Glioma CpG Island Methylator Phenotype (G-CIMP): Biological and Clinical Implications

Tathiane M Malta[1,2], Camila F de Souza[1,2], Thais S Sabedot[1,2], Tiago C Silva[2], Maritza QS Mosella[2], Steven N Kalkanis[1], James Snyder[1,3], Ana Valeria B Castro[1,#], Houtan Noushmehr[1,2,#,*]

[1] Department of Neurosurgery, Henry Ford Hospital, Detroit, MI, USA

[2] Department of Genetics, Ribeirao Preto Medical School, University of São Paulo, Sao Paulo, SP, Brazil

[3] Department of Neurology, Henry Ford Hospital, Detroit, MI, USA

 # these authors contributed equally

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CORRESPONDING AUTHOR INFORMATION

Houtan Noushmehr, PhD

Associate Scientist/Professor

Department of Neurosurgery

Henry Ford Hospital

E-mail: hnoushm1@hfhs.org

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ABSTRACT

Gliomas are a heterogenous group of brain tumors with distinct biological and clinical properties. Despite advances in surgical techniques and clinical regimens, treatment of high-grade glioma remains challenging and carries dismal rates of therapeutic success and overall survival. Challenges include the molecular complexity of gliomas as well as inconsistencies in histopathological grading, resulting in an inaccurate prediction of disease progression and failure of standard therapy. The updated 2016 World Health Organization (WHO) classification of tumors of the central nervous system reflects a refinement of tumor diagnostics by integrating genotypic and phenotypic features, thereby narrowing defined subgroups. The new classification recommends the molecular diagnosis of *IDH* mutational status in gliomas. *IDH*-mutant gliomas are prompt to manifest the CpG Island Methylator Phenotype (G-CIMP). Notably, the recent identification of clinically relevant subsets of G-CIMP tumors (G-CIMP-high and G-CIMP-low) provided a further refinement in glioma classification that is independent of grade and histology. This scheme is useful for predicting patient outcome and may be translated into effective therapeutic strategies tailored to each patient. In the present review, we highlight the evolution of our understanding of G-CIMP subsets and how recent advances in characterizing gliomas' genome and epigenome may influence future basic and translational research.

KEY WORDS

CpG island methylator phenotype (CIMP), DNA methylation, glioma progression, *IDH* mutation, molecular subtypes, G-CIMP (Glioma-CIMP)

ABBREVIATIONS

2-HG - 2-hydroxyglutarate (2-HG)

α-KG - α-ketoglutarate

CNS - Central nervous system

CGI - CpG Island

CpG - 5'-C-phosphate-G-3'

CTCF - CCCTC-binding factor

G-CIMP - Glioma CpG island methylator phenotype

GBM - Glioblastoma (World Health Organization grade IV)

GWAS - Genome-wide association study

HDAC - Histone deacetylase

IDH - Isocitrate dehydrogenase

LGG - Lower-grade glioma (here defined by diffusely infiltrative low-grade or intermediate-grade glioma (World Health Organization grade II or III))

MGMT - O6-methyl-guanine DNA methyltransferase

NAD - Nicotinamide adenine dinucleotide

OS - Overall survival

TMZ - Temozolomide

WHO - World Health Organization

Gliomas: an overview

Gliomas are a heterogenous group of brain tumors with distinct biological and clinical properties [1,2]. Classically, glioma subtypes and grading were exclusively defined by histological features. However, this classification strategy does not reflect the heterogeneity of the tumor, is prone to subjectivity and discordance among neuropathologists, falls short of predicting disease course and cannot reliably guide treatment [2]. Therefore, multiple research efforts have focused on identifying molecular signatures that define more discrete subgroups in glioma and have a greater impact and relevance in the clinical setting [1–5]. Corroborating the importance of molecular markers in cancer classification, researchers found that 1 in 10 cancer patients would be better classified by molecular taxonomy rather than by the current system based on the primary tissue of origin and stage of disease [6].

The 2016 update to the WHO classification of tumors of the central nervous system (CNS) represents a shift in tumor diagnostics by integrating molecular and phenotypic features into the classification of tumors, and thereby narrowing defined subgroups [1]. Among the genetic alterations associated with diffuse gliomas, *IDH* mutation, histone H3 K27M status, and the integrity status of chromosomes 1 and 19 (1p and 19q) are now integrated with the traditional histology/grade-based glioma classification [1,7]. Gliomas harboring mutations in *IDH1/2* as well as codeletion of 1p and 19q chromosome arms (1p/19q) have shown favorable prognostic and/or predictive values in relation to their counterparts (*IDH*-wildtype or 1p/19q intact or non-codel) [8–12]. Some epigenomic markers also have shown prognostic and/or predictive values [13]. Patients harboring gliomas that carry *MGMT* promoter methylation demonstrate increased overall survival (OS) and time to progression of the disease after treatment with temozolomide (TMZ) or radiation [14–17]. *MGMT* promoter methylation status is used to guide therapeutic management for anaplastic gliomas with wildtype *IDH1/2* and in glioblastoma (GBM)

of the elderly [14–17]. Another important milestone highlighting the clinical importance of epigenetic signatures in gliomas was the discovery of the Glioma-CpG Island Methylator Phenotype (G-CIMP) [18]. Patients carrying G-CIMP (G-CIMP positive) tumors have shown a better prognosis than those not carrying that phenotype (G-CIMP negative). G-CIMP positive (G-CIMP+) tumors was closely related to *IDH*-mutation. Nearly all *IDH*-mutant gliomas were G-CIMP+ and had a favorable prognosis [18,19]. However, comprehensive methylation profiling in a large cohort of glioma revealed that not all *IDH*-mutant/G-CIMP+ tumors had the same prognosis [7]. Based on the extent of global methylation, this study uncovered two subsets of *IDH*-mutant/G-CIMP+ gliomas, one that presented a low degree of methylation and poorer outcome (G-CIMP-low) and another subset depicting high methylation and good OS as usually described for *IDH*-mutant/G-CIMP+ (G-CIMP-high) [7,18,19]. G-CIMP+ subsets (-low and -high) have shown distinct biological features and clinical implications that will be further explored in this review. Following a brief introduction to epigenomics in cancer and in neuro-oncology, we will detail the evolution of our understanding that led to the identification of the G-CIMP subsets. We will also describe how recent advances in methylation-based biomarkers that characterize gliomas may influence future basic and translational research. Our review will conclude with current and upcoming clinical applications, focusing on the potential translational importance of G-CIMP subsets.

