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REVIEW

Unraveling the Glioma Epigenome—From Molecular Mechanisms to Novel Biomarkers and Therapeutic Targets

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Abstract

Epigenetic regulation of gene expression by DNA methylation and histone modification is frequently altered in human cancers including gliomas, the most common primary brain tumors. In diffuse astrocytic and oligodendroglial gliomas, epigenetic changes often present as aberrant hypermethylation of 5′-cytosine-guanine (CpG)-rich regulatory sequences in a large variety of genes, a phenomenon referred to as glioma CpG island methylator phenotype (G-CIMP). G-CIMP is particularly common but not restricted to gliomas with isocitrate dehydrogenase 1 (IDH1) or 2 (IDH2) mutation. Recent studies provided a mechanistic link between these genetic mutations and the associated widespread epigenetic modifications. Specifically, 2-hydroxyglutarate, the oncometabolite produced by mutant IDH1 and IDH2 proteins, has been shown to function as a competitive inhibitor of various α-ketoglutarate (α-KG)-dependent dioxygenases, including histone demethylases and members of the ten-eleven-translocation (TET) family of 5-methylcytosine (5mC) hydroxylases. In this review article, we briefly address (i) the basic principles of epigenetic control of gene expression; (ii) the most important methods to analyze focal and global epigenetic alterations in cells and tissues; and (iii) the involvement of epigenetic alterations in the molecular pathogenesis of gliomas. Moreover, we discuss the promising roles of epigenetic alterations as molecular diagnostic markers and novel therapeutic targets, and highlight future perspectives toward unraveling the "glioma epigenome."

INTRODUCTION

Cancer development and progression are driven by complex aberrations involving structural genetic alterations, such as point mutations, deletions, insertions, translocations, amplifications and other genetic rearrangements, as well as various types of epigenetic changes resulting in aberrant gene expression because of altered patterns of DNA methylation and post-translational modification of histone proteins [for recent reviews see (71, 117)]. The molecular mechanisms underlying the transforming and growth-promoting effects of cancer-associated genetic and epigenetic changes are still poorly understood in their entire complexity, as is the mechanistic interplay between both types of alterations in cancer cells. However, recent data have provided intriguing new insights by the identification of genetic changes affecting genes directly functioning as epigenetic regulators, for example, DNA and histone methyltransferases, or by linking mutations in genes encoding the isocitrate dehydrogenase 1 (IDH1) and 2 (IDH2) with specific metabolic changes that influence the function and activity of various epigenetic regulators, thereby leading to global alterations in DNA methylation and chromatin structure. Interestingly, the molecular characterization of epigenetic alterations in cancer has not only improved our understanding of disease-driving molecular pathomechanisms but has also resulted in the identification of valuable novel biomarkers for improved tumor diagnostics, as well as a better prediction of therapy response and prognosis. Moreover, as alterations in DNA methylation and histone modifications can be reversed by treatment with specific drugs, such epigenetic changes represent promising molecular targets for novel therapeutic approaches.

In this review article, we will (i) briefly summarize the basic principles of epigenetic control of gene expression with a particular focus on DNA methylation and histone modification; (ii) introduce the most commonly used methods to investigate such epigenetic alterations at the level of individual genes up to the entire genome; and (iii) focus on recent progress concerning the pathomechanistic roles and clinical significance of epigenetic alterations in primary brain tumors, in particular gliomas.

BASIC PRINCIPLES OF EPIGENETIC CONTROL OF GENE EXPRESSION

"An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (12). DNA methylation and post-translational modification of histone proteins are the best-characterized epigenetic mechanisms that determine the access to genetic information as required

for DNA replication, transcription and repair. Physiologically, epigenetic control plays a crucial role in X chromosome inactivation (64), genomic imprinting (77), silencing of retrotransposons (4), telomere length control (15), centromere functionality (120) and recently was also shown to impact alternative splicing of pre-mRNAs (85).

