Glioma epigenetics: From subclassification to novel treatment options

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ABSTRACT

Gliomas are the most common malignant primary brain tumors, of which glioblastoma is the most malignant form (WHO grade IV), and notorious for treatment resistance. Over the last decade mutations in epigenetic regulator genes have been identified as key drivers of subtypes of gliomas with distinct clinical features. Most characteristic are mutations in IDH1 or IDH2 in lower grade gliomas, and 

2.1. Epigenetic subtypes of gliomas

Insights into the molecular landscape of diffuse gliomas have revealed characteristic genetic and epigenetic profiles which have clarified their etiologic evolution [5,10–16] and allowed their classification into distinct molecular subtypes that have been integrated into the 2016 WHO classification (Fig. 1) [17]. Mutations in the epigenetic modulator genes isocitrate dehydrogenase 1 or 2 (IDH1 or IDH2), and in the histone genes H3F3A or HIST1H3B have become key biomarkers for tumor classification and emphasize the important role of epigenetic alterations as drivers in the evolution and biology of gliomas [10,12,18–20].

A point mutation in IDH1 or IDH2 (IDHmt) is characteristic for lower grade gliomas (WHO grade II/III), which are most prevalent in young adults. IDHmt gliomas are further subdivided into two major subtypes: oligodendrogliomas, with codeletion of chromosomal arms 1p/19q (1p/19q codel) that are usually associated with an activating mutation in the promoter of TERT, and astrocytomas, without 1p/19q codel. The latter are almost always associated with a mutation in TP53, and a mutation in ATRX that leads to loss of its nuclear expression, and diagnostically can be determined by immunohistochemistry [20]. Low grade gliomas without IDH mutation are termed IDH wild-type (IDHwt)
astrocytomas and are considered a provisional entity by the 2016 WHO classification. Upon further genetic analyses they may be classified into other entities [21]. In glioblastomas IDHmt are infrequent (< 10%) [10], and are usually observed in younger patients whose tumors may have progressed from an IDHmt non-codeleted lower grade glioma WHO grade II or III [22]. The histone mutation H3K27 M is characteristic for pediatric midline high grade glioma and the H3G34R/V mutation for hemispheric high grade glioma in children and young adults [23]. Most interestingly, these epigenetic driver mutations are associated with characteristic DNA methylation profiles, display characteristic age distributions and tumor locations that is suggestive of brain development related associations and are considered different diseases Fig. 1 [12,23].

### 2.2. DNA methylation

The most commonly studied epigenetic alterations in cancer comprise changes in DNA methylation, in particular methylation at the 5th position of cytosines at CpG sites, resulting in 5-methylcytosine, also known as the “fifth base” of DNA. There are several DNA methyltransferases involved in DNA methylation, of which all use S-adenosyl-l-methionine as source of methyl groups. DNMT1 preferentially methylates hemi-methylated DNA and is responsible for maintenance DNA methylation patterns during replication, while DNMT3A, DNMT3B, and DNMT3L act on unmethylated DNA and are responsible for de novo methylation [24,25]. DNA demethylation involves the ten-eleven translocation family of enzymes TET(1–3) that convert 5mC to 5-hydroxymethylcytosine (5hmC) [26]. Additional epigenetic DNA modifications are known, however, their identification is technically more challenging and their function is less well studied [27–29]. Cancer development in general is associated with global DNA demethylation (hypomethylation) affecting intergenic regions, DNA repetitive sequences, gene bodies, including regulatory sequences; and aberrant de novo methylation of CpG islands (hypermethylation) in promoter regions of tumor suppressor genes [reviewed in [30]]. CpG islands refer to regions with high density of CpGs within a sequence and are often located in the regulatory region of promoters and are unmethylated in non-cancerous tissue [31]. Epigenetic gene silencing following CpG island methylation is mediated through methyl-CpG-binding domain (MBD) proteins such as MECP2 that recruit histone-modifying and chromatin-remodeling complexes to the methylated sites. DNA methylation profiles of cancer are highly characteristic and retain some traits of cell of origin. They have been successfully employed for re-defining/ refining classification of brain tumors [12,32,33] or to determine the origin of metastasis of unknown primary cancer [34]. Most of the aforementioned studies reviewed here have been performed on the Illumina DNA methylation BeadChip platform that interrogates genome-wide DNA methylation and allows in addition gene copy number analysis. Hence, there are multiple efforts to develop molecular classifying algorithms based on data derived on the Illumina DNA methylation platform for WHO classification of brain tumors (e.g. The Heidelberg platform for next generation neuropathology can be used at: MolecularNeuropathology.org) [35]. Aberrant methylation of CpG islands in gene promoters leads to gene silencing affecting cancer relevant pathways associated with the hallmarks of cancer [36]. In glioblastomas activation of the WNT pathway is mediated by aberrant promoter methylation of multiple negative regulators, such as the gene encoding the WNT inhibitory factor 1 (WIF1) or the family of secreted frizzled-related proteins (sFRPs), dickkopf (DKK), and naked (NKDs) [37,38]. Similarly, negative regulators of the Ras pathway are silenced, such as the Ras association (RalGDS/AF-6) domain family member RASSF1A [39].

