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Research Article

Intermediate- and Low-Methylation Epigenotypes Do Not Correspond to CpG Island Methylator Phenotype (Low and -Zero) in Colorectal Cancer

Pawel Karpinski[1], Michael Walter[6], Elzbieta Szmida[1], David Ramsey[7], Blazej Misiak[1], Joanna Kozlowska[1], Marek Bebenek[4], Zygmunt Grzebieniak[3], Nikolaus Blin[1,5], Lukasz Laczmanski[2], and Maria M. Sasiadek[1]

Abstract

Background: Most recent genome-wide studies on the CpG island methylation in colorectal cancer (CRC) have led to the discovery of at least 3 distinct methylation clusters. However, there remains an uncertainty whether the CRC clusters identified in these studies represent compatible phenotypes.

Methods: We carried out comprehensive genome-scale DNA methylation profiling by Illumina Infinium HumanMethylation27 of 21 DNA pools that represent 84 CRC samples divided according to their high-, intermediate-, and low-methylation epigenotypes (HME, IME, and LME, respectively) and 70 normal-adjacent colonic tissues. We have also examined the relationship among 3 epigenotypes and chromosomal gains and deletions (assessed by Comparative Genomic Hybridization) in a group of 100 CRC samples.

Results: The HME subgroup showed features associated with CpG island methylator phenotype – high (CIMP-high) including methylation of specific CpG sites (CpGs) as well as significantly lower mean number of chromosomal imbalances when compared with other epigenotypes. The IME subgroup displayed the lowest number of methylated CpGs (717 vs. 2,399 and 2,679 in HME and LME, respectively) and highest mean number of chromosomal imbalances when compared with HME (P, 0.001) and LME (P, 0.004). A comparison between the methylation profiles of 3 epigenotypes revealed more similarities between the HME and LME (1,669 methylated CpGs overlapped) than HME and IME (673 methylated CpGs overlapped).

Conclusion: Our results provide evidence that IME and LME CRCs show opposite features to those that have been previously attributed to CIMP-low and CIMP-0 CRCs.

Impact: These discrepancies should be considered when interpreting the data from a particular epigenotyping method. *Cancer Epidemiol Biomarkers Prev; 22(2); 201–8. ©2012 AACR.*

Introduction

It is now well established that sporadic colorectal cancers (CRC) are characterized by significant heterogeneity with regard to global epigenomic status including CpG island methylation (1). The existence of a distinct epigenotype in CRC, referred to as CpG island methylator phenotype (CIMP), was reported for the first time in 1999 (2). Since then, a CIMP-specific marker panel has been developed (Weisenberger 1-panel method) and further extended (Ogino 1-panel method), which allowed for comprehensive studies of CIMP (3, 4).

CIMP-high tumors have been associated with distinct molecular and clinical characteristics including high CpG island methylation rates, older age, proximal tumor location, microsatellite instability (MSI), frequent *BRAF* V600E mutation, and extensive methylation of the 3p22 region (5, 6). Subsequent studies by Ogino and colleagues have shown the existence of tumors enriched for *KRAS* mutations that displayed less extensive methylation of CIMP-related markers (designated as CIMP-low; ref. 7). Finally, tumors with infrequent CIMP-related marker methylation (designated as CIMP-0) have been characterized by a frequent loss of heterozygosity at 18q and *TP53* mutations (8, 9).

Recent unsupervised hierarchical clustering of genome-wide selected marker panel (Yagi 2-panels method) has confirmed the existence of 3 distinct clusters in CRC referred to as the high-, intermediate-, and low-methylation epigenotypes (HME, IME, and LME, respectively) (10). However, it is not clearly established whether HME, IME, and LME are equivalent to CIMP-

Authors' Affiliations: Departments of [1]Genetics and [2]Endocrinology and Diabetology; [3]Second Department of General and Oncological Surgery, Wroclaw Medical University, Poland; [4]First Department of Surgical Oncology, Lower Silesian Oncology Center, Wroclaw, Poland; Divisions of [5]Molecular Genetics and [6]Medical Genetics, Institute of Human Genetics, University of Tuebingen, Tuebingen, Germany; and [7]Department of Mathematics and Statistics, University of Limerick, Limerick, Ireland

