Epigenetics of neurological cancers

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Epigenetic mechanisms involving DNA methylation, histone modifications and noncoding RNAs regulate and maintain gene-expression states. Similar to genetic mutations, alterations in epigenetic regulation can lead to uncontrolled cell division, tumor initiation and growth, invasiveness and metastasis. Research in brain cancer, particularly gliomas, has uncovered global and gene-specific DNA hypomethylation, local DNA hypermethylation of gene promoters and the de-regulation of microRNA expression. Understanding epigenetic dysregulation in brain cancers has provided new tools for prognostication, as well as suggesting new approaches to therapy. There is significant interest in new sequencing-based technologies that map genetic and epigenetic alterations comprehensively and at high resolution. These methods are being applied to brain tumors, and will better define the contribution of epigenetic defects to tumorigenesis.

Review
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Epigenetics is defined as mitotically heritable changes in gene expression that are not due to changes in the primary DNA sequence. Epigenetic mechanisms include enzymatic modification of DNA or associated histone proteins that together regulate and maintain gene-expression states, and have important roles in chromosome structure and stability. Epigenetic mechanisms are essential for mammalian development. The discovery of altered epigenetic profiles in human neoplasia has led to new integrative models in which both genetic and epigenetic mechanisms contribute to and interact in cancer, and perhaps many other common human diseases. Owing to their reversible nature, epigenetic alterations are being targeted for reversal by therapeutic agents in cancer clinical trials. Here, we discuss the contribution of epigenetic mechanisms to brain development and tumorigenesis, how these epigenetic modifications are used for diagnosis and prognosis, and how their reversal may have therapeutical value.

Epigenetics

DNA methylation involves the covalent, enzymatic addition of a methyl group to cytosine in DNA. In adult tissues, methylation occurs almost exclusively at a cytosine followed by guanine, or CpG dinucleotide. Presumably as a result of deamination of methylated CpGs over evolutionary time, CpG dinucleotides are fivefold under-represented in the human genome. CpG islands are an exception, as they are regions of several hundred base pairs to a few kilobases in length that contain a relative abundance of CpGs and higher GC content [1]. DNA methylation in promoter regions blocks transcription and suppresses activity of transposable elements, regulates or maintains gene imprinting and is critical for chromosome stability [2,3].

DNA methyltransferase proteins, the DNMTs, catalyze the addition of the methyl group to DNA and maintain DNA methylation patterns. DNMT3A and DNMT3B are *de novo* methyltransferases, which establish new methylation marks at unmethylated sites, while DNMT1 is the maintenance methyltransferase and maintains the pattern of DNA methylation after DNA replication [4,5]. DNMT3L, another member of the DNMT family, shares homology to DNMT3A and DNMT3B through a cysteine-rich region, but lacks a catalytic domain [4]. Recent structural analysis in mice has demonstrated that DNMT3A and DNMT3L can form a complex that has two active sites and can bind DNA [6]. Ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1) colocalizes with DNMT1 throughout S phase and may facilitate DNMT1 interaction with chromatin [7]. Furthermore, UHRF1 contains an SRA domain that has a strong preference for hemimethylated DNA, suggesting that it might help recruit DNMT1 during DNA replication [7].

The regulation of *de novo* and maintenance methylation has been a focus for many years; however, passive and active demethylation also play a vital role in cellular function and development of an organism [8,9]. Active DNA demethylation also takes place during postnatal stages at specific gene loci, in post-mitotic neurons upon induction of synaptic activity [10], and in T cells upon anti-CD3 mediated activation [11].

Keywords
■ DNA methylation ■ epigenetics ■ glioma ■ histone deacetylase ■ histone modification ■ microRNA ■ SAHA/vorinostat

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Thus, DNA demethylation appears to be a more dynamic and regulated process than previously appreciated. To date, an enzyme capable of directly removing a methyl group from cytosine has not been identified; by contrast, indirect mechanisms of active demethylation via DNA repair mechanisms involve deaminases, glycosylases such as MBD4, the DNA repair exonuclease XPG and the GADD45 family of proteins [12–14].

In addition to 5-methycytosine, 5-hydroxymethylcytosine (HMC) is found in genomic DNA from T2, T4 and T6 bacteriophages [15], but also in mouse brain and human embryonic stem cells, whilst being curiously absent from a human cancer cell line [16,17]. In mammals, the enzyme TET1 can convert 5-methylcytosine to HMC [17]. It may be that HMC can lead to genomic demethylation, either through a passive mechanism via the inability of DNMT1 to bind [18] or maintain methylation during mitosis, or through an active mechanism acting as an intermediate for a glycosylase [13]. Loss of TET1 and its family member TET2 are associated with malignancies, and a TET1 fusion to the histone methyltransferase MLL has been described in acute myeloid leukemia [19–21].

While modification of DNA by CpG methylation generally leads to gene silencing, post-translational modifications of histone proteins including acetylation, methylation, phosphorylation, ubiquitination or sumoylation can lead to either gene activation or repression, depending on which specific amino acid residue is modified. One of the best studied is the acetylation status of lysine residues, a reversible process catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs). The HAT-mediated addition of an acetyl group conveys structural changes to chromatin by decreasing the interaction between the negatively charged DNA backbone and the positively charged histone tail. This decrease in interaction can lead to a less compacted nucleosome, which permits greater access of transcription-factor complexes to the DNA (Figure 1A). Therefore, histone acetylation is a mark of actively transcribed genes and genes primed for induction [22,23]. Conversely, HDACs remove the acetyl group, potentially leading to repression of gene transcription.

