CpG island methylator phenotype in adenocarcinomas from the digestive tract: Methods, conclusions, and controversies

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Author contributions: Sánchez-Vega F, Gotea V, Chen YC and Elnitski L have all read and approved the final manuscript; Elnitski L developed the idea and supervised the research; Sánchez-Vega F, Gotea V and Chen YC generated and analyzed data; and all authors contributed to the writing and editing of the final manuscript; Sánchez-Vega F and Gotea V contributed equally to this work.

Conflict-of-interest statement: None.

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Manuscript source: Invited manuscript

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Received: June 2, 2016
Peer-review started: June 6, 2016
First decision: September 2, 2016
Revised: November 3, 2016
Accepted: January 2, 2017
Article in press: January 3, 2017
Published online: March 15, 2017

Abstract
Over the last two decades, cancer-related alterations in DNA methylation that regulate transcription have been reported for a variety of tumors of the gastrointestinal tract. Due to its relevance for translational research, great emphasis has been placed on the analysis and molecular characterization of the CpG island methylator phenotype (CIMP), defined as widespread hypermethylation of CpG islands in clinically distinct subsets of cancer patients. Here, we present an overview of previous work in this field and also explore some open questions using cross-platform data for esophageal, gastric, and colorectal adenocarcinomas from The Cancer Genome Atlas. We provide a data-driven, pan-gastrointestinal stratification of individual samples based on CIMP status and we investigate correlations with oncogenic alterations, including somatic mutations and epigenetic silencing of tumor suppressor genes. Besides known events in CIMP such as BRAF V600E mutation, CDKN2A silencing or MLH1 inactivation, we discuss the potential role of emerging actors such as Wnt pathway deregulation through truncating mutations in RNF43 and epigenetic silencing of WIF1. Our results highlight the existence of molecular similarities that are superimposed over a larger backbone of tissue-specific features and can be exploited to reduce heterogeneity of response in clinical trials.

Key words: CpG island methylator phenotype; CpG island; Promoter; DNA methylation; Hypermethylation; Gastrointestinal cancer

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Core tip: Awareness of the CpG island methylator phenotype (CIMP) is growing for all adenocarcinomas. Here, we summarize previous work on the topic and discuss unanswered questions regarding commonalities and differences of CIMP tumors from esophageal, gastric, and...
colorectal adenocarcinomas, where data has been made available from the Cancer Genome Atlas. Our analysis includes a review of our pan-cancer method to stratify tumors based on CIMP and addresses the most frequent mutations found in those samples. We include new data implicating truncating mutations in RNF43 and silencing of WIF1. We also describe in detail the methylation of CpG sites within the MLH1 promoter across these tumor types.

Sánchez-Vega F, Gotea V, Chen YC, Elniaski L. CpG island methylator phenotype in adenocarcinomas from the digestive tract: Methods, conclusions, and controversies. World J Gastrointest Oncol 2017; 9(3): 105-120 Available from: URL: http://www.wjgnet.com/1948-5204/full/v9/i3/105.htm DOI: http://dx.doi.org/10.4251/wjgo.v9.i3.105

INTRODUCTION
Aberrant patterns of DNA methylation occur in human cancers with the most notable being a widespread and pronounced gain of methylation at CpG islands in tumor cells. A prominent increase in global levels of CpG island methylation observed across multiple samples was first reported in a subset of patients with colorectal cancer (CRC) and it is now a clinically recognized characteristic of many types of tumor, referred to as the CpG island methylator phenotype (CIMP). In this commentary, we discuss the classification and functional ramifications of CIMP across four types of gastrointestinal adenocarcinomas (esophageal, gastric, colon and rectal), using data from The Cancer Genome Atlas (TCGA) to address lingering questions and identify novel areas of inquiry to spur future investigation. Finally, we explore CIMP’s potential application to cancer diagnostics and subtyping, while emphasizing that much remains unknown regarding the molecular mechanisms of tumor-associated DNA methylation, including CIMP generation and maintenance.

CpG islands play a crucial biological role in development and disease by acting as transcriptional regulatory elements in the genome and controlling the expression of ubiquitously expressed genes. Approximately 50% of all CpG islands are located within promoter regions, and approximately 70% of all annotated promoters are associated with a CpG island. Hypermethylation of CpG dinucleotides within these regions results in the establishment or reinforcement of repressive chromatin and the steric occlusion of transcription factor binding, reducing gene expression. When promoters of tumor suppressor genes are methylated, repression can represent a critical “hit”, in the terminology of the double-hit theory of gene inactivation, conferring a selective advantage to affected cancer cells. For example, the heterozygous silencing of BRCA1 via DNA methylation plays a critical role in breast cancer oncogenesis and tumor proliferation. Other well-known examples of silencing involve MLH1 in CRCs and MGMT silencing in gliomas. In the case of MLH1, methylation-derived silencing inhibits DNA repair, which leads to microsatellite instability (MSI) and cascades into many other downstream functional consequences.

Researchers have identified reproducible, tissue-specific patterns of CpG island promoter hypermethylation in various types of tumors. The specificity of hypermethylation appears to result from the precise targeting of CpG islands by polycomb repressors, resulting in the preferential deposition of DNA methyl groups during oncogenesis. Because these patterns are frequently occurring in cancer patients, they have been used as novel, clinically relevant molecular markers for cancer diagnosis and prognosis. To cite two examples, hypermethylation of the GSTP1 promoter in more than 90% of prostate adenocarcinomas has been used to improve diagnosis of this disease, whereas hypermethylation of SET pseudogene 9 allows researchers to differentiate among different stages of CRC.

The demonstration that tumors exhibiting CIMP represent a distinct clinical subtype of CRC provided the first evidence that, by subdividing cancers into methylation subclasses, clinicians could potentially refine treatment outcomes. Numerous studies have since demonstrated the presence of CIMP in additional cancer types. However, little overlap has been detected among these CIMP incarnations, indicating the tissue-specific nature of the effect. Current models indicate that tumorigenesis affects DNA methylation at CpG islands where repressive H3K27me3 modifications are already present, providing a more permanent layer of suppression in differentiated cells and explaining the origin of tissue-specific patterns. According to such models, aberrant DNA methylation is not a stochastic outcome, but a targeted, albeit aberrational process. In this light, it becomes reasonable to speculate that distinct tumor types could use similar cellular pathways to target their own characteristic CpG islands for DNA methylation. Mechanistic congruity among different tumor types would allow us to understand multicancer and pan-cancer processes from a unified molecular perspective. However, testing this hypothesis requires us to use consistent methods to assess DNA methylation across tumor types and to analyze large numbers of samples to provide statistical power. In the rest of this article, we provide examples of such analyses.