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Corresponding Author: Pawel Karpinski, Department of Genetics, Wroclaw Medical University, ul. Marcinkowskiego 1, 50-368 Wroclaw, Poland. Phone: 48-71-7841256; Fax: 48-71-7840063; E-mail: polemiraza@poczta.fm

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high, CIMP-low, and CIMP-0 (9, 11). Therefore, our study aimed at clarifying the issue of whether differences among these epigenotypes exist. Using genome-scale methylation profiling of 27,578 CpG sites (Illumina Infinium HumanMethylation27), we profiled pooled 84 CRC samples previously divided according to their HME/IME/LME epigenotypes (12). We further characterized chromosomal imbalances in 100 CRCs (also divided according to their epigenotype status) by means of comparative genomic hybridization (CGH). Finally, we related our findings to the very recent DNA methylation analysis of CIMP-high, CIMP-low, and non-CIMP tumors.

Our results show that IME CRCs show relatively low methylation frequency of CpG sites and a high mean number of chromosomal imbalances when compared with HME and LME CRCs. HME and LME subclasses display more related pattern of DNA methylation and level of chromosomal imbalances when compared with IME CRCs.

Materials and Methods

Human CRC tissue samples and human CRC cell lines

Surgically resected frozen tissues of 233 colorectal cancers and matched normal colon samples were obtained from the 2nd Department of General and Oncological Surgery, Wroclaw Medical University and the 1st Department of Surgical Oncology, Lower Silesian Oncology Center, Wroclaw. The CRC patient group consisted entirely of Polish individuals (all Caucasians). Only patients with primary, sporadic colorectal cancer who had not received preoperative therapy were included in the study. Informed consent was obtained from all the patients. The study was accepted by the Wroclaw Medical University Ethics Committee.

DLD1 and SW480 cell lines were obtained from the International Institute of Molecular and Cell Biology, Laboratory of Cell Biology (Warsaw, Poland). HTC116, HT29, CACO-2, and LOVO were obtained from the Institute of Human Genetics, Division of Molecular Genetics (Tübingen, Germany). The cell lines were authenticated by using the panel of 11 STRs (AmpFlSTR SGM Plus; Applied Biosystems). The number of repeats for each locus was verified in the CLIMA database (13).

Tumor samples with at least 40% viable neoplastic cell content were used for DNA isolation using the Gentra Puregene Tissue Kit (Qiagen) according to the manufacturer's instructions. Prior pooling and CGH study DNA quality was verified by NanoDrop measurements, visual inspection of electrophoretic gel, and β-globin gene amplification (14).

Bisulfite modification and epigenotyping

Bisulfite treatment of 1 μg genomic DNA obtained from resected frozen tissues and cell lines was carried out using the EpiTect kit (Qiagen). Epigenotyping was carried out by a combined bisulfite restriction assay (COBRA) as previously described (12). We quantified DNA methylation in 7 specific promoters originally described by Yagi and colleagues (10). Briefly, after the analysis of the methylation of a panel of 3 markers (*CACNA1G*, *SLC30A10*, and *LOX*), HME tumors were defined as those with at least 2 methylated markers. The remaining tumors were screened using 5 markers (*SLC30A10*, *ELMO1*, *FBN2*, *THBD*, and *HAND1*); IME tumors were defined as those with at least 3 methylated markers. Tumors not classified as HME or IME were designated as LME. Primer sequences were previously described (12).

Analysis of other molecular classifiers

Detection of *BRAF* V600E in tumor tissues was carried out using the mutant allele-specific PCR amplification described by Sapio and colleasgues (15). Mutations at codon 12 of the *KRAS* gene were detected by PCR–RFLP as described by Miranda and colleagues (16). Mutations at codon 13 of the *KRAS* were assessed by SNaPshot minisequencing (primer sequences on request). Microsatellite instability (MSI) was determined by pentaplex PCR, using the quasimonomorphic markers BAT-26, BAT-25, NR-21, NR-22, and NR-24, as described in detail by Buhard and colleagues (17).