#### 2.3. The glioma CpG Island methylator phenotype associated with IDH1 or IDH2 mutations

Gliomas with mutations in the metabolic genes IDH1 or IDH2 display a striking signature of DNA hypermethylation that is completely different from IDHwt gliomas, and has been termed Glioma CpG Island Methylator Phenotype (G-CIMP) [11]. This fairly recent discovery has indicated a novel driver mechanism in tumor development, consequently IDHmt gliomas are now considered a different disease as reflected in the WHO 2016 classification [17]. IDH mutations are early lesions in the development of gliomas and cluster in the substrate binding site of these enzymes, at codon 132 of IDH1 or codon 172 of IDH2, respectively [40,41]. These mutations are always heterozygous and confer a gain of function that favors a neomorphic reaction catalyzing the conversion of α-ketoglutarate into D-2-hydroxyglutarate (2HG) [41]. 2HG acts as a so-called oncometabolite by accumulating to high concentrations that inhibit α-ketoglutarate-dependent enzymes. α-Ketoglutarate-dependent enzymes comprise epigenetic modifiers such as the enzyme TET2 involved in DNA demethylation or the lysine-specific histone demethylase KDM2A [28,29,42–45]. However, α-ketoglutarate-dependent enzymes are also involved in other cellular functions that are inhibited by 2HG, such as the DNA repair enzymes of the ALKBH family, thereby altering response to chemotherapy [46], or HIF1α regulating proteins, affecting hypoxia sensing/signalising [47]. Obviously, the cell metabolism is seriously disturbed. Respective vulnerabilities have been identified and proposed as treatment opportunities [45,48]. Similarly, acute myeloid leukemia (AML) display CIMP in presence of a mutation in IDH1 or IDH2, with a preference for IDH2, or a mutation in TET2. These mutations are mutually exclusive, and

<table>
<thead>
<tr>
<th>MGMT meth</th>
<th>&lt;5%</th>
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<td>Young adults</td>
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<td>• DNA hypomethylation</td>
<td>• Gain CHR 7</td>
<td>• G-CIMP</td>
<td>• G-CIMP</td>
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<td></td>
<td></td>
<td>• Loss CHR10</td>
<td>• ATRX mt</td>
<td>• TERTp-mt</td>
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<td>• TERTp-mt</td>
<td>• TP53mt</td>
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### Fig. 1. Major genetic and epigenetic subgroups of gliomas. Characteristic epigenetic alterations are written in bold. CHR, chromosome; H3, histone 3; G-CIMP, glioma CpG island methylator phenotype.
have been shown to preclude hematopoietic differentiation [44]. These insights have contributed to the elucidation of the underlying mechanisms of CIMP. IDH mutations are considered the drivers for the development of G-CIMP through the production of the oncomethylator 2HG that among other effects mediates DNA hypermethylation through inhibition of TET2 [44,49]. Moreover, IDHmt gliomas regardless of tumor grade display a distinct immune phenotype characterized by reduced expression of immune response signatures and by less infiltration of tumor-associated immune cells [50–52]. This may be mediated by various mechanisms, including G-CIMP-associated hypermethylation of immune response related genes in the tumor, as shown recently for CD274 (PD-L1) in IDHmt glioma [50], and by down-regulation of leukocyte chemotaxis [51,52]. Altogether the plethora of G-CIMP associated silenced genes, affecting multiple cancer relevant pathways, may hold opportunities for novel therapeutic approaches.

