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REVIEW ARTICLE

Overview of DNA methylation in adult diffuse gliomas

Kosuke Aoki[1] · **Atsushi Natsume**[1]

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Abstract
Adult diffuse gliomas form a heterogeneous group of tumors of the central nervous system that vary greatly in histology and prognosis. A significant advance during the last decade has been the identification of a set of genetic lesions that correlate well with histology and clinical outcome in diffuse gliomas. Most characteristic driver mutations consist of *isocitrate dehydrogenase 1* (*IDH1*) and *IDH2*, and *H3 histone family member 3A*, which are strongly associated with DNA and histone methylation patterns. A well-characterized DNA methylation aberration is on the O6-methylguanine-DNA methyltransferase promoter. This aberration is associated with an improved response to the DNA alkylating agent, temozolomide. Methylation alterations are used for classification or treatment decisions of diffuse gliomas. This supports the importance of considering epigenomic aberrations in the pathogenesis of gliomas. Recent DNA methylation analyses revealed a small group of IDH mutant diffuse gliomas exhibiting decreased DNA hypermethylation resulting in substantial unfavorable prognosis comparable to glioblastoma. Thus, DNA methylation patterns may become a new standard that replaces the conventional grading system based on histological diagnosis. In this review, we summarize recent developments regarding the contributions of methylation patterns to the pathogenesis of adult diffuse glioma, the interactions between methylation patterns and driver mutations, and potential epigenomic targeted therapies.

Keywords Diffuse glioma · DNA methylation · G-CIMP · MGMT promoter methylation

Introduction

Diffuse gliomas, the most frequent primary brain tumors, are a heterogeneous group of brain tumors with distinct histological and clinical features. Classification and tumor grading of diffuse gliomas were originally defined by histological diagnosis [1]. However, this classification strategy is subjective and does not reflect intratumoral heterogeneity and interobserver variation of the histological diagnosis, suggesting that it cannot reliably guide patient care [2, 3]. The advent of microarray and next-generation sequencing enabled genome-wide genomic and transcriptomic sequencing and has revealed several genetic alterations that clearly classify diffuse gliomas into discrete subtypes with characteristic molecular and clinical features. This knowledge led to the adoption of an integrated diagnosis with molecular information in the World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS), revised in 2016 [4].

Mutations of *isocitrate dehydrogenase 1 (IDH1)* and *IDH2* occur in the majority of lower grade gliomas (LGGs) [5, 6]. IDH mutant LGGs are subclassified into two groups based on the presence (oligodendroglioma) or absence (astrocytoma) of chromosome 1p and 19q co-deletion (1p19q) [7, 8]. Glioblastomas (GBM) are also classified based on the IDH mutation. IDH mutant GBM has genetic alterations and an age distribution similar to LGGs, and largely consists of secondary GBM that originates from a preexisting LGG [5, 9]. IDH mutations lead to the production of 2-hydroxyglutarate (2-HG) that inhibits the activity of histone and DNA demethylases, resulting in the hypermethylation of DNA and histones that drive the disease phenotype.

Other diffuse gliomas frequently have mutations in genes that encode chromatin-regulating enzymes. *H3 histone family member 3A* (*H3F3A*) and *histone cluster 1 H3 family member b/c* (*HIST1H3B/C*) encode the H3.3 and H3.1 histone H3 variants, respectively. *H3K27M*, which results from

✉ Kosuke Aoki
aoki-ngy@umin.ac.jp

[1] Department of Neurosurgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

replacement of the 27th lysine (K) residue of these genes by methionine (M), is a characteristic mutation for pediatric midline high-grade gliomas, whereas *H3G34R/V*, which results from replacement of the 34th glycine (G) residue by arginine (R) or valine (V), is found frequently in hemispheric high-grade gliomas in children and young adults [10–12]. The *H3F3A* mutation inhibits methyltransferases resulting in aberrant genome-wide DNA methylation patterns.

