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Aberrant DNA methylation patterns in microsatellite stable human colorectal cancers define a new marker panel for the CpG island methylator phenotype

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Abstract

A distinct group of colorectal carcinomas (CRCs) referred to as the "CpG island methylator phenotype" (CIMP) shows an extremely high incidence of de novo DNA methylation and may share common pathological, clinical or molecular features. However, there is limited consensus about which CpG islands (CGIs) define a CIMP, particularly in microsatellite stable (MSS) carcinomas. To study this phenotype in a systematic manner, we analyzed genome-wide CGI DNA methylation profiles of 19 MSS CRC using methyl-CpG immunoprecipitation (MCIp) and hybridization on 244K CGI oligonucleotide microarrays, determined KRAS and BRAF mutation status and compared disease-related DNA methylation changes to chromosomal instability as detected by microarray-based comparative genomic hybridization. Results were validated using mass spectrometry analysis of bisulfite-converted DNA at a subset of 76 individual CGIs in 120 CRC and 43 matched normal tissue samples. Both genome-wide profiling and CpG methylation fine mapping segregated a group of CRC showing pronounced and frequent de novo DNA methylation of a distinct group of CGIs that only partially overlapped with previously established classifiers. The CIMP group defined in our study revealed significant association with colon localization, either KRAS or BRAF mutation, and mostly minor chromosomal losses but no association with known histopathological features. Our data provide a basis for defining novel marker panels that may enable a more reliable classification of CIMP in all CRCs, independently of the MS status.

Abbreviations: CGI, CpG island; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CNV, copy number variation; CRC, colorectal carcinoma; ES cells, embryonic stem cells; GO, gene ontology; MCIp, methyl-CpG immunoprecipitation; MSI, microsatellite instable; MSS, microsatellite stable.

Claudia Gebhard and Roger Mulet-Lazaro contributed equally to this work.

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KEYWORDS
colorectal cancer, DNA methylation, epigenetics, gene regulation, neoplasia

What's new?

The CpG island methylator phenotype (CIMP) is an important feature of colorectal carcinoma (CRC). No consensus exists, however, on a marker panel that defines CIMP in CRC, neither for microsatellite stable (MSS) nor for microsatellite instable disease. Here, to better understand associations between markers and CIMP CRC, the authors analyzed genome-wide CpG island DNA methylation profiles in CRC. Genome-wide profiling and CpG methylation fine mapping revealed frequent *de novo* DNA methylation of a group of CpG islands. The CIMP group, the basis for a novel marker panel, was associated with colon localization, *KRAS* and *BRAF* mutation, and minor chromosomal losses.

1 | INTRODUCTION

Carcinogenesis is generally associated with both genetic and epigenetic alterations that likely act in concert to drive disease initiation and progression. Epigenetic lesions often include an aberrant redistribution of DNA methylation, resulting in global hypomethylation and a regional hypermethylation in CpG islands (CGIs). Whereas the former is thought to favor chromosomal instability (CIN) and the inappropriate activation of oncogenes, the latter may lead to the permanent silencing of tumor-suppressor genes.[1] Certain tumors show an exceptionally high incidence of CGI de novo DNA methylation (mCGI) and may constitute distinct subclasses sharing common pathological, clinical or molecular features. This so-called "CpG island methylator" phenotype (CIMP) was first discovered in colorectal carcinomas (CRCs) as a class of tumors showing frequent mCGI of several promoters and included the majority of sporadic CRC with microsatellite instability (MSI) related to *hMLH1* methylation.[2] A refined definition of CIMP marker regions based on a larger candidate gene panel revealed the tight association of MSI CIMP with activating mutations in the BRAF oncogene.[3] This panel of five markers was further extended to an eight-marker panel, which allowed for comprehensive studies of CIMP.[4] However, the existence and clinical relevance of the CIMP status have been a constant matter of debate,[5] and the use of different methods and variable marker panels to define CIMP has led to markedly divergent estimates of CIMP frequency and its association with other genetic markers.[6] Notably, there is largely conflicting data about the existence of CIMP in sporadic, microsatellite stable (MSS) CRC, which are frequently associated with KRAS rather than BRAF mutations.[7] The selection of currently used CIMP markers has primarily been based on candidate gene approaches and MSI CRC[8] questioning the adequacy of CIMP to describe MSS CRC. Some studies actually provided evidence supporting different hypermethylation profiles in genetically different CRCs,[9] suggesting that MSS CRC may have a unique hypermethylation signature that is poorly represented in current marker panels.[9] Due to this lack of consensus, a precise panel of marker genes allowing a standardized identification of CIMP in all groups of CRC remains to be defined.

