

Biomarkers, Genomics, Proteomics, and Gene Regulation

Novel Application of Structural Equation Modeling to Correlation Structure Analysis of CpG Island Methylation in Colorectal Cancer

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The CpG island methylator phenotype (CIMP-high, CIMP1) is a distinct phenotype associated with microsatellite instability (MSI) and BRAF mutation in colon cancer. Recent evidence suggests the presence of KRAS mutation-associated CIMP subtype (CIMP-low, CIMP2). We used cluster analysis, principal component analysis (PCA), and structural equation modeling (SEM), a novel strategy, to decipher the correlation structure of CpG island hypermethylation. Using a database of 861 colon and rectal cancers, DNA methylation at 16 CpG islands [CACNA1G, CDKN2A (p16/ink4a), CHFR, CRABP1, HIC1, IGF2, IGFBP3, MGMT, MINT-1, MINT-31, MLH1, NEUROG1, p14 (CDKN2A/arf), RUNX3, SOCS1, and WRN] was quantified by real-time PCR. Tumors were categorized into three groups: Group 1 with wild-type KRAS/BRAF (N = 440); Group 2 with mutant KRAS and wild-type BRAF (N = 308); and Group 3 with wild-type KRAS and mutant BRAF (N = 107). Tumors with mutant KRAS/BRAF (N = 6) were excluded. In unsupervised hierarchical clustering analysis, all but six markers (CACNA1G, IGF2, RUNX3, MGMT, MINT-1, and SOCS1) were differentially clustered with CIMP-high and CIMP-low according to KRAS and BRAF status. In SEM, the correlation structures between CIMP, locus-specific CpG island methylation, and MSI differed according to KRAS and BRAF status, which was consistent with PCA

results. In conclusion, KRAS and BRAF mutations appear to differentially influence correlation structure of CpG island methylation. Our novel data suggest two distinct perturbations, resulting in differential locus-specific propensity of CpG methylation. (Am J Pathol 2010, 177:2731–2740; DOI: 10.2353/ajpath.2010.100361)

Epigenetic alterations are important mechanisms in human carcinogenesis. A number of tumor suppressor genes are aberrantly silenced by promoter CpG island methylation in colorectal cancer. A subset of colorectal cancers exhibit widespread promoter CpG island methylation, ie, the CpG island methylator phenotype (CIMP),^{1,2} which is a major cause of microsatellite instability (MSI) in sporadic colorectal cancer through epigenetic silencing of a mismatch repair gene *MLH1*.^{3–5} Colorectal cancers with high-level CIMP (ie, CIMP-high) show distinct characteristics including associations with older age, female sex, proximal tumor location, the presence of synchronous colorectal cancer,⁶ *BRAF* mutation, wild-type *TP53*, stable chromosomes, and inactive *WNT/β-catenin* *CTNNB1*,^{3,7–12} independent of MSI.^{4,5,13}

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Experimental and human correlative data suggest that DNMT3B (DNA methyltransferase 3B) may contribute to locus-specific CpG island methylation and CIMP-high in colorectal tumors.^{14–16} Therefore, the best preventive or therapeutic approaches for CIMP-high tumors will be very different from those for non-CIMP-high tumors.

In contrast to CIMP-high, the existence of CIMP-low as a distinct phenotype remains controversial. CIMP-low in colorectal cancer has been associated with *KRAS* mutation,^{11,17,18} low-level locus-specific methylation,¹⁹ and poor prognosis.²⁰ CIMP-low and other proposed subtypes [“CIMP2”¹⁰ and “intermediate-methylation epigenotype (IME)”²¹] appear to share overlapping characteristics. Experimental evidence suggests contribution of *KRAS* or *BRAF* activation to locus-specific CpG island methylation,^{22,23} although there are conflicting data.²⁴ However, the interrelationship between *KRAS* and *BRAF* mutations and various methylation markers have not been deciphered using a large number of tumors. Because *KRAS* and *BRAF* are commonly-mutated human oncogenes, it is of particular interest to understand how *KRAS* and *BRAF* mutations relate to locus-specific CpG island methylation in cancer cells, which may confer drug sensitivity or resistance.

In this study, we used a database of 861 colorectal cancers and biostatistical techniques including cluster analysis, principal component analysis (PCA) and structural equation modeling (SEM), the latter of which is a novel strategy to decipher the correlation structure of CpG island methylation in cancer. We have found that the correlation structure of locus-specific methylation varies according to *KRAS* and *BRAF* mutational status. The consistent data by cluster analysis, PCA and SEM increase confidence in our conclusions. Our novel data suggest a possible role of *KRAS* and *BRAF* mutation status in modifying propensity for CpG island methylation in a locus-specific manner during carcinogenic process.

