
High levels of microsatellite instability (MSI-H)	10.6	22	208
Concordance of <i>hMLH1</i> methylation status with MSI status	92.6	187	202
CpG island methylation of additional markers			
Frequency of hypermethylation			
<i>P16</i> gene †	19.5	39	200
<i>N33</i> gene	93.8	180	192
<i>MINT1</i> †	10.1	21	208
<i>MINT2</i> †	22.6	47	208
<i>MINT25</i>	28.3	51	180
<i>MINT27</i>	39.6	67	169
<i>MINT31</i> †	20.3	42	207
CIMP with three markers ($\geq 60\%$)	10.1	21	208
<i>KRAS</i> proto-oncogene/ <i>BRAF</i> gene			
<i>KRAS</i> codon 12 or 13 mutation	39.2	80	204
<i>KRAS</i> codon 12 or 13 G-to-A transition	—	56	—
Codon 12a G-to-A	—	5	—
Codon 12b G-to-A	—	26	—
Codon 13b G-to-A	—	25	—
<i>BRAF</i> mutation	6.5	13	199
<i>BRAF</i> V600E nt 1799 T-to-A transversion	4.5	9	199
Discordant <i>KRAS</i> and <i>BRAF</i> V600E mutation status	100	9	9
<i>P53</i> gene			
Exon 5-8 mutation	55.2	112	203
G-to-A transition	—	40	—
C-to-T transition	—	31	—

*Number in parenthesis is the number of *APC* mutations. The total number of *APC* mutations exceeds the number of tumors due to the occurrence of multiple mutations in 41 tumors. The total number of point mutations includes the number of G-to-A transition mutations.

†Five markers used to define CIMP based on cluster analysis (see Fig. 2).