EVALUATING CIMP: FROM GENE PANELS TO GENOME-WIDE METHYLATION PROFILES
A quick overview of important milestones in the study of CIMP within the context of gastrointestinal cancers is provided in Table 1. Given the diversity of methods for assessing DNA methylation, profiling has been performed over a wide range of technical depths and breadths. Initially, the implementation challenges of wide-scale methylation profiling limited the scope of CIMP evaluation. Researchers working on CRC employed panels of genes...
using a low-throughput approach, such as methylation-specific PCR. These panels varied in size from four[27] to several dozen genes[28], and invariably included subsets of the sequences originally employed by Toyota et al.[29]. Although other CIMP-tumor characterizations have emerged, CRC remains the most heavily investigated tumor type with respect to CIMP subtypes. A variety of gene panels are still in use[30], some of which include MLH1[31-35] due to its aforementioned connections to MSI[36,37].

Following an increase in the scope of methylation studies, individual CpG sites started being used to detect aberrant methylation across multiple cancer types. For example, CDKN2A profiling has been used in at least 10 cancer types[32], and MLH1 profiling has been extended to pancreatic cancer[38], leukemia[39], ovarian cancer[40], endometrial cancer[41], gastric cancer[42], and lung cancer[43]. Although these sites are consistently differentially methylated in multiple tumor types, none of them are informative enough to classify samples as CIMP in an independent manner.

The limitations of these early ascertainment methods and lack of extensive overlaps across tumor types, coupled with a variable range of methylation at any given CpG site, fueled a debate over the relevance of CIMP in cancer[44]. The advent of array-based platforms for measuring DNA methylation, such as the Illumina Infinium HumanMethylation27 and HumanMethylation450 arrays[45], helped end this debate[46]. Recent genome-wide experiments using high-throughput data have not only corroborated the biological relevance of CIMP to CRC diagnostics and survival rates, but have led to finer subdivisions of methylation levels, such as CIMP-low and CIMP-zero[47-49]. These classifications better reflect global patterns of hypermethylation, which often fail to fit within “high” or “low” classes in colorectal and other cancers. For example, our early studies of gynecological tumor epigenomes showed a finely increasing signal of CpG island hypermethylation among ovarian and endometrial tumors, rather than a binary methylation signature[50]. This signature represented an intermediate ranking between the fully methylated and unmethylated states, where the CIMP intermediate group corresponded to the serous subtype with TP53 mutations. This observation weighed heavily into our recently demonstrated method to stratify DNA methylation patterns of most cancer types collected by TCGA at the time for CIMP classification. Categories that we defined include CIMP+, CIMP-intermediate (CIMPi), and CIMP-. Such broad-scale analyses provide a means of subtyping individual tumor collections into relatively homogenous methylation subgroups, notwithstanding the fact that each subgroup can contain a gradient in methylation levels. The absence of a highly dichotomous methylation pattern suggests that a complex interplay of factors determines CIMP status, including tumor heterogeneity and clonality[52], multiple somatic/germline mutations[53], copy number variation, and mutation heterozygosity[54].

Within the ongoing effort to better the understanding of cancer biology, we argue that evaluating methylation on an epigenome-wide scale should be favored over the analysis of a few, select loci. For example, large-scale analyses have revealed the now widely recognized phenomenon that DNA methylation occurs at genes with a role in early development and morphogenesis, leading to the discovery that polycomb binding is a precursor to aberrant DNA methylation[55,56]. Also, a number of recent studies have highlighted important similarities in terms of somatic alterations and epigenetic patterns across cancers of different organs and tissues[57-59]. This type of multi-cancer or pan-cancer approach benefits from increased statistical power compared with smaller studies of individual cancer types, which, however, are better suited to capturing tissue-specific features. Researchers can harness the advantages of both approaches by studying related cancer types that occur in tissues derived from common cell lineages. A good example of this approach is provided by previous multi-cancer analyses of tumors of the gastrointestinal tract[60].

### GENOMIC CHARACTERISTICS ASSOCIATED WITH CIMP IN GASTROINTESTINAL TUMORS

TCGA has used patterns of mutation to classify colo-
rectal sample genomes into two large groups, non-hypermethylated and hypermutated\(^{[63]}\). Colon and rectal tumor samples in the former class largely possess CIMP-low phenotypes and have almost indistinguishable molecular signatures in terms of copy number variation, mRNAs, and miRNAs. By contrast, hypermutated samples are predominantly tumorigenic of the colon. Roughly three-quarters exhibit CIMP-high status, as well as ML H1 silencing and MSI, whereas the other quarter are characterized by mutations in other mismatch repair genes such as MLH3 and mutations in POLE. The contrast between samples exhibiting high chromosomal instability (CIN) and samples exhibiting large mutational load is not unique to CRCs, as it has been described in other cancer types, including the endometrioid vs. serous subtypes of both ovarian and endometrial cancers\(^{[50,62]}\). Consistent with these observations, the importance of CIMP as a mutually exclusive alternative to CIN has been underscored in describing dysfunctional events in tumor genomes\(^{[63]}\). As we have reported previously\(^{[51]}\), the MSI vs. CIN duality largely corresponds to a CIMP+ vs. CIMP- dichotomy. This can be extrapolated to a pan-cancer dichotomization of tumors into a "mutator" class, characterized by a large number of somatic mutations, closer to CIMP+, and a "copy number" class, characterized by an abundance of copy-number alterations but lacking excessive mutations, closer to CIMP-. This duality has been previously referred to as the cancer genome hyperbola\(^{[57]}\).