Materials and Methods

Study Group

We used the databases of two large prospective cohort studies; the Nurses' Health Study (NHS, $N = 121,700$ women followed since 1976),²⁵ and the Health Professionals Follow-up Study (HPFS, $N = 51,500$ men followed since 1986).²⁵ A subset of cohort participants developed colorectal cancer during prospective follow-up. Previous studies on the cohorts have described baseline characteristics of cohort participants and incident colorectal cancer cases and confirmed that our colorectal cancers were well representative as a population-based sample.²⁵ We collected paraffin-embedded tissue blocks from hospitals where participants had undergone resections of primary colorectal cancers. Among our cohort studies, there was no significant difference in demographic features between cases with tissue available and those without available tissue.²⁵ Based on availability of adequate tissue specimens and results (on CpG island methylation and *KRAS* and *BRAF* sequencing), a total of 861 colorectal cancer cases were included in this study. Histopathological features including tumor differenti-

ation, mucinous features, and signet ring cells were examined by a pathologist (S.O.). Poor differentiation was defined as the presence of <50% glandular area. Considering the importance of MSI testing in screening for Lynch syndrome, we provide Supplemental Table 1 (available at <http://ajp.amjpathol.org>) to describe cases with MSI-high and unmethylated *MLH1* in tumor. We have previously analyzed all of the 861 tumors for statuses of MSI, *KRAS*, *BRAF*, DNMT3B and the 16 CpG island methylation markers.^{4,15} However, our current study represents new analyses of principal component analysis (PCA) and structural equation modeling (SEM) on the tumor database. To our knowledge, an application of SEM to correlation structure analysis of CpG island methylation has not been done by any research group. Informed consent was obtained from all study subjects. Tissue collection and analyses were approved by the Harvard School of Public Health and Brigham and Women's Hospital Institutional Review Boards.

DNMT3B Immunohistochemistry, KRAS and BRAF Sequencing, and Microsatellite Instability (MSI) Analysis

DNMT3B immunohistochemistry was performed as previously described.¹⁵ DNA was extracted from paraffin tissue, and PCR-Pyrosequencing targeted for *KRAS* codons 12 and 13,²⁶ and *BRAF* codon 600 were performed.¹⁷ MSI status was determined using D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487.²⁷ MSI-high was defined as the presence of instability in $\geq 30\%$ of the markers, and MSI-low/microsatellite stability (MSS) as 0 to 29% unstable markers.

Real-Time PCR (MethyLight) for Quantitative DNA Methylation Analysis

Sodium bisulfite treatment on DNA and subsequent real-time PCR (MethyLight²⁸) was validated as previously described.²⁹ We quantified DNA methylation in 16 CpG islands,⁴ including the 5 CpG island methylator phenotype (CIMP)-specific promoters (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*)³ and 11 other CpG islands [*CDKN2A* (p16), *CHFR*, *CRABP1*, *HIC1*, *IGFBP3*, *MGMT*, *MINT-1*, *MINT-31*, *MLH1*, p14 (*CDKN2A/ARF*), and *WRN*].^{4,19} We used the database of 861 colorectal cancer cases with available molecular data, including methylation data on the 16 preselected CpG islands. *COL2A1* was used to normalize for the amount of template bisulfite-converted DNA.²⁹ Primers and probes were previously described.^{3,4} The PCR condition was initial denaturation at 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. 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 Sssl-treated human genomic DNA (presumably fully methylated) and multiplying this value by 100. While methylation

positivity at each locus for CIMP classification was set as $PMR \geq 4$ as previously validated,²⁹ PMR value at each locus was used as a continuous variable to assess locus-specific methylation. CIMP-high was defined as methylation positivity at ≥ 6 of 8 methylation markers (*CACNA1G*, *CRABP1*, *CDKN2A* (p16), *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*), CIMP-low as 1 to 5 of the 8 markers, and CIMP-0 as 0/8 methylated markers.⁴

Statistical Analysis

We used the SAS system for Windows (version 9.1.3; SAS Institute, Cary, NC) for the all statistical analyses. The statistical significance level was set at $P = 0.05$. The χ^2 test or the F-test was used to assess statistical significance of differences in the categorical variables or the continuous variables, respectively, between tumor groups. The correlations among the 16 CpG island methylation markers, CIMP (high versus low versus CIMP-0), and MSI score were studied by Spearman's rank correlation, adjusted for age, sex, body mass index (BMI), family history of colorectal cancer (present versus absent), disease stage (ordinal; I, II, III and IV), tumor location (proximal versus distal), and DNMT3B. The Spearman's rank correlation coefficient was designated as ρ . To estimate the correlation coefficient for either *KRAS* or *BRAF* mutation eliminating the effect of the other mutation, the correlation coefficients among the 16 CpG islands and *KRAS* (or *BRAF*) mutation were calculated after exclusion of *BRAF* (or *KRAS*) mutation.

To explore the latent correlation structure of CIMP and locus-specific CpG island methylation, we performed principal component analysis (PCA) and cluster analysis. We used the VARCLUS procedure of SAS (SAS Institute, Cary, NC) to cluster the 15 CpG island markers excluding *MLH1* for each group of colorectal cancers. We excluded *MLH1* from the cluster analysis because *MLH1* methylation has been known to be in the intermediate path from CIMP-high to MSI-high.³⁻⁵ In contrast to standard clustering methods, which divide observations into clusters based on similar values, VARCLUS divides numeric variables based on similar correlation structure (ie, pairwise correlation values). This can be regarded as an analogy of the k-means method using correlation coefficients, and the resulting clusters are similar to those created by factor analysis with oblique rotation. However, the VARCLUS produces disjoint clusters as opposed to fuzzy clusters produced by factor analysis which have more straightforward biological interpretations. To assess the reliability of clustering results, we calculated approximated unbiased P value (AUp) via multiscale bootstrap resampling,³⁰ which is commonly used in phylogenetic analysis. We generated 1000 bootstrap samples to estimate AUp. P value of a cluster is a value between 0 and 1, which indicates how strong the cluster is supported by data.