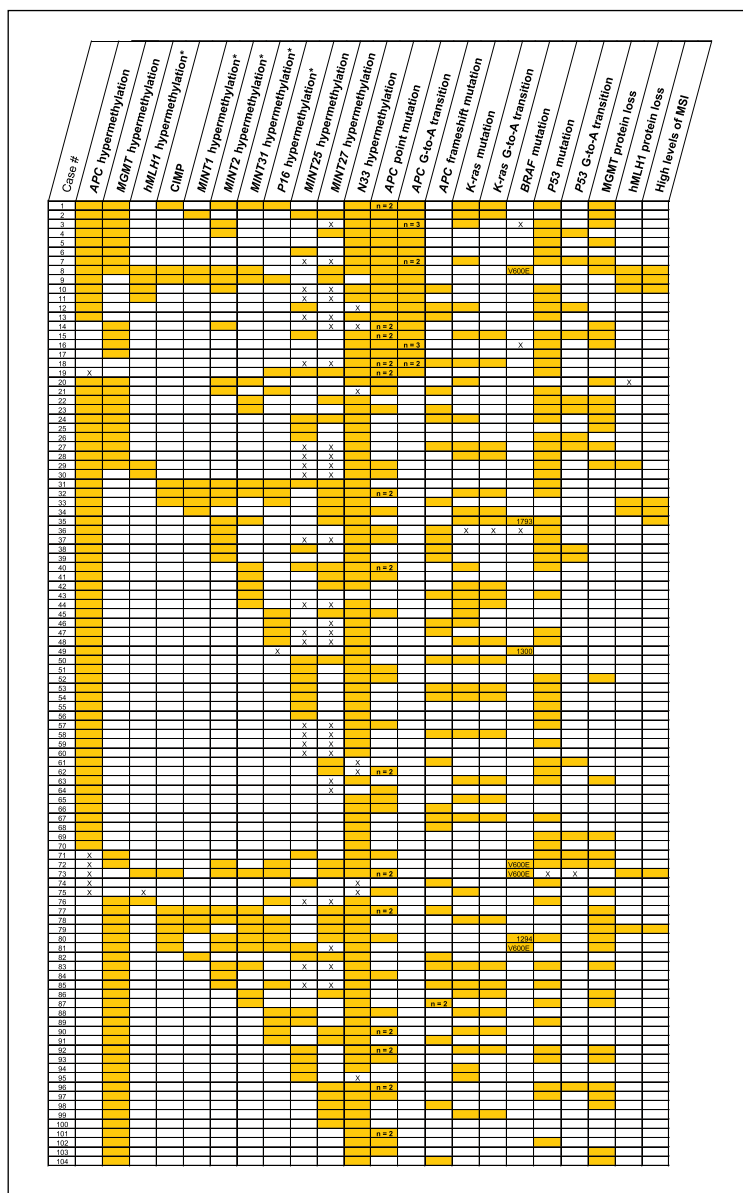


Fig. 3. Diagram of epigenetic and genetic alterations in 208 CRCs arranged by methylation and mutation status. The CIMP markers from Fig. 2 are indicated by asterisk in the column headings. Shading, the presence of the molecular alteration; X, unavailable data; open cell, absence of the alteration. Numbers in the APC columns indicate when two or more mutations are present, and numbers in the BRAF column indicate the type of or nucleotide with mutation. The intertumoral heterogeneity of the alterations and the epigenetic-genetic associations are apparent. The strong relationship between hypermethylation of APC with hypermethylation of MGMT or hMLH1 and G-to-A transition mutation in APC is evident in cases 1 to 11.

Mutations of KRAS proto-oncogene, BRAF, and P53 suppressor gene. Mutation of the KRAS gene at codon 12 or 13 was found in 39.2% of the CRC. Among the 80 mutations, 70.0% were a G-to-A transition at codon 12a (GGT→AGT, $n = 5$), at 12b (GGT→GAT, $n = 26$), or at 13b (GGC→GAC, $n = 25$), but no tumor had a G-to-A transition at 13a (GGC→AGC). C-to-T transition at 13c does not occur, and therefore strand bias for G:C→A:T mutation is not a consideration. Among all of the molecular alterations we studied, KRAS mutation was associated with stage, occurring with higher frequency in more advanced tumors: 15.8% of stage I, 37.7% in stage II, 38.4% in stage III, and 57.1% in stage IV ($P = 0.03$).

BRAF mutation was identified in 6.5% of the tumors, with the V600E T-to-A transversion at nucleotide 1799 accounting

for 9 of the 13 mutations. The other BRAF mutations were G469V G-to-T transversion (case 80), T5991 C-to-T transition (case 35), F468S T-to-C transition (case 49), and N581S A-to-G transition (case 166). KRAS and BRAF V600E mutations, but not the other BRAF mutations, were mutually exclusive (Fig. 3), as expected (37, 38, 45, 46).

Mutation in exons 5 to 8 of the P53 gene was present in 55.2% of the carcinomas. Of the 112 P53 mutations, 35.7% were a G-to-A transition, and seven of these were located in a non-CpG site. C-to-T mutation occurred in 31 tumors. Thus, there was no evidence of strand bias in G:C→A:T mutations (40 G-to-A and 31 C-to-T on sense strand).

Associations among hypermethylation and gene mutations. Table 3 and Fig. 4 summarize the relationships among the

Fig. 3. Continued.

epigenetic and genetic alterations identified in multivariable analysis. Hypermethylation of specific genes was associated with the pattern of gene mutations in subgroups of CRC.

Hypermethylation of *APC* in concert with either *MGMT* or *hMLH1* was strongly associated with G-to-A transition mutation in *APC* (cases 1-19 in Fig. 3; OR, 26.8; $P < 0.0002$; Table 3B; Fig. 4). A G-to-A transition was found in 50.0% of 22 CRC with *APC* hypermethylation concurrent with either *MGMT* or *hMLH1* hypermethylation, but in only 4.4% of 182 carcinomas without concurrent methylation of these gene pairs. *APC* hypermethylation was not associated, however, with *APC* mutations in general (Table 3A) or with *APC* mutations of other subtypes. C-to-T transitions representing G-to-A on the antisense strand as occurs with *MGMT* deficiency (16) as well as C-to-T transitions on the sense strand had no associations. Further evidence for the influence of specific, rather than generalized, methylation on mutation pattern was provided by the lack of association of

hypermethylation of the other genes and markers with mutation of *KRAS* (Table 3C and D).