Methylation of histones is another form of epigenetic regulation that is reversible. Mono-, di- and tri-methylation on histone H3 at lysine 4 (H3K4) is associated with transcriptional activation, while the di- and tri-methylation on histone H3 at lysine 9 (H3K9me2 and H3K9me3) is indicative of transcriptional inhibition [24]. Another repressive mark, histone H3 lysine 27 trimethylation (H3K27me3), is placed by the polycomb group complex (PcG). PcG proteins are responsible for the initiation of heritable long-term silencing of gene expression [25]. H3K27me3 in cancer cells can recruit DNA methyltransferases to gene promoters, potentially leading to *de novo* methylation and gene silencing (Figure 1C) [26–28]. However, a subset of inactive genes marked by H3K27me3 in cancer cells do not undergo *de novo* methylation (Figure 1D). Genes with H3K27me3 but without DNA methylation have also been observed in normal cells [29]. Histone methylation is also reversible by histone demethylases. Lysine-specific histone demethylase 1 (LSD1/KDM1) can remove mono- and dimethylated lysines, while the JmjC-domain histone methylase family (JHDMs) can remove mono-, di- and tri-methyl groups from different lysines on histone H3 [30,31].

Another mechanism that is potentially epigenetic and modulates gene expression involves small RNAs, in particular microRNAs (miRNAs). MiRNAs are short RNAs (~21–22 nucleotides) that bind to the 3′ untranslated region of target mRNAs and prevent translation of the mRNA [32]. MiRNAs can have multiple mRNA targets, and individual mRNA can be targeted by multiple miRNAs. MiRNAs have the capacity to fine-tune gene-expression programs and cell function through cell-type-specific and temporal regulation of their expression, which itself can be deregulated through epigenetic mechanisms in brain tumors.

Epigenetics in the CNS

DNA methylation plays an integral part in the development and function of the mammalian CNS, and research in this area may shed light on the causes and consequences of aberrant epigenetic mechanisms in brain tumors. Early studies reported that in mice *Dnmt1* mRNA is expressed at an unexpectedly high level in postmitotic neurons, suggesting a role for DNMT1 in postnatal brain function [33]. Coupled with a later finding that DNMT3A is also expressed in the postnatal brain, it appears that DNA methylation might be important for neuronal maturation in the adult CNS [34]. Conditional removal of DNMT1 in mouse CNS precursor cells leads to global demethylation and perinatal death from a defect in neural control of respiration [35]. Thus, in addition to the unanticipated function in postmitotic cells of the adult brain, DNA methyltransferases are important for the normal development of the CNS.