Structural equation modeling (SEM), a statistical technique for testing and estimating causal relationships,³¹ was used to assess the independent effects of the CIMP status on CpG island methylation and MSI status. SEM has three advantages. First, it can simultaneously model both latent and observed variables. Second, it allows for

effect decomposition; because the total causal effect is the sum of the values of all of the paths between two variables, an indirect effect is determined by subtracting the direct effect from the total. The third advantage is a path diagram, which graphically explains the hypothetical causality. Because we could not observe the true state of CIMP-high, we regarded methylation level of CpG island markers as a surrogate for the true state of CIMP-high. The correlations and path coefficients of *MLH1*, the other CpG island markers, and MSI were computed while controlling for possible confounding effects (sex, age, BMI, family history, tumor location, and DNMT3B status). We used DNMT3B as a potential confounder according to the aim of this study; our goal was to explore the relationships of *KRAS* and *BRAF* mutations with CIMP and CpG island methylation markers. The maximum likelihood method was used to estimate the parameters of SEM, and approximated t -tests were conducted to test individual path coefficients, following the t distribution with degrees of freedom of $n - k - 1$ (n : sample size; k : the parameters of the path model). We calculated asymptotic 95% confidence interval (CI) for the direct effects and bias-corrected and accelerated bootstrap CIs for the total and indirect effects based on 1000 bootstrap replicates. In addition, we performed multiple group analysis to test invariance of the structure across groups. Of the several fit indices available for the overall model test, we selected the goodness-of-fit index (GFI), the adjusted GFI (AGFI), and the root mean square error of approximation (RMSEA) as the fit index for the overall model test. The data probably do not fit the model if the AGFI is negative or much larger than 1 and RMSEA is 0.10. Good models have an RMSEA of 0.05 or less.

Results

CpG Island Methylation, KRAS and BRAF Status in Colorectal Cancers

Using the database of 861 clinically and molecularly-annotated colorectal cancers in the two prospective cohort studies, we examined DNA methylation in the 5 CpG islands (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*) selected as CIMP (CpG island methylator phenotype) surrogate markers,³ and in 11 other CpG islands [*CDKN2A* (p16), *CHFR*, *CRABP1*, *HIC1*, *IGFBP3*, *MGMT*, *MINT-1*, *MINT-31*, *MLH1*, p14 (*CDKN2A/ARF*) and *WRN*]. We determined CIMP status using eight markers, including the five markers in the Weisenberger panel,³ *CDKN2A*, *CRABP1*, and *MLH1*, as previously validated.⁴ To evaluate the effect of *KRAS* and *BRAF* mutations on the correlation structure of the 16 individual CpG island methylation markers, we categorized tumors into four groups (Table 1): Group 1 with both wild-type *KRAS* and *BRAF*; Group 2 with mutant *KRAS* and wild-type *BRAF*; Group 3 with wild-type *KRAS* and mutant *BRAF*; and Group 4 with both mutant *KRAS* and *BRAF*. Because the sample size of Group 4 was very small ($N = 6$), we made comparisons between Groups 1, 2, and 3 ($N = 855$), excluding Group 4 for further analyses.

Table 1. Clinical, Pathological, and Molecular Characteristics of Colorectal Cancer Cases According to *KRAS* and *BRAF* Mutational Status