Here, we performed a global analysis of aberrant DNA methylation across the large majority of human CGI for 19 sporadic MSS CRC, identified a segregate subgroup of CIMP-positive MSS CRC and defined a new candidate marker panel that can classify all CIMP CRCs.

2 | MATERIALS AND METHODS

2.1 | Sample collection and processing

CRC clinical sample collection and staging, and processing of clinical samples and control cells are described in the Supporting information.

2.2 | Sample analyses

Microsatellite analysis, KRAS and BRAF mutation analysis, methyl-CpG immunoprecipitation (MCIp) microarray, mass spectrometry analysis of bisulfite-converted DNA (using the MassARRAY system) and microarray-based comparative genomic hybridization (aCGH) were performed as described in the Supporting Information.

2.3 | Statistics

All statistical testing of enrichment data (motifs or attributes) was performed using a cumulative hypergeometric distribution (Fisher exact test). Statistical testing of differences in CpG content, mRNA level and H3K27me3 distributions was done using the two-sided Mann-Whitney *U* test. Differences were considered statistically significant when *P* values were $< .05$. Correlation between clinicopathological and molecular parameters was tested using a two-sided Fisher's exact test.

3 | RESULTS AND DISCUSSION

3.1 | Unsupervised clustering of genome-wide DNA methylation data segregates CIMP CRC samples

To globally characterize aberrant de novo DNA methylation in CRC, we analyzed the methylation status of 23 000 CGIs (mCGIs) of the human genome in 19 MSS CRCs samples (compared to normal aged colon), using MCIp coupled with microarray analysis.[10] A previously described set of 10 comparative MCIp methylation profiles[11] was extended by nine additional MSS CRC samples, and KRAS and BRAF mutational status were determined (Tables S1 and S2).

Individual probe signals were combined to assign methylation ratios (sample MCIp/reference MCIp) to whole CGI regions.[11] As a normal control (reference), we used colon from three healthy aged donors (median age 59). Since we were unable to obtain DNA from colon of healthy young donors, we could not directly study the influence of age on DNA methylation. Hence, we also included monocyte DNA of younger origin (median age 23) in our comparison. Compared to monocytes, a large set of CGI (~1400) was preferentially methylated in aged colon, whereas only a small set of genes (~100) showed tissue-specific methylation in blood monocytes. Since colon-specific methylation targets comprised many CGI that were previously shown to be de novo methylated during aging,[12] and the majority of these CGI were even more strongly methylated in several CRC and also frequently de novo methylated in primary acute leukemia samples (Figure S1), we concluded that most of these regions ("age" group) are prone to age-related de novo methylation in normal colon.

A hierarchical two-dimensional unsupervised clustering of hypermethylation data was conducted to identify distinct subsets of methylation behavior (Figure 1A). The initial set of 23 000 CGI was reduced to CGI regions on autosomes (to exclude sex bias). Around 5000 CGI regions showed a clear MCIp enrichment in at least three independent samples. Five out of eight KRAS-mutated CRC clearly emerged