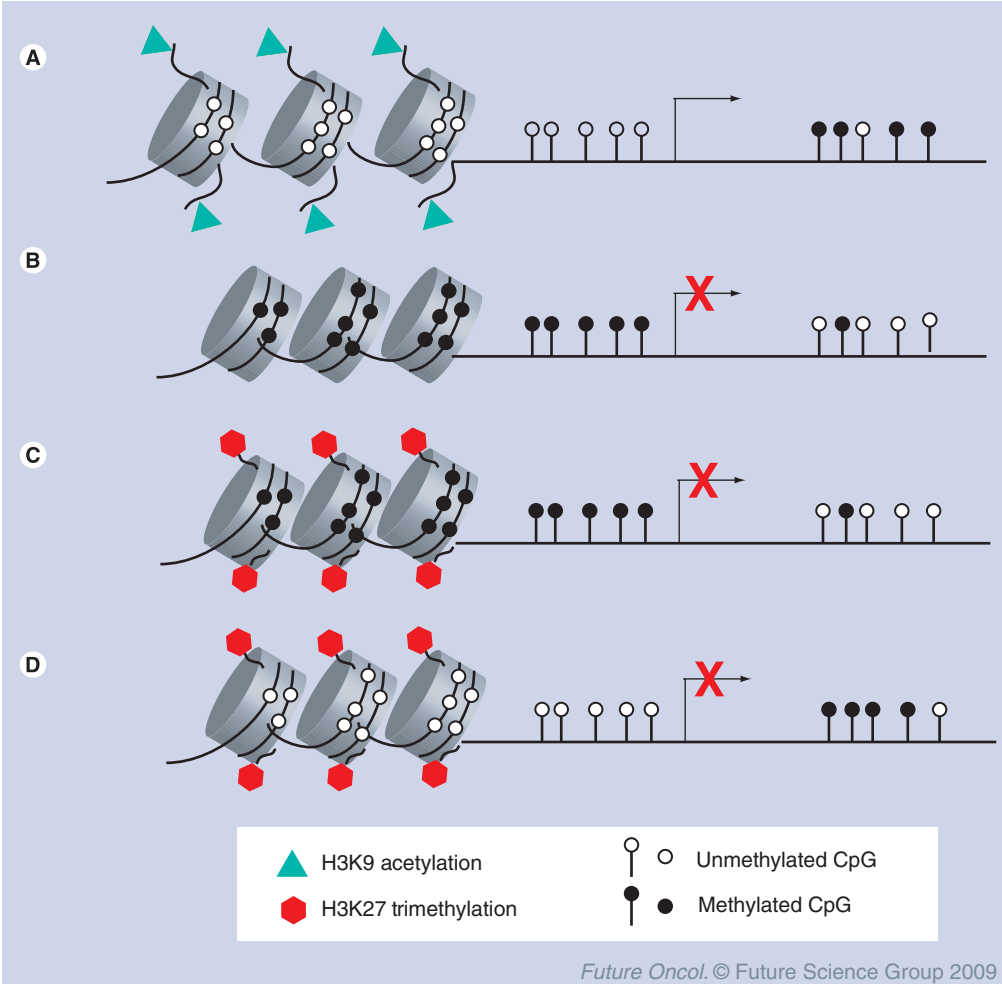

Figure 1. DNA methylation and histone modifications at promoters of normal and cancer cells. (A) At promoters of genes that are actively transcribed, the tails of histone proteins are marked with acetylation and the CpG dinucleotides are unmethylated. **(B)** Silenced genes can be marked by aberrant methylation of CpG dinucleotides alone, or **(C)** by histone H3 lysine 27 trimethylation and methylation of CpG dinucleotides or **(D)** By histone H3 lysine 27 trimethylation in the absence of CpG methylation. Initial reports in cancer cell lines suggest this pattern **(D)** is found in far fewer genes compared with aberrant DNA methylation. It is quite possible that other histone modifications change coordinately or inversely with DNA methylation, but have not yet been studied in gliomas.

The *de novo* DNMTs, DNMT3A and DNMT3B, are expressed in the developing murine CNS, with DNMT3B found during embryonic day E11 to E15, while DNMT3A is expressed from early neurogenesis into the postnatal CNS [34]. Interestingly, DNA methylation can also influence the timing of CNS development through regulation of the JAK–STAT pathway [36,37]. STAT3, an important member of this pathway, also plays an important role in gliomagenesis [38–40].

Proteins that bind to methylated DNA are also involved in epigenetic regulation of CNS development and function. Mutations in methyl CpG binding protein 2 (*MECP2*) are known to cause Rett syndrome, a developmental disease associated with mental retardation [41]. Mice with generalized or CNS-specific deletions of *Mecp2* have virtually indistinguishable phenotypes, suggesting the function of MeCP2 is primarily related to development of the CNS [42–44]. In one of these mouse models, point mutations in *Mecp2* lead to increased histone H3 acetylation in the cerebellum and cortex [44]. Methyl CpG binding domain protein 2 (MBD2) and MeCP2 also interact with HDACs to induce heterochromatin formation and gene silencing [45,46]. Taken together, these data provide further evidence that DNA methylation and its effectors are vital for CNS development and function.

Similar to methylation of DNA, histone modifications are also important in CNS functions, such as learning and memory [47]. The CREB-binding protein (CBP) exhibits HAT

activity, and mutations of CBP underlie at least 50% of cases of Rubinstein–Taybi syndrome, a mental retardation disorder that also carries a risk of cancer, including medulloblastoma and glioma [48–50]. Histone methylation is also an important epigenetic mechanism for CNS function. Postmortem brain from individuals that had Huntington's disease and Friereich's ataxia exhibit misregulation of H3K9 methylation [51,52]. *Mll1/KMT2A*, a histone methyltransferase and component of the trithorax chromatin remodeling complex, is essential for postnatal neurogenesis [53]. Similar to histone methylation, the demethylation of histones has a role in CNS function, as an X-linked mental retardation and autism gene, *SMCX/KDM5C*, is a Jumonji-containing protein that encodes an H3K4 demethylase [54,55]. These data suggest that specific post-translational histone modifications are crucial for the normal development and function of the CNS.

Gliomas & epigenetics

While the role of epigenetics in the development and progression of gliomas, particularly of glioblastoma multiforme (GBM), has been studied for many years, many new findings are still emerging. Alterations in DNA methylation, histone modifications and miRNA expression are found in GBMs, although how they arise and what effects they have on tumorigenesis remain under active investigation.

DNA hypomethylation in gliomas

Hypomethylation of genomic DNA has been found in many different cancers [56], including GBMs, where up to 80% exhibit demethylation compared with normal brain [57]. The amount of global DNA hypomethylation in GBMs can vary, from very little change to as much as 50% relative to normal brain [57], which is equivalent to approximately 10 million CpG sites in a single tumor. Loss of methylation is found at repetitive elements, as well as at single copy loci [57]. One particular gene, encoding the cancer-testis antigen MAGEA1, was demethylated and expressed in a subset of primary GBMs and in glioma cell lines [57]. GBMs with severe loss of global methylation are also among the most proliferative of these aggressive tumors [57,58]. While these studies demonstrate a role for the maintenance of global methylation levels, the exact cause of hypomethylation in GBMs and other tumors is not well understood. So far, loss of DNMT proteins has not been observed in tumor cells. However, the potential for counterbalancing of methyltransferase activity through active DNA demethylation, or involvement of histone modifications in potential downstream DNA hypomethylation, have not been ruled out.