Samples selection and pooling before microarray analysis

Samples were selected for microarray analysis on the base of DNA quality and epigenotype. After epigenotyping of 233 CRCs, great care was taken during samples selection to avoid disturbance in distribution of clinical and molecular features specific for HME/IME/LME epigenotypes. Finally, 20 HME, 32 IME, 32 LME CRCs, and DNA extracted from 70 normal colon tissues (adjacent to 10 HME, 30 IME, and 30 LME tumors) were selected for subsequent experiments (Table 1). DNA pools were constructed according to guidelines described by Docherty

Table 1. Characteristics of DNA pools used in genome-wide methylation analysis

	HME, *n* (%)	IME, *n* (%)	LME, *n* (%)	Normal, *n* (%)
Number of samples	20	32	32	70
Number of pools	6	6	6	3
Age mean ± SD	66 ± 12	64 ± 12	63 ± 11	64 ± 11
Female	12 (60)	19 (60)	17 (53)	31 (44)
Male	8 (40)	13 (40)	15 (47)	39 (56)
Proximal	16 (80)	5 (16)	9 (28)	26 (37)
Distal	4 (20)	27 (84)	23 (72)	44 (63)
BRAF mutant	12 (60)	0 (0)	0 (0)	—
BRAF wild-type	8 (40)	32 (100)	32 (100)	—
KRAS mutant	0 (0)	14 (44)	12 (37)	—
KRAS wild-type	20 (100)	18 (56)	20 (63)	—
MSI	10 (50)	0 (0)	0 (0)	—
MSS	10 (50)	32 (100)	32 (100)	—

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and colleagues (18, 19). Six biologically independent DNA pools were designed for each CRC epigenotype. Normal colon tissue was represented by 3 independent DNA pools. All 21 pools were verified for the epigenotype status by COBRA (see Supplementary Table S1 for details).

DNA methylation mircoarray analysis

Bisulfite treatment of DNA pools was carried out using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's protocol. The Infinium Methylation-27 Assay was used to detect methylation at 27,578 CpG sites, spanning 14,495 genes (20). After bisulfite conversion, each sample was whole-genome amplified (WGA) and enzymatically fragmented. The bisulfite-converted WGA-DNA samples were purified and applied to the BeadChips. Allele-specific primer annealing was followed by single-base extension using DNP- and Biotin-labeled ddNTPs. After hybridization, staining, and washing, the intensities of the individual beads were scanned in an Illumina Bead Array Reader. Subsequently quality control and statistical analysis was carried out using various Bioconductor packages (v.2.10.0; ref. 21). To assess the quality of the arrays, the signal intensities of control probes together with additional parameters (signal distributions of intensities, average methylation values, coefficient of variation of replicate beads, and scatterplots) were analyzed. Taken together, all arrays were of good quality and were included in the subsequent data analysis. The data were normalized using "shift and scaling normalization" (22). Then, 3,981 previously reported, polymorphism-containing or nonspecific probes were masked before the statistical analysis (23). The intensities of methylated and nonmethylated probes were used to calculate the methylation level (β-values) and the log ratio of the intensities (*M* values). To find differentially methylated probes the *M* values were used to calculate the coefficients of the linear model, which describe the methylation profile of the corresponding locus, and the *P* values were extracted (21, 24). Subsequently, the *P* values were corrected for multiple testing using the Benjamini–Hochberg false discovery rate. All probes with difference in β values that were equal to or greater than 0.20 and *P* less than or equal to 0.05 were retained (20). All Infinium DNA methylation data are deposited at the NCBI Gene Expression Omnibus under accession number GSE37740.