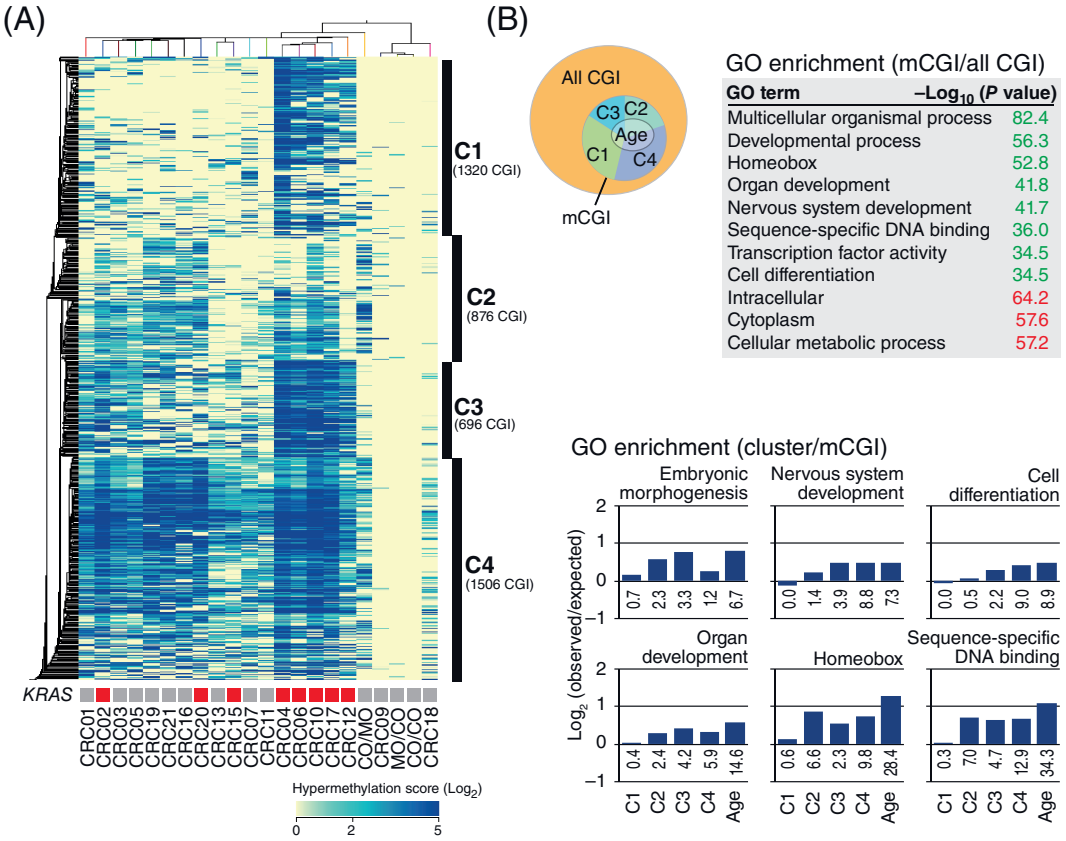

FIGURE 1 Global DNA hypermethylation data segregates CRC samples. (A) Comparative MCIp data for CpG island (CGI) regions clearly hypermethylated (mCGI) in at least three independent samples were used for the hierarchical two-dimensional unsupervised clustering (here: Manhattan distance and Ward's linkage rule). All tested clustering approaches separated a group of five CRC samples (CRC4, 6, 10, 12 and 17) that were characterized by high levels of de novo DNA methylation, likely representing the CGI methylator phenotype (CIMP). As indicated below the cluster diagram, all five samples were characterized by the presence of a KRAS mutation (red boxes). Based on the potentially age-related origin of de novo methylation (as indicated by the hypermethylation data for normal colon), CGI regions were divided into five groups (C1-4 as indicated and the age group). (B) Gene ontology analysis reveals strong associations of the mCGI group with developmental processes as compared to all CGI regions (all CGI). $-\text{Log}_{10}$ *P* values (hypergeometric) for the enrichment/depletion of the indicated terms are shown (green or red coloring of *P* values indicates enrichment or depletion, respectively). Separate analysis of individual subgroups (C1-C4, "age") reveals different compositions with the C1 group being least associated with developmental terms. Bars indicate ratios of observed vs expected term distributions, and the numbers represent $-\text{Log}_{10}$ *P* values (hypergeometric) for the enrichment of each term

as a separate subgroup of MSS CRC, comprising samples with the highest frequency of de novo DNA methylation. This group was consistently segregated using different hierarchical clustering approaches (Manhattan-Ward's, Pearson centered-average and Euclidean-centroid) and likely constitutes CIMP in MSS CRC (MSS-CIMP). The clustering analysis also revealed four major groups of CGI (C1-C4) that showed variable specificity, with C1 and C3 being least associated with the "age" group and highly associated with the MSS-CIMP group. In general, aberrant de novo methylation was a common feature of CRC.