These driver gene mutations alter genome-wide or focal DNA methylation patterns, and clearly classify diffuse gliomas into distinct subtypes with characteristic clinical features, including age distribution, tumor location, and prognosis. This suggests the importance of epigenomic changes in the initiation and progression of diffuse gliomas. Several classification schemes based on genome-wide DNA methylation were proposed and could provide further refinement in each defined subgroup of diffuse gliomas. Here, we review recent developments regarding the contributions of DNA methylation patterns to the pathogenesis of adult diffuse glioma, the interactions between methylation patterns and driver mutations, and potential epigenomic targeted therapies, including inhibitors of DNA methylation and IDH mutant enzymes.

DNA methylation

One of the most commonly studied epigenetic alterations in malignant tumors is DNA methylation. DNA methylation is the covalent transfer of a methyl group to the 5′ position of the cytosine ring, primarily at a cytosine-phosphate-guanine (CpG) dinucleotide, resulting in 5-methylcytosine. The DNA methylation status results from the action of methyltransferases and demethyltransferases. DNA methyltransferase 1 (DNMT1) is responsible for maintenance of the DNA methylation pattern after DNA replication, whereas DNMT3A, DNMT3B, and DNMT3L are responsible for de novo methylation [13–15]. The ten-eleven translocation family of enzymes 1–3 are involved in DNA demethylation by conversion of 5-methylcytosine to 5-hydroxymethylcytosine (Fig. 1) [16]. CpG islands are short interspersed DNA sequences that deviate significantly from the average genomic pattern by an elevated G + C base composition, and are located preferentially at gene promoters [17].

One of the most important characteristics of cancer is the decrease in global DNA methylation (demethylation) affecting intergenic regions, DNA repetitive sequences, and gene bodies, including regulatory sequences. By contrast, hypermethylation of CpG islands in promoter regions is also a frequent phenomenon [18]. Such hypermethylation is an important mechanism of inducing transcriptional silencing of tumor suppressor and DNA repair genes, and may be a critical step during tumor formation [19, 20]. In