Samples selection and CGH

Samples were selected for CGH on the basis of epigenotype, DNA quality, and availability. Eventually, 20 HME, 40 IME, and 40 LME CRCs were selected for subsequent analysis (Table 2). CGH was carried out as described by Kallioniemi and colleagues, with minor modifications described by Tonnies and colleagues (25, 26). The telomeric, peri-centromeric, heterochromatic regions, as well as chromosomes X and Y were excluded from analysis. Chromosomal alterations of each sample were examined in 10 metaphases. To verify the results, 23 cases were analyzed by reverse labeling. The Mann–Whitney test was used to compare the mean number of gains in different CRC subgroups.

Table 2. Clinical and molecular characteristics of 100 CRCs selected for CGH and the mean of copy number changes estimated by CGH using HME, IME, and LME CRC clusters

Clinical and molecular Characteristics	Total, *n* (%)[a]	HME, *n* (%)[a]	IME, *n* (%)	LME, *n* (%)	*P* (HME vs. IME)	*P* (HME vs. LME)	*P* (IME vs. LME)	*P* (HME vs. IME vs. LME)
	100 (100)	20 (20)	40 (40)	40(40)				
Age, mean ± SD	65 ± 11	67 ± 13	65 ± 10	65 ± 11	0.517	0.611	0.868	0.775
Female	48 (48)	13 (65)	18 (45)	17 (42)	0.177	0.170	1.000	0.271
Male	52 (52)	7 (35)	22 (55)	23 (58)				
Proximal	28(28)	15 (75)	8 (20)	5 (12)	5.88×10^{-5}	2.54×10^{-6}	0.546	2.95×10^{-6}
Distal	72 (72)	5 (15)	32 (80)	35 (88)				
BRAF mutant	15 (15)	14 (70)	0 (0)	1 (2)	5.88×10^{-9}	2.54×10^{-8}	1.000	2.95×10^{-11}
BRAF wild-type	85 (85)	6(30)	40 (100)	39 (98)				
KRAS mutant	30 (30)	2 (10)	21 (52)	11 (27)	0.003	0.180	0.04	0.00461
KRAS wild-type	70(70)	18 (90)	19 (48)	29 (73)				
MSI	14 (14)	11 (55)	2 (5)	1 (2)	2.64×10^{-5}	4.89×10^{-6}	1.000	3.22×10^{-7}
MSS	86 (86)	9 (45)	38 (95)	39 (98)				
CGH mean changes ± SD	7.6 ± 6.5	4.5 ± 5.5	10.4 ± 6.9	6.25 ± 5.6	0.001	0.142	0.004	0.001
CGH mean amplifications ± SD	3.7 ± 3.6	1.9 ± 2.8	4.7 ± 3.7	3.4 ± 3.6	0.001	0.045	0.064	0.003
CGH mean deletions ± SD	3.9 ± 4.0	2.5 ± 3.6	5.7 ± 4.5	2.8 ± 3.0	0.007	0.418	0.003	0.003

[a]Data adapted from Kozlowska et al. (27).

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Results

Genome-wide methylation analysis of three epigenotypes

We carried out a comprehensive DNA methylation profiling of 21 DNA pools that consisted of 84 CRC samples divided previously according to their epigenotypes (HME, IME, and LME) and 70 normal-adjacent colon tissue by the use of the Illumina Infinium HumanMethylation27 DNA assay (12). The characteristics of the selected samples are shown in Table 1. After exclusion of polymorphism-containing and nonspecific probes, methylation status of 23,837 CpG sites was compared between each CRC epigenotype and normal-adjacent tissue (23). We retained for further analysis only the CpG sites (CpGs) that displayed a difference in β values of more than 0.20 and P value less than or equal to 0.05 (after correction for multiple testing; ref. 20). Using these criteria, we identified 3421 CpGs differentially methylated between all CRC epigenotypes and normal colonic tissues. The DNA methylation pattern of these CpGs was further investigated by principal components analysis (PCA). As shown in Fig. 1A, HME, IME, and LME epigenotypes clearly show distinct methylation profiles. The comparison of differentially methylated CpGs (Fig. 1B) revealed that the HME and LME displayed comparable number of differentially methylated CpGs (2,399 and 2,679, respectively), whereas IME showed a lower total number of differentially methylated sites (n, 717) when compared with the other epigenotypes. Substantial overlap (49% of all differentially methylated CpGs) between HME and LME was observed, whereas HME and IME subgroups displayed the simultaneous methylation of 19% of all differentially methylated CpGs (Fig. 1B).