3.2 | Aberrant silencing in CIMP CRC is mediated by age-independent mechanisms

As observed in previous studies, genes associated with mCGI were generally enriched for gene ontology (GO) terms related to developmental processes (Figure 1B). Separate GO term analyses of the four major CGI clusters (C1-C4, Figure 1A) as well as putative age-related de novo methylation targets ("age" group) revealed significant differences between these groups. The "age" group was most strongly enriched for development-related attributes, whereas the MSS-CIMP-specific C1 cluster showed the least association (Figure 1B). Among the five groups, C1 and age also differed significantly in their CpG content, mRNA expression and ES cell H3K27 trimethylation but not so much in their CGI size (Figure S2A-D). The "age" group had the lowest median CpG content (Figure S2A), and neighboring genes were rarely expressed in normal colon (Figure S2C). In addition, "age" group of CGI had the highest association with the Polycomb group (PcG) repressor mark H3K27me3 in ES cells (Figure S2D), in line with previous studies.[13] In contrast, the MSS-CIMP-specific C1 cluster showed average CpG content, higher mRNA expression in normal colon and lower median H3K27me3 in ES cells (Figure S2A-D). In addition, the C1 group comprised more promoter regions (Figure S2E) and showed a higher evolutionary conservation compared to the age group (Figure S2F). De novo motif analyses showed that the age group was depleted for five previously identified ubiquitous transcription factor motifs associated with DNA methylation protection (Sp1, NRF1, YY1, GFY and GABP) and enriched for several cell type-specific consensus motifs (Figure S3), suggesting that the "age" group is particularly enriched for developmental and differentiation-associated genes that are usually not expressed in colon. In contrast, the MSS-CIMP-specific C1 cluster showed only little association with sequence motifs (Figure S3). These differences suggest separate (yet unknown) mechanistic pathways for the aberrant silencing of MSS-CIMP-specific C1 cluster genes compared to the age-dependent methylation pathway.

3.3 | CIMP CRCs have fewer chromosomal losses

CRC is often characterized by CIN. Previous work suggested that CIMP and CIN may represent alternative routes for cancer development,[14] although a considerable overlap between CIMP and CIN has also been shown.[15] Since disease-related de novo methylation often causes gene repression that may complement chromosomal losses or mutations, we related DNA methylation profiles with chromosomal changes in CRC samples. Unbalanced chromosomal changes were analyzed by aCGH (Figure S4 and Table S3). The global distribution of copy number variation (CNV) in our CRC was comparable to that of previous studies.[16] The relation of CIMP and KRAS status with losses at commonly deleted chromosomal regions (8p23-p12, 15q11-q15, 17p13-p11 and 18) suggests an association of both KRAS mutation and CIMP with the appearance of fewer chromosomal losses.

3.4 | CGI methylation compensates for copy number variation in CIMP CRCs

Since DNA methylation may participate in compensating chromosomal gains,[17] we globally related the distribution of MCIp enrichment with copy number alterations. As a prime example for dosage compensation, the distribution of methylation (MCIp signal) ratios in male and female X-chromosomes was compared to that observed across all chromosomes of an individual (Figure S5A). In female CRC samples, dosage compensation at one X chromosome resulted in a marked shift of methylation ratios compared to all chromosomes. In contrast, the gain of X chromosomes in male CRC only induced a slight increase in overall DNA methylation, likely reflecting the copy number gain relative to the male reference sample. Similar observations were made at autosomes with unbalanced chromosomal changes; in individual samples with stable diploid chromosome 8, shown as representative example, the distribution of methylation ratios was similar to that of all other chromosomes, whereas samples with chromosome 8p deletions and 8q amplifications showed a corresponding loss or gain of methylation in the respective regions relative to all other chromosomes (Figure S5B). Both methylation gains and losses roughly corresponded to relative copy number changes, suggesting that the mechanisms responsible for aberrant DNA methylation in individual samples are largely independent of chromosome copy numbers.

3.5 | A new marker panel identifies CIMP CRCs better than previous known markers

To validate our microarray analysis and to test potential marker regions using an independent approach, we selected a set of 83 regions for mass spectrometry analysis of bisulfite-converted DNA (MassARRAY) from CRC samples and matched normal colon tissue. The set comprised a number of previous marker regions (literature set), representative examples of C2, C3, C4 and age group (validation set) as well as a panel of MSS-CIMP-specific C1 cluster regions (novel CIMP candidate set). Seven regions were excluded due to poor quality spectra. For the remaining 76 amplicons, mean methylation ratios of individual regions obtained by the MassARRAY and MCIp microarray approaches were highly concordant (median Pearson coefficient of .81). To validate previous and novel candidate markers for CIMP, we analyzed an additional set of CRCs, so that we had in total a number of 120 CRC (102 MSS CRCs, 10 MSI CRCs and the MS status of 8 patients was not available) together with 43 matched normal colon tissues and 11 samples of healthy human blood monocytes.