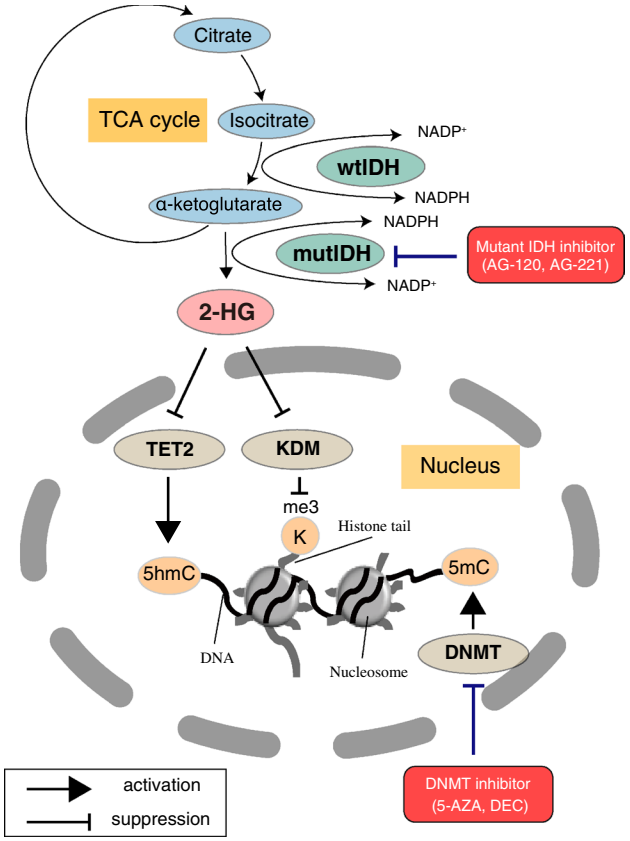

Fig. 1 Mutant isocitrate dehydrogenase (IDH)-induced methyltransferase of histones and DNA. Wild-type IDH converts isocitrate into α-ketoglutarate (α-KG), producing NADPH. Mutant IDH converts α-KG into 2-hydroxyglutarate (2-HG), consuming NADPH. 2-HG inhibits the activity of histone lysine demethylase and ten-eleven translocation enzymes (DNA demethylases), resulting in the hypermethylation of histones and DNA. Inhibition of DNA methyltransferase and mutant IDH results in demethylation of DNA and/or histones. *wtIDH* wild-type IDH, *mutIDH* mutant IDH, *3me* trimethylation, *5mC* 5-methylcytosine, *5hmC* 5-hydroxymethylcytosine, *5-AZA* 5-azacytidine, *DEC* 5-aza-2′-decitabine

GBM, aberrant promoter DNA methylation patterns of genes involved in key biological pathways have been reported. For example, the retinoblastoma, receptor tyrosine kinase (RTK)/phosphoinositide 3-kinase (PI3K), p53, and WNT pathways are affected by hypermethylation of CpG island promoters [21–27].

Glioma CpG island methylator phenotype (G-CIMP) and IDH mutation

Originally, the CIMP was defined by the genome-wide hypermethylation of CpG islands, first described in the context of colorectal cancers [28]. The concept of CIMP quickly became the focus of several cancer studies, including gliomas. A large fraction of gliomas exhibit substantial DNA

hypermethylation and have been termed as G-CIMP [29, 30]. CIMP-positive tumors carry distinct clinicopathological and molecular features; the G-CIMP-positive subtype is closely associated with mutations of *IDH1* and *IDH2*, and an improved prognosis [30].

Somatic mutations in IDH are observed in a wide spectrum of human cancers, most commonly diffuse gliomas, myeloid malignancies, chondrosarcomas, and intrahepatic cholangiocarcinomas [5, 31–33]. IDH is an important metabolic enzyme in the tricarboxylic acid cycle. Wild-type IDH catalyzes the oxidative decarboxylation of isocitrate into α-ketoglutarate (α-KG), which is associated with the regulation of histone and DNA demethylation in normal cells (Fig. 1). IDH gene mutations are drivers for the development of gliomas and map to specific arginine residues within the catalytic pocket of the enzyme; mutations in *IDH1* and *IDH2* occur mostly at arginines 132 and 172, respectively [34, 35]. Mutant IDH converts α-KG to the so-called "oncometabolite" 2-HG, which is a competitive inhibitor of the protein family of α-KG-dependent dioxygenases, including histone lysine demethylase and the ten-eleven translocation family enzymes involved in DNA demethylation (Fig. 1) [36, 37]. Because αKG regulates histone and DNA demethylation, inhibition of α-KG by 2-HG results in cellular hypermethylation. Mutant IDH changes the cellular redox environment by altering the ratio of NADPH to $NADP^+$, and is also involved with hypoxia inducible factor-1α regulatory proteins and nucleic acid metabolism [38]. Furthermore, IDH mutant gliomas display a characteristic tumor immune microenvironment exhibiting a lower number of tumor-infiltrating lymphocytes and perturbation of nuclear factor of activated T cells transcriptional activity and polyamine biosynthesis, resulting in suppression of T cell activity [39].

O6-Methylguanine-DNA methyltransferase (MGMT) promoter methylation

MGMT is a DNA repair protein involved in cellular defenses against mutagenesis and toxicity from alkylating agents, including temozolomide. Within diffuse gliomas, most well-characterized DNA methylation aberrations are not only G-CIMP, but also MGMT promoter DNA methylation. Patients harboring gliomas with MGMT promoter DNA methylation demonstrate increased overall survival and time to progression of the disease after chemotherapy and/or radiotherapy [40, 41]. Approximately 80% of IDH mutant secondary high-grade gliomas (anaplastic astrocytoma or GBM) have MGMT methylation, suggesting a strong correlation between the two [42]. Some studies have used the MGMT methylation status as a stratification tool in clinical trials [43]. Although there is a strong correlation between IDH mutation and MGMT methylation in gliomas, the role of MGMT methylation status on the benefit from temozolomide therapy in IDH mutant glioma is less clear [44]. IDH mutant grade II and III diffuse gliomas (LGGs), in contrast to GBM, usually carry two copies of chromosome 10 on which MGMT resides (10q26.3). Thus, MGMT may not be silenced completely, resulting in resistance of MGMT to temozolomide therapy [45].