Even if it is conceptually helpful, the simple high-level dichotomy assessed by mutations or copy number alterations fails to adequately represent all of the mechanisms of diversity in gastric tumors. For example, a comprehensive molecular study carried out by TCGA subdivided gastric tumors into four distinct subgroups\(^{[64]}\). Two distinct CIMP-high tumor subgroups were identified: One associated with Epstein-Barr virus (EBV), and one associated with MSI. Among 10 different cancer types analyzed by TCGA, the EBV-CIMP subgroup exhibited the highest frequency of DNA hypermethylation at gene promoters, highlighting the interplay, causative or correlational, between environmental exposures such as viral infection and DNA methylation of the tumor genome. Studies involving other infectious agents also suggest potentially relevant associations between presence of pathogens, gastric cancer prognosis, and CIMP status. For example, in patients infected with *Helicobacter pylori*, CIMP+ tumors exhibit higher rates of recurrence and metastasis than CIMP- tumors\(^{[65]}\).

Of the four types of gastrointestinal cancer examined in the present article, esophageal cancers have been the least thoroughly studied with regards to CIMP stratification. However, CIMP and its associated driver mutations have been investigated in the context of some esophageal tumor subtypes\(^{[66]}\). In particular, subsets of tumors exhibiting high levels of methylation have been reported in both esophageal adenocarcinoma and Barrett’s esophagus, a precursor lesion to esophageal adenocarcinoma\(^{[67]}\). Moreover, the overall amounts of DNA hypermethylation in Barrett’s esophagus predict progression to esophageal adenocarcinoma\(^{[68,69]}\). Genes such as CDKN2A, APC, CDH1, TAC1 and MGMT have been reported to exhibit increased methylation in esophageal adenocarcinomas, esophageal squamous cell carcinomas and Barrett’s esophagus when compared to normal esophageal DNA\(^{[70]}\). By contrast, MLH1 promoter methylation has been reported in esophageal squamous cell carcinomas, but not adenocarcinomas\(^{[70,71]}\), confirming differences in methylation profiles between esophageal subtypes.

### ANALYSES OF CIMP IN GASTROINTESTINAL CANCERS

Here, we investigated CIMP in four types of gastrointestinal adenocarcinoma (GIAD) samples provided by TCGA: Esophageal adenocarcinoma (EAC), which is a subset of esophageal carcinoma (or ESCA, using the TCGA nomenclature); stomach adenocarcinoma (STAD); colon adenocarcinoma (COAD); and rectal adenocarcinoma (READ). Using a previously described approach\(^{[51]}\), we assessed mean methylation levels in tumor and healthy adjacent tissues and ranked samples using unsupervised clustering. Specifically, we measured DNA methylation levels at a set of informative probes (i.e., sets of loci that were differentially methylated between tumor and normal samples at statistically significant levels) using statistical selection criteria applied independently for each tumor collection (Table 2; see research). We then evaluated CIMP status by classifying samples according to average methylation levels across the set of informative probes. This type of CIMP stratification, in which samples with similar methylation intensity levels are grouped together, reduces heterogeneity within the full tumor collection and facilitates the identification of functional somatic alterations that may play a shared role across different cancer types (and subtypes).

| Cancer type | Differentially methylated probes | Control samples | Tumor samples | CIMP- | CIMPi | CIMP+ |
|-------------|---------------------------------|----------------|--------------|-------|-------|-------|
| EAC         | 6717                            | 11             | 87           | 26    | 31    | 30    |
| STAD        | 1110                            | 2              | 260          | 109   | 95    | 56    |
| COAD        | 2656                            | 38             | 274          | 96    | 92    | 86    |
| READ        | 1255                            | 7              | 96           | 31    | 39    | 26    |

CIMP: CpG island methylator phenotype; CIMPi: CIMP intermediate; COAD: Colon adenocarcinoma; EAC: Esophageal adenocarcinoma; READ: Rectal adenocarcinoma; STAD: Stomach adenocarcinoma.
After clustering based on average methylation levels across the probes, samples were categorized into three distinct groups: CIMP+, CIMPi, and CIMP-. CIMP- samples had CpG island methylation profiles that were closer to those observed in normal samples, whereas CIMP+ samples showed a reproducible pattern of DNA hypermethylation with respect to non-cancer controls (Figure 1A). CIMPi samples displayed methylation levels that fell between the CIMP+ and CIMP- groups. In subsequent analyses, we compared CIMP- and CIMP+ samples and excluded the intermediate group, to avoid borderline cases and to guarantee that the tumors being compared were sufficiently different from a molecular point of view.

In a previous study, we showed that our CIMP+ and CIMP- assignments largely coincided with independent assignments by the TCGA for an overlapping sample set of CRC tumors[51]. Here, we compared our CIMP classification with the four molecular subtypes defined by TCGA for gastric tumors: (1) EBV+; (2) MSI; (3) genomically stable (GS); and (4) CIN[64] (Table 3). We observed a significant association between CIMP+ status and the EBV+ and MSI subtypes, in agreement with the extreme CIMP reported for these subtypes by TCGA. Highlighting the previously mentioned incompatibility of CIMP and CIN, CIN samples were significantly skewed
toward CIMP- status. However, other samples also occupied the CIMP- category, including GS samples, which displayed few alterations in DNA methylation and lacked MSI.

In addition to evaluating CIMP in each of the four cancer types independently, we combined all of the data into a single set. Here, the intersection of the loci selected in the four previous, independent analyses was considered informative ($n = 151$, Figure 1B). In this new classification of samples (Figure 1C), CIMP labels remained largely consistent with the previously assigned labels. Importantly, when samples in the pooled data set were ranked according to their average level of DNA methylation across the set of selected probes, they tended to cluster by CIMP status rather than tissue of origin. This novel finding implies commonalities in the underlying generation of aberrant methylation across cancer types.

**CIMP AND MLH1 PROMOTER HYPERMETHYLATION**

Early studies of CIMP established that the MLH1 promoter is consistently hypermethylated in CRC\(^5\). This observation has since been extended to other cancer types\(^72\), and its importance is highlighted by the inclusion of MLH1 in many gene panels used to evaluate CIMP. The strong association between CIMP and MLH1 promoter hypermethylation continues to be reinforced by recent studies with large sample sizes, such as a pan-cancer analysis performed by our group\(^53\) using a catalog of 479 somatic functional events (Ciriello et al\(^57\), 2013). In this previous work, we investigated a cohort of 3299 samples that spanned 9 different cancer types and found that MLH1 promoter silencing was the single genomic functional event that displayed the strongest statistical association with CIMP.