Unsupervised hierarchical clustering for the 76 selected regions (Figure 2) revealed with few exceptions that the validation set (labeled in black) clearly segregated normal tissues (monocytes and colon) and cancer samples. Most of these regions (except the top rows in the cluster analysis which represent regions with colon-specific DNA methylation) include potential biomarkers for cancer, showing low levels or absent DNA methylation in all control samples and a high penetrance of aberrant methylation frequency in CRC.

The newly defined marker regions that constitute our novel CIMP candidate panel (labeled in red) clearly segregated the CIMP samples, including those five samples previously detected by the comparative MCIp approach. This novel candidate marker panel enabled a better distinction than established CIMP markers from the literature (labeled in green) (Figure S6).

3.6 | CIMP CRCs are associated with KRAS or BRAF mutations and colon localization

The descriptive analyses of CIMP, clinical and histopathological parameters revealed a CIMP frequency of 19.2% (23/120) in our whole CRC cohort. The presence of CIMP was significantly associated with tumor localization (preferentially in the left and right sided colon; $P = .0015$) and either KRAS or BRAF V600E mutation status ($P < .001$), but not with age, sex, histologic grade, lymph node metastasis, lymphatic or venous invasion (Table 1). The observation that CIMP in our cohort of CRC correlated with either KRAS or BRAF mutation, which belong to one same pathway, suggests that this genetic defect is directly linked to the yet unknown mechanisms responsible for CIMP aberrant DNA methylation profile. Global profiling studies as well as studies focusing on defined CIMP marker panels have identified similar genotype/epigenotype relationships.[18-22]

Although our new CIMP marker panel was defined based on MSS CRC, all BRAF-mutated MSI CRC were also detected as hypermethylated. Thus, our new marker panel did not only segregate hypermethylated MSS CRC (13/23) but also hypermethylated MSI CRC (8/23). Our data, therefore, suggest that traditional markers may underestimate the frequency of CIMP and that our novel candidate marker panel could help to reach a consensus on how to score CIMP in all subgroups of CRC. Accordingly, previous studies have also reported sets of hypermethylated genes in both MSS and MSI CRC cases.[23]