DNA methylation-based classification of diffuse gliomas

The cancer methylome consists of not only somatically acquired changes in DNA methylation, but also characteristics retaining some traits of the cell of origin. This enables one to determine the origin of metastasis of unknown primary cancers [46, 47]. Furthermore, the DNA methylation profile of cancers can be used to subclassify CNS tumors, that were considered previously to be homogeneous diseases, into discrete subtypes with characteristic molecular and clinical features [48, 49]. Recently, Capper et al. defined 82 methylation-based CNS tumor classes, encompassing new and known tumor groups, using tissue samples from more than 2800 individuals with cancer [50]. The results of this classification scheme suggested that methylation profiling may offer an avenue for expanding and improving CNS tumor diagnoses.

Ceccarelli et al. performed a large genome and methylome analyses using over 1000 diffuse gliomas from The Cancer Genome Atlas dataset and found seven discrete subtypes with distinct molecular and clinical features (Table 1) [51]. This DNA methylation-based classification recapitulated the aforementioned diffuse glioma classification based on IDH mutations and 1p19q co-deletion. In IDH wild-type tumors, gliomas were further classified into four subgroups: classic-like, mesenchymal-like, LGm6-GBM, and pilocytic astrocytoma (PA)-like. Most classic-like and mesenchymal-like tumors belonged to "classical" and "mesenchymal" gene expression-based subgroups, respectively, which were described by Verhaak et al. [52]. The PA-like subgroups contained a larger fraction of LGG compared to "non-PA-like" IDH-wild-type clusters, including classic-like, mesenchymal-like, and LGGm6-GBM tumors. The PA-like subgroup exhibited distinct clinical features including markedly longer survival and younger age, and was characterized by a relatively low frequency of typical GBM genetic alterations. By contrast, non-PA-like IDH-wild-type tumors carried a poor prognosis and frequent GBM-like genetic alterations such as epidermal growth factor receptor amplification, telomerase reverse transcriptase promoter mutation, and chromosome seven gain and ten loss. Thus, most non-PA-like IDH-wild-type tumors, regardless of histological diagnosis, are classified into WHO grade IV according to cIMPACT-NOW

Table 1 Characteristics of DNA methylation subtypes described by Ceccarelli et al.

DNA methylation subtypes				
G-CIMP-high	G-CIMP-low	Codel	PA-like	Classic-like/ mesenchymal-like/LGm6-GBM
IDH mutation status				
IDH mutant	IDH mutant	IDH mutant	IDH wild-type	IDH wild-type
1p19q co-deletion				
1p19q intact	1p19q intact	1p19q co-deletion	–	–
Age at diagnosis—median (25th and 75th percentile)				
36 (30–49)	36 (30–44)	45 (36–54)	32 (30–49)	60 (52–69)
Glioblastoma—% of tumors				
6%	60%	0%	0%	85%
Characteristic genetic alterations				
–	RTK/PI3K cell cycle	–	–	*EGFR*amp, *pTERT*mut, seven gain, ten loss
Overall survival (month)—median (95% confidence interval)				
87.4 (65.7–130.7)	32.4 (22.7–NR)	95.5 (78.2–NR)	133.7 (25.5–NR)	13.8 (12.2–15.4)

amp amplification, *G-CIMP* glioma CpG island methylator phenotype, *mut* mutation, *NR* not reach, *PA* pilocytic astrocytoma, *RTK/PI3K* receptor tyrosine kinase/phosphoinositide 3 kinase, *pTERT* TERT promote