Using data gathered in genome-wide methylation analysis, we explored the methylation of 10 CpGs (*B3GAT2*, *FOXL2*, *KCNK13*, *RAB31*, *SLIT1*, *FAM78A*, *FSTL1*, *KCNC1*, *MYOCD*, and *SLC6A4*) that have been recently proposed by Hinoue and colleagues as a new "two-panel method" to differentiate CIMP-high and CIMP-low subtypes (9). Using this approach, HME subgroup was classified as CIMP-high whereas IME and LME subgroups were classified as CIMP-0 and CIMP-low, respectively (Supplementary Table S2). Similar classification of HME, IME, and LME, based on Infinium DNA methylation data, was obtained when we looked at the methylation of CIMP-specific markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, *SOCS1*, *CDKN2A*, *CRABP1*, and *MLH1*) previously proposed by Ogino and colleagues (Supplementary Table S3; ref.4). Finally, the epigenotyping proposed by Yagi and colleagues could not be fully explored using our Infinium DNA methylation data, because of lack of probe for 1 of the HME/IME markers (*SLC30A10*) on the Infinium HumanMethylation27 array (Supplementary Table S4; ref. 10). Detailed array data for all above-mentioned markers containing β values and detection P values for all 21 pools are presented in Supplementary Table S5.

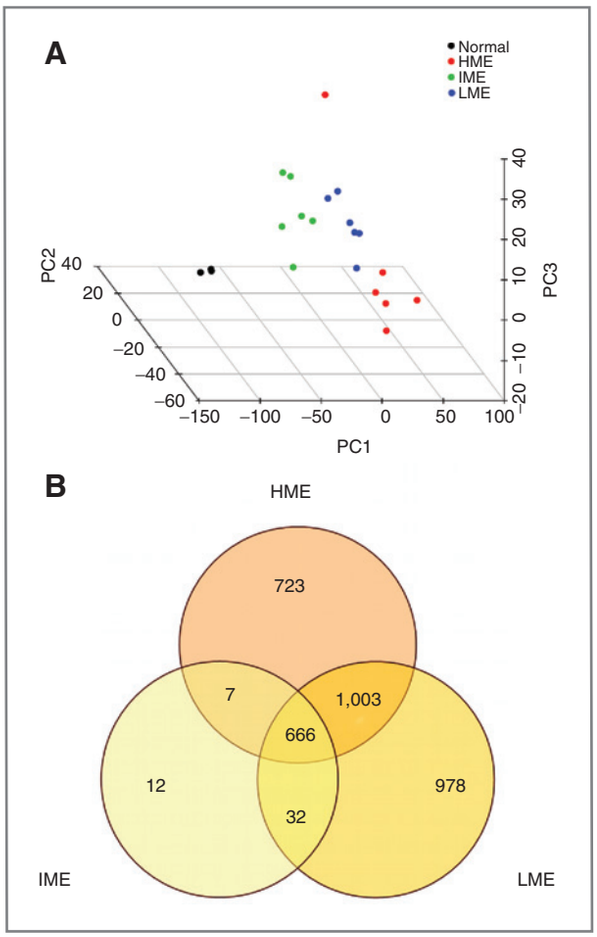

Figure 1. Genome-scale methylation profiling of HME, IME, and LME DNA pools. A, principal components analysis of the methylation data of 3421 CpGs differentially methylated between CRC epigenotypes and normal colonic tissues. Each dot represents a single DNA pool. B, schematic representation of set of 3421 CpGs in a Venn diagram showing the relationship of differentially methylated CpGs in the 3 epigenotypes.