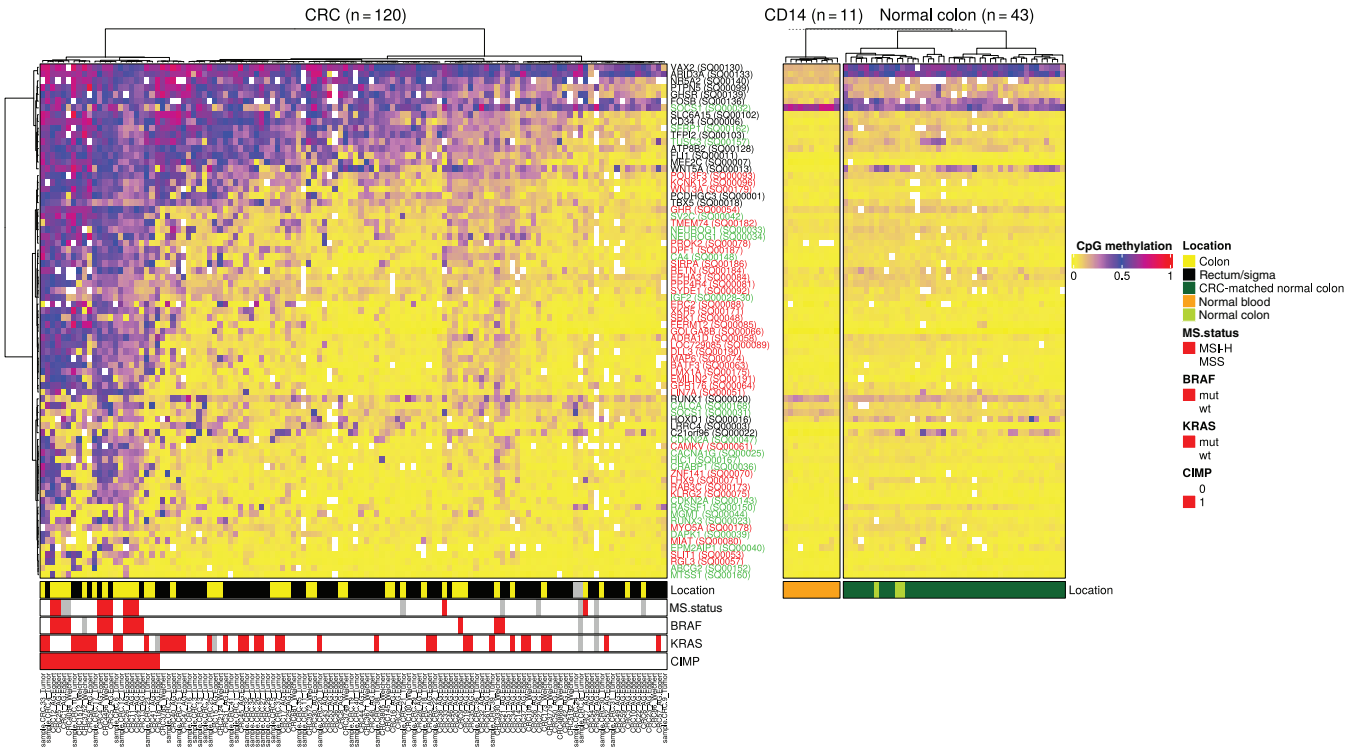

FIGURE 2 Hierarchical clustering of regional DNA methylation data obtained by MALDI-TOF MS. CGI regions selected for validation purposes from the C2, C3, C4 and age group (labeled in black), candidates for a refined CIMP panel (C1 cluster regions, labeled in red) as well as previously defined CIMP markers (labeled in green) were analyzed using bisulfite conversion and subsequent MALDI-TOF MS in 120 CRC samples and 54 normal controls (including 40 matched normal colon tissues, 3 normal colon samples from healthy donors and 11 samples of human blood monocytes). Mean methylation ratios were subjected to hierarchical two-dimensional unsupervised clustering (Manhattan distance and Ward's linkage rule). DNA methylation values are depicted by a color scale as indicated (methylation ranges from yellow [0 = non-methylated] to red [1 = fully methylated]). Sample characteristics including KRAS, BRAF and MSI/MSS status as well as tissue type are represented in color-coded rows below the diagrams (red: mutated; gray: not determined)

TABLE 1 Clinicopathological and molecular characteristics of MSS CRC in relation to CIMP

Group	Negative	Positive	*P*	Test statistic	Effect size (CI)
Observations	97	23			
Age					
Median (MAD)	66.00 (11.86)	71.00 (7.41)	.13	0.27	−0.37 (−0.83; 0.097)
Mean (SD)	64.79% (11.66)	68.87% (8.64)			
Range	37-90	52-87			
Missing	1.0% (1)	0.0% (0)			
Tumor localization					
Colon	28% (27)	65% (15)	**.0015**	9.4	0.21 (0.07; 0.61)
Rectum/sigma	70% (68)	35% (8)			
Missing	2.1% (2)	0% (0)			
Gender					
F	31% (30)	43% (10)	.33	0.76	0.59 (0.21; 1.7)
M	68% (66)	57% (13)			
Missing	1% (1)	0% (0)			
Lymphnode.metastases					
pN0	59% (57)	74% (17)	.24	1.2	0.51 (0.15; 1.5)
pN1-2	41% (40)	26% (6)			
Missing	0% (0)	0% (0)			
Lymphatic.invasion					
L0	14% (14)	22% (5)	.43	0.25	0.44 (0.036; 3.3)
L1	13% (13)	8.7% (2)			
Missing	72% (70)	70% (16)			
Venous.invasion					
V0	22% (21)	30% (7)	.3	0.9	0 (0; 2.8)
V1	7.2% (7)	0% (0)			
Missing	71% (69)	70% (16)			
Tumor stage					
pT2	14% (14)	4.3% (1)	.3	1	3.9 (0.53; 172)
pT3-5	81% (79)	96% (22)			
Missing	4.1% (4)	0% (0)			
KRAS					
Mut	28% (27)	43% (10)	.14	1.6	0.49 (0.17; 1.4)
Wt	69% (67)	52% (12)			
Missing	3.1% (3)	4.3% (1)			
BRAF					
Mut	3.1% (3)	48% (11)	**<.001**	33	0.034 (0.0053; 0.16)
Wt	95% (92)	48% (11)			
Missing	2.1% (2)	4.3% (1)			
MS.status					
MSI-H	2.1% (2)	35% (8)	**<.001**	23	0.038 (0.0036; 0.22)
MSS	92% (89)	57% (13)			
Missing	6.2% (6)	8.7% (2)			
BRAF/KRAS					
Mut	31% (30)	91% (21)	**<.001**	27	0.023 (5.4e−04; 0.16)
Wt	66% (64)	4.3% (1)			
Missing	3.1% (3)	4.3% (1)			