DNA hypermethylation in gliomas

In addition to local and global hypomethylation, locus-specific hypermethylation is also found in GBMs. CpG islands in 5′-promoter regions are predominantly unmethylated in normal cells, but undergo hypermethylation in cancer cells (**Figure 1B**) [59], although the causative mechanism(s) is not fully understood. Many different pathways are affected by hypermethylation-related gene silencing in gliomas, including cell-cycle regulation, apoptosis, DNA repair, angiogenesis, drug resistance and invasion. For example, the tumor suppressor genes *PTEN* and *RB* are known to undergo DNA hypermethylation-induced silencing in GBM cell lines and hypermethylation in primary tumors [60,61]. *PDCD4*, a tumor suppressor that can suppress expression of target genes, is itself silenced in many cancers including gliomas through methylation of the 5′ CpG island [62]. Another candidate tumor suppressor, *LRRC4*, is silenced by CpG island promoter hypermethylation in both primary gliomas and glioma cell lines [63]. The imprinted gene *PEG3*, which has tumor-suppressor activity in glioma cell lines, exhibits both promoter hypo- and hyper-methylation in primary gliomas [64]. The changes in levels of *PEG3* methylation are inversely correlated with gene expression, suggesting that the methylation status of the exonic CpG island can influence gene expression [64]. Hypermethylation of the promoter of the DNA repair protein ERCC1 may have a role in the development of resistance to cisplatin in gliomas, since the levels of *ERCC1* mRNA are associated with sensitivity to cisplatin in glioma samples [65] and hypermethylation of *ERCC1* in glioma cell lines and primary gliomas may confer resistance through suppression of *ERCC1* mRNA and protein. *CD133*, a cell-surface marker that is expressed on neural progenitor cells and putative cancer stem cells, is silenced in both colon cancer and glioma cell lines by promoter CpG island methylation [66]. However, the data from primary gliomas and subsequent xenograft models are not as consistent as the cell lines, suggesting that the role of *CD133* promoter methylation and expression in gliomas is not yet fully understood.

Many of these genes were chosen for investigation using a candidate gene approach, but it remains possible that a subset of glioma genes might be inactivated primarily by methylation,

and thus, only through methylation screening could such genes be identified. Such methylation screens have uncovered new potential tumor suppressors in *RUNX3* and *TESTIN* [67,68]. Another screen found that *BEX1* and *BEX2*, novel X-linked genes, are hypermethylated and silenced in gliomas [69]. When re-expressed in cells, these genes led to increased sensitivity to chemotherapy-induced apoptosis [69]. While these screens identified candidate genes in glioma cell lines, complementary approaches have investigated aberrant DNA methylation in primary glioma samples. A study looking at 87 primary GBMs using a bead array system found 32 genes that exhibit changes in their methylation profile compared with a normal brain sample [70]. In this study, *HOXA11*, *CD81*, *PRKCDBP*, *TES*, *MEST*, *TNFRSF10A* and *FZD9* were hypermethylated in more than 50% of the samples [70].

An integrated genomic and epigenomic approach using copy-number analysis and DNA-methylation scanning identified both *SLC5A8* and *WNK2* methylation in primary human gliomas [71,72]. Methylation of *SLC5A8* is correlated with decreased expression, while re-expression of exogenous *SLC5A8* led to inhibition of colony formation *in vitro* [71]. Mouse models of oligodendroglioma also exhibited silencing of *mSLC5A8*, suggesting a possible evolutionary conserved pathway for glioma formation or progression [71].

WNK2 is a member of the WNK family (*WNK*1–4), which is known to have genetic point mutations in many types of solid tumors [73–75]. In gliomas, *WNK2* exhibits aberrant methylation of its promoter and concurrent silencing of gene expression. Reintroduction of *WNK2* into glioma cell lines leads to decreased colony formation, which along with many examples of point mutations in other common cancer types, supports a role for WNK2 as a suppressor of cell growth [72]. On a pathway level, *WNK2* epigenetic silencing may synergize with genetic amplification of *EGFR*, a potent oncogene in gliomas, as WNK2 suppresses the activity of MEK1 through the Rho–GTPase pathway mechanism, and reduces EGFR signaling [76]. These studies underscore the importance of integrative genetic and epigenetic screens to discover novel mutations or alterations, and how they give rise to brain cancers.

Hypermethylation of the O^6-methylguanine-DNA-methyltransferase (MGMT) promoter is associated with increased sensitivity to combined chemo- and radio-therapy. MGMT is a cellular DNA repair protein that removes alkylation damage at the O^6 position of guanine. Glioma patients are treated with chemotherapeutic drugs such as temozolomide (Temodar®) that induce alkylation damage at the O^6 position of guanine, among other sites on DNA. If left unrepaired, the lesions lead to DNA crosslinks and induce apoptosis. While high levels of MGMT are correlated with resistance to alkylating agents in glioma cell lines and xenografts [77–81], promoter hypermethylation of *MGMT* is associated with increased patient survival when coupled with radiation and treatment with temozolomide [82,83]. The Cancer Genome Atlas Project analyzed *MGMT* methylation status in 91 GBMs, and discovered a link between *MGMT* promoter hypermethylation and the mismatch repair pathway [84]. Recurrent GBMs with methylated *MGMT* exhibited a high incidence of G–C to A–T transition mutations at non-CpG sites (81%), also seen in mismatch repair genes (MMR), similar to those reported for *p53* [85]. This is in contrast to tumors with unmethylated *MGMT*, which do not have a high incidence of G–C to A–T transitions at non-CpG sites (23%), suggesting that *MGMT* methylation and MMR deficiency in the context of treatment influence the mutational spectrum [84].