Since promoter hypermethylation is usually associated with gene silencing\(^10,8\), one could compare the effects of MLH1 promoter hypermethylation and disabling gene mutations, addressing parallels with loss-of-function. Indeed, MLH1 promoter silencing replicates the phenotype of MLH1 loss-of-function mutations in hereditary nonpolyposis colon cancer, which displays dinucleotide repeat instability\(^73\). Moreover, research in cell lines demonstrates that reversing MLH1 promoter hypermethylation increases transcription of the gene and restores mismatch repair capacity\(^11,74\). It is therefore tempting to hypothesize that MLH1 promoter hypermethylation, which is strongly associated with CIMP and displays the functional hallmarks of a loss-of-function mutation, is a causal event in the onset of CIMP. However, previous studies, including our own, have shown that CIMP can be observed in the absence of MLH1 promoter hypermethylation or mutation\(^51,61,75\), implying either a relationship that is correlational but not causal, or multiple mechanisms underlying CIMP development.

Only recently has experimental evidence emerged to help elucidate the role of MLH1 promoter hypermethylation in CIMP. In CRC, Fang et al\(^76\) have shown that the common BRAF V600E mutation leads to elevated levels of the protein MAFG. In turn, MAFG binds to the promoter of MLH1 and other genes, where it recruits a heterodimeric partner, BACH1; a chromatin remodeling factor, CHD8; and a DNA methyltransferase, DNMT3B - ultimately resulting in increased methylation at the target sites. These results suggest that mutations such as BRAF V600E orchestrate aberrant methylation patterns; therefore, MLH1 promoter hypermethylation might be thought of as part of the CIMP onset process rather than an initiating event.

Many interesting genes may fit into a model in which, following the onset of somatic mutations, a cascade of downstream methylation events occurs. For instance, CDKN2A promoter hypermethylation is also linked to BRAF mutations, through increased expression of the DNA methyltransferase DNMT3B\(^77\). Similarly, hypermethylation and silencing of the INK4-ARF locus (also known as CDKN2A and CDKN2B) occurs through KRAS activation of ZNF304, which recruits the DNA methyltransferase, DNMT1\(^78\).

### MLH1 PROMOTER METHYLATION IN GASTROINTESTINAL TUMOR DATA FROM TCGA

We analyzed GIAD data supplied by TCGA to learn more about the relationship between MLH1 promoter methylation and CIMP. First, we identified 41 probes from the Illumina Infinium HumanMethylation450 array...
located in the extended MLH1 promoter, operationally defined as 1.5 kb upstream and 500 bp downstream of the transcription start site (TSS) (Figure 2A). We then examined methylation levels for each cancer type, comparing CIMP+ to CIMP- samples, and found that COAD and STAD tumors displayed the strongest differences (Figure 2B). We next looked at the positions of differential methylation. A set of 24 probe sites were differentially methylated between the CIMP+ and CIMP- groups in COAD tumors, and an extended region of 38 probe sites were differentially methylated in STAD tumors (after Bonferroni correction for 41 positions). By contrast, we found no significantly differentially methylated positions in READ samples, and only three in EAC samples. The strongest association between MLH1 promoter hypermethylation and CIMP occurred in COAD tumors (Figure 2B): One-third (20/60) of CIMP+ samples in COAD exhibited MLH1 promoter hypermethylation, in contrast to less than 3% of CIMP- samples (2/71) \( (P = 2.1 \times 10^{-6}, \text{Fisher’s exact test}) \). At the other extreme, no READ CIMP+ samples exhibited hypermethylation of the MLH1 promoter.

We also examined the association between mutations that disable MLH1 and the presence of CIMP. First, we collected all somatic mutations mapped to MLH1 in samples whose CIMP status had been determined (Table 4 and Figure 2C). The most detrimental somatic alterations in MLH1 are frameshift mutations, which render large fractions of the protein product nonfunctional. We observed frameshift mutations in all three CIMP classes (CIMP+, CIMPi and CIMP-), without a significant bias toward CIMP+ samples. In fact, several truncating mutations within the DNA mismatch repair functional domain of the protein occurred in CIMPi and CIMP- samples. These data suggest that loss of function alterations at MLH1 might not be sufficient for the onset of CIMP.

| Sample classification | CIMP+ | CIMPi | CIMP- | Control |
|-----------------------|-------|-------|-------|---------|
| Missense mutation     |       |       |       |         |
| Truncating mutation   |       |       |       |         |
| Inframe mutation      |       |       |       |         |

Figure 2 MLH1 promoter methylation and somatic mutations. A: Diagram of the MLH1 promoter region and the adjacent gene, EPM2AIP1, obtained from the UCSC Human Genome Browser. The probes in this region from the Illumina Infinium HumanMethylation450 array are shown with color bars relative to the CpG island present at this locus: The north shore (orange), the CpG island (red), and the south shore (dark red); B: Heat maps of GIADs showing DNA methylation status across a large genomic region that encompasses the MLH1 promoter. Probes are displayed from left to right, and samples are ordered from top to bottom by average methylation across the region. Color side bars indicate CIMP status: CIMP+ (gold), CIMP intermediate (CIMPi; magenta), CIMP- (green), and control tissue (blue); C: Distribution of 16 somatic mutations in the coding region of MLH1. Color boxes correspond to different functional domains, as specified in the cBioPortal at MSKCC[99], and the vertical axis shows the number of mutations affecting a given codon. GIADs: Gastrointestinal adenocarcinomas; CIMP: CpG island methylator phenotype.
In addition, cancer, where it is associated with EBV-positive status hypermethylation has been reported in colorectal and gastric across several tumor types (Table 5), which is indicative of significantly negatively correlated with mRNA expression as hypergeometric test). The database exhibited concordant differential methylation in contrast, only 2.4% of genes not included in the TSGene genes included samples across all four cancer types (Table 5). These levels significantly different between CIMP+ and CIMP- site in the promoter region that exhibited methylation suppressor genes (4.1%) contained at least one probe the TSGene database We explored this concept by searching for known tumor functional vulnerabilities as well; moreover, the silencing promoter hypermethylation and result in comparable suppressor genes could potentially be silenced through to the appearance of CIMP suggests that other tumor evidence pointing to SUPPRESSOR GENES HYPERMETHYLATION OF TUMOR CIMP AND PROMOTER.