Clinical, pathological, or molecular feature	All cases	Group 1 (Wild-type <i>KRAS/BRAF</i>)	Group 2 (Mutant <i>KRAS</i> , wild-type <i>BRAF</i>)	Group 3 (Wild-type <i>KRAS</i> , mutant <i>BRAF</i>)	Group 4 (Mutant <i>KRAS/BRAF</i>)	<i>P</i> value
Total N	861	440	308	107	6	
Sex						<0.0001
Male, n (%)	385 (45)	202 (43)	158 (51)	24 (22)	1 (17)	
Female, n (%)	476 (55)	238 (54)	150 (49)	83 (78)	5 (83)	
Mean age ± SD, years	66.6 ± 8.4	66.0 ± 8.6	67.1 ± 8.3	67.6 ± 7.3	64.9 ± 6.5	0.075
Mean body mass index ± SD	26.6 ± 4.7	26.6 ± 4.6	26.4 ± 4.6	26.5 ± 5.4	29.2 ± 4.1	0.94
Family history of colorectal cancer						0.98
Absent, n (%)	649 (75)	331 (75)	232 (75)	81 (75)	5 (83)	
Present, n (%)	212 (25)	109 (25)	76 (25)	26 (25)	1 (17)	
Smoking status						0.70
Never, n (%)	342 (40)	182 (42)	113 (38)	45 (42)	2 (33)	
Current or past, n (%)	506 (60)	254 (58)	186 (62)	62 (58)	4 (67)	
Tumor location						<0.0001
Proximal colon, n (%)	365 (44)	137 (33)	138 (47)	87 (83)	3 (50)	
Distal colon, n (%)	270 (33)	168 (40)	88 (30)	13 (13)	1 (17)	
Rectum, n (%)	189 (23)	116 (27)	67 (23)	4 (3.8)	2 (33)	
Tumor stage						0.0007
I, n (%)	194 (26)	112 (29)	65 (24)	16 (16)	1 (17)	
II, n (%)	247 (32)	128 (33)	75 (27)	40 (39)	4 (67)	
III, n (%)	222 (29)	107 (28)	91 (33)	23 (23)	1 (17)	
IV, n (%)	101 (13)	36 (9.4)	42 (15)	23 (23)	0	
Tumor grade						<0.0001
Low, n (%)	766 (91)	399 (92)	275 (95)	78 (73)	4 (67)	
High, n (%)	78 (9)	33 (8)	14 (5)	29 (27)	2 (33)	
CIMP status*						<0.0001
CIMP-0, n (%)	400 (46)	255 (58)	136 (44)	7 (7)	2 (33)	
CIMP-low, n (%)	333 (39)	150 (34)	156 (51)	25 (23)	2 (33)	
CIMP-high, n (%)	128 (15)	35 (8.0)	16 (5)	75 (70)	2 (33)	
MSI status						<0.0001
MSI-low/MSS, n (%)	727 (85)	385 (89)	285 (93)	54 (50)	3 (50)	
MSI-high, n (%)	124 (15)	48 (11)	20 (6.6)	53 (50)	3 (50)	
DNMT3B expression						0.0005
(-), n (%)	645 (85)	327 (87)	225 (86)	69 (71)	3 (60)	
(+), n (%)	115 (15)	49 (13)	36 (14)	29 (28)	2 (40)	

The percentage number (%) indicates the proportion of cases with a specific clinical, pathological, or molecular feature within each group. Groups 1–3 were used in further analyses; Group 4 was excluded from further analysis.

*CIMP-high was defined as ≥6/8 methylated markers using the 8-marker CIMP panel [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*].

CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSS, microsatellite stability; SD, standard deviation.

Correlations between the 16 CpG Island Markers and *KRAS* and *BRAF* Mutations

We assessed the relationship between *KRAS/BRAF* mutations and each of the 16 CpG island methylation markers after adjusting for clinical features (Table 2; for unadjusted data, see Supplemental Table 2 at <http://ajp.amjpathol.org>). Except for *MGMT*, all of the other 15 CpG island markers positively correlated with *BRAF* mutation (adjusted ρ , 0.15 to 0.59; $P < 0.001$). Only *WRN* showed a modest positive correlation with *KRAS* mutation (adjusted ρ , 0.17; $P < 0.0001$). Three other markers (*IGFBP3*, *MIINT-1*, and *p14*) were positively but weakly associated with *KRAS* mutation (adjusted ρ , 0.10 to 0.11; $P < 0.034$), while *MLH1* was negatively correlated with *KRAS* mutation (adjusted ρ , -0.14; $P = 0.0012$). These results implied that not all methylation markers exhibited similar correlations with *BRAF* or *KRAS* mutation, and methylation patterns appeared to be locus-specific.

CIMP-High Cluster and CIMP-Low Cluster According to *KRAS* and *BRAF* Mutation Status

To examine differences in the correlation structures between groups, next we performed a clustering analysis of methylation markers in each tumor group (Figure 1, A–C). Table 3 shows which markers were clustered with CIMP-high or CIMP-low status in each tumor group. Replication rates were consistently higher within each group than across groups. This implied that these clusters were specific to *KRAS* and *BRAF* mutational status and that the specific correlation structure of the methylation markers was highly variable according to *KRAS* and *BRAF* status. Only *CACNA1G* clustered as a CIMP-high surrogate marker in all three groups ($AUp \geq 0.5$), and it clustered with *RUNX3* reproducibly in *BRAF*-wild-type tumors (Group 1, $AUp = 1.0$; Group 2, $AUp = 0.55$). *CHFR* and *WRN* clustered with high reproducibility only in the *BRAF*-mutant group (Group 3, $AUp = 0.91$). These results remained essentially un-

Table 2. Correlation between Each of the 16 CpG Island Methylation Markers and *KRAS* or *BRAF* Mutation

CpG island methylation marker	<i>KRAS</i> mutation		<i>BRAF</i> mutation	
	ρ^*	<i>P</i> value	ρ^*	<i>P</i> value
<i>CACNA1G</i>	0.0094	0.83	0.40	<0.0001
<i>CDKN2A</i> (p16)	-0.012	0.77	0.40	<0.0001
<i>CHFR</i>	-0.028	0.52	0.30	<0.0001
<i>CRABP1</i>	0.0096	0.83	0.40	<0.0001
<i>HIC1</i>	-0.022	0.61	0.14	0.0042
<i>IGF2</i>	-0.0076	0.86	0.43	<0.0001
<i>IGFBP3</i>	0.097	0.026	0.19	0.0001
<i>MGMT</i>	0.059	0.18	0.0046	0.93
<i>MINT-1</i>	0.10	0.017	0.26	<0.0001
<i>MINT-31</i>	0.014	0.75	0.34	<0.0001
<i>MLH1</i>	-0.14	0.0012	0.38	<0.0001
<i>NEUROG1</i>	0.066	0.13	0.47	<0.0001
p14 (<i>CDKN2A</i> /ARF)	0.11	0.015	0.28	<0.0001
<i>RUNX3</i>	0.0046	0.92	0.59	<0.0001
<i>SOCS1</i>	-0.092	0.035	0.21	<0.0001
<i>WRN</i>	0.17	<0.0001	0.37	<0.0001

*A ρ value represents Spearman's partial correlation coefficient adjusted for age, sex, BMI, smoking status, tumor stage, and location.

changed when we analyzed methylation marker variables as bivariate at various cut-off points (data not shown), confirming the robustness of our findings.