2.4. Clinically relevant epigenetic biomarkers

2.4.1. IDH status

Determination of the IDH mutation status is key for integrated glioma classification. An antibody specific for the most common mutant, IDH1R132H that accounts for >90% of all IDH mutations in gliomas, facilitates diagnosis [40,53]. Alternatively, the associated G-variant, IDH1R132H that accounts for >90% of all IDH mutations in young adults, a histone H3K27M mutation needs to be considered, the reliable measurement of 2HG in easily accessible longitudinal follow-up of patients, although this has not reached the clinic yet [54,55]. The oncomethylator 2HG can be detected in patients by magnetic resonance spectroscopy and may be used for longitudinal follow-up of patients, although this has not reached the clinical yet [54,55]. The reliable measurement of 2HG in easily accessible body fluids is hampered by the blood brain barrier and has not yielded a useful clinical test [56]. In IDHwt glioma of brain midline structures of young adults, a histone H3K27M mutation needs to be considered, which can be detected by immunohistochemistry with a H3K27M-specific antibody [20].

2.4.2. MGMT promoter methylation status predictive biomarker in glioblastoma

The DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT) is the most prominent epigenetically silenced gene in gliomas [57–59]. In glioblastomas promoter methylation of MGMT is predictive for benefit from the alkylating agent temozolomide, as shown in several phase III clinical trials, and the MGMT methylation status has become the first predictive biomarker in neuro-oncology [57,60–63]. MGMT repairs the most toxic lesion, O6-methylguanine, induced by alkylating agents, thereby blunting the treatment effects. This is reflected by the fact that patients with an unmethylated MGMT basically do not profit from the addition of temozolomide concomitant and adjuvant to radiotherapy [57]. Routine determination of the MGMT status allows for stratified treatment, and is used for selecting glioblastoma patients for clinical trials omitting temozolomide in the experimental arm [64–66]. The role of the MGMT methylation status on benefit from temozolomide in IDHmt lower grade gliomas is less clear [6], although most (>80%) show methylation at the MGMT promoter, probably as part of G-CIMP [67]. However, unlike glioblastomas who usually lose one copy of chromosome 10 on which MGMT resides, IDHmt lower grade gliomas usually retain both copies and MGMT may not be completely silenced, resulting in residual repair capacity of MGMT contributing to resistance to temozolomide therapy [68]. The most commonly used tests to determine the MGMT methylation status employ methylation specific PCR [69], as reviewed elsewhere [61]. A MGMT status classifier (MGMT-STM27) is available for samples analyzed on the Illumina DNA methylation platform (HM27 K, HM450 K, EPIC) [68,70] and is widely used, including in clinical trials [6,15,71].

2.5. Histone code

The DNA molecule is packed in the nucleus of the cell by anchoring proteins called histones. The formed complex of DNA and 8 histone components of the nucleosome is referred to as chromatin. The chromatin is the essential environment through which transcription factors and signalling pathways alter gene activity. Histones serve not only for spatial organization of the DNA double helix, but also exhibit covalent marks that orchestrate chromatin accessibility. Each of the 8 core histones has a N-terminal tail hanging from the nucleosome that is subjected to multiple post-translational modifications at specific residues, comprising methylation, acetylation, sumoylation, ubiquitination, phosphorylation, and others. In fact, over 500 distinct histone modifications have been described, whereas the function of only a fraction of them has been studied (reviewed in [72]). The histone marks are recognized by a class of epigenetic proteins called readers, which in concert with recruited proteins remodel particular genomic regions to modulate target gene expression. The histone marks may be removed by erasers and added by writers, thereby dynamically regulating the chromatin code [73]. Active genes have been associated with tri-methylation of lysine 4 (H3K4me3) and acetylation of lysine 9 (H3K9ac), while inactive genes may be decorated with H3K9me3 and H3K27me3. However, many active and inactive genes have overlapping patterns of histone modifications. In fact bivalent histone marks are a hallmark of embryonic stem cells (H3K27me3/H3K4me3) that keep genes in a poised state for rapid changes and may predispose important regulatory genes to inactivation by aberrant DNA hypermethylation, resulting in malignant transformation and tumor progression [74].