Epigenetic modifications in cancer

Within the nucleus of a cell, the three-billion base pairs of the human DNA sequence are stored and organized as chromatin, which is made up of repeating units called nucleosomes. Nucleosomes are formed by octamers of histone proteins that are prone to chemical modifications such as methylation and acetylation. Several epigenetic mechanisms may operate in synchrony to modify the packaging of the genome, including DNA methylation, histone modifications, nucleosome remodeling, small and long non-coding RNA, protein:DNA interactions via chromatin modifying transcription factors, and three-

dimensional chromatin architecture [20]. The resulting epigenomic landscape determines the accessibility of regulatory elements to the DNA, thereby modulating the transcriptional regulation given by transcription factors [20]. Epigenetic mechanisms are implicated both in physiological and pathological events, such as tissue-specificity and carcinogenesis, respectively [20]. In the process of malignant transformation, gene inactivating mutations have been shown to control the epigenomic landscape, implying a crosstalk between the genome and the epigenome [21]. In the present review, we will focus on the first epigenetic modification to be linked to cancer and probably the most extensively studied epigenetic modification in mammals - DNA methylation [22].

DNA methylation

DNA methylation status results from the action of methylating or demethylating enzymes. DNA methylation is the covalent transfer of methyl groups to the 5' position of the cytosine ring, mainly in the context of the dinucleotide cytosine-p-guanine (CpG), resulting in 5-methylcytosine (5mC). DNA methyltransferases (DNMTs), known as the DNA methylation "writer" enzymes, catalyze the transfer of the methyl group to 5' cytosine [23]. On the other hand, demethylation of 5mC is promoted by the TET1/2 (Ten-Eleven Translocation) methylcytosine dioxygenases, known as the DNA methylation "erasers", to generate 5-hydroxymethylcytosine (5hmC) [24]. Some stretches of DNA contain frequent CpG sites, defined as CpG islands (CGI), which are preferentially located at the 5' end of genes overlapping gene promoters [25]. CpG sites are also found in gene bodies and in other regions that are named, relative to their proximity to CGIs, as CpG shores (2kb regions flanking CGIs), CpG shelves (>2kb regions flanking CpG shores), and open sea regions (>4kb to the nearest CGIs) [26]. Intergenic regions that are enriched for CpG, but located in open seas, may encompass distal genomic regulatory elements such as enhancers, silencers and insulators. These transcriptional regulatory elements contain recognition sites for DNA-binding transcription factors, which function either to enhance or repress transcription. Specifically, enhancers are

elements able to activate target genes from distal locations independent of their orientation [27], while insulators are boundary elements that possess the ability to block, or insulate, the signals of either enhancers or silencers [28]. The interplay between protein–DNA complexes defines the spatial organization of the human genome. As a result, chromatin loops are formed, mediated by insulator proteins such as the CCCTC-binding factor (CTCF), facilitating or blocking enhancer-promoter interactions. However, epigenomic alterations, such as gain of DNA methylation at the binding site of a regulatory element, could disrupt those interactions [29].

Unlike the CpG sites dispersed throughout the genome that are usually methylated, CGIs located at promoter regions are generally unmethylated. In physiological conditions, CGI methylation usually occurs as a mechanism of gene repression in specific regions such as the inactive X-chromosome, imprinted genes and germline cell-specific genes [30]. In cancer, DNA methylation becomes aberrant and is mostly characterized by focal hypermethylation around the promoters of genes and gene bodies and global hypomethylation among non-promoter elements [31,32] (Figure 1). Promoter hypermethylation is an important mechanism of epigenetic silencing of tumor suppressor genes [33,34]. Methylation in non-promoter regions is proposed to play a major role in intratumoral expression heterogeneity [35]. In contrast, global hypomethylation largely affects intergenic and intronic regions of the genome that may also result in chromosomal instability. Besides affecting gene expression and chromosomal status, aberrant DNA methylation also modulates isoform expression and facilitates mutational events in adult stem and progenitor cells [13,32,34].

CIMP: CpG island methylator phenotype

Classically, CIMP is defined by genome-wide hypermethylation of CpG islands (CGI) (Figure 1). Tumors carrying this phenotype were first described and validated in the context of colorectal cancers, also known as colorectal CIMP [36,37]. Since then, CIMP has also been described in several tumors,

including gliomas [18,38]. Interestingly, the CIMP+ tumor subset exhibits distinct epidemiological, clinicopathological and molecular features in relation to its CIMP- counterpart. CIMP may have a prognostic significance (both favorable or unfavorable) in terms of OS or a predictive value of therapeutic response in certain tumors [38–40]. Despite tissue-specific differences, evidence suggests that CIMP may be a universal feature across different tumors. Addressing this issue, researchers applied a genome-wide unbiased and unsupervised hierarchical clustering of cancer-specific methylated CGI genes across 15 tumors types; however, gliomas were not included in the cohort [41]. They found a set of 89 shared hypermethylated CpG island probes that allowed the segregation of 12 tumor types into CIMP+ and CIMP- subgroups, according to the presence or absence of methylated probes, respectively [41].