The presence of *APC* hypermethylation or the presence of MSS or MSI-L in the absence of CIMP was associated with mutation of *P53* (60.9%, 103 of 169, versus 9.1%, 2 of 22; OR, 15.9; $P < 0.0002$; Table 3G; Fig. 4). The inverse relationship of *P53* mutation and CIMP has been reported in previous studies (35), as has the inverse relationship between *P53* mutation and MSI-H (47–50), but the association of *P53* mutation with *APC* hypermethylation has not been reported previously.

CIMP in concert with MSI-H due to hypermethylation of *hMLH1* was associated with mutation of *BRAF*, as in previous studies (50–54). In addition, we identified the novel association of CIMP and loss of *MGMT* protein due to *MGMT* methylation with *BRAF* mutation (38.1%, 8 of 21, versus 1.2%, 2 of 165; OR, 4.5; $P < 0.0002$; Table 3E; Fig. 4). Only 2 of 13 CRC with *BRAF* mutation lacked CIMP, MSI-H, or loss of

Table 2. Summary of G-to-A transition mutations in *APC* gene

Case	Codon	G in nucleotide sequence	Protein sequence change
1	1578b	GAA TGT ATT	Cys→Tyr
2	1550c	CAA GAG AAA	Glu→no change
3	1400c	CGT TCG ATT	Ser→no change
	1513c	GAT GAG CCA	Glu→no change
	1561c	GAA AAG GAC	Lys→no change
4	1530a	CAG GAA AAT	Glu→Lys
5	1540c	TCA GAG CAG	Glu→no change
6	1466b	AGT GGA CCT	Gly→Glu
7	1357a	TCA GGA GCG	Gly→Arg
	1534a	AAT GGG AAT	Gly→Arg
8	1312a	ATT GGA ACT	Gly→Arg
9	1338c	CTG CAG GGT	Gln→no change
10	1470a	CAA GCT GCA	Ala→Thr
11	1377a	TAT GTT CAG	Val→Ile
12	1321b	GTG AGC GAA	Ser→Asn
13	1426b	GAT AGC CCT	Ser→Asn
14	1374a	CCT GAA CAC	Glu→Lys
15	1416b	AGT GGC ATT	Gly→Asp
16	1348b	GCC AGG CAC	Arg→Lys
	1460a	ACT GCT GAA	Ala→Thr
	1475a	GCT GCA GTT	Ala→Thr
17	1435b	AGC AGA AGT	Arg→Lys
18	1385b	TTT AGC AGA	Ser→Asn
	1521a	GTG GAA TTA	Glu→Lys
19	1317a	GCT GAA GAT	Glu→Lys

MGMT. CIMP was also strongly associated with *BRAF* V600E T-to-A transversion (18.2%, 6 of 33, versus 0.7%, 1 of 153; OR, 48.5; $P < 0.0002$; Table 3F; Fig. 4), as described previously (51–54). Only one of five CRCs with *BRAF* V600E mutation and MSI-H had methylation of *MGMT* and loss of *MGMT* protein expression, whereas all four tumors with *BRAF* V600E mutation that were MSS or MSI-L had lost *MGMT* expression (Fig. 3). These findings raise the possibility that MSI-H/mutator phenotype and *MGMT* expression have alternative relationships with *BRAF* mutation.