A recent study using the TCGA GBM and LGG datasets exposed that at least one of a set of 36 genes involved in chromatin organization was targeted by genetic alterations in 54% of gliomas [16]. Interestingly, most gliomas with alterations in this set of genes belonged to the molecular subgroup of IDHmt non-codeleted gliomas (n = 230, 87%) [16]. Among the genes predicted to be potential glioma drivers, several have epigenetic functions, such as ATRX (n = 226 mutations, 25% of all cases), SETD2 (n = 24, 1%), ARID2 (n = 20, 1%), DNMT3A (n = 11, 1%), SMARCA4 (n = 29, 3%), and ARID1A (n = 15, 1%) [16].

In addition to histone modifications, genes coding for histones are mutated in high grade glioma of children and adolescents [23]. Nearly 30% of pediatric high grade gliomas (WHO grades III and IV) harbor point mutations at specific sites (K27M/G and G34R/V) of histone 3 (H3) gene variants (H3F3A, approximately 85%; H3T113B/C mutually exclusive, 14%; and other more rare variants [75]). Of note, there are thirty H3 variants in the human genome, and therefore the mutant allele is present only in a minor proportion (7.6–17.6%) of the total H3 proteins [76]. Lysines (K) are key residues in histone tails subjected to post-translational modifications by methylation or acetylation. Therefore, substitution of K27 with methionine or isoleucine in H3 has an impact on the epigenetic state of cells. Mechanistically it was shown that mutated histones H3K27M/I directly bind to histone-lysine N-methyltransferase (EZH2), thereby inhibiting the enzymatic activity of the Polycomb repressive complex 2. The transgene encoding H3K27M was demonstrated to efficiently reduce H3K27me3 levels in vitro and in vivo [76]. Whereas the global levels of H3K27me3 are diminished in H3K27M mutated gliomas, certain genes were shown to consistently preserve their H3K27me3 mark [77].

2.6. Chromatin organization

Gene expression is also regulated by the overall 3D chromatin organization, which is modulated by several factors, including DNA methylation and histone marks. The structure of the chromatin is composed of loops or topology associated domains (TADs), which are conserved evolutionarily, and are largely cell type invariant. The organization of TADs is responsible for placing the enhancer regions and promoter regions into spatial proximity in order to activate target gene expression. Originally TADs have been considered to be invariant building blocks of chromosomes [78]. However, recent findings by the
group of Bernstein suggested that the architecture of TADs is disturbed in IDHmt glioma, allowing aberrant interactions between strong enhancers and oncogenes (Fig. 2). As a result of the reorganization of TADs, the transcription of tumor promoting factors (e.g. oncogenes and anti-apoptotic factors) is enhanced and promotes neoplastic growth [79]. These observations were associated with the finding that IDH1mt gliomas exhibit hypermethylation at cohesin and CCCTC-binding factor (CTCF)-binding sites, which inhibits the binding of insulator protein that is crucial for proper organization of TADs.

Overall, the functional impact of G-CIMP goes well beyond promoter hypermethylation-related effects and may be responsible for disrupting chromosomal topology and allowing aberrant regulatory interactions that may induce oncogene expression [79].

3. Targeting the glioma epigenome

Several different approaches of targeting epigenetic alterations have been or are being tested in clinical trials: those targeting mutant IDH either by small molecule inhibitors, or as target for vaccination in the respective patient population; and those targeting epigenetic modifiers affecting large parts of the epigenome such as BETi, HDACi, DNMTi, and EZH2i (Fig. 3). Respective clinical trials, as available on clinicaltrials.gov, are summarized in Table 1.