update 3 [53]. In IDH mutated and non-1p19q-co-deleted tumors (astrocytoma IDH-mut), they are further classified into two subgroups based on the extent of DNA methylation: G-CIMP-high and -low. The G-CIMP-low subgroup exhibits a substantial unfavorable prognosis comparable to GBM, and carries genetic abnormalities in cell cycle and RTK/PI3K genes such as *CDKN2A, CDK4*, and *PIK3R1*. These have been reported to be genetic alterations with unfavorable prognostic value in astrocytoma IDH-mut [54].

The updated WHO classification of CNS tumors represents a shift in tumor diagnosis by integrating histological and molecular findings [4]. However, in LGGs, the grading system based on histological criteria remains mainly focused on tumor proliferation activity. Regarding proliferative activity, there are no strict criteria for delineating mitotic index cut-off values to distinguish between grade II and III tumors. This could result in substantial interobserver variability in determining the grade [2, 3]. Some studies reported that, according to the WHO classification revised in 2016, the role of tumor grade in patient survival was not well-characterized in LGG, after accounting for the IDH mutation status and 1p19q co-deletion [34, 55, 56]. However, each LGG subgroup classified based on IDH mutation and 1p19q co-deletion has a highly variable clinical course. Based on these results, a more objective grading system using defined diffuse glioma subgroups is required. In diffuse gliomas, the G-CIMP-low subgroup may be the new standard that replaces the conventional grading system based on histological diagnosis.

DNA hypomethylation and tumor malignancy

To investigate the temporal dynamics of methylation patterns of G-CIMP, de Souza et al. compared DNA methylation profiles using primary and recurrent samples of LGGs from 77 patients (200 tumors). The results showed that some primary G-CIMP-high cases exhibited a demethylation pattern after disease recurrence that was observed primarily in G-CIMP-low tumors. This result suggested that the G-CIMP-high subgroup was a predecessor to the G-CIMP-low subgroup [51, 57]. A dramatic loss of DNA methylation during the progression and/or recurrence of IDH mutant LGGs was also reported in other papers [58, 59].

Mechanisms by which DNA hypomethylation is associated with tumor malignancy involve altered cis-regulatory elements as well as promoter hypomethylation that lead to transcriptional upregulation of genes [58]. CpG sites are found not only in gene promoters, but also in gene bodies or cis-regulatory elements such as enhancers, silencers, and insulators [60, 61]. These regulatory elements contain binding sites for transcription factors and act to increase or decrease transcription. Interplay within the protein-DNA complex forms a three-dimensional folded chromatin loop called a topology associated domain (TAD). TADs are mediated by insulator proteins containing CCCTC-binding factors (CTCFs). The chromatin structure is composed of loops, and CTCFs block communication between enhancers and promoters in intergenic regions (Fig. 2). In IDH