Discussion

Our study emphasizes the heterogeneity of both hypermethylation and epigenetic-genetic interactions in molecular subgroups of CRC. The characteristics of hypermethylation of *APC*, a key gene in CRC, differed from those of the other genes and markers we evaluated. *APC* hypermethylation was more

frequent in older patients, but hypermethylation of other genes and markers in our panel was not age-related. *APC* hypermethylation did not cluster with methylation of the markers that defined the CIMP, as is evident in Fig. 2. Although age-related, *APC* hypermethylation was also tumor specific, occurring only rarely in colorectal mucosa, in contrast to other genes with age-related methylation that are frequently methylated in the mucosa as well (55). Although a recent study (56) suggested that *APC* hypermethylation had little effect on colorectal carcinogenesis, another study (44) reported that *APC* hypermethylation was discordant with methylation of other genes, as we found, and that the phenotype of tumors with *APC* hypermethylation was different from those with hypermethylation of other genes. These results emphasize the importance of identifying associations to clarify CIMP-positive and other subgroups of CRC that have hypermethylation of the type not involved in CIMP.

The influence of epigenetic alterations on genetic events has been the subject of speculation and hypotheses addressed at both tumor initiation and tumor progression, including the role of epigenetics in potentiation and modulation of the effects of genetic changes (13). Among the small number of published studies that have addressed directly the topic of epigenetic-genetic interactions (57), Esteller et al. reported an association between *MGMT* hypermethylation and G:C→A:T transitions in both *KRAS* and *P53* (18, 19). Their studies led to the proposal that methylation-induced loss of *MGMT* protein expression, and therefore its intracellular function, influenced the mechanism and the type of subsequent mutations in these other genes involved in progression through failure of adduct repair that should have been accomplished by *MGMT*. Our study shows that hypermethylation of *APC* in concert with hypermethylation of either *hMLH1* or *MGMT* is strongly associated with G-to-A transition in non-CpG sites of the *APC* gene, but not with C-to-T transition. To our knowledge, ours is the first report of the relationship in tumors of the epigenetic mechanism of concordant gene hypermethylation that includes *APC* to mutation type in that gene in the *WNT* signaling pathway. In addition, we found that *APC* hypermethylation was associated with *P53* mutation, whereas CIMP was inversely related to *P53* mutation, as in previous studies (35, 50, 58).

In contrast to the strong association of G-to-A mutation of *APC* with concurrent *MGMT* and *APC* hypermethylation, we found no relationship to loss of *MGMT* gene product expression. Other investigators also found no specific

Associated Findings	Type of Mutation			
	<i>APC</i> G-to-A	<i>P53</i>	<i>BRAF</i>	<i>BRAF</i> V600E
<i>APC</i> hypermethylation	■	■		
<i>MGMT</i> hypermethylation	■			
No <i>MGMT</i> expression			■	
<i>hMLH1</i> hypermethylation	■			
MSI-H			■	
MSS/MSI-L		■		
CIMP			■	■
No CIMP		■		
Odds Ratio	26.8	15.9	4.5	48.5
P value	<0.0002	<0.0002	<0.0002	<0.0002

Fig. 4. Diagram illustrating the subgroups of CRC with epigenetic-genetic interactions identified through multivariable logistic regression model. Hypermethylation of *APC* concurrently with either *MGMT* or *hMLH1* hypermethylation was associated with G-to-A transition mutation in *APC*, whereas *APC* hypermethylation or absence of CIMP in MSS/MSI-L tumors was associated with *P53* mutation. CIMP and MSI-H or CIMP in the absence of MSI-H but with loss of *MGMT* expression was associated with *BRAF* mutation. CIMP was also associated with *BRAF* V600E. ORs were adjusted by tumor stage, and all P values are based on 5,000 permutations of the original data set.