As an additional analysis of methylation marker correlation structure, we also performed principal component analysis (PCA) using the 15 CpG island methylation markers (excluding *MLH1*, described above) in each tumor group according to *KRAS* and *BRAF* mutational status (see Supplemental Figure 1 at <http://ajp.amjpathol.org>). In all groups, at least 12 of 15 principal components were needed to account for 90% of the total variability, and the eigenvalues of the first four (in Group 2 and Group 3) or three (in Group 1) principal components were greater than 1.0. This supports our conclusion that the correlation structure of specific methylation markers varies within each tumor group, while the global structures of the three tumor groups differ significantly according to *KRAS* and *BRAF* status.

Causal Modeling of CIMP and Locus-Specific CpG Island Methylation by Structural Equation Modeling (SEM)

Based on the results of the previous studies^{3-5,10,11,17,18} and our clustering analysis, we constructed causal models as represented in Figure 2, A-C. Two latent variables ("CIMP-high" and "CIMP-low," or one latent variable "CIMP-high" in Group 3) were adopted to explain the relationship between CIMP, *MLH1*, and MSI. These causal models were also based on the facts that *KRAS* and *BRAF* mutations, and CpG island methylation occur early in colorectal carcinogenic process whereas MSI occurs relatively late,³²⁻³⁵ and the assumption that the latent "CIMP-high" or "CIMP-low" status in colonic cells influences methylation in locus-specific CpG islands including *MLH1*. Grouping tumors by *KRAS* and *BRAF* mutational status seemed reasonable, given a recent study that has shown clinically significant level of CpG

island methylation appears to be later than *BRAF* mutation events.³⁶

Figure 2, A-C shows only 19 or 18 variables of interest so that the results are easier to read; other clinical and pathological variables (sex, age, family history of colorectal cancer, stage, tumor location, and DNMT3B status) were also included in the models to adjust path coefficients. In each tumor group, we recategorized the 15 CpG islands to a latent class (CIMP-high or CIMP-low) according to the size of the path coefficient between CIMP status and each of the 15 markers, which corresponded to the factor loading. There was only one latent CIMP class in Group 3, in agreement with the results by clustering and PCA. Our models exhibited a good fit for tumor Group 1 and Group 2 (GFI = 0.94, AGFI = 0.90, 0.91 and RMSE = 0.04); for Group 3, the fit indices showed lower values (GFI = 0.8, AGFI = 0.73 and RMSE = 0.07) due to the smaller number of model parameters. RMSE for Group 3 was relatively larger than that for other groups; however, it was shown that the model would fit the data modestly. We observed that this categorization of the 15 CpG islands in each tumor group almost matched with the results by the cluster analysis. This implied that the indicator of CIMP status defined by the observed 8 CIMP markers would reflect the latent CIMP status. The χ^2 difference tests for the multiple group analysis indicated that there was a significant difference in the factor loadings between the tumor groups ($P < 0.000001$).

This SEM analysis revealed an interesting potential pathogenetic relationship. Specifically, in *BRAF*-wild-type tumors (Group 1 and Group 2), there appeared to be a significant relationship ($P < 0.02$) between CIMP-high and MSI which was not mediated through *MLH1* methylation. Besides MSI caused by *MLH1* methylation in *BRAF*-mutated CIMP-high tumors (Figure 2C), MSI has been known to be caused by different mechanisms in different tumors. Hereditary nonpolyposis colorectal cancer (HNPCC) due to a germline mutation plus a second hit in a mismatch repair gene has been implicated in approximately 2% of colorectal cancers in the general population.³⁷ Certainly, HNPCC could not explain the majority of colorectal cancers in Group 1 and Group 2. Additional studies are necessary to elucidate mechanisms of the potential link between CIMP-high and MSI in *BRAF*-wild-type tumors.

In agreement with the results of the cluster analysis and PCA, correlation structures of CIMP status and locus-specific CpG island methylation by SEM differed between Group 1, Group 2 and Group 3. Thus, colorectal preneoplastic or neoplastic cells might have had differential propensity of CpG island methylation in a locus-specific manner, according to *KRAS* and *BRAF* mutational status.