| Sample                  | Cancer type | CIMP class | Mutation                     | Mutation type             | AA pos. | Aff. AAs | VEST score |
|-------------------------|-------------|------------|------------------------------|---------------------------|---------|----------|------------|
| TCGA-A6-6780-01         | COAD        | CIMP+      | chr:37038192.C>A            | Missense substitution     | 67      | 1        | 0.994      |
| TCGA-CA-6719-01         | COAD        | CIMP+      | chr:37067243.C>A            | Missense substitution     | 385     | 1        | 0.701      |
| TCGA-CM-6171-01         | COAD        | CIMP+      | chr:37070349.C>T           | Frameshift deletion       | 495     | 262      | -          |
| TCGA-EI-6917-01         | READ        | CIMP+      | chr:37058999.C>T           | Missense substitution     | 265     | 1        | 0.981      |
| TCGA-BR-6452-01         | STAD        | CIMP+      | chr:37107536.A>G           | 3' UTR                    | -       | -        | -          |
| TCGA-FF-AABE-01         | STAD        | CIMP+      | chr:37090086.C>T           | Nonselective substitution | 659     | 98       | -          |
| TCGA-A6-6318-01         | COAD        | CIMPi      | chr:37050844.C>A           | Missense substitution     | 16      | 1        | 0.901      |
| TCGA-AD-6889-01         | COAD        | CIMP+      | chr:37070334.A>G           | Frameshift insertion      | 195     | 562      | -          |
| TCGA-AZ-6601-01         | COAD        | CIMP+      | chr:37076732.C>T           | Missense substitution     | 385     | 1        | 0.952      |
| TCGA-CM-4746-01         | COAD        | CIMP+      | chr:37059602.A>G           | Frameshift deletion       | 286     | 471      | -          |
| TCGA-ER-6884-01         | READ        | CIMP+      | chr:37059695.A>G           | Acceptor splice site      | 264     | 493      | -          |
| TCGA-BR-6802-01         | STAD        | CIMP+      | chr:370703348.A>G          | Frameshift deletion       | 195     | 562      | -          |
| TCGA-FI-6874-01         | STAD        | CIMP+      | chr:37050312               | Frameshift deletion       | 154     | 603      | -          |

| Sample                  | Cancer type | CIMP+      | Mutation                     | Mutation type             | AA pos. | Aff. AAs | VEST score |
|-------------------------|-------------|------------|------------------------------|---------------------------|---------|----------|------------|
| TCGA-A6-6781-01         | COAD        | CIMP-      | chr:37038384.A>G            | Frameshift deletion       | 195     | 562      | -          |
| TCGA-CM-6674-01         | COAD        | CIMP+      | chr:37058999.C>T           | Frameshift deletion       | 265     | 492      | -          |
| TCGA-F4-6856-01         | COAD        | CIMP+      | chr:37089123.GAA>          | In-frame deletion         | 615     | 1        | -          |
| TCGA-RS-A6KZ-01         | EAC         | CIMP+      | chr:37034874.T>C           | 5' UTR                    | -       | -        | -          |
| TCGA-CG-5723-01         | STAD        | CIMP+      | chr:3703350.G>G            | Frameshift deletion       | 213     | 544      | -          |

1 Computed using the VEST tool[81], which evaluates only the effect of missense substitutions. AA pos.: Amino acid position; Aff. AAs: Number of affected amino acids (the MLH1 protein contains 756 residues); CIMP: CpG island methylator phenotype; CIMP: CIMP intermediate; COAD: Colon adenocarcinoma; EAC: Esophageal adenocarcinoma; READ: Rectal adenocarcinoma; STAD: Stomach adenocarcinoma; VEST: Variant effect scoring tool.

CIMP AND PROMOTER
HYPERMETHYLATION OF TUMOR SUPPRESSOR GENES

The evidence pointing to MLH1 inactivation as a corollary to the appearance of CIMP suggests that other tumor suppressor genes could potentially be silenced through promoter hypermethylation and result in comparable functional vulnerabilities as well; moreover, the silencing of these genes could represent actionable clinical targets. We explored this concept by searching for known tumor suppressor genes that exhibited concerted promoter hypermethylation in all four GIAD cancer types. Using the TSGene database[79], we found that 26 of 634 tumor suppressor genes (4.1%) contained at least one probe site in the promoter region that exhibited methylation levels significantly different between CIMP+ and CIMP- samples across all four cancer types (Table 5). These genes included ERBB4, WT1, WIFI, and RASSF2. By contrast, only 2.4% of genes not included in the TSGene database exhibited concordant differential methylation in CIMP+ samples across the four cancer types (P = 0.007, hypergeometric test).

Furthermore, in affected tumor suppressor genes, such as DPNAS5, RASSF2 and WIFI, promoter methylation was significantly negatively correlated with mRNA expression across several tumor types (Table 5), which is indicative of epigenetic silencing. DNFAS5 is a tumor suppressor gene involved in apoptosis and response to DNA damage[80,81]. Its hypermethylation has been reported in colorectal and gastric cancer, where it is associated with EBV-positive status[82,83]. In addition, WIFI and RASSF2, whose methylation and expression levels were significantly correlated across all four cancer types in our study, have been described in the context of CIMP in gastrointestinal adenocarcinomas[60,65,64-66]. These data suggest that, in a subset of genes, selective pressure may favor loss-of-function events caused by DNA methylation, facilitating tumor growth.