Glioma epigenomic molecular signatures

G-CIMP: Glioma-CpG island methylator phenotype

The glioma-CIMP (G-CIMP) subtype was first described by Noushmehr *et al.* in GBM and was further validated in lower-grade glioma (LGG) [18,19]. The G-CIMP+ subtype occurred frequently in LGG samples, whereas in GBM it was mostly associated with secondary or recurrent (treated) tumors [18,19]. By integrating the DNA methylation data with four gene expression clusters previously described in GBM by Verhaak *et al.* (i.e. proneural, neural, classical, and mesenchymal) [42,43], the authors found that G-CIMP+ subtypes were highly enriched among the proneural subtypes and in younger patients, compared to G-CIMP- tumors. Later on, the G-CIMP subtype was also described in pediatric glioma patients [44]. Importantly, G-CIMP+ was shown to be closely associated with *IDH*-mutant gliomas in several studies [18,19].

By performing a large multi-platform genomic analysis across 1,122 lower- and high-grade

primary adult gliomas, our team, as part of Ceccarelli *et al.*, uncovered 7 discrete subtypes, with distinct biological features and clinical outcomes [7]. This DNA methylation-based classification refined and recapitulated previous glioma stratification subgroups based on *IDH* mutation and chromosomes 1p/19q codeletion status. The subgroups were mainly driven by *IDH1/2* mutation status and classified as 1- *IDH*-wildtype, enriched for GBM, further segregated as classic-like, mesenchymal-like, LGm6-GBM and PA-like subgroups; and 2- *IDH*-mutant, enriched for LGG, further clustered as 1p/19q codel and non-codel. The *IDH*-mutant non-codel cluster was further refined into two distinct subgroups, based on the extent of methylation: G-CIMP-low (6% of *IDH*-mutant) and G-CIMP-high (55% of *IDH*-mutant) with a low or high degree of DNA methylation, respectively [7].

Prognostic value of G-CIMP+ and its subsets

The G-CIMP DNA methylation showed relevant prognostic value, independently of known OS predictors in adult diffuse glioma, such as grade and age [18]. The favorable prognostic value of G-CIMP+ in both LGGs and GBMs has been reported in many other studies [7,18,19,44–46]. Notably, *IDH*-mutant G-CIMP+ GBM presented favorable survival and molecular similarities to LGG; on the other hand, LGG carrying IDH-wildtype G-CIMP- presented molecular and clinical behavior similar to GBM [7,19,44,45]. The refinement of G-CIMP+ stratification revealed that not all *IDH*-mutant G-CIMP+ tumors had the same prognosis. The G-CIMP-low subset had the poorest OS among the *IDH*-mutant gliomas (median survival G-CIMP-low=2.7 years, G-CIMP-high=7.2 years, cox-regression $p<0.001$; and vs codel=7.9 years, $p<0.001$) resembling the behavior observed in IDH-wildtype gliomas (median survival 1.2 years) [7].

Relationship between G-CIMP+/subsets and established prognostic and/or predictive molecular biomarkers

IDH mutations, codeletion of 1p/19q, *MGMT* promoter methylation, and G-CIMP+ are

independent, favorable prognostic biomarkers [47]. However, combining some of them has shown to improve their individual prognostic value [7,18,47]. For instance, patients harboring a triple combination of 1p19q codeletion, *IDH* mutation and *MGMT* methylation had significantly better OS than those carrying the *MGMT* methylation biomarker alone [48]. In addition, some of them (codeletion of *1p19q* and *MGMT* promoter methylation) have also been established as predictive biomarkers and have been used in decision making for chemotherapy and/or radiation treatment [49,50]. The relationship between G-CIMP and the aforementioned biomarkers will be explored in the following sections.

IDH mutation

One of the most relevant clinical discoveries in neuro-oncology involves IDH1 mutations in glioma for their role as a prognostic marker and potential as a drug target [1,7,10,19,44,51–53]. The *R132H IDH1* mutation, an amino acid substitution at a single arginine residue in the active site of the enzyme, is highly prevalent in grade II and III gliomas and appears in secondary GBMs, which develop from lower-grade tumors [10]. Although much less common, mutations in *IDH2*, the mitochondrial homolog of the cytosolic *IDH1*, have also been identified in gliomas [54].

Due to the close relationship between *IDH* mutations and G-CIMP+, it was suggested that G-CIMP+ prognostic value was mainly due to its relation to *IDH* mutations [51]. The mechanisms associated with favorable prognosis in *IDH*-mutant G-CIMP+ tumors are still under investigation [13,18]. However, a meta-analysis of a G-CIMP gene list with prior gene expression analyses suggested that G-CIMP+ tumors may be less aggressive because of silencing of key mesenchymal genes [18].

The predictive value of *IDH* mutation for treatment response is still controversial [4,49,50,55]; however, a phase III clinical trial reported that *IDH*-mutant anaplastic gliomas benefited from early combination of procarbazine, lomustine, and vincristine [50]. The predictive value of G-CIMP+ and its

subsets is still unknown.

1p/19q

1p/19q codel has an established favorable prognostic and predictive value in gliomas [46,49] and is found exclusively in *IDH*-mutant tumors [56]. The interaction between 1p/19q status and G-CIMP+ has also been reported [12,57]. *IDH*-mutant G-CIMP+ codel tumors, for example, were associated with a better OS than *IDH*-mutant G-CIMP+ non-codel (mean survival G-CIMP+ codel=9.9 years and G-CIMP+ non-codel=4.4 years) [57]. In our pan-glioma cohort, 9.1% of the *IDH*-mutant G-CIMP+ non-codel subgroup was represented by G-CIMP-low subtype with the remainder by the G-CIMP-high subtype [7]. As described, among the *IDH*-mutant G-CIMP+ non-codel tumors, the G-CIMP-high subset had similar survival to the *IDH*-mutant codel tumors [7].