Discussion

This study represents the first large-scale investigation that used causal modeling to assess the complex inter-relationship between *KRAS* and *BRAF* mutations, the CpG island methylator phenotype (CIMP) and locus-specific CpG island methylation. In particular, an application of structure equation modeling (SEM) to correlation struc-

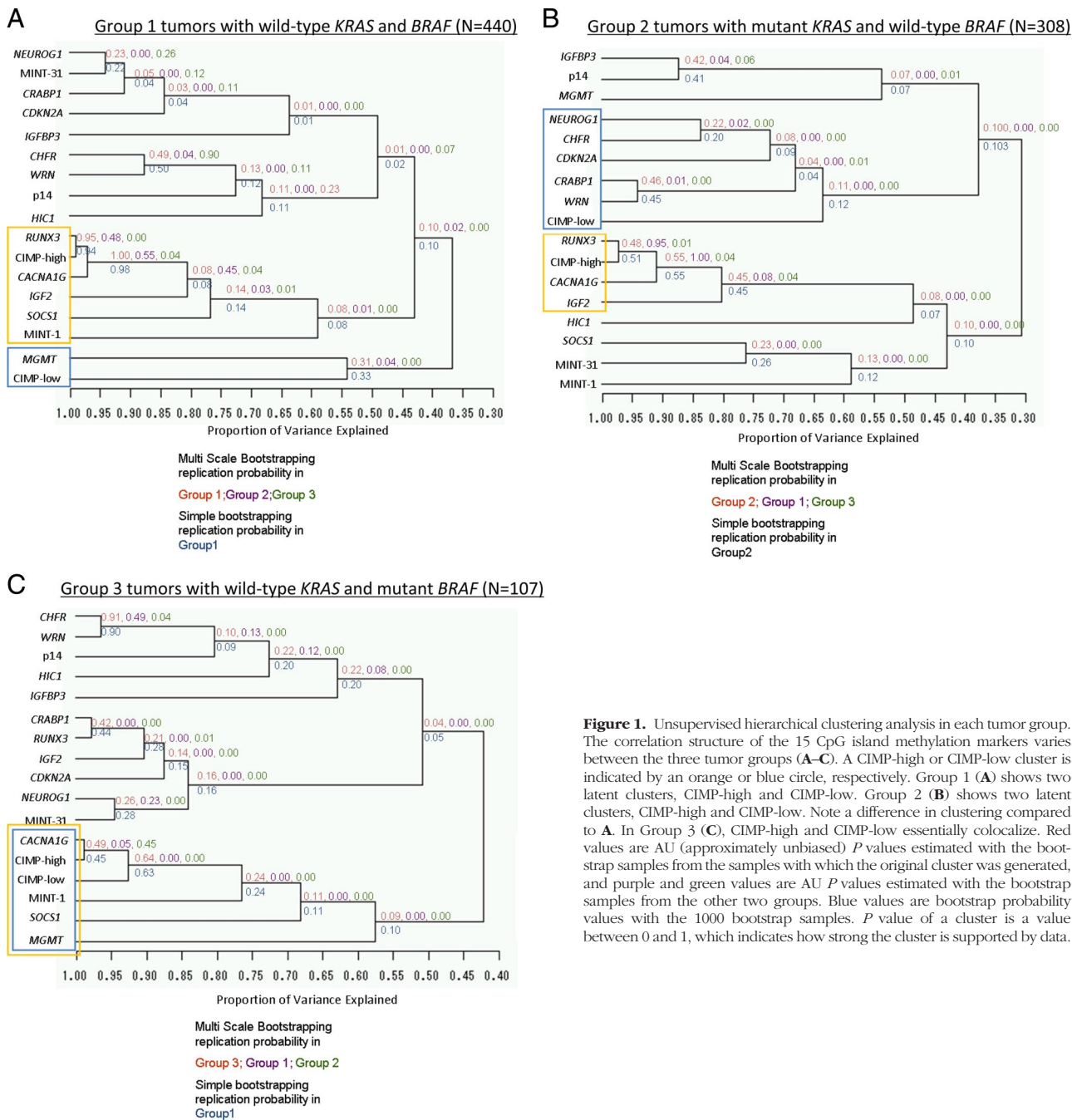


Figure 1. Unsupervised hierarchical clustering analysis in each tumor group. The correlation structure of the 15 CpG island methylation markers varies between the three tumor groups (A–C). A CIMP-high or CIMP-low cluster is indicated by an orange or blue circle, respectively. Group 1 (A) shows two latent clusters, CIMP-high and CIMP-low. Group 2 (B) shows two latent clusters, CIMP-high and CIMP-low. Note a difference in clustering compared to A. In Group 3 (C), CIMP-high and CIMP-low essentially colocalize. Red values are AU (approximately unbiased) *P* values estimated with the bootstrap samples from the samples with which the original cluster was generated, and purple and green values are AU *P* values estimated with the bootstrap samples from the other two groups. Blue values are bootstrap probability values with the 1000 bootstrap samples. *P* value of a cluster is a value between 0 and 1, which indicates how strong the cluster is supported by data.

ture analysis of CpG island methylation is quite novel. We examined the database of 855 colorectal cancers by cluster analysis, principal component analysis (PCA), and SEM, a combination of techniques that has not been used in any of the previous studies on CIMP.^{38–66} Our sample size was large enough to perform SEM analysis using a number of variables, while adjusting for potential confounding by clinical and pathological variables including DNMT3B expression. The 16 methylation markers included the five markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*) that were selected from 195 CpG islands³ and validated as the CIMP-high surrogate markers.⁴

We have shown that the interrelationship between CIMP and locus-specific methylation at the 16 CpG islands substantially differ according to background of *KRAS* and *BRAF* mutational status. Our data suggest a possible role of *KRAS* and *BRAF* mutations in differentially modifying a propensity of CpG island methylation in a locus-specific manner. Although unlikely given the high statistical significance, there is a possibility that the observed differential associations might have occurred by chance. Because our current analysis is exploratory by nature, a validation by independent dataset is necessary.