3.1. Inhibitors of mutant IDH (mtIDHi)

Inhibitors of mutant IDH are currently tested in several trials for patients with IDHmt gliomas with the aim to block the production of the oncometabolite 2HG to normalize the function of α-ketoglutarate dependent enzymes. Encouraging results from first preclinical studies suggested differentiation promoting effects and attenuation of growth in vitro and in vivo (subcutaneous xenografts) of glioma cells with an endogenous heterozygous IDH mutation, while no appreciable changes of overall DNA methylation was observed [80]. However, later reports indicated that inhibition of IDHmt may not inhibit growth of IDHmt glioma cells or propagation of orthotopic tumor xenografts [48,81]. On the contrary, inhibition of IDHmt was suggested to promote the growth of most tested IDHmt glioma lines in vitro [48]. A recent report adds to this controversy. 10% (6/50) of a set of paired samples (first resection/recurrence) was found to lose the IDHmt allele upon malignant progression. Overall G-CIMP was preserved, however, exhibiting some alterations in DNA methylation [82]. The latter may overlap with “G-CIMP-low” where progression related specific loss of methylation has been postulated to enhance expression of cell cycle related genes associated with worse prognosis [16]. The progression related loss of the IDHmt allele questions its importance for tumor maintenance and consequently the suitability as single drug target. This question is also of relevance for the vaccination trials targeting IDH1R132H, for which encouraging preclinical studies have been presented [83]. Interestingly, it has been suggested that reversing the inhibitory effect of 2HG on STAT1 mediated expression of IFN-γ–inducible chemokines may improve the vaccination approach [51]. The complexity of the different effects on tumor biology linked to targeting the IDHmt renders the outcome predictions difficult. Thus, the results of the clinical trials are eagerly awaited.

3.2. EZH2 inhibitors (EZH2i)

EZH2 is an interesting target within the polycomb repressor complex 2 (PRC2) in pediatric glioma, since the H3K27M mutation has been
shown to inhibit PRC2 activity [76]. The EZH2i Tazemetostat is currently tested in pediatric glioma with a gain of function mutation in EZH2 or loss of function mutations in the chromatin remodeling complex subunits SMARCB1 or SMARCA4 (Table 1). However, these mutations are rare in pediatric gliomas [18,75,84].

Preclinical studies of the EZH2i Tazemetostat show a mixed picture. While the EZH2i Tazemetostat was suggested to lack activity in pediatric glioma cells in vitro independent of H3.3 mutations [85], a recent study provided evidence that Tazemetostat may affect growth of primary H3K27M-positive glioma cells in presence of functional p16INK4A [86].

Short-term EZH2 depletion in glioblastoma cells without H3 or IDH mutations, has been associated with reduced proliferation [87], while recent results suggest that prolonged EZH2 inhibition may cause a switch in cell fate, enhancing proliferation and DNA damage repair, resulting in tumor progression [88].

3.3. DNA methylation inhibitors (DNMTi)

Preclinical studies have suggested efficacy of DNA methylation inhibitors (DNMTi) in in vitro and in vivo models of IDHmt glioma [81,89]. However, this has not translated into successful treatments with DNMTi in glioma patients, possibly because 5-Azacytidine and Decitabine are S-phase-specific and have relatively short half lives [90]. The novel second-generation hypomethylating drug guadecitabine with better pharmacodynamic characteristics is currently tested in a phase III study in AML [91]. However, it remains controversial whether general demethylation is desired in glioma, as unwanted proto-oncogenes may be activated, and demethylation of the repair gene MGMT may render glioblastomas resistant to alkylating agents that are part of the standard of care. Interestingly, low dose demethylating agents impact immune regulation and may induce innate immune response by reactivating retroviruses [92,93].

3.4. Histone deacetylase inhibitors (HDACi)

The underlying rationale of using HDACi as cancer therapeutics is to reverse dysregulated target gene expression by modulating histone acetylation marks [94]. Dynamic regulation of the chromatin state is mediated by mechanisms such as covalent modification of chromatin, which includes histone acetylation and methylation, and ATP-dependent chromatin remodeling. Histone 3 lysine acetylation is a mark of active enhancers that control the expression of associated distal genes. Generally, exposure to HDACi results in hyperacetylation of histones, which globally affects gene expression.