MGMT promoter methylation

MGMT promoter methylation has an established prognostic and predictive value and is used in therapeutic decision planning in gliomas [17,49,58]. *MGMT* promoter methylation has also been strongly associated with G-CIMP as well as with *IDH* mutation regardless of glioma grade [7,11,59–61]. From our published data, we estimated that among the TCGA *IDH*-mutant cohort, 91.8% of glioma specimens presented *MGMT* promoter methylation compared to 40.0% among *IDH*-wildtype tumors ($p < 0.001$) [7]. Notably, G-CIMP-low samples presented a lower proportion of *MGMT* promoter methylation (68.0%) compared to G-CIMP-high (88.8%, $p = 0.008$, unpublished data) [7].

Interestingly, better OS associated with *MGMT* promoter methylation was reported in patients harboring G-CIMP+ but not G-CIMP- GBM [47]. In addition, the predictive value of *MGMT* promoter methylation was described only in the context of *IDH1/2* wildtype in anaplastic gliomas [49]. These findings prompt researchers to suggest that the prognostic significance and the predictive value of the *MGMT*

promoter methylation in those tumors was dependent on the G-CIMP status and *IDH* mutation, respectively [47,49,60]. Moreover, some authors proposed that G-CIMP+ status may have a better prognostic value than *MGMT* promoter methylation in anaplastic oligodendroglial brain tumors [60].

Potential drivers associated with G-CIMP

Putative driver mechanisms underpinning the aberrant CpG methylation that occurs in CIMP tumors are still under surveillance in gliomas. Some potential cancer-specific mutated driver genes encompass *IDH1/2 and H3F3A* [18,19,44,62]. Hotspot mutations in *IDH1/2* genes provoke IDH to display a neomorphic enzymatic activity that leads to the reduction of α-KG to 2-hydroxyglutarate (2-HG), an oncometabolite that functions as a competitive inhibitor of α-KG [53,63,64]. The accumulation of 2-HG impairs the activity of α-KG-dependent-dioxygenases such as histone and DNA demethylases (e.g. TET enzymes), leading to global hypermethylation (CIMP) as well as impairment of cell differentiation [19,63,65]. Another consequence of *IDH* mutations is the alteration of other metabolic pathways components, such as the coenzyme NAD+ [66]. NAD+ has key role in intracellular signaling pathways implicated in cancer cell growth. It was shown that *IDH*-mutant cells presented reduced expression of NAPRT1 (nicotinate phosphoribosyltransferase 1), a rate-limiting enzyme within the NAD+ salvage system. Reduced expression of NAPRT1 led to lower basal NAD+ levels, rendering the *IDH*-mutant cell more vulnerable to death [66,67]. Furthermore, *IDH* mutations also result in the methylation of histone markers that, by themselves, contribute to DNA methylation [68]. Histone *H3F3A* mutations were also reported to drive the global aberrant pattern of methylation in a subset of pediatric GBM by unknown mechanisms [44]. Interestingly, in a subset of GBM, the authors found recurrent and mutually exclusive mutations either in *IDH1* or *H3F3A* (affecting amino acids K27 or G34) with distinct clinical, genomic, and epigenomic features [44].

Recent large-scale genome-wide association studies (GWAS) provided evidence for a defined germline variant located on chromosome 9p21.3 which was found to be enriched among G-CIMP tumors [69] and a variant mapped to an intronic region located on chromosome 2q33.3, 50K base pairs from *IDH1* [70]. Despite lack of functional experiments, these findings, in combination with known somatic alterations, offer potential insights in the role of genetic variants into the biology and etiology of G-CIMP tumor development and possibly progression.

Figure 2 summarizes the major milestones in integrating genomics and epigenomics data to uncover glioma molecular and clinical phenotypes and that led to G-CIMP subsets characterization (either directly or indirectly).

G-CIMP+ subsets and glioma progression/recurrence

WHO grades II and III *IDH*-mutant non-codel (astrocytic) gliomas habitually recur and unpredictably undergo malignant transformation to highly aggressive and treatment-resistant grade IV GBM [1,71]. Remarkably, the analysis of a small cohort of primary and recurrent matched tumor samples, composed of LGG and GBM, revealed that some G-CIMP-high tumors exhibited a demethylated pattern, after relapse, similar to those observed in G-CIMP-low gliomas, suggesting a progression from the G-CIMP-high to the G-CIMP-low subset [7]. A recent work from our own laboratory confirmed and expanded the finding of the epigenetic shift of G-CIMP-high to G-CIMP-low upon tumor recurrence in TCGA and non-TCGA samples [72]. Interestingly, G-CIMP-low at recurrence appeared in 12% of all gliomas and shared epigenomic features resembling *IDH*-wildtype primary GBM [72]. Genome-wide decreases in methylation levels associated with progression have also been reported by other studies [73,74].

The hypothesis that G-CIMP-high tumors may relapse as G-CIMP-low gliomas suggests that

variations in DNA methylation could be key determinants of the mechanisms that drive glioma progression. Notably, the majority of CpG sites that underwent significant DNA demethylation in G-CIMP-low recurrent tumors were primarily found within intergenic (open sea) regions [7] (Figure 1). CTCF, a methylation-sensitive insulator protein, has an important role in stabilizing enhancer-gene interactions in intergenic regions, as mentioned previously in this review. In IDH-mutant gliomas, it was shown that hypermethylation at CTCF binding sites reduced CTCF binding. The consequent loss of insulation led to aberrant enhancer-gene interaction that ultimately resulted in the upregulation of a glioma oncogene [75]. Since the discovery of IDH-mutant glioma subtypes, our group began to investigate the potential role of DNA methylation status at a single-base pair resolution, using Whole-Genome Bisulfite Sequencing (WGBS). Confirming previous data [7], our unpublished findings revealed that, compared to G-CIMP-high (as well as to non-tumoral brain specimens), G-CIMP-low presented DNA hypomethylation at some CTCF binding sites. Collectively, these findings support the hypothesis that the loss of methylation at the CTCF binding sites will also influence chromatin architecture mediated by the insulator binding disruption that in turn will deregulate genes nearby (Figure 3) (Sabedot TS et al, unpublished data). We also found that the hypomethylated intergenic regions were enriched for OLIG2 and SOX-family motif binding sites [7], which have been described as neurodevelopmental transcription factors essential for GBM propagation [76]. SOX-family genes are transcription factors that are also involved in the induction and maintenance of stem cell pluripotency [77], promote self-renewal of neural stem cells in the nervous system [78], and within brain tumors regulate the plasticity between glioma stem cell and non-stem cell states [79]. Accordingly, G-CIMP-low tumors displayed abnormalities in cell cycle pathway genes such as *CDK4* and *CDKN2A* supporting the association between stem cell signaling pathways and tumor proliferation in glioma.–Therefore, loss of CpG methylation at these functional genomic elements, known to be associated with normal development and pluripotency, defines a possible