Analysis of chromosomal imbalances in 3 epigenotypes

To assess the mean number of chromosomal imbalances in each epigenotype, we carried out CGH using the selected 20 HME, 40 IME, and 40 LME CRCs (Table 2, see Supplementary CGH data for details; ref. 12). IME showed a clearly higher mean number of total chromosomal imbalances (10.4) compared with the other CRC subtypes (4.5 in HME; P, 0.001; and 6.25 in LME, P, 0.004). Next, we compared the mean number of amplifications and deletions between epigenotypes. In relation to HME and LME, the mean number of amplifications was highest among IME CRCs (1.95 and 3.45 vs. 4.75, respectively). A similar observation was made in the context of deletions where IME displayed on average twice as many deletions (5.7) as in HME (2.5) and LME (2.8). The difference between HME and LME was much less pronounced and manifest itself via borderline difference in the mean number of amplifications (P, 0.045).

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Epigenotyping of CRC cell lines by COBRA

We epigenotyped 6 CRC cell lines (DLD1, HTC116, HT29, CaCo2, SW480, and LOVO) that have been characterized previously according to their CIMP status and/or epigenotype (HME, IME, or LME) status (10, 28). DLD1, HTC116, HT29, and LOVO were classified as HME, whereas CaCo2 and SW480 were grouped as IME (Fig. 2, Table 3). The methylation level of each marker in each cell line is presented in the Supplementary Table S6.

Discussion

The methylator phenotype (CIMP) in CRC was identified by Toyota and colleagues after selecting particular CpG islands (CpGis) that displayed methylation in cancer-specific manner (so-called MINT loci; ref. 2). This allowed to distinguish subgroups of CRCs with prominent number of methylated CpGis, specific molecular features (*BRAF* mutation and MSI), and preferential localization in the proximal colon (later called CIMP-high or CIMP1; refs. 1, 2). Subsequently, after screening methylation of 92 CpGis in 187 CRCs, a new CIMP-specific marker panel was proposed by Weisenberger and colleagues and further enriched with new markers by Ogino and colleagues (3, 4). Parallel to the searches for marker candidates, a subgroup of CRCs with less extensive CpGis methylation enriched for the *KRAS* mutants was revealed (CIMP-low or CIMP2; refs. 7, 29). Finally, non-CIMP tumors (CIMP-0) have been correlated with a frequent loss of heterozygosity (LOH) at 18q that is regarded as 1 of the signs of chromosomal instability in CRC (8). Subsequent studies have focused on genome-wide searches for methylation markers suitable to distinguish between CIMP-low and CIMP-0 (9, 10). On the basis of methylated DNA immunoprecipitation (MeDIP) and re-expression studies conducted on 2 CRC cell lines (HTC116 and SW480), Yagi and colleagues recently proposed a marker panel, which led to the classification of CRC cases into 3 distinct epigenotypes: HME, IME, and LME (10). Application of this marker panel and COBRA has recently enabled us to accurately epigenotype 233 CRC cases (12).

Although at least 3 marker panels (described by Ogino and colleagues, Shen and colleagues, and Yagi and colleagues) for CRC epigenotyping are currently in use, there is no consensus whether clustered tumors represent comparable phenotypes with exception of the CIMP-high subgroup (4, 9, 10, 29). Indeed, attributes of CIMP-high/CIMP1/HME tumors (MSI, *BRAF* V600E, proximal localization, and older age) seem to be constant across the number of studies (1). In contrast, some previous studies had implied that there is no certainty whether IME/CIMP2/CIMP-low are equivalent, presumably because of limited genetic and clinical features supporting the existence of this subtype (9). One recent genome-scale DNA methylation study provided methylation profiles of CIMP-high, CIMP-low, and non-CIMP subgroups using the Illumina Infinium DNA methylation assay consisting of 27,578 CpG sites (9). These data prompted us to examine whether pooled HME, IME, and LME CRCs will display comparable methylation profiles by using an identical DNA methylation platform. Surprisingly, the IME subgroup displayed the lowest number of