Notes: Values in bold correspond to statistically significant results (*P* value < 0.05).

3.7 | A 10-marker methylation panel effectively identifies all CIMP CRC patients

To facilitate clinical application of this panel, we selected a subset of 10 markers predicted to be the most informative by an ensemble of machine learning approaches (Table S6). Indeed, this reduced panel had the same discriminatory power as the 76 amplicons together (Figure 3A). To validate its applicability, we used this 10-marker panel to cluster Illumina 450K methylation array from 394 CRC patients in The Cancer Genome Atlas (TCGA) (READ and COAD projects). CIMP cases were defined by K-means clustering of the top 5000 most variable CpG sites (Figure S7). Our novel panel, consisting of only 10 regions, achieved a 95.1% sensitivity and 99.7% specificity in the classification of these CIMP cases (Figure 3B).

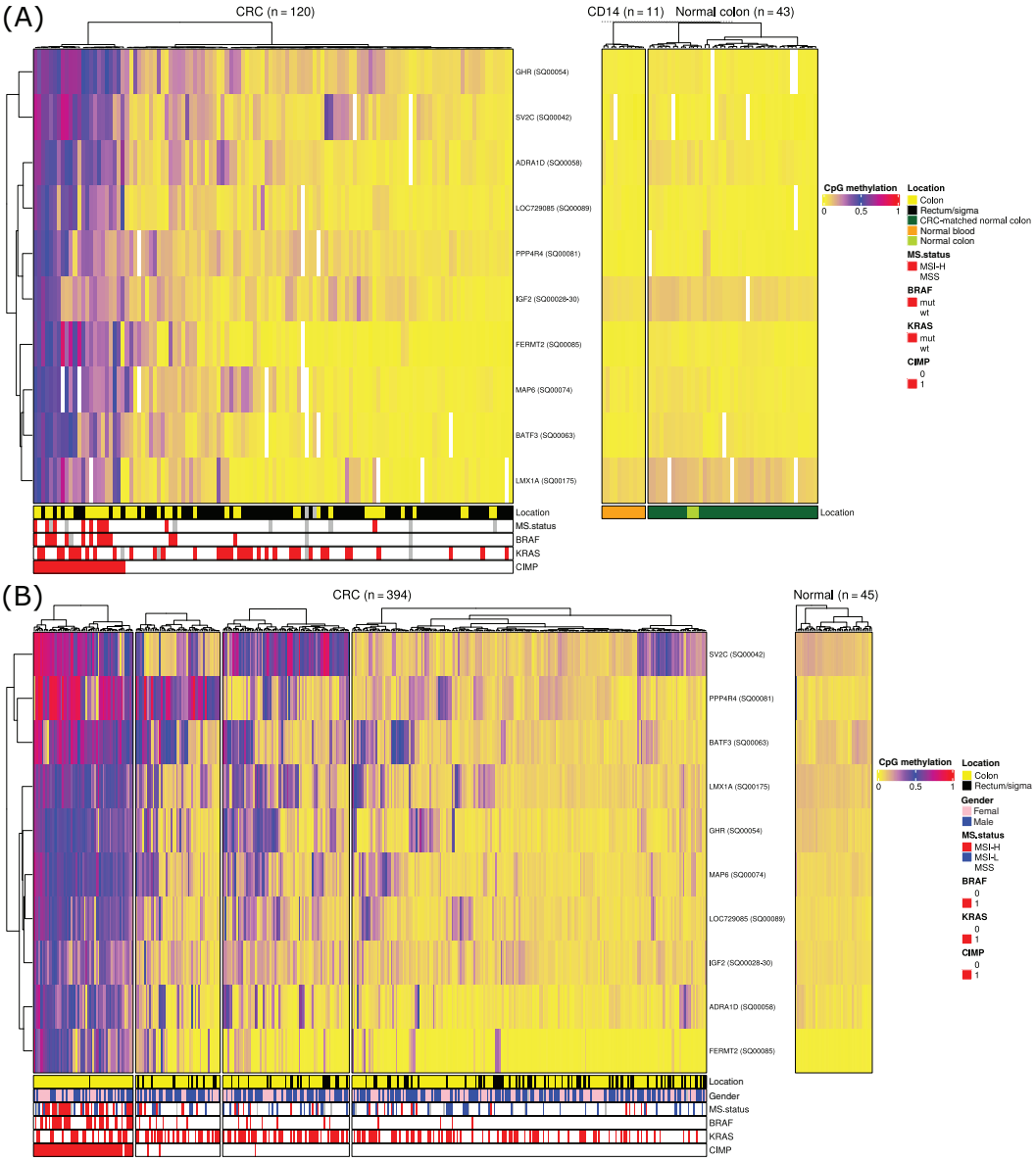

FIGURE 3 A 10-marker methylation panel effectively identifies CIMP patients in CRC. (A) Hierarchical clustering of in-house methylation data from 120 CRC patients and 54 normal samples. The 10 most informative CGI regions from the original set were selected by machine learning approaches. Mean methylation ratios of CRC patients were subjected to hierarchical two-dimensional unsupervised clustering (Manhattan distance and Ward's linkage rule). DNA methylation values are depicted by a color scale as indicated (methylation ranges from yellow [0 = non-methylated] to red [1 = fully methylated]). Sample characteristics are represented in color-coded rows below the diagrams (red: mutated; gray: not determined). CIMP cases clustered separately as shown for the entire panel of 76 amplicons, confirming that the reduced panel shows identical discriminatory power. (B) Hierarchical clustering of TCGA methylation data from 394 CRC patients and 45 matched normal colon samples. Individual CpG sites contained in the regions of the CIMP panel (10-marker panel) were binned, and the average methylation ratios were calculated. The resulting matrix was subjected to hierarchical two-dimensional unsupervised clustering as described in (A)

4 | CONCLUSIONS AND FUTURE PERSPECTIVES