Table 3. Associations between composite binary predictor variables created through logic regression and mutation of *APC*, *KRAS*, *BRAF*, or *P53* genes**A. APC mutation**

Created composite variable	APC mutation present	No APC mutation found	Total	OR*	P [†]
APC hypermethylation present or (neither MGMT protein expression nor CIMP found)	65	22	87	2.9	0.11
APC hypermethylation absent and (presence of MGMT protein expression or of CIMP)	56	50	106		
Total	121	72	193		

B. APC G-to-A transition

Created composite variable	APC G-to-A present	No APC G-to-A found	Total	OR*	P [†]
(Concurrent APC and MGMT hypermethylation) or (concurrent APC and hMLH1 hypermethylation)	11	11	22	26.8	<0.0002
(Concurrent APC and MGMT hypermethylation not found) or (concurrent APC and hMLH1 hypermethylation not found)	8	174	182		
Total	19	185	204		

C. KRAS mutation

Created composite variable	KRAS mutation present	No KRAS mutation found	Total	OR*	P [†]
CIMP present	4	16	20	3.3	0.25
CIMP not found	74	97	171		
Total	78	113	191		

D. KRAS G-to-A transition

Created composite variable	KRAS G-to-A present	No KRAS G-to-A found	Total	OR*	P [†]
MSI-H present and CIMP not found	7	5	12	4.4	0.35
MSS/MSI-L or CIMP present	48	131	179		
Total	55	136	191		

E. BRAF mutation

Created composite variable	BRAF mutation present	No BRAF mutation found	Total	OR*	P [†]
(Concurrent CIMP and MSI-H) or (CIMP present and MGMT protein expression not found)	8	13	21	4.5	<0.0002
CIMP not found or (concurrent MSS/MSI-L and MGMT protein expression)	2	163	165		
Total	10	176	186		

F. BRAF V600E T-to-A transversion

Created composite variable	BRAF V600E mutation present	No BRAF V600E mutation found	Total	OR*	P [†]
CIMP present	6	27	153	48.5	<0.0002
CIMP not found	1	152	33		
Total	7	179	186		

G. P53 mutation

Created composite variable	P53 mutation present	No P53 mutation found	Total	OR*	P [†]
APC hypermethylation present or (MSS/MSI-L present and CIMP not found)	103	66	169	15.9	<0.0002
No APC hypermethylation found and (MSI-H or CIMP present)	2	20	22		
Total	105	86	191		

H. P53 G-to-A transition

Created composite variable	P53 G-to-A present	No P53 G-to-A found	Total	OR*	P [†]
MSS/MSI-L present and CIMP not found	36	124	160	11.6	0.13
MSI-H or CIMP present	0	31	31		
Total	36	155	191		

*ORs were adjusted by tumor stage.

[†]All *P* values are based on 5,000 permutations of the original data set.

association with MGMT protein expression (22), although they found a weak relationship between reduced or absent MGMT expression by immunohistochemistry and G:C→A:T mutations in various genes. The methylation of *MGMT* may be more strongly associated with G-to-A transitions than is reduced protein expression because methylation indicates stable loss of expression, detects heterogeneous loss of expression, or indicates effects of expression level differences that are mechanistically important but not discernible by nonquantitative immunohistochemical methodology. In addition, G-to-A transitions among G:C→A:T mutations had an association with methylation in our study, whereas C-to-T transitions did not. *In vitro* studies of MGMT-deficient cells have shown that G-to-A transitions accounted for 94% of G:T→A:T transition hotspots after exposure to a methylating agent, and in T-lymphocytes of normal individuals, 72% of hotspots (16). Thus, G-to-A transition may be influenced preferentially by *MGMT* gene or MGMT protein alterations. We found in the small number of tumors with *BRAF* mutation in our study that MGMT protein expression was usually present in those with MSI-H but lost in those with MSS or MSI-L, raising the possibility of a role of MGMT in *BRAF* mutation occurrence or selection.

We found that MSI-H was inversely associated with *P53* mutation, as in previous studies (47–50), and that *hMLH1* methylation status and MSI-H status were strongly concordant (92.6% of our CRC, Table 1). Our sample size, however, was insufficient to distinguish among the possible effects on *APC* mutation of hypermethylation of multiple genes that include *hMLH1* and the mutator phenotype/MSI-H that results from *hMLH1* hypermethylation with loss of the gene product. We found a strong inverse relationship of *P53* mutation to CIMP and an association of *BRAF* mutation with CIMP, MSI-H, and hypermethylation of *hMLH1*, as reported previously (35, 46, 53, 55, 58). There was no such relationship between CIMP and *APC* or *KRAS* mutation, also as reported previously (43, 44, 50, 59). Thus, the effect of CIMP status on gene mutation is quite different among genes, providing additional evidence that suggests specificity of some epigenetic-genetic interactions resulting in molecular subgroups of CRC.