mechanism of glioma progression [7,72].

In addition, Bai *et al.* found that key developmental transcription factors that are regulated by Polycomb Repressive Complex 2 (PRC2) in human stem cells became hypermethylated during glioma progression resulting in gene silencing, which could ultimately cause GBM cells to enter a continuous state of stem cell-like self-renewal [74]. Mazor *et al.* reported specific hypomethylation events that may contribute to the increased cell proliferation upon progression from LGGs to GBMs [73]. Recently, we derived a metric to measure the degree of de-differentiation of tumors based on DNA methylation and found a strong association between a high undifferentiated score and glioma molecular subtypes associated with worse clinical outcome (i.e., G-CIMP-low, classic-like, mesenchymal-like, LGm6-GBM, and PA-like). Interestingly, G-CIMP-low patients showed higher undifferentiated scores compared to G-CIMP-high patients, resembling those found in primary *IDH*-wildtype tumors (Malta TM et al, manuscript in preparation). Moreover, recurrent G-CIMP-low gliomas presented higher undifferentiated scores compared to their primary G-CIMP-high counterparts [72]. Taken together, these findings suggest that a stem cell-like phenotype may be involved in unfavorable clinical outcome and reinforce the importance of epigenetic alterations occurring in intergenic regions that may disrupt important regulatory elements affecting oncogenesis and tumor progression (Figure 4).

G-CIMP detection

The diagnosis of G-CIMP was reported by Noushmehr *et al.*, 2010 [18]. It was based on an epigenetic biomarker panel consisting of seven hypermethylated loci (*ANKRD43*, *HFE*, *MAL*, *LGALS3*, *FAS-1*, *FAS-2*, and *RHO-F*) and one hypomethylated locus, *DOCK5*, validated *in silico*. A sample was considered G-CIMP+ if at least six genes displayed a combination of *DOCK5* DNA hypomethylation and/or hypermethylation of the remaining genes in the panel. The utilization of the MethyLight assay to

detect the aforementioned biomarkers showed perfect concordance with the results obtained by array platforms and may be suitable for clinical utilization [18].

Later on, G-CIMP-high and -low subsets were defined and validated *in silico*, by a panel of 163 DNA-methylated probe signatures, using methylation arrays [7,72]. Our group developed a concise DNA methylation biomarker panel, derived from those probes, consisting of 14 methylated probes allowing the identification of the seven glioma subgroups previously reported [7] and validated *in silico* (unpublished data). Eight of those probes distinguished between G-CIMP+ subsets (-low and -high) in the context of *IDH*-mutant glioma specimens; however, their detection by more readily available assays warrants investigation.

The longitudinal investigation of the molecular features involved in the transition between G-CIMP-high to -low gliomas also allowed the identification of a set of candidate biomarker signatures that enabled the prediction of G-CIMP-high tumors that would progress to G-CIMP-low during relapse. The epigenetic biomarker panel, validated *in silico*, was composed of seven hypomethylated CpG sites that currently warrant clinical validation [72].

Epigenomic and chromatin targeted-therapies

Epigenetic mechanisms are heritable and potentially actionable, making them an attractive target for the treatment of diseases, including cancer [80]. Many of the epigenome-targeting drugs have proved beneficial and safe in hematologic malignancies [81,82]. However, studies in solid tumors are limited [52,80,83]. Epigenetic drugs approved or in clinical trials have been thoroughly explored in recently published reviews [52,80,84].

In gliomas, few trials from this class have been completed [81,82,84,85]. To our knowledge, there are

no clinical trials specifically addressing G-CIMP+ subtypes. However, preclinical and clinical studies using drugs targeting DNA methylation directly, or the mechanisms involved in G-CIMP, are underway. Representing epigenetic drugs with broad actions are hypomethylating agents such as the inhibitors of DNA methyltransferase (DNMT), the histone deacetylase (HDAC) and the bromodomain and extra-terminal motif proteins (e.g. BRD4 inhibitor) [80].

DNMT inhibitors or DNA demethylating agents (e.g., 5-Azacytidine, 5-Aza-2-deoxycytidine-decitabine) promote dose-dependent global demethylation activity by depleting or degrading the DNA methyltransferases. This may reverse aberrant expression of genes related to nearly all pathways involved in cancer initiation and progression (i.e., tumor suppressor genes, oncogenes, genes associated with stemness or pluripotency, apoptosis, cell-cycle and, immune response) [86,87]. Moreover, DNMT inhibitors were also shown to reverse tumoral immune evasion through viral infection mimicry and up-regulation of viral defense pathways [88–90]. For instance, in an *IDH*1-mutant glioma xenograft, low doses of the demethylating agent, decitabine, restored the activity of DNMT1 and, consequently, reversed DNA methylation marks in promoters of differentiating genes. This change resulted in the loss of stem-like features and in the arrest of the glioma growth in that model [91]. Although preclinical studies using these drugs in gliomas seem promising, a corresponding clinical trial using decitabine on adult glioma has not been reported. Considering the potential adverse impact of loss of methylation in the progression or recurrence of gliomas, reported previously [72,73], it seems reasonable to take G-CIMP subsets into account during clinical trial design.