CIMP AND ASSOCIATED SOMATIC MUTATIONS

An outstanding question that remains is the causal connection between somatic mutations and the onset of CIMP. Over the years, extensive association analyses in colon and rectal cancers have been performed to address this problem[25,51]. The results have highlighted the diverse mutation spectrum across tissues, which refutes the hypothesis of a universal driver mutation being responsible for altered DNA methylation levels[51]. Mutations associated with CIMP have been found in CDKN2A, IDH1/2, TET2 and RB1, among other genes[25]. In addition, as discussed, mutations in BRAF directly lead to hypermethylation at specific loci[76,77], and their effects probably extend to myriad targets across the genome.

We further explored the association between somatic mutations and DNA methylation using data from TCGA. For this purpose, we compared the recurrence of somatic mutations in CIMP+ and CIMP- samples across the entire GIAD cohort. A decision tree analysis pointed to several alterations associated with CIMP+ status (Figure 3A and B). This approach ranks mutations in descending order of statistical significance based on their presence or absence in CIMP+ samples. The top-scoring mutation was a 1-bp deletion at chr17:56,435,161 (Figure 3A), which was present in 21 of 22 STAD CIMP+ samples (Figure 3B). This mutation causes a frameshift in the last exon of RNF43, a tumor suppressor that encodes a RING-type...
E3 ubiquitin ligase (p.G659fs*41). *RNF43* is upregulated in colon cancer\(^8\) and inhibits Wnt/β-catenin signaling in pancreatic cancer cells\(^9\). Two other top-scoring mutations affect *APC*, a tumor suppressor whose inactivation is associated with the onset of colon cancer. One was a nonsynonymous C-to-T substitution at chr5:112,175,639, and the second was an AA insertion at chr5:112,175,951. Although these alterations were present in a relatively small number of samples (14 in total), they were observed almost exclusively in CIMP+ tumors (13 out of 14). Not surprisingly, we also found a *BRAF* V600E mutation (A-to-T change at chr7:140,453,136) that was significantly associated with CIMP+ status (Figure 3A).

Together with a common *KRAS* mutation (C-to-T change at chr12:2,539,281; p.G13D), these represent the only two mutations significantly associated with CIMP+ in COAD samples; this is consistent with their already characterized presence in COAD\(^{14,78}\). Finally, a T insertion at chr1:6257785 affecting *RPL22* was also significantly associated with CIMP+ status across GIAD samples, although the number of affected samples was relatively small (6 out of 7 were CIMP+). In the future, these associations may be explored further to investigate their potential functional role in the context of aberrant DNA methylation.

We also compared mutations in CIMP+ and CIMP- samples by aggregating point mutations at the gene level (Table 6). Amid the top scorers in this analysis, we found

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Figure 3  Binary decision trees for separating gastrointestinal adenocarcinomas into CpG island methylator phenotype categories. Recursive partitioning of GIADs from TCGA using binary classification trees based on CIMP status and mutational profiles. Results are provided for A: The combined GIAD data set at the individual mutation level; B: The STAD and COAD data sets at the individual mutation level; C: The combined GIAD data set at the mutated gene level; D: The STAD and COAD data sets at the mutated gene level. Red and green branches illustrate whether a specific mutation is present or absent (or whether a given gene is mutated or not) in the corresponding subset of tumors. Terminal nodes show the number of samples and the associated CIMP+ vs CIMP- fractions, as well as the proportion of different cancer types represented in each subset. GIADs: Gastrointestinal adenocarcinomas; TCGA: The Cancer Genome Atlas; CIMP: CpG island methylator phenotype.
A signal of somatic mutations in coding genes could consist exclusively of CIMP+ samples. Our trees from individual wild-type (Figure 3C). In fact, tumors with mutated chromatin remodeling genes such as ARID1A, which is an important member of the SWI/SNF complex, and histone methyltransferase genes such as KMT2D (MLL2) and KMT2C (MLL3). These two MLL complexes are involved in H3K27 demethylation and H3K4 methylation, which regulate the transcription of many developmental genes, including the HOX gene family[90]. The list of genes whose mutation levels were associated with CIMP status was also significantly enriched for genes from the RTK/RAS/PI(3)K signaling pathway (FDR < 4 × 10^{-5}), including ERBB2, ERBB3, ERBB4, KRAS, PIK3CA, NRAS, and PTEN. These results suggest that the cumulative signal of somatic mutations in coding genes could contribute to CIMP.

Finally, we applied binary decision trees to identify combinations of mutated genes that correlate with CIMP+ or CIMP- status (Figure 3C and D). Using the pooled GIAD data set, our tree shows that KMT2D mutations recur in gastroesophageal (i.e., STAD and EAC) samples (Figure 3C). In fact, tumors with mutated KMT2D and wild-type TP53 consist exclusively of CIMP+ samples (n = 21). We observed a second set of samples (including representatives from all four histologies) that contained SOX7 mutations and lacked KMT2D mutations; all 11 of these tumors were CIMP+. Our trees from individual cancers (Figure 3D) show that KRAS and BRAF mutations in COAD, as well as RNF43, PIK3CA, and KRAS mutations in STAD, are associated with CIMP+ status.

### CONFOUNDING FACTORS IN THE EVALUATION OF CIMP

Basing CIMP classification on mean methylation levels in tumor vs normal tissues allows us to separate cancer-related features from tissue-of-origin signals, but it also makes stratification vulnerable to a number of potential technical and biological artifacts. For example, our classification algorithm relies on the assumption of having a sufficiently large and sufficiently heterogeneous set of controls for each individual tumor type in order to guard against potentially confounding variables such as age, gender, race or anatomic location. Since only two non-tumor control samples were available for STAD, we may have encountered false positives in the probe selection process for this cancer type[91]. Another confounding effect may come from tumors’ stimulation of the immune response, leading leukocytes (including T cells, NK cells, and macrophages) to infiltrate cancerous tissues and skew the methylation signature[91]. Additionally, tumor
We examined our CIMP classifications using the measure of tumor purity calculated with ABSOLUTE, a computational method based on the analysis of somatic DNA alterations\(^{92}\). As a proof of principle, we reclassified CIMP status for the STAD data set using only high-purity (i.e., \(\geq 50\%\)) samples, as determined by the purity estimates available through TCGA\(^{94}\). We then compared sets of selected probes and CIMP designations before and after filtering for purity. After removing low-purity samples, the number of differentially methylated probes increased from 1110 to 1610. This result is consistent with the removal of samples that added background noise and masked the methylation signal of tumor cells. Since the new set of differentially methylated probes encompassed the original probe set, the inclusion of low-purity samples does not appear to have appreciably impacted our precision for feature selection, although it may have impoverished recall due to an increased number of false negatives. After using the new probe set, only five samples changed status from CIMP+ to CIMP-.