Gliomas & histone modification changes

To date, there have only been a few studies investigating the role of histone modifications in tumorigenesis and progression, particularly for gliomas. Changes in some post-translational histone modifications are common in cancers, including the loss of H4K16 acetylation (H4K16ac) and H4K20 trimethylation (H4K20me3) [86], and genes encoding histone modifying enzymes can be mutated genetically. In addition, low levels of H3K18 acetylation (H3K18ac) are correlated with prostate tumor recurrence [87]. These results suggest an important role for maintaining normal levels of histone acetylation. There are 18 known mammalian HDACs, which can be classified into four main groups based on structure, cellular localization and homology. In GBMs, class II and IV HDAC mRNAs are downregulated relative to lower grade astrocytomas and normal brain tissue [88]. One study also observed increased H3 acetylation in GBM compared with normal brain [88]. However, this is in opposition to the results reported for other types of cancer [86,87], which exhibit a loss of histone H3 acetylation. Even though the role of histone acetylation in GBMs is not fully understood, new therapies (discussed

in more detail below) involving HDAC inhibitors (HDACi) are currently in clinical trials and others are in development [89–96].

Histone modifications and DNA methylation are interdependent at the level of the marks, but also at the level of protein–protein interaction between the histone- or DNA-modifying enzymes. For example, suppression of DNMT1 or DNMT3b in glioma cell lines led to re-expression of three genes, *DUSP5*, *SDC2* and *TMTC1* [97], but surprisingly, DNA hypermethylation at these promoters was unchanged. Instead, a reduction of H3K9me2, H3K9me3 and H3K27me3 levels was observed for the three genes [97]. Although it is not clear how altered DNA methyltransferase levels impacted these specific histone modifications, a mechanism involving local decreased DNA methylation is unlikely.

Deregulation of miRNAs in gliomas

Studies investigating the roles of miRNAs in gliomas are just emerging. While not strictly defined as an epigenetic mechanism, in model organisms miRNAs regulate heterochromatin formation, and in mammals miRNA can influence expression of many genes without altering the genetic sequence. MiRNA profiling has shown that miR-21 is overexpressed in primary GBMs, early-passage GBM cultures, and GBM cell lines [98]. miR-21 is overexpressed in a variety of cancers, including breast, lung, colon, prostate, pancreas, ovarian and stomach cancer [99,100]. miR-21 targets the *p53*, *TGF*-β, and mitochondrial apoptosis tumor-suppressive pathways [101]. Knockdown of miR-21 leads to repression of cell growth, increased apoptosis, and cell-cycle arrest, as well as decreased ability to migrate and invade [101,102]. Expression of another miRNA, miR-7, is downregulated in GBMs, and reintroducing miR-7 into GBM cell lines results in decreased viability and invasiveness [103]. As a potential tumor suppressor, miR-7 inhibits the Akt pathway and suppresses EGF receptor expression [103]. However, others, miR-124a and miR-137 for example, exhibit decreased expression in both anaplastic astrocytomas and glioblastomas compared with normal brain tissue [104]. Treatment of glioblastoma cell lines with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza) resulted in increased expression of miR-137, suggesting that its expression might be regulated by DNA methylation [104].

miR-128 is associated with epigenetic alternations by way of its interaction with a histone-modifying enzyme, and is downregulated in GBMs [105]. Re-expression of miR-128 in glioma cell lines leads to a decrease in *Bmi-1* expression [105]. *Bmi-1* is a member of the PcG family and also plays a role in stem-cell renewal [106,107]. The silencing of *Bmi-1* via miR-128 results in a global reduction of H3K27me3 [105]. While these studies focus on specific targets for individual miRNAs, in general, multiple targets exist for individual miRNA. These studies underscore the importance of investigating miRNAs in brain cancers, both in terms of how they are dysregulated and the mRNAs they target.

Pediatric brain cancers & epigenetics

Medulloblastoma is a malignant tumor in the cerebellum that occurs primarily in children and carries 5-year survival rates of 70–80% for standard risk patients [108]. Sporadic forms of medulloblastoma are associated with loss of chromosomal arm 17p where *p53* resides, although *p53* mutations are not commonly associated with medulloblastoma [109]. Methylation screens of the 17p region led to the discovery of hypermethylated in cancer 1 (*HIC1*), a zinc finger transcriptional repressor that itself is activated by p53 and is hypermethylated in a variety of pediatric and adult tumors [110,111]. *HIC1* targets at least two different genes, the class III HDAC *SIRT1* and a proneural transcription factor, *ATOH1* [112,113]. Hypermethylation and gene silencing of *HIC1* is commonly found in medulloblastomas of all stages and pathology [114–116], and treatment with 5-aza can reactivate *HIC1* [114,116].

Epigenetic screens have been used to identify numerous other genes that are misexpressed in medulloblastoma, such as *SPINT2*, *S100* and *MCJ*. *SPINT2*, a serine protease inhibitor and putative tumor suppressor, exhibits promoter hypermethylation in 34.3% of medulloblastomas [117]. The hypermethylation leads to reduced *SPINT2* expression, which is reversible by treatment with 5-aza [117]. Re-expression of *SPINT2* leads to reduced cell motility, proliferation and anchorage-independent growth with an increase of overall survival time in xenograft models [117]. The *S100* family of proteins is associated with malignant progression. In medulloblastoma cell lines, two members of this family (*S100A6* and *S100A10*) exhibit promoter hypermethylation [118]. The hypermethylation of *S100A6* is associated with the aggressive large cell/anaplastic morphophenotype [118]. *MCJ*, a member of the DNAJ protein family, is silenced via promoter methylation in medulloblastomas [119]. Interestingly, the methylation patterns of the

MCJ promoter fall into two categories – complete methylation of the promoter CpG island or limited methylation concentrated at the 5′ end of the of CpG island [119]. Furthermore, epigenetic loss of *MCJ* expression can result from biallelic promoter methylation or methylation on one allele combined with genetic loss of the other allele [119]. Other genes such as *DIKKOPF-1* and *COL1A2* are also silenced via DNA methylation in medulloblastomas [120,121].