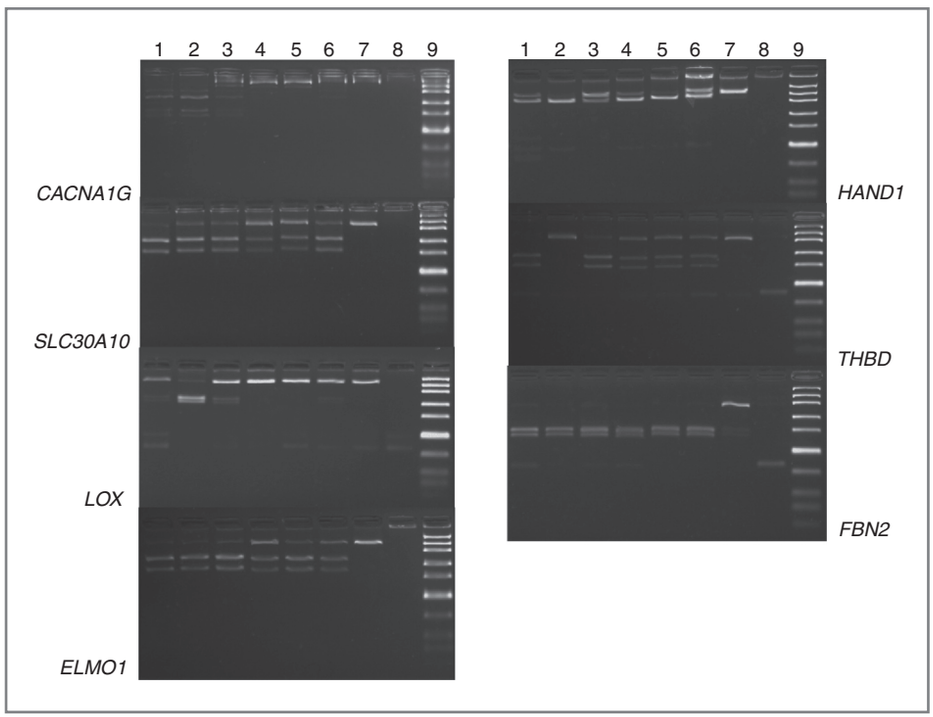

Figure 2. Epigenotyping of CRC cell lines by combined bisulfite restriction assay (COBRA): lane 1, DLD1; lane 2, HTC116; lane 3, HT29; lane 4, CACO2; lane 5, SW480; lane 6, LOVO; lane 7, DNA pool combined from 50 normal colon tissues; lane 8, blank control (H_2O); and lane 9, GeneRuler Ultra Low Range DNA Ladder (Fermentas UAB). The level of methylation of markers for each lane is presented in the Supplementary Table S6.

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Table 3. Epigenetic and cytogenetic status of 6 selected colorectal cancer cell lines

Cell line	Epigenotyping by COBRA (this study)	Epigenotyping by Yagi et al. (10)	Epigenotyping by Hinoue et al. (9)	Ploidy (32–35)	Numerical abnormalities (±)(32–35)	Translocations (32–35)	Copy number changes (32–35)
DLD1	HME	HME	CIMP-high	Diploid	0	1	3
HTC116	HME	HME	CIMP-high	Near-diploid	1	5	4
HT29	HME	N.D	CIMP-high	Triploid	6	8	20
CaCo2	IME	IME	CIMP-0	Aneuploid	18	16	N.D
SW480	IME	IME	CIMP-0	Hyperdiploid	6	15	18
LOVO	HME	HME	N.D	Near-diploid	3	1	4

Abbreviation: N.D., not defined

methylated CpGs when compared with HME and LME. Moderate CpGis methylation frequencies were shown by Hinoue and colleagues as a feature of non-CIMP tumors, whereas CIMP-low was the second most methylated subgroup of tumors on the basis of clustering analyzes (9). Further, we showed a substantial overlap between HME and LME-associated markers (Fig. 1B). Such relation had been previously reported for CIMP-high and CIMP-low subgroups (9). By the use of CIMP-high and -low defining markers (described by Hinoue and colleagues), we classified the HME subgroup as CIMP-high whereas the IME and LME subgroups were classified as CIMP-0 and CIMP-low, respectively (Supplementary Table S2; ref. 9). Similar classification was achieved when we applied markers described by and colleagues (Supplementary Table S3; ref. 4). We admit that methylation of the IME-specific Yagi markers in the IME/LME pools displayed by the Infinium DNA methylation assay (Supplementary Tables S4 and S5) is not as clear as methylation of the same markers revealed by COBRA (Supplementary Table S1). This may be partially explained by a different location of CpG dinucleotides analyzed by COBRA and the Infinium DNA methylation assay (data not shown). It is likely that methylation of markers in epigenotypes that display less dense methylation across CpG islands (such as IME/LME) is more dependent on the exact location of analyzed CpGs (29). Therefore, all of the correlations here reported or negated need to be confirmed in other independent studies.