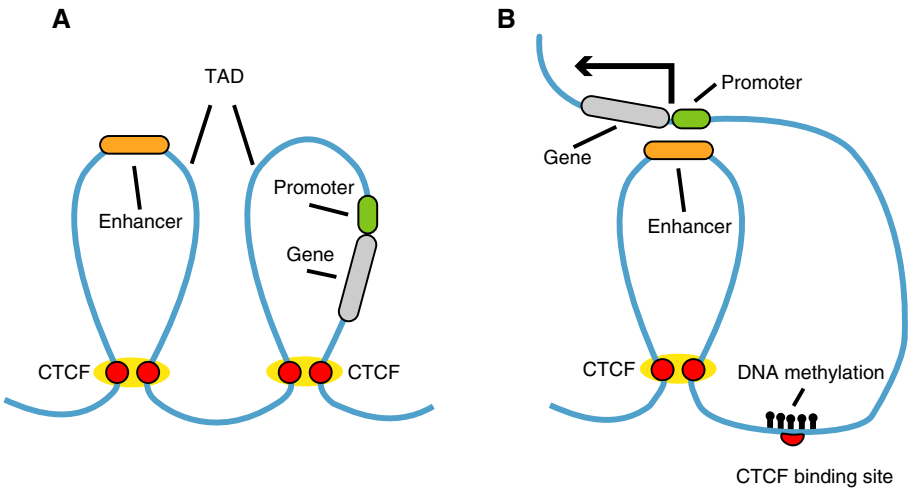

Fig. 2 Chromatin disorganization associated with DNA methylation in CCCTC-binding factor (CTCF) binding sites. **a** Topology associated domains (TADs) are mediated by insulator CTCF proteins that block the interaction between enhancers and promoters. **b** DNA methylation within CTCF binding sites reduces the capacity of CTCF binding. This leads to aberrant enhancer-gene interactions and the upregulation of oncogenes

mutant gliomas, hypermethylation at CTCF binding sites reduces the CTCF binding capacity. This leads to aberrant enhancer–gene interactions and the upregulation of oncogenes such as platelet-derived growth factor receptor-α in glioblastoma [62]. It is speculated that the G-CIMP-low subgroup exhibits a loss of genome-wide DNA methylation, including CTCF binding sites, influencing chromatin architecture by disrupting insulator binding [63].

DNA methylation targeted therapies

Genetic and epigenetic alterations, including mutations of *IDH* and *H3F3A*, are associated with tumor initiation and progression in diffuse gliomas. Furthermore, tumor cells routinely use the epigenomic process to escape from chemotherapy and host immune responses. Hence, a growing emphasis of recent drug discovery efforts has been on targeting the epigenome, including DNA methylation [64].

There are two classes of drugs targeting the epigenome: broad reprogrammers and drugs targeting focal regions (targeted therapy). Broad reprogrammers include DNA methylation inhibitors (DNMTi), histone deacetylase inhibitors, and bromodomain and extra-terminal motif protein inhibitors. Epigenomic targeted therapies include inhibitors of enhancer of zeste 2 polycomb repressive complex 2 (EZH2) and IDH. EZH2 targets within the polycomb repressor complex, which H3K27M inhibits [65].

DNMTi promote global DNA demethylation in a dose-dependent manner by depleting or degrading DNA methyltransferases, resulting in large-scale changes in gene expression. However, they generally did not tend to affect cancer-specific gene expression [60, 66]. DNMTi, such as 5-azacytidine and 5-aza-2′-decitabine, are effective against hematologic neoplasms and are approved by the United States Food and Drug Administration (FDA) for treating the myelodysplastic syndrome, which can progress to a rapidly growing cancer of bone marrow cells called acute myeloid leukemia (AML) [67, 68]. However, the effects of DNMTi are diverse and generally have a slow onset. Additionally, low-dose 5-azacytidine or 5-aza-2′-decitabine treatment can induce long-lasting decreases in self-renewal and tumorigenicity of tumor-initiating cells with minimal cytotoxic effects [69]. These results suggest mechanisms other than the inactivation of tumor-suppressor genes and the activation of crucial oncogenes must exist by which DNMTi can target cancer. For example, DNMTi induce a cell-autonomous immune activation response by permitting the expression of endogenous retroviruses that were silenced previously by DNA methylation [70, 71]. This antiviral response may underlie some of the antitumor activity of these drugs. A next generation DNA hypomethylating agent, guadecitabine, inhibits DNMT with better pharmacodynamic characteristics; a phase 3 study is currently being conducted in AML to delineate its effectiveness [72]. As for diffuse gliomas, a phase 1 study for 5-azacytidine is underway for patients with hematologic or solid tumor malignancies, including GBM.