DNA methylation is not the only widespread epigenetic alteration found in medulloblastoma. A genome-wide SNP screen identified genomic regions that are altered in copy number, affecting genes that add, remove or 'read' histone lysine methylation [122]. Restoration of expression of one of these genes, *L3MBTL*, led to a decrease in proliferation of medulloblastoma cell lines [122]. The genetic alteration of such epigenetic-modifying genes leads to a global loss of H3K9me2, and might also result in a genome-wide redistribution or global loss of other histone methylation at specific lysine residues. Thus, alteration of multiple epigenetic mechanisms, either directly through epigenetic 'mutations' or indirectly through genetic alterations of genes encoding epigenetic modifying enzymes, contributes to the formation of medulloblastomas.

Prognosis & epigenetics

Despite the identification of a large number of epigenetic alterations in brain tumors, very little progress has been made in finding marks with prognostic value. The most well studied epigenetic mark for prognosis of treatment for gliomas is the methylation status of *MGMT*. Hypermethylation of the *MGMT* promoter region, when sufficiently dense, leads to *MGMT* silencing in gliomas, lymphomas, breast cancer, prostate cancer and retinoblastoma [123]. Hypermethylation of the *MGMT* promoter region along with radiation and treatment with temozolomide, is associated with longer survival of patients with GBMs and those low-grade gliomas treated with temozolomide only [82,124]. A subset of GBM patients receiving this therapeutic regimen can survive up to, and perhaps beyond 5 years [83]. It should be noted that the typical assay used to assess *MGMT* methylation, methylation-sensitive PCR (MSP), does not measure precisely within the core promoter, nor is it quantitative. These issues undoubtedly contribute to the observed lack of correlation between methylation, assessed by MSP, and *MGMT* expression, assessed by reverse transcription (RT)-PCR, immunohistochemistry or enzymatic activity. In contrast to GBM, hypermethylation of *MGMT* occurs later in progressive oligodendrogliomas, suggesting that its prognostic effect in oligodendrogliomas might be different than for GBMs and low-grade gliomas [125].

A second DNA methylation-related prognostic marker of GBM and astrocytoma patient survival is the level of p-SMAD2, an indicator of TGFβ activity, as high levels are associated with poor survival [126]. TGFβ is an inhibitor of proliferation in various cell types including astrocytes and is a putative tumor suppressor [127]. However, some tumors such as gliomas lose TGFβ-mediated suppression of proliferation and instead TGFβ then promotes proliferation, angiogenesis, invasion and metastasis [127]. The investigation into a potential 'switch' that causes TGFβ to undergo transition from tumor suppressor to oncogene, uncovered a correlation between *PDGF*-β induction and high p-SMAD2 levels. This induction is dependent on the methylation status of the *PDGF*-β promoter, as a hypermethylated promoter does not lead to induction by TGFβ and these tumors are typically less aggressive [126]. Thus, the methylation status of *PDGF*-β may influence the TGFβ signaling pathway in gliomas, and could be useful as a prognostic marker.

A third potential prognostic epigenetic mark is the methylation status of the target of methylation induced silencing 1 (*TMS1/ASC*) gene, a gene that can activate the NF-κB pathway [128]. In typical GBM patients compared to long-term survivors, *TMS1/ASC* promoter hypermethylation was found significantly more often in long-term survivors [128]. A mechanism by which promoter hypermethylation of *TMS1/ASC* associates with long-term survival requires further investigation.

A fourth epigenetic mark with a potential prognostic significance was recently identified in a retrospective study of 27 GBMs, which found methylation of the tumor suppressor *p15* promoter in 37% of the samples. *p15* is found on chromosome 9p, a region often deleted in GBM patients, and a region significantly associated with risk of developing GBM [129]. Patients with methylation of *p15* had a significant shorter overall survival (16.9 vs 23.8 months) [130]. This study provides another possible prognostic marker for GBM, pending replication on a larger sample set.

Therapeutics & epigenetics

With the knowledge that many loci exhibit epigenetic alterations in brain cancers, it is no surprise that therapies that can reverse these changes are being pursued. In particular, the DNA methylation inhibitor decitabine

(5-aza-2′-deoxycytidine; Dacogen®, MGI Pharma Inc., MN, USA) and the HDACi suberoylanilide hydroxamic acid (SAHA; vorinostat, Patheon, Inc., Ontario, Canada) are being used for cancer patients, although only vorinostat is currently in clinical trials for GBM.

Theoretically, modulation of DNA methylation could provide therapeutic benefit by reversing hypermethylation and restoring expression of silenced genes in gliomas. Treatment of gliomas in culture with decitabine decreased cell proliferation and increased apoptosis [131]. Treatment of glioma cell lines with decitabine and Taxol® (paclitaxel; Bristol-Myers-Squibb, NY, USA) can lead to silencing of telomerase and decreased cell viability [132]. However, decitabine treatment can also activate *MGMT*, which might increase resistance to alkylating agents such as temozolomide, depending on the relative balance of local and global effects. An alternative to decitabine involves reduction of DNMT1 levels through double-stranded (ds) RNA knockdown experiments [133]. This approach, coupled with either paclitaxel or temozolomide, results in decreased glioma cell viability [133]. Thus, in cell lines, the anti-tumor effect appears to predominate over potential pro-resistance effects when DNA methyltransferase is inhibited. Nevertheless, more appropriate *in vitro* and *in vivo* models are needed to confirm these results, as the epigenetic profile of glioma cell lines is substantially different from that of primary gliomas.