Vorinostat has been tested in phase II trials of recurrent glioblastomas, first as single agent that has shown good tolerability. Moreover, the study also indicated that the drug affected target pathways in glioblastomas [95]. Taken to the next step, vorinostat was tested in combination with the protease inhibitor bortezomib that however, in the dosing scheme used showed no efficacy [96]. In newly diagnosed glioblastoma the addition of vorinostat to the standard of care of radiochemotherapy did not meet the primary endpoint of efficacy in a phase I/II trial. However, indications from molecular subgroup analyses may provide criteria for future patient selection [97].
Discoveries of the last decade have completely changed our view on the genomic and epigenetic landscape of human gliomas. Driver mutations in epigenetic regulator genes have clarified their etiology and defined molecular subtypes with distinct biology. Consequently, epigenetic biomarkers play now a central role in tumor classification and decision making for stratified therapies. A whole arsenal of epigenetic drugs promises to target epigenetically deregulated pathways by interfering on several levels. However, the complex interplay between gene expression, DNA methylation, histone modifications, and chromatin organization presents challenges for rational trial designs. Moreover, the dynamic epigenetic changes in response to therapy (resistance) or the interaction with the tumor microenvironment yield challenges and opportunities. At present a number of trials have been initiated and await completion (Table 1). The insights from basic and preclinical research, and clinical trials reviewed here, suggest that epigenetic drugs combined with conventional therapy, such as alkylating drugs or tyrosine kinase inhibitors may act synergistically to eliminate refractory tumor cells and potentially inhibit the expansion of resistant clones.

4. Conclusions

Importantly, these resistant clones were shown to be sensitive to epigenetic drugs. Dirks and co-workers have reported that Temozolomide-resistant clones displayed epigenetic changes that rendered them sensitive to the inhibitor of Menin-MLL (MI-2-2) [106]. Furthermore, the group of Bernstein demonstrated that Dasatinib-tolerant persisters were dependent on the H3K27me3 demethylases KDM6A/B and were therefore highly sensitive to the KDM6A/B small molecule inhibitor GSK4 [107]. Overall, the insights of these preclinical studies suggest that epigenetic drugs combined with conventional therapy, as alkylating drugs or tyrosine kinase inhibitors may act synergistically to eliminate refractory tumor cells and potentially inhibit the expansion of resistant clones.

### 3.5. BET inhibitors (BETi)

Another way to target enhancer elements is to inhibit readers of the acetylated histone tails (Fig. 3). Bromodomain and extra-terminal tail (BET) proteins are chromatin readers, they recognize and bind to the H3K9 and H3K27 acetyl marks, recruit mediator complex and promote transcription of target genes. BET proteins were shown to be essential for high-level expression of oncogenes. Moreover, BETi were demonstrated to reduce the transcription of oncogenes by attenuating enhancer activity at so-called super-enhancers [98]. Super-enhancers are referred to as large clusters of transcriptional enhancers driving expression of genes that control cell identity and disease including cancer [99]. In an in vivo RNAi screen, testing chromatin regulators required for survival of glioblastoma cells in an intact environment, BRD4 was among the top hits [100]. Preclinical studies in orthotopic mouse glioblastoma xenografts have reported efficiency of several BETi, such as the tool drug JQ1, I-BET151, and OTX015 [101–103]. Moreover, IDHmmt primary glioma cells were reported to be very sensitive to BETi JQ1 and GS-626510 with half-maximal inhibitory concentrations 1 000 times lower than the one of Temozolomide [104].

A phase IIA trial for dose optimization of OTX015 in recurrent glioblastoma patients was terminated in 2015 due to lack of efficacy in this patient population [105]. Other BETi with different pharmacokinetic properties are currently in preclinical evaluation.

### 3.6. Potential of epigenetic drugs in treatment resistance

The combinations of epigenetic drugs with conventional chemo/radiation therapy or with targeted compounds are still in the early stages of preclinical research in glioma. Recent reports suggested that both conventional chemotherapy [106] and tyrosine kinase inhibitors [107] may facilitate the growth of pre-existing resistant clones.

#### Table 1

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<th>Clinical trial identifier*</th>
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conventional therapies will be required. Furthermore, the glioma myelo-mine, in particular in IDH-m/GMP positive gliomas, still awaits exploration for potential vulnerabilities of cancer relevant pathways that may be amenable to specific treatments, beyond Temozolomide for glioblastomas with MGMT promoter methylation. Further insights on the effects of epigenetic drugs on the modulation of the tumor immune environment; and the exploration of 2HG-dependent metabolic vul-nerabilities in IDHm gliomas may provide additional opportunities. We are just at the beginning of an exciting new era.

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