Drugs targeting chromatin organization, such as HDAC and bromodomain family protein inhibitors and bromodomain may potentiate DNA-damaging therapies by increasing chromatin accessibility, but their use is still under scrutiny in cancer [92]. Considering the potential negative impact of

low methylation in chromatin organization, this drug could be considered in the treatment of patients harboring G-CIMP-low or those with risk to G-CIMP-low progression.

Targeting *IDH*-mutant tumors, including G-CIMP+ gliomas, with *IDH*-mutant enzyme inhibiting agents is currently under investigation in clinical trials [52,66,80]. The mechanistic intent of these drugs involve the metabolic pathways altered in *IDH*-mutant tumors (i.e. decrease of 2-HG in hematologic or salvage of NAD+ in solid tumors) [66]. However, a preclinical study has not identified a decrease or epigenetic changes in *IDH*-mutant cancer initiating cells exposed to an IDH-R132 inhibitor drug despite marked reduction in 2-HG [66]. They postulated that the activity of this class of drug may be limited to a subset of tumors. Moreover, NAD+ metabolic depletion was recently shown to be an attractive therapeutic target in *IDH1*-mutant cells vulnerable to a cytotoxic response when exposed to a nicotinamide phosphoribosyltransferase (NAMPT) inhibitor. The same response was not seen in *IDH1*-wildtype cell lines [66]; however, a later preclinical model demonstrated that NAMPT inhibitors enhanced the cytotoxic effects of TMZ in GBM cells [93].

Immune modulation is also affected by epigenomic alterations and has been shown to be an attractive target for pharmacotherapy in cancer [94]. The identification and targeting of disease-specific neoantigens has demonstrated success in other diseases renewing excitement for immunotherapy in glioma. An immunogenic epitope vaccine targeting mutant *IDH1* in glioma with promising preclinical work is now in phase I clinical trials [95,96]. Several other clinical trials are underway in glioma including phase III targeted immunotherapy trials [97]. The use of immune checkpoint inhibitors targeting programmed cell death protein 1 (PD1) and/or its ligand 1 (PD-L1) has also shown activity in several cancer types and is currently being tested in GBM clinical trials. The *PD-L1* promoter methylation and *PD-L1* lower expression in *IDH*-mutant gliomas compared to *IDH*-wildtype point to an epigenetic regulation of *PD-L1* and indicate that patient harboring *IDH*-mutant gliomas may not benefit from the

monotherapy with drug targeting the blocking of the PD1/PD-L1 pathway [98].

Co-administration of epigenomic agents (*e.g.*, DNA-demethylating agents and HDAC inhibitors) has demonstrated improved efficacy of immunotherapy in many tumor types by increasing tumoral immune-response, enhancing the expression of immune blockade checkpoints, and by reducing cellular adaptation that leads to drug resistance [99]. Given the potential epigenetic regulation of PD-L1 expression, patients harboring *IDH*-mutant (G-CIMP+) tumors may benefit from combined treatment modalities including demethylating agents and PD1/PD-L1 inhibitors. The relationship between G-CIMP subsets and response to immunotherapy, either as a predictive marker or epigenetic therapy target, is largely unknown and worthy of further investigation.

Final remarks and perspectives

Advances in glioma research have highlighted the significance of epigenomic alterations. The incorporation of genetic markers into the traditional WHO histopathological classification of CNS tumors reflects widespread adoption of the latest scientific and clinical advances in molecular neuro-oncology into clinical practice [1]. Moreover, evidence suggests that comprehensive analysis, such as unsupervised glioma subtyping based on gene expression or on G-CIMP status, rather than individual molecular markers, may improve prognostic and predictive outcomes [100]. Currently, the guidelines for the detection of established prognostic biomarkers are based on individual marker assays: for example, immunohistochemistry or DNA sequencing for mutations in *IDH1-R132H* and variants and H3-K27M, fluorescent in-situ hybridization or microsatellite PCR-based loss of heterozygosity analyses for codeletion of chromosomal arms 1p and 19q, and real- time methylation-specific PCR for MGMT promoter methylation [61]. However, the advent of genome-wide analysis technologies has allowed the concurrent detection of methylation pattern, genomic and copy number alterations in tumor specimens [61].

Remarkably, the identification of a methylation-based classifier of glioma specimens enabled the researchers to capture already known prognostic markers such as *IDH* mutation and 1p19q codeletion [7]. In addition, it uniquely enabled the identification of a subset of tumors within the *IDH*-mutant/G-CIMP+ group (i.e., G-CIMP-low) that presented a prognostic disadvantage in relation to its counterparts (*IDH*-mutant G-CIMP-high non-codels and codels). In addition to refining glioma classification, the identification of G-CIMP+ subsets also shed light on the role of demethylation of specific genes in the pathogenesis of glioma progression, independently of grade or *IDH* status [72].

In summary, the detection of G-CIMP+ and subsets (-high and -low) builds on the 2016 WHO molecular effort to further refine glioma classification (Figure 5) and provides insight into disease course and treatment opportunity [72]. The prognostic significance of G-CIMP+ across all glioma types has been confirmed in many studies [7,18,46,47]. Moreover, in addition to their prognostic value, the identification of G-CIMP subsets led us to define predictive candidate biomarkers for tumor recurrence as G-CIMP-low [72]. It is possible that, as for other established therapeutic predictive markers in gliomas, such as 1p/19q codeletion and *MGMT* promoter methylation, G-CIMP+ subsets will be used for patient counseling and be part of algorithms used for clinical trial design and for therapeutic decisions [4] . However, the utility of these classifiers and biomarkers in planning treatment strategies and designing clinical trials remains to be fully validated.