Inhibition of HDACs could also provide a therapeutic benefit alone, or in combination with temozolomide. HDACs catalyze the removal of acetyl groups from histone lysine residues, as well as nonhistone proteins. The HDAC family consists of 18 members in humans that are divided into five different classes. HDACs of class I (HDACs 1, 2, 3 and 8), class IIA (HDACs 4, 5, 7 and 9), class IIB (HDACs 6 and 10) and class IV (HDAC 11) all contain zinc in their active sites, while the class III HDACs (sirtuins) do not contain zinc. The removal of acetyl groups on core histone proteins can lead to more condensed chromatin that is not as accessible to transcription factors, which can result in gene silencing.

HDACis have been used to treat many different malignancies, though it is not yet clear exactly how HDACi functions on a cellular level, nor which HDAC or other protein is their most important target for anti-tumor activity [134]. Similarly, it is not yet known how extensively

Table 1. Clinical trials using epigenetic therapy.

Trial	HDAC class targeted	Disease	Clinical trial phase	Status	Protocol ID
Vorinostat, isotretinoin, carboplatin	I, II	Recurrent GBM	I, II	Active	NCT00555399
Vorinostat, bevacizumab, temozolomide	I, II	Recurrent GBM	I, II	Active	NCT00939991
Vorinostat, borezomib	I, II	Recurrent GBM	II	Active	NCT00641706
Vorinostat, temozolomide	I, II	Malignant gliomas	I	Active	NCT00268385
Vorinostat, temozolomide, radiation	I, II	GBM	I, II	Temporarily closed	NCT00731731
Vorinostat	I, II	Progressive or recurrent GBM	II	Closed	NCT00238303
Vorinostat, bevacizumab, irinotecan	I, II	Recurrent GBM	I	Closed	NCT00762255
Panobinostat, bevacizumab	I, II	Recurrent high-grade glioma	I, II	Active	NCT00859222
Panobinostat	I, II	Malignant brain tumor	II	Active	NCT00848523
Valproic acid, temozolomide, radiation	I, IIa	GBM	II	Active	NCT00313664
Valproic acid	I, IIa	High-grade glioma in children	II	Active	NCT00879437
Valproic acid, etoposide	I, IIa	Neuronal tumors & brain metastases	I	Active	NCT00513162
Valproic acid	I, IIa	Recurrent or refractory CNS tumors in children	I	Closed	NCT00107458
Romidepsin	I, II, IV	Recurrent high-grade glioma	I, II	Closed	NCT00085540

GBM: Glioblastoma multiforme; HDAC: Histone deacetylases.

HDACi changes the global landscape of acetylation specifically, or how it may alter the entire epigenome secondary to modifying acetylation. One speculation is that HDAC inhibition results in increased acetylation in promoters and activates critical growth regulating or apoptotic genes [135]. Another speculation is that HDAC inhibition generically opens chromatin genome-wide to increase DNA damage by chemotherapeutic agents [135]. In any case, it is known that HDACi treatment synergizes with DNA damaging agents such as temozolomide in arresting glioma cells *in vitro* [91].

Clinical trials for glioma patients have included four different HDACi therapies (Table 1). One of these, vorinostat, is an inhibitor of class I and class II HDACs and binds to the zinc ion in the active site of HDACs [96]. Vorinostat has been approved by the US FDA for the treatment of cutaneous T-cell lymphoma [136]. Preclinical studies found that vorinostat induces apoptosis and inhibition of cell growth in glioma cells *in vitro*, *ex vivo* and *in vivo* [94,137,138]. These and other studies led to the inclusion of vorinostat in seven clinical trials for primary GBM patients, with promising results reported from a Phase II study [139]. In recurrent GBMs, treatment with vorinostat alone resulted in modest 6-month progression-free survival [139]. Histone acetylation and gene-expression analysis determined that vorinostat was affecting its intended target within the tumor [139], although the effect on nonhistone protein acetylation sites was not assessed.

Trials are also currently underway for two additional HDACis, valproic acid (Depakene; Depakote, Abbott Laboratories, IL, USA) and panobinostat (LBH589; Novartis, Basel, Switzerland). Valproic acid is active against class I and IIA HDACs and is being tested against GBM in combination with temozolomide plus radiation, and in a broader second trial against neuronal tumors and brain metastases in combination with etoposide. The use of valproic acid and radiation is also being tested in children with high-grade gliomas. Panobinostat, a potent inhibitor of class I and II HDACs [140], is also in active clinical trials for gliomas. One trial is examining the effects of panobinostat in patients with recurrent malignant gliomas, while another trial is testing its effects in combination with bevacizumab in recurrent high-grade gliomas. A Phase I/II trial was conducted with depsipeptide (Romidepsin; FK-228, Gloucester Pharmaceuticals, MA, USA). Depsipeptide is active against class I HDACs, and depsipeptide monotherapy was tested against high-grade gliomas. Another HDACi not yet in clinical trials is pivaloyloxymethyl butyrate (AN-9), a derivative of butyrate. Butyrate is an aliphatic acid HDACis effective against class I and IIA HDACs. AN-9 demonstrates efficacy in GBM cell culture and animal models, with studies showing that AN-9 sensitized GBM mouse xenografts to radiation and showed decreased tumor growth and increased survival [91,141]. In addition, there are several HDACi that have shown efficacy against other forms of cancer in cell lines but have not yet been tested for gliomas, including belinostat (PXD101; Topotarget, Copenhagen, Denmark) [142,143] and OSU-HDAC42 [144].