To determine whether relations between IME/LME and CIMP-low/CIMP-0 holds true also for well-defined setting, we epigenotyped by COBRA 6 CRC cell lines that have been characterized previously according to their epigenotype status and/or CIMP status (Fig. 2, Table 3; refs. 10, 28). Our results were concordant with those described by Yagi and colleagues; however, when we compared our epigenotyping to the CIMP status of the cell lines described by Hinoue and colleagues. it became evident that all IME cell lines were defined as CIMP-0 (10, 28). This evaluation indicated that the lack of equivalency between IME/CIMP-low and LME/CIMP-0 is likely caused by a different set of markers used in CIMP/epigenotype determination rather than the pooling error or some other methodological issues. This could be supported by some differences between performance of Ogino and Yagi markers on the classification of tumors to CIMP-low/CIMP-0 or IME/LME subgroups, which has been previously shown by Yagi and colleagues (10). One possible cause is that Yagi and colleagues used cell lines to derive specific markers whereas markers decribed by Ogino and colleagues and Hinoue and colleagues were developed by screens of large groups of CRCs (3, 31). Moreover, 1 of the cell lines (SW480) used by Yagi and colleagues to identify markers to distinguish IME from LME exemplify the aneuploid cell line exhibiting complex karyotype with many numerical and structural abnormalities (Table 3; refs. 32–35). To provide evidence that use of SW480 as a marker "donor" for IME could favor tumors with copy number changes, we conducted CGH analysis of 100 previously epigenotyped CRCs (12). Indeed, IME tumors displayed apparently higher mean number of total chromosomal imbalances as well as significantly higher mean number of amplifications and deletions when compared with HME and LME (Table 2). Because a number of studies assigned indicators of chromosomal instability (such as LOH of selected loci and enrichment of *TP53* mutations) to the CIMP-0 phenotype, our results provide a molecular link between IME and CIMP-0 and explain the reason for their similarities (8, 36, 37). Concerns, however, can be raised regarding significant enrichment of IME for *KRAS* mutations, which have been regarded as an attribute of CIMP-low (7, 29). Interestingly, very recent findings suggest that *KRAS* mutation do not impose a unique methylation profile on CIMP-low and, therefore, the *KRAS* mutation cannot be regarded as the cardinal classifier of CIMP-low/IME, which is also underlined by the fact that *KRAS* mutations are present, to some extent, in CIMP-0/LME tumors (9, 12, 38).

In summary, our data show that IME tumors display features that have been assigned to CIMP-0, whereas LME tumors can be compared, to some extent, with CIMP-low

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phenotype except for lower *KRAS* mutations frequency. These discrepancies should be considered when interpreting the data from a particular epigenotyping method; however, more studies leading to validation of these findings are needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P. Karpinski, N. Blin, M.M. Sasiadek
Development of methodology: P. Karpinski, E. Szmida, B. Misiak, J. Kozlowska, L. Laczmanski, M.M. Sasiadek
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Karpinski, M. Walter, E. Szmida, B. Misiak, J. Kozlowska, M. Bebenek, Z. Grzebieniak
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Karpinski, M. Walter, D. Ramsey, B. Misiak, N. Blin, M.M. Sasiadek
Writing, review, and/or revision of the manuscript: P. Karpinski, D. Ramsey, N. Blin, M.M. Sasiadek
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Karpinski, L. Laczmanski
Study supervision: M.M. Sasiadek

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