It remains unclear, whether defects on the level of genotype or epigenotype co-evolve during clonal selection of cancer cells, or whether one is the consequence of the other. We and others showed that CGIs that are usually protected against de novo methylation are characterized by the combinatorial binding of transcription factors or RNA polymerase II, suggesting that disturbances of transcriptional networks frequently observed in cancer cells may have a profound impact on the cancer epigenome.[11,24] This is further supported by studies showing that genes involved in chromatin remodeling are prevalently mutated in CIMP CRC.[18,25] On the other hand, overexpression of BRAF mutant in a CIMP-negative and BRAF-wild-type colon carcinoma cell line failed to induce CIMP, suggesting that the signals provided by BRAF mutant may not be sufficient for the induction of CIMP.[26]

The exact mechanism underlying CIMP remains elusive.[15] Clearly, further studies are needed to clarify whether DNA hypermethylation events are a cause or a consequence of CRC development. Our new marker panel could provide a novel basis to define CIMP, regardless of the MSI/MSS status, and might aid future investigations to define the frequency and role of CIMP in CRC.

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CONFLICT OF INTEREST

Wilko Weichert has attended Advisory Boards and served as speaker for Roche, MSD, BMS, AstraZeneca, Pfizer, Merck, Lilly, Boehringer, Novartis, Takeda, Bayer, Amgen, Astellas, Eisai, Illumina, Siemens, Agilent, ADC, GSK and Molecular Health. Wilko Weichert receives research funding from Roche, MSD, BMS and AstraZeneca. All of this is outside of the submitted work. The other authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The microarray data presented in our study have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE25229. Other data that support the findings of our study are available from the corresponding authors upon request.

ETHICS STATEMENT

The study was approved by the local ethics committee of the University of Regensburg (Nr.05/003) and by the local ethic committee of the University Medical Center Göttingen (Nr.9/8/08) and by the local ethic committee of the University of Heidelberg (S206/05). A written informed consent was obtained.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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