Table 3. Results of the Cluster Analysis of the 15 CpG Island Methylation Markers (H, Clustered with CIMP-High Status; L, Clustered with CIMP-Low Status)

CpG island methylation marker	Group 1 (Wild-type <i>KRAS</i> / <i>BRAF</i>)	Group 2 (Mutant <i>KRAS</i> , wild-type <i>BRAF</i>)	Group 3 (Wild-type <i>KRAS</i> , mutant <i>BRAF</i>)
<i>CACNA1G</i>	H	H	H, L
<i>CDKN2A</i> (p16)		L	
<i>CHFR</i>		L	
<i>CRABP1</i>		L	
<i>HIC1</i>		H	
<i>IGF2</i>	H	H	
<i>IGFBP3</i>			
<i>MGMT</i>	L		H, L
<i>MINT-1</i>	H	H	H, L
<i>MINT-31</i>		H	
<i>NEUROG1</i>		L	
p14 (<i>CDKN2A</i> / <i>ARF</i>)			
<i>RUNX3</i>	H	H	
<i>SOCS1</i>	H	H	H, L
<i>WRN</i>		L	

In this clustering analysis, splitting criterion was specified as the eigenvalues of all clusters being greater than one. Blank indicates no clustering with CIMP-high or CIMP-low.

CIMP, CpG island methylator phenotype.

In SEM, we assumed certain causal relations. It has been well-known that *MLH1* methylation causes most sporadic MSI-high colorectal cancers.³⁻⁵ In addition, we could assume that the “latent CIMP-high or CIMP-low status” might cause methylation in locus-specific CpG islands including *MLH1*. MSI has been known to occur relatively late in carcinogenic process, in contrast to *KRAS* and *BRAF* mutations and CpG island methylation; all of the latter changes could be seen in early adenomas and polyps.³²⁻³⁵ It is also reasonable to classify tumors by *KRAS* and *BRAF* mutational status before clustering, PCA and SEM analyses. A recent study has shown clinically significant level of CpG island methylation appears to be later than *BRAF* mutation events.³⁶ Thus, in carcinogenic process, *KRAS* and *BRAF* mutations appear to occur early, followed by CpG island methylation, and then followed by MSI. Thus, our temporal assumptions and models appear to be appropriate.

A comprehensive examination of genetic and epigenetic alterations is important in cancer research. The relations between CIMP, MSI, and mutations in *KRAS* and *BRAF* have been evaluated in a number of previous studies.^{3-5,10,11,18,38,39} Thus far, cluster analyses have been used to evaluate CpG island methylation markers,^{3,4,10,21,54,65,66} and multivariate logistic regression analyses have been used to assess the independent relationship of CIMP with each of clinical, pathological, and other molecular features, using a large number of tumors.^{4,5} Nonetheless, no previous study has used PCA or SEM, and our current study is the first to demonstrate differential correlation structures of locus-specific CpG island methylation according to *KRAS* and *BRAF* mutational status.

Whether there is a causal relationship between mutations in *KRAS* and *BRAF* and CIMP remains enigmatic and has been a subject of active investigations. In particular, the relationship between *BRAF* mutation and CIMP-high,^{3-5,10,11} and that between *KRAS* mutation and CIMP-low^{11,17,18} (CIMP2¹⁰ or IME²¹) have recently drawn much attention. It is speculated that *KRAS* and *BRAF* mu-

tations may contribute to CpG island methylation in colorectal cancer.³⁹ Although literature data are currently scant, there has been experimental evidence that suggests contribution of *KRAS* or *BRAF* mutation to locus-specific CpG island methylation.^{22,23} In one study using colon epithelial cell line NCM460, a transfection of mutant *BRAF* appears to induce *MLH1* methylation,²³ and in another study using NIH 3T3 cells, transformation by activated RAS caused DNA methylation at specific CpG islands.²² Consistent with these previous findings,^{22,23} we have shown that the correlation structures of CIMP status and CpG island methylation markers differ substantially by *KRAS* and *BRAF* mutational status. As noted above, the specific differences could represent a chance variation, but the overall correlation structure is reproducibly modified as *KRAS*/*BRAF* status varies. It is thus plausible that *KRAS* and *BRAF* mutational status differentially influence the propensity of CpG island methylation in a locus-specific manner.

The relationship between *WRN* methylation and *KRAS* mutation warrants discussion. It has been shown that *KRAS* mutation transgenic mouse develop senescent lung adenomas which display classic senescence markers including up-regulation of *CDKN2A* (p16), and that progression to adenocarcinoma appears to require premalignant cells to bypass senescence.⁶⁷ *WRN* helicase is important in maintenance of proper telomere function, and epigenetic silencing of *WRN* may be one of mechanisms to bypass senescence in colorectal neoplasia.