### Table 6: Genes differentially mutated between CpG island methylator phenotype+ and CpG island methylator phenotype-gastrointestinal adenocarcinoma samples

| Gene     | CIMP+ | % CIMP+ | Count | CIMP- | % CIMP- | P% Diff | P-value | FDR | Pathway             |
|----------|-------|---------|-------|-------|---------|---------|---------|-----|---------------------|
| KMT2D    | 35    | 20.30%  | 10    |       |         | 4.30%   | 16.00%  | 6.22E-07 | 2.24E-05 | Chromatin           |
| ARID1A   | 60    | 34.90%  | 32    |       |         | 13.90%  | 21.00%  | 1.15E-06 | 2.24E-05 | Chromatin           |
| RNF43    | 42    | 24.40%  | 17    |       |         | 7.40%   | 17.10%  | 3.04E-06 | 3.79E-05 | Wnt                 |
| CSF3R    | 19    | 11.00%  | 2     |       |         | 0.90%   | 10.20%  | 4.19E-06 | 3.79E-05 | ERK                 |
| SOX7     | 14    | 8.10%   | 0     |       |         | 0.00%   | 8.10%   | 4.86E-06 | 3.79E-05 | ERK                 |
| PIK3CA   | 48    | 27.90%  | 26    |       |         | 11.30%  | 16.70%  | 2.62E-05 | 1.70E-04 | PIK3/RAS            |
| PAX6     | 17    | 9.90%   | 2     |       |         | 0.90%   | 9.00%   | 3.96E-05 | 2.21E-04 | Differentiation     |
| ATM      | 37    | 21.50%  | 17    |       |         | 7.40%   | 14.20%  | 5.05E-05 | 2.46E-04 | DNA damage          |
| KRAS     | 52    | 30.20%  | 32    |       |         | 13.90%  | 16.40%  | 1.04E-04 | 4.53E-04 | PIK3/RAS            |
| EGR1     | 15    | 8.70%   | 2     |       |         | 0.90%   | 7.90%   | 1.63E-04 | 6.37E-04 | Differentiation     |
| GATA3    | 19    | 11.00%  | 5     |       |         | 2.20%   | 8.90%   | 2.22E-04 | 7.87E-04 | NF-KB               |
| KMT2C    | 38    | 22.10%  | 22    |       |         | 9.50%   | 12.60%  | 6.15E-04 | 2.01E-03 | Chromatin           |
| ALDH2    | 10    | 5.80%   | 1     |       |         | 0.40%   | 5.40%   | 1.18E-03 | 3.30E-03 | Metabolic           |
| CDK12    | 18    | 10.50%  | 6     |       |         | 2.60%   | 7.90%   | 1.18E-03 | 3.30E-03 | PIK3/RAS            |
| SAFA8    | 15    | 8.70%   | 4     |       |         | 1.70%   | 7.80%   | 1.44E-03 | 3.73E-03 | Chromatin           |
| BCO2     | 19    | 11.00%  | 7     |       |         | 3.00%   | 8.00%   | 1.68E-03 | 4.09E-03 | Chromatin           |
| PTEN     | 24    | 14.00%  | 11    |       |         | 4.80%   | 9.20%   | 1.97E-03 | 4.32E-03 | PIK3/RAS            |
| AXIN2    | 21    | 12.20%  | 9     |       |         | 3.90%   | 8.30%   | 2.00E-03 | 4.32E-03 | Wnt                 |
| CTCF     | 14    | 8.10%   | 4     |       |         | 1.70%   | 6.40%   | 2.73E-03 | 5.41E-03 | Chromatin           |
| PALB2    | 11    | 6.40%   | 2     |       |         | 0.90%   | 5.50%   | 2.77E-03 | 5.41E-03 | DNA repair          |
| ERBB3    | 18    | 10.50%  | 7     |       |         | 3.00%   | 7.40%   | 2.96E-03 | 5.49E-03 | PIK3/RAS            |
| ERBB4    | 29    | 16.90%  | 17    |       |         | 7.40%   | 9.50%   | 4.05E-03 | 6.97E-03 | PIK3/RAS            |
| FBXW7    | 32    | 18.60%  | 20    |       |         | 8.70%   | 9.90%   | 4.11E-03 | 6.97E-03 | Notch               |
| CIC      | 23    | 13.40%  | 12    |       |         | 5.20%   | 8.20%   | 6.55E-03 | 1.06E-02 | Proliferation       |
| HLA-A    | 17    | 9.90%   | 8     |       |         | 3.50%   | 6.60%   | 1.13E-02 | 1.71E-02 | Immune              |
| MSH6     | 19    | 11.00%  | 10    |       |         | 4.30%   | 6.70%   | 1.14E-02 | 1.71E-02 | MMR                 |
| ERBB2    | 15    | 8.70%   | 8     |       |         | 3.50%   | 5.30%   | 2.98E-02 | 4.21E-02 | PIK3/RAS            |
| CASP8    | 13    | 7.60%   | 6     |       |         | 2.60%   | 5.00%   | 3.02E-02 | 4.21E-02 | Wnt                 |
| SMAD4    | 27    | 15.70%  | 20    |       |         | 8.70%   | 7.00%   | 4.05E-02 | 5.45E-02 | Apoptosis           |
| TFE3     | 6     | 3.50%   | 1     |       |         | 0.40%   | 3.10%   | 4.53E-02 | 5.90E-02 | Wnt                 |
| APC      | 82    | 47.70%  | 87    |       |         | 37.70%  | 10.00%  | 5.24E-02 | 6.60E-02 | Wnt                 |
| NRAS     | 10    | 5.80%   | 5     |       |         | 2.20%   | 3.60%   | 6.55E-02 | 7.74E-02 | PIK3/RAS            |
| SMARCB1  | 10    | 5.80%   | 5     |       |         | 2.20%   | 3.60%   | 6.55E-02 | 7.74E-02 | Chromatin           |
| IGFBP7   | 3     | 1.70%   | 0     |       |         | 0.00%   | 1.70%   | 7.70E-02 | 8.65E-02 | DNA Damage          |
| TBL1XR1  | 6     | 3.50%   | 2     |       |         | 0.90%   | 2.60%   | 7.76E-02 | 8.65E-02 | Wnt                 |

\(^1\)Results are based on a combined set of 179 CIMP+ and 154 CIMP+ gastrointestinal adenocarcinoma samples. \(P\)-values were computed using a two-tailed Fishers' exact test. Only genes with FDR < 10% are shown. CIMP: CpG island methylator phenotype.
vice versa. Thus, our CIMP classification system is robust in the presence of varying sample purity.