In the current study, we found no association between *MGMT* hypermethylation and G-to-A or C-to-T mutations in *KRAS* and *P53*. Our results corroborate those of other investigators (21, 22), but contrast with the studies of Esteller et al. (18, 19) who first reported these associations. We also found a clear association of *APC* mutation type (i.e., G-to-A transition) with hypermethylation of *APC* concurrent with *MGMT* or *hMLH1* hypermethylation. However, Esteller et al. (5) reported that *APC* promoter hypermethylation is biased toward CRC with genetically intact *APC*, whereas other investigators (21, 22) found no correlation between *APC* methylation status and *APC* mutations. The discordance in results of the reported studies may be explained by several factors. Technical issues such as the use of different assays could lead to different sensitivities in detecting

the mutations, and target sequences for the detection of both mutations and methylation abnormalities differ among studies (51). Standardization of methylation markers and criteria for classification, as has been done for MSI (39), is not in place for hypermethylation studies. Alternatively, the discordance could reflect intertumoral differences in molecular mechanisms and clinical-pathologic characteristics, including stage of disease, in the patient populations studied. For example, correlation between dietary factors and specific mutations of *KRAS* (59–61) and *APC* (62) in CRC has been reported. Thus, differences in environmental factors among study populations may affect the methylation and mutation events that are found and reported.

The mechanisms responsible for the epigenetic-genetic associations we found remain to be determined. Alkylation of DNA at the *O*⁶ position of guanine is an important step in the formation of G-to-A mutations in cancer. Human colorectal DNA has long been known to contain *O*⁶-methylguanine and *N*⁷-methylguanine adducts that arise from exposure to methylating factors and agents (16, 63, 64). The sources of these exposures are unknown but can potentially be dietary/lifestyle and occupational characteristics, endogenous alkylating agents, and *in situ* formation typically mediated by the bacterial or chemical nitrosation of amines (65–67).

Although *APC* mutation is considered to be the initiating alteration in the development of many CRC, this alteration is uncommon in small sporadic adenomas (68, 69). On the other hand, hypermethylation of *MGMT* and *p16* are present at relatively high frequency even in small adenomas (17, 26, 28) and in aberrant crypt foci that are the earliest precursor lesion (29), whereas hypermethylation of *hMLH1* is not frequent in these early lesions (48, 70). One recent hypothesis linking epigenetics and genetic alterations is that colon cancer cells with *APC* gene inactivation are protected from methylation-induced cytotoxicity (71) so that lack of *APC* function due to epigenetic silencing may prevent cell death after exposure to DNA-methylating agents. As a consequence, mutagenic adducts including *O*⁶-methylguanine could accumulate in the DNA, followed by development of G-to-A mutations. Hypermethylation of *APC* with *MGMT* or *hMLH1* thus may produce synergistic effects on this specific type of *APC* mutation and may explain the association we found. On the other hand, *APC* may be merely a marker gene for the secondary effects of global hypermethylation on occurrence of mutation, because 24% (6 of 25) of the G-to-A mutations we found were silent and therefore would not affect *APC* function or provide selective advantage to the tumor cells through changes in that specific gene. The remainder of the G-to-A transitions in *APC* produced amino acid substitutions rather than truncation of the gene product. Additional studies to include the temporal order of the early epigenetic and genetic events are needed to determine the specific mechanisms by which the interactions affect the neoplastic process in large bowel mucosa and the premalignant lesions in which CRC develop.

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Yutaka Suehiro, Chi Wai Wong, Lucian R. Chirieac, et al.

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