As mentioned previously, *IDH* mutations are drivers in diffuse gliomas through 2HG production, leading to DNA and histone hypermethylation. Because the effects of 2HG on chromatin and cell differentiation are largely reversible, IDH mutant enzyme inhibiting agents may be useful for treating IDH mutant malignancies [73]. First-generation IDH mutant enzyme inhibitors demonstrated activity in AML [74, 75]. Recently, the IDH1 inhibitor, ivosidenib (AG-120), and the mutant IDH2 inhibitor, enasidenib (AG-221), induced clinical responses in phase 1/2 trials and were approved by the FDA for patients with relapsed/refractory IDH mutant AML [76, 77]. In diffuse gliomas, the mutant IDH1 inhibitor induced near-complete 2HG inhibition in vitro and in vivo, but not all IDH mutant glioma cell lines were sensitive to these inhibitors, possibly because of no appreciable

changes in genome-wide DNA methylation [78, 79]. Some basic studies suggested that inhibition of mutant IDH may induce proliferation of IDH mutant glioma cell lines in vitro [78]. Decreased DNA hypermethylation was associated with the malignant phenotype and decreased overall survival in an astrocytoma IDH mutant, as mentioned previously. Genome-wide or focal demethylation with DNMTi or IDH mutant inhibiting drugs could inactivate tumor-suppressor genes, activate oncogenes, and/or promote demethylation of MGMT, which could lead to resistance to the alkylating agent, temozolomide, in GBM. Moreover, while mutation of IDH initiates gliomagenesis, and is retained upon glioma recurrence, mutant IDH and 2HG might not be required for clonal expansion at tumor recurrence [80]. This raises questions about its importance for tumor maintenance, and the suitability of targeting IDH mutants for treatment. By contrast, one clinical study showed the effectiveness of IDH inhibitors. Specifically, AG-120 monotherapy was associated with a favorable safety profile and prolonged stable disease in a previously treated non-enhancing *IDH1* mutant glioma patient population [81]. Furthermore, additional IDH inhibitors were designed and have entered clinical trials. These include the *IDH1*-mutant inhibitors IDH-305, DS-1001b, and BAY-1436032, and a pan inhibitor of mutant IDH1 and IDH2 enzymes, vorasidenib (AG-881), which fully penetrates the blood–brain barrier. The results of these clinical trials are eagerly awaited.

Conclusion

Diffuse gliomas are increasingly understood to involve epigenetic alterations in addition to genetic modifications. Driver mutations in epigenetic regulator genes have clarified the etiology of diffuse gliomas and defined their molecular subtypes. The advent of genome-wide analysis technologies has revealed comprehensive DNA methylation patterns, as well as genomic and transcriptomic alterations in diffuse gliomas. DNA methylation-based classification schemes not only confirmed the aforementioned genetic subtypes according to IDH mutations and 1p19q co-deletion, but also identified the characteristic subtype "G-CIMP-low", which exhibited decreased DNA hypermethylation and poor prognosis compared to its G-CIMP-high counterpart. Longitudinal studies suggested that the G-CIMP-low subgroup was a successor to the G-CIMP-high subgroup. It is expected that an analysis of the effect of demethylating specific genes will improve our understanding of the pathogenesis of glioma, regardless of its histological grade.

Researchers are rapidly developing drugs targeting the epigenome, including DNA methylation and mutant IDH, for treating IDH mutant tumors. While not all *IDH* mutant glioma cell lines were sensitive to these inhibitors, IDH inhibitors prolonged stable disease in patients with non-enhancing *IDH1* mutant glioma. In general, non-enhancement in magnetic resonance imaging is a characteristic feature of LGG, not GBM. LGG accounts for 94 and 40% of G-CIMP-high and -low, respectively (Table 1). Thus, the extent of DNA methylation is possibly associated with whether or not the IDH inhibitor is effective.

Although this review focuses on aberrations in DNA methylation, additional epigenetic alterations contribute to the pathogenesis of glioma. These alterations include aberrant epigenetic regulation and altered histone modification patterns. A thorough examination of epigenetic alterations may reveal novel avenues of treatment, consequently increasing survival rates in patients with diffuse gliomas.

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