DNA methylation

DNA may be methylated at the 5′-position of cytosine usually within cytosine-guanine (CpG) dinucleotides. Approximately 70%–80% percent of all CpG dinucleotides are methylated in human somatic cells. A concentration of CpG sites is seen in the so-called CpG islands, which are usually short CpG-rich DNA regions located in more than half of all human gene promoters, and in so-called CpG island shores, which are located upstream of promoter-associated CpG islands or in regions of large repetitive sequences, such as centromeres and retrotransposon elements (19, 28, 138). There are three main DNA methyltransferases that catalyze cytosine methylation in CpG dinucleotides. Acting as *de novo* methyltransferases, DNMT3A and DNMT3B target unmethylated CpGs. In contrast, DNMT1 targets hemimethylated DNA to maintain the methylation pattern following DNA replication (19). The effects of DNA methylation depend on its localization in the genome. For example, CpG methylation of repetitive sequences has been associated with increased genome stability. Hypermethylation of promoter-associated CpG-rich sequences may repress transcription and, hence, gene expression, whereas methylated CpGs located in the genes themselves are associated with active genes (123). The molecular mechanisms by which CpG methylation influences the regulation of gene expression are not fully understood. First models suggested that CpG methylation in promoter regions may directly block the binding of transcription factors to their target sequences in the DNA (58). However, more recent studies demonstrated that promoter methylation inhibits the binding of the transcriptional machinery by recruiting methylated CpG-binding proteins and modifying chromatin structure via further protein interactions (87, 136).

5-methylcytosine (5mC) generated by DNA methyltransferases may be further modified by hydroxylation to 5-hydroxymethylcytosine, a modification that is mediated by the ten-eleven-translocation (TET) family of α-ketoglutarate (α-KG)- and Fe(II)-dependent dioxygenases (124). In Purkinje neurons, for example, 5-hydroxymethylcytosine is approximately 40% as abundant as 5mC, suggesting an important biological function (70). However, the precise functional roles of 5mC hydroxylation are still poorly understood and a matter of active research (26, 147). It has been suggested that 5-hydroxymethylcytosine induces passive demethylation by preventing DNMT1-mediated maintenance methylation and inducing active demethylation by DNA repair mechanisms. Very recent data in fact show that oxidation of 5mC and 5-hydroxymethylcytosine by TET proteins, followed by thymine-DNA glycosylase (TDG)-mediated base excision of the resulting 5-carboxylcytosine, constitutes an important pathway for active DNA demethylation (53). Moreover, several methyl-CpG-binding proteins display reduced affinity for 5-hydroxymethylcytosine, which, in turn, may have effects on transcriptional regulation.

Post-translational histone modifications

Chromatin is not just an inert structure required for the proper package of DNA within the cell nucleus but represents a highly dynamic and instructive DNA scaffold (9). The histones H2A, H2B, H3 and H4 associate as octamers with DNA to form a nucleosome, the fundamental repeating unit in chromatin (3). Histones may be post-translationally modified, mainly in their N-terminal tails. The list of such modifications is constantly growing and comprises acetylation (ac), methylation (me), phosphorylation (ph), sumoylation (sumo), ubiquitination (ub) and others. Histone modifications influence the overall structure of chromatin and positively or negatively modulate the binding of effector molecules (9). Thereby, several enzymes that add or remove histone modifications interact to produce a chromatin structure that has a more activating or a more repressive effect on gene transcription. Histone acetylation, for example, acetylation of lysine 56 on histone H3 (abbreviated H3K56ac) and acetylation of lysine 16 on histone H4 (H4K16ac), seems to be generally associated with activation of transcription. On the other hand, histone methylation may either repress gene expression (H3K9me2, H3K27me3) or activate transcription (eg H3K4me2, H3K4me3), depending on the specific sites of methylation (78). The consequences of histone modifications depend on the genomic context. For instance, H3K6ac and H3K6me are both associated with active genes, the former being enriched in promoter sequences, the latter being enriched in the bodies of active genes (95).

Interplay between DNA methylation and histone modifications

Different types of histone modifications are strongly interconnected with each other in the regulation of gene expression. Histone modifications may crosstalk *in situ* (at the same residue), *in cis* (on the same histone) and *in trans* (on different histones) (95). For instance, H3K9 methylation, related to gene repression, blocks H3K9 acetylation that occurs on promoters of active genes and vice versa (100). As an example of a histone crosstalk *in cis*, H3S10ph is required for H3K14 acetylation (82). On the other hand, H3S10ph inhibits H3K9 methylation and vice versa (110). H3K4me seems to crosstalk with H4K16 acetylation *in trans* (29). Moreover, bidirectional complex and as yet poorly understood molecular interactions are taking place between histone modifications and DNA methylation. For example, H3K9 trimethylation is required for DNMT3B-dependent DNA methylation of pericentromeric repeats (76). On the other hand, H3K4 methylation was shown to be mutually exclusive to *de novo* DNA methylation by DNMT3A/B (89, 92, 104). The ubiquitin-like protein containing plant homeo domain (PHD) and ring finger domains 1 (UHRF1) protein recognizes H3K9me marks and, after binding to hemimethylated DNA, recruits DNMT1 to allow for maintenance of DNA methylation in heterochromatin following replication (118). In addition, UHRF1 induces histone deacetylation and transcriptional repression by recruiting histone deacetylase 1 (HDAC1) after binding to methylated CpG (132). These are only some examples of the complex epigenetic interplay between histone modifications and DNA methylation in the regulation of gene expression (for a more comprehensive overview see (95)).