An attractive idea for the therapeutic application is the combined treatment with a DNA methylation inhibitor and HDACi, as *in vitro* studies in gliomas and other tumor cells show they act synergistically [145–147]. Changes in gene expression or cell viability are observed when treating the cancer cell lines with either 5-aza or HDACi alone [145–147]. However, treatment with 5-aza and HDACi leads to substantially increased gene activation [145,147] or increased radiosensitivity [146]. Trials with valproic acid and decitabine are underway in acute myeloid leukemia and solid tumors. While the *in vitro* results are encouraging, there are no current clinical trials in gliomas for combined DNA methylation inhibitors and HDACi.

Epigenome analysis

As previously discussed, epigenome-wide screens have been employed to detect cancer-specific changes in DNA methylation, histone modifications and miRNA expression, typically by microarray approaches. However, 40–50% of the genome is composed of repetitive elements that cannot be studied by microarray, and thus nearly half of the genome has been ignored despite having important regulatory functions. However, recent advances in high-throughput sequencing present a new unbiased methodology to uncover epigenetic alterations in brain cancers. Original epigenetic screens in brain tumors were carried out with restriction landmark genome scanning, a technique that utilizes methylation-sensitive enzymes [148–153] that assessed approximately 1000 sites for each enzyme combination. More recent techniques for mapping genome-wide DNA methylation include methylated DNA immunoprecipitation or methylation-sensitive restriction enzymes, coupled with either DNA microarrays or massively parallel sequencing [154–157]. Alternatively, the gold-standard

bisulfite conversion has been used in reduced representation bisulfite sequencing (RRBS) [158,159]. These array and sequencing technologies can also be applied to mapping histone modifications and small RNAs [160,161]. Thus, there are many possibilities for uncovering new epigenetic alterations with prognostic value in human brain tumors.

Future perspective

With the advances in high-throughput sequencing, it appears that a much more detailed and comprehensive analysis of glioma epigenetics is possible in the next few years. In particular, genome-wide maps of DNA methylation, histone modifications and miRNA expression can be generated from primary tumors and compared with normal brain tissue. The hope is that these genome-wide comparisons can uncover epigenetic marks that might offer better prognosis and/or targets for therapeutics, as well as advancing our understanding of the epigenetic and genetic underpinnings of drug response and gliomagenesis.

Epigenetic therapies for gliomas are currently limited to HDACi. However, it remains to be seen if other histone modifications are aberrant in gliomas and whether these could be reversed for therapeutic benefit. In addition, the lack of an effective DNA demethylating agent with low toxicity and ease of delivery to the brain has prevented more widespread use of DNA methylation-blocking therapies. However, as previously mentioned, clinical trials investigating the combined effects of decitabine and valproic acid in other tumor types are ongoing. This combined approach of altering DNA methylation and histone acetylation, which results in synergistic effects *in vitro* at lower doses, might reduce toxicity and increase efficacy sufficiently to promote clinical trials in brain tumors. Furthermore, the use of small RNAs as therapeutic agents is becoming more realistic with improved delivery modalities, and could be used to target the mRNA corresponding to epigenetic modifying enzymes. Currently, there are multiple clinical trials of miRNA- and siRNA-based therapies against malignancies, though none for gliomas. With more specific knowledge of critical pathways affected by epigenetic alterations, new small RNA-type therapies may be designed to be more target-specific. Recent work to design better HDACi drugs has led to OSU–HDAC42, an HDACi that may be more potent than SAHA [144].

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Executive summary

Gliomas & epigenetics

- Genome-wide scans and candidate approaches have identified genes that exhibit promoter hyper- and hypo-methylation in gliomas compared with normal brain.
- Relatively little is known about changes in post-translational histone modifications in primary gliomas, although histone deacetylase (HDAC) inhibitors are already in clinical trails for glioblastoma multiforme (GBM) patients.
- miRNA expression can be regulated by DNA methylation and microRNAs can also target proteins that modulate histone modifications in gliomas.
- Understanding the epigenetic mechanisms present in brain development may shed light on their contribution to gliomagenesis.

Prognosis

- Promoter region methylation of *MGMT* is a associated with a longer survival of patients with GBMs and low-grade gliomas treated with temozolomide and radiation.
- New epigenetic marks of potential prognostic significance include the promoter methylation status of *PDGF*-β, *TMS1/ASC* and *p15*.

Therapeutics

- HDAC inhibitors are promising therapeutics for treatment of gliomas.
- Vorinostat is in clinical trials for GBM patients.
- Combined DNA methylation inhibitors and HDAC inhibitors are synergistic in gene activation in glioma and other cancer cell lines, but have not yet been tested in glioma clinical trials.

Epigenome analysis

- Recent advances in high-throughput sequencing allow high-resolution comprehensive mapping of DNA methylation and histone modifications in normal brain and glioma genomes. As these methods are sequencing based, genetic mutations can be discovered simultaneously.

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