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FIGURE LEGENDS:

Figure 1. CIMP subsets in human cancer. This illustration depicts aberrant DNA methylation changes at specific genomic locus in normal and tumor cells, especially in CIMP tumors. Each DNA strand represents one individual methylome. Methylated CpG sites in normal state are represented in blue; non-CIMP tumor DNA methylation gain in yellow, and aberrant DNA hypermethylation in CIMP tumors in red (modified from Weisenberg 2014) [37].

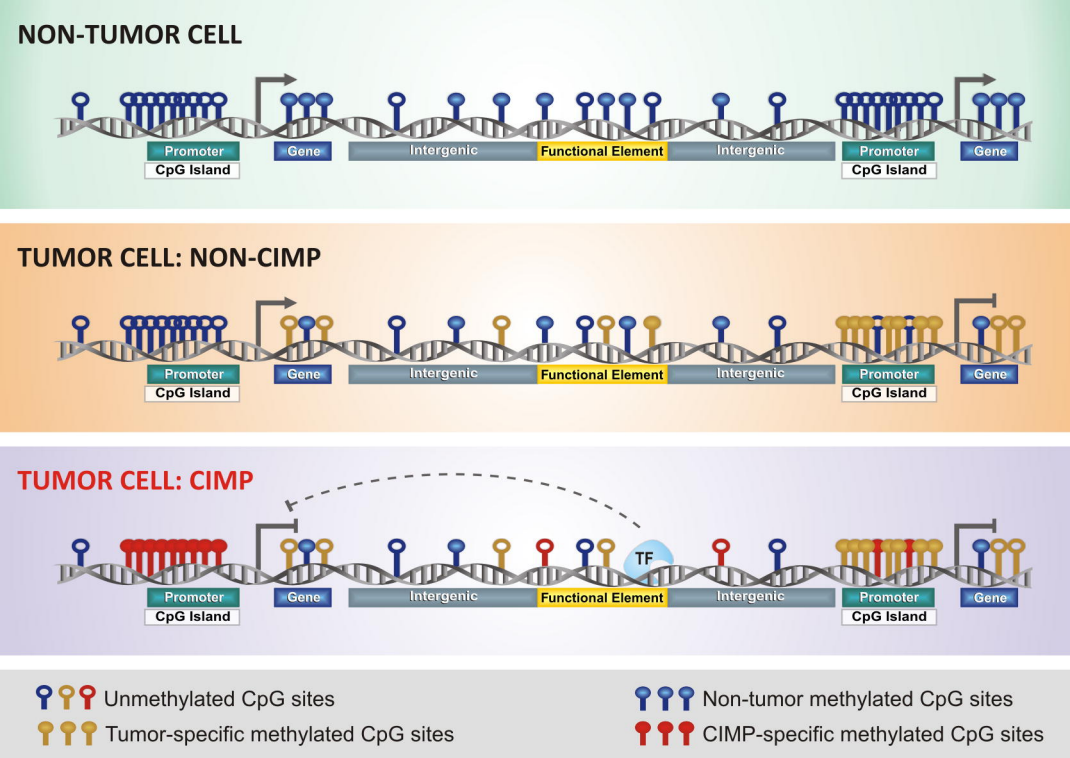
Figure 2. Summary of major milestones in the integration of large-scale genomic and epigenomic data that uncovered molecular and clinical phenotypes associated with pediatric and adult gliomas (timeline). Each milestone is indicated by marker papers that reported key molecular findings with clinical implications, along with a bullet summarizing their contribution. The timeline is divided by the 2016 WHO publication period (before and after) (Modified from the original copyfree design by Freepik).

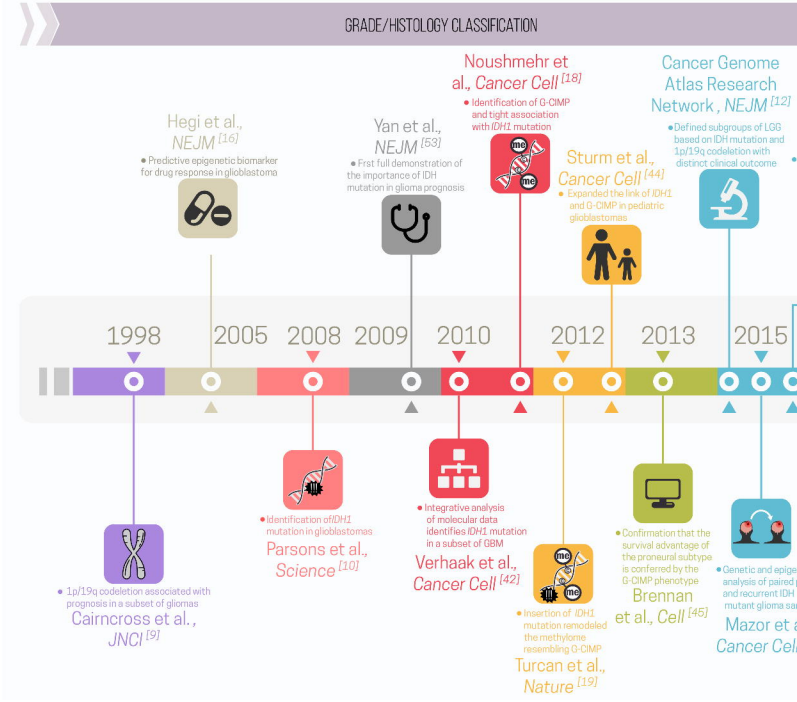
Figure 3. Chromatin changes in the progression of G-CIMP-high to G-CIMP-low tumors. This illustration shows a model of chromatin reorganization during the progression from G-CIMP-high to G-CIMP-low. G-CIMP-low (lower panel) shows loss of DNA methylation at specific loci causing disruption of CTCF binding sites, reorganization of chromatin, and dysregulation of gene expression (upper panel).