In summary, in our large database of colorectal cancers, the correlation structures of the 16 methylation markers and CIMP status differed significantly according to *KRAS* and *BRAF* mutational status. Therefore, our data support a possible role of *KRAS* and *BRAF* status in differentially modifying cellular propensity for locus-specific CpG island methylation. Further studies are necessary to elucidate a mechanistic link between *KRAS* or *BRAF* mutation, CIMP, and locus-specific CpG island methylation in colorectal cancer.

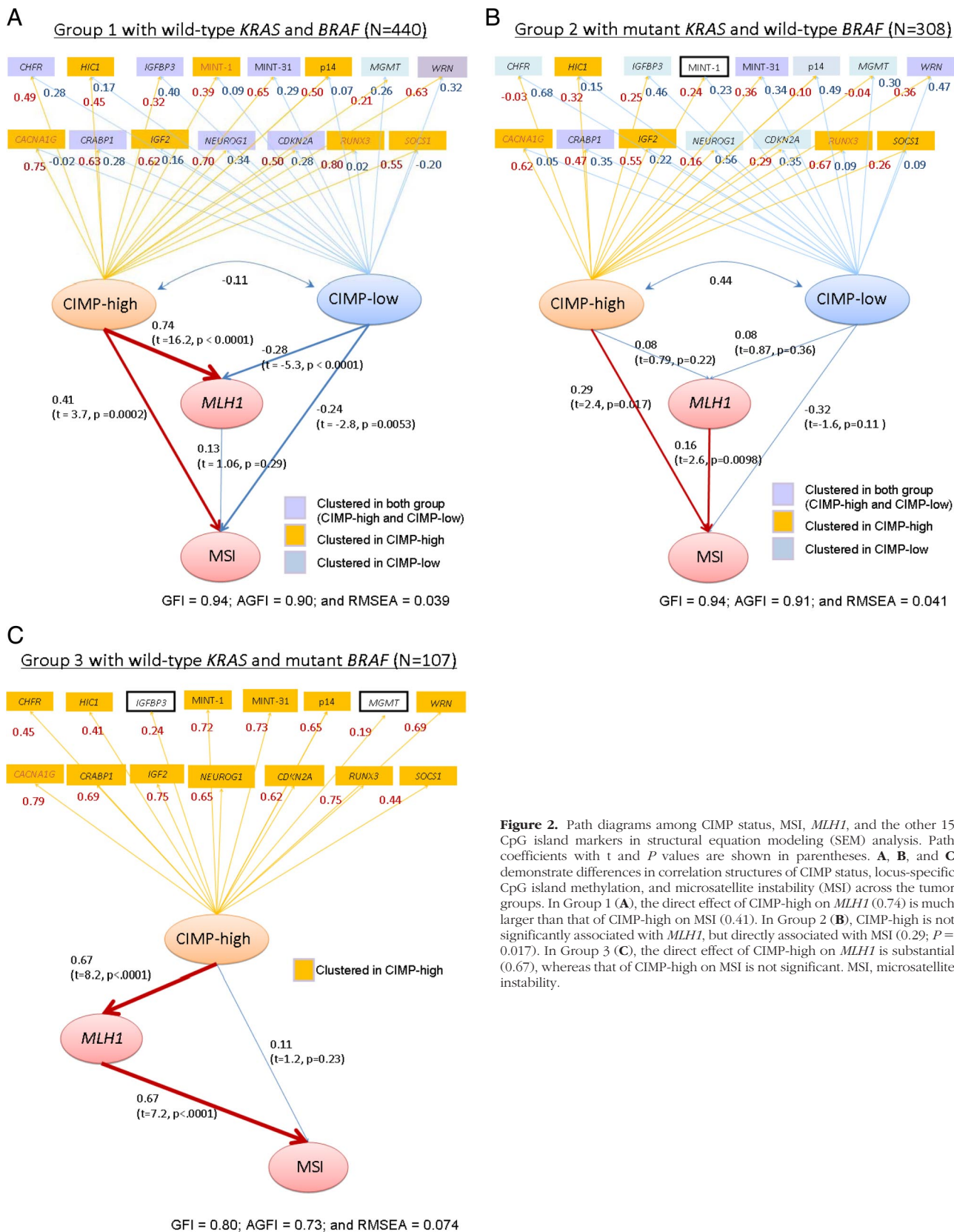


Figure 2. Path diagrams among CIMP status, MSI, *MLH1*, and the other 15 CpG island markers in structural equation modeling (SEM) analysis. Path coefficients with t and P values are shown in parentheses. **A**, **B**, and **C** demonstrate differences in correlation structures of CIMP status, locus-specific CpG island methylation, and microsatellite instability (MSI) across the tumor groups. In Group 1 (**A**), the direct effect of CIMP-high on *MLH1* (0.74) is much larger than that of CIMP-high on *MSI* (0.41). In Group 2 (**B**), CIMP-high is not significantly associated with *MLH1*, but directly associated with *MSI* (0.29; $P = 0.017$). In Group 3 (**C**), the direct effect of CIMP-high on *MLH1* is substantial (0.67), whereas that of CIMP-high on *MSI* is not significant. MSI, microsatellite instability.

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