CONCLUSIONS AND PERSPECTIVES

Ever since the original study in CRC by Toyota et al,[29], evaluation of CIMP status in cancer has been an active area of research. CIMP stratification has direct implications for patient treatment[24]. Because DNA methylation is potentially reversible, it represents an attractive target for therapies that can be tailored to individual cancer epigenomes[20,94]. Nucleoside analogs, such as 5-azacytidine, can be incorporated into DNA to reversibly block DNA methylation, and their effectiveness is being tested in numerous clinical trials.

In this commentary, we have provided evidence that supports refining the molecular profiles of gastrointestinal tumors based on CIMP status, to look beyond traditional tissue-of-origin interpretations. Our analysis of four types of gastrointestinal tumors not only confirms known CIMP associations but also leads to several new observations relevant to current models of DNA methylation and cancer. For example, we report recurrence of a frameshift mutation in RNF43 that is significantly associated with CIMP status in stomach and, to a lesser extent, colon tumors. A recent study linked RNF43 mutations to MSI in colorectal and endometrial tumors, which are Wnt-dependent[95]. The tumor suppressor function of this gene qualifies its mutations to be potential drivers of STAD, although mechanistic links to DNA methylation remain inconclusive. In addition, RNF43 mutations had been identified in endometrioid and mucinous ovarian carcinomas[96]; we have shown the former tumor subtype is largely CIMP[95]. The RNF43 frameshift mutation that we highlighted in STAD samples in this paper is located within a 7-bp, CG-rich tract, and it may be created by the mismatch repair deficiency responsible for the MSI phenotype. Thus, the mutation's connection to CIMP status may occur downstream of MSI. However, RNF43-truncating mutations, which are common in MSI+ colorectal tumors, display mutual exclusivity with inactivating APC mutations[95], suggesting a more direct role in onogenesis. Furthermore, our results point to additional events that could target the Wnt signaling pathway, such as epigenetic silencing of WIF1, which is consistently observed across the four GIAD types, or several of the somatic mutations highlighted in Table 6.

We believe that subdividing samples according to CIMP status has the potential to reduce heterogeneity within cancer subtypes and lead to more uniform molecular and phenotypic characteristics, thus producing more uniform response rates in clinical trials. Whether employed within cohort analyses or individual-level assessments, CIMP profiles have the potential to orient researchers and clinicians toward the biological properties of a tumor through their associations with MSI phenotypes, specific mutational profiles, and the repression of important tumor suppressor genes. Each of these avenues could potentially identify complementary therapeutic modalities. Guided in this way, researchers may identify new candidates for synthetic lethal therapeutic targeting, in which bottlenecks in pathways necessary for tumor cell survival can be targeted, resulting in more precise interventions than many of the current standard-of-care regimens.

RESEARCH

Data

We downloaded level 3 DNA methylation data from TCGA’s data portal (https://tcga-data.nci.nih.gov/tcga/). Data had been acquired using the Illumina HumanMethylation 450K platform and pre-processed following TCGA standard protocols. We further normalized the data from each sample using the BMIQ method[97], which corrects for technical differences between type I and type II probes in the Illumina HumanMethylation platform. We also downloaded level 3 RNA-Seq data from the Broad Institute TCGA Genome Data Analysis Center (standard run dated 06/01/2015, http://dx.doi.org/10.7908/C1251HBG). For EAC, COAD, and READ, we used log2, normalized RSEM RNA-SeqV2 values. For STAD, we used log2: RPKM RNA-Seq values, since RSEM estimates were not available. In addition, somatic mutation data for all four cancer types were downloaded through the bulk download interface of the TCGA portal (https://tcga-data.nci.nih.gov/tcga/findArchives.htm). Finally, Cpg island and transcript annotation data were downloaded from the UCSC genome browser (cpgIslandExt track for Cpg islands, and refFlat and knownGene tracks for transcripts).

Algorithms and statistical analysis

All statistical analyses were done using the R statistical package. We used Cpg island annotations from UCSC for hg19 and gene annotations provided by Illumina for the HumanMethylation 450K platform. Promoter regions were defined as 2-kb regions encompassing the TSS of protein-coding loci (1.5 kb upstream of the TSS and 500 bps downstream of the TSS). Our DNA methylation analysis was restricted to probes located within Cpg islands. Within each individual cancer type, we discarded probes with low variance across samples (SD < 0.1, based on normalized β values between 0 and 1), as well as probes located on the X and Y chromosomes.

Discriminative probes were selected by requiring minimal methylation in control samples (average methylation in controls < 0.05) and increased methylation in tumor samples (average methylation in tumors > 0.25). After a set of discriminative probes had been chosen separately for each tumor type, samples were classified into CIMP categories using k-means clustering on the vector of average methylation values computed across the set of selected probes (k = 3, initial centroids chosen to match population quartiles). Binary decision trees were computed using the R package “partykit”[98-100].

Probe selection, CIMP classification, and decision tree analysis were performed as published in our previous
pan-cancer study. We computed Spearman correlation values between expression values for each of the 28 genes in Table 5 and methylation values for probes in the corresponding TSSs. P-values were adjusted using the Bonferroni correction to account for the multiple probes associated with each gene.

ACKNOWLEDGMENTS

We thank Kristin Harper for editorial assistance. This work was funded by the Intramural program of the National Human Genome Research Institute, the National Institutes of Health.

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