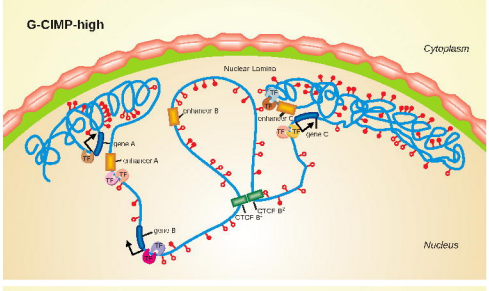
Figure 4. Overview of major discoveries that define G-CIMP-high and G-CIMP-low glioma subtypes. G-CIMP-high and G-CIMP-low tumors share the following genomic alterations: IDH mutant-1p/19q intact, TERT promoter wild type, and ATRX and TP53 mutant. However, G-CIMP-low subset defined a subgroup of *IDH*-mutant 1p/19q intact gliomas associated with DNA demethylation. Changes in chromatin architecture led to the imbalance between insulators and enhancers and the consequent activation of cell-cycle related genes, increase in stemness features, and poor clinical outcome compared

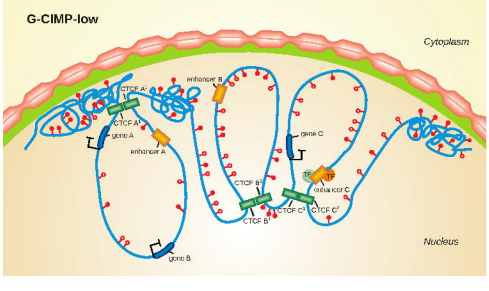
to G-CIMP-high gliomas (cartoon representation, not to scale).

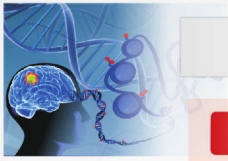
Figure 5. Perspectives in incorporating G-CIMP subsets into the current 2016 WHO glioma classification algorithm. A simplified diagram for glioma classification based on histological, genetic and epigenetic features. The incorporation of G-CIMP subsets further refined glioma classification. NGS: next-generation sequencing; PCR: polymerase chain reaction; FISH: fluorescent in situ hybridization; LOH: loss of heterozygosity; SNP: single-nucleotide polymorphism; wt: wildtype (Modified from the original copyfree design by Freepik).


NON-TUMOR CELL
Promoter
CpG Island
Gene
Intergenic
Functional Element
Intergenic
Promoter
CpG Island
Gene
TUMOR CELL: NON-CIMP
Promoter
CpG Island
Gene
Intergenic
Functional Element
Intergenic
Promoter
CpG Island
Gene
TUMOR CELL: CIMP
TF
Promoter
CpG Island
Gene
Intergenic
Functional Element
Intergenic
Promoter
CpG Island
Gene
Unmethylated CpG sites
Tumor-specific methylated CpG sites
Non-tumor methylated CpG sites
CIMP-specific methylated CpG sites


GRADE/HISTOLOGY CLASSIFICATION
1998
2005
2008
2009
2010
2012
2013
2015
Cairncross et al., JNCI [9]
• 1p/19q codeletion associated with prognosis in a subset of gliomas
Hegi et al., NEJM [16]
• Predictive epigenetic biomarker for drug response in glioblastoma
Parsons et al., Science [10]
• Identification of IDH1 mutation in glioblastomas
Yan et al., NEJM [53]
• First full demonstration of the importance of IDH mutation in glioma prognosis
Verhaak et al., Cancer Cell [42]
• Integrative analysis of molecular data identifies IDH1 mutation in a subset of GBM
Noushmehr et al., Cancer Cell [18]
• Identification of G-CIMP and tight association with IDH1 mutation
Turcan et al., Nature [19]
• Insertion of IDH1 mutation remodeled the methylome resembling G-CIMP
Sturm et al., Cancer Cell [44]
• Expanded the link of IDH1 and G-CIMP in pediatric glioblastomas
Brennan et al., Cell [45]
• Confirmation that the survival advantage of the proneural subtype is conferred by the G-CIMP phenotype
Cancer Genome Atlas Research Network, NEJM [12]
• Defined subgroups of LGG based on IDH mutation and 1p/19q codeletion with distinct clinical outcome
Mazor et a
Cancer Cell
• Genetic and epige
analysis of paired
and recurrent IDH
mutant glioma sa


G-CIMP-high
Cytoplasm
Nuclear Lamina
enhancer B
enhancer C
gene A
enhancer A
TF
gene C
CTCF B'
CTCF B''
gene B
Nucleus


G-CIMP-low
Cytoplasm
CTCF A'
CTCF A''
gene A
enhancer A
enhancer B
gene C
TF
enhancer C
CTCF B'
CTCF B''
CTCF C'
CTCF C''
gene B
Nucleus



GLIOMA IDHmut 1p/19q-intact

TP53 and ATRX mutant; TERT intact

	G-CIMP-high		G-CIMP-low	
DNA methylation levels (genome-wide)		Loss of DNA methylation		Published
Epigenetically active cell cycle pathway		Proliferation		
Patient overall survival rate		Poor prognosis		

Progression?

Epigenetic stemness signature		Stem cell-like		Unpublished
Epigenomic Landscape		Reorganization		

Diagnostic criteria/ methods
Astrocytoma/Oligodendroglioma/ Oligoastrocytoma/Glioblastoma
Adult Glioma
Histology
Histo pathologic
IDH1/2 status
IDH1/2 mutant
IDH1/2 wt
Immunohistochemistry NGS PCR
Genomic
1p/19q status
1p/19q intact
1p/19q codel
FISH LOH assay SNP array
G-CIMP status
G-CIMP high
G-CIMP low
NGS Methylation-specific PCR Methylation array
Epigenomic
Classification criteria