COMMONLY USED METHODS FOR THE DETECTION OF EPIGENETIC DNA AND HISTONE ALTERATIONS

Methods for the detection of 5mC

The investigation of methylated cytosines in genomic DNA relies mainly on the following three principles: (i) sodium bisulfite conversion of nonmethylated cytosines to uracil; (ii) use of methylation-sensitive and nonsensitive isoschizomeric restriction enzymes; and (iii) immunoprecipitation of methylated DNA by antibodies against 5mC or affinity-binding to methylated DNA-binding enzymes. These pretreatment methods can be combined with different analytical platforms, including microarrays and high-throughput sequencing to enable genome-wide DNA methylation analysis (Figure 1, Table 1).

Methods used for the targeted detection of promoter methylation of individual genes

Several of the most commonly used methods for locus-specific DNA methylation analysis are based on the treatment of DNA with sodium bisulfite, resulting in a conversion of nonmethylated cytosines to uracil, whereas methylated cytosines remain unaltered. Modified DNA is used as a template for amplification in the methylation-specific PCR (MSP) technique, a rapid and very sensitive method for the detection of methylated DNA (55). MSP is the most frequently used technique for the diagnostic determination of promoter hypermethylation of the O^6-methylguanine-DNA methyltransferase gene (*MGMT*) in glioblastomas (142). PCR is performed using primer sets designed to distinguish methylated from unmethylated DNA. This method is even useful for the investigation of DNA methylation in formalin-fixed paraffin embedded tissues (FFPE).

MethyLight is a fluorescent-based real-time PCR method used to detect and quantify DNA methylation in bisulfite-converted DNA (31). The method is highly sensitive and no post-PCR manipulations, such as gel electrophoresis, are required. However, similar to MSP, this method generates no information about the precise regional methylation pattern of the genomic DNA at the individual CpG sites investigated.

Combined bisulfite restriction analysis (COBRA) is a quantitative technique for methylation detection that also depends on bisulfite-converted DNA (148). The genomic region of interest is amplified with primers not discriminating methylated from nonmethylated DNA but encompassing a restriction enzyme site, which is affected by the bisulfite treatment. The PCR product is digested with the respective enzyme and quantified using gel electrophoresis and densitometry. COBRA is compatible with

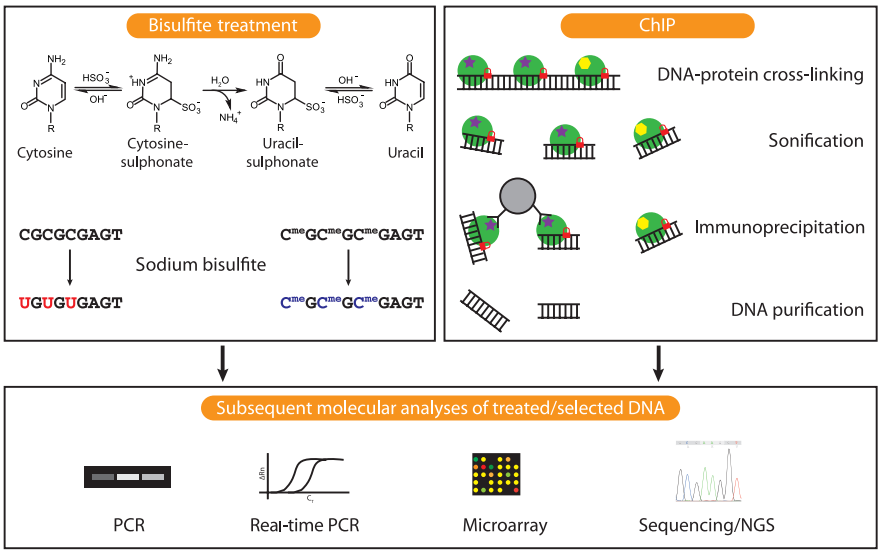

Figure 1. Commonly used methods to analyze epigenetic alterations. Sodium bisulfite treatment, which is frequently used for the investigation of DNA methylation, converts cytosine to uracil, while 5-methylcytosine (5mC) and 5-hydroxymethylcytosine are protected against this conversion. Subsequent analyses of the bisulfite-treated DNA using PCR [methylation-specific PCR (MSP)], real-time PCR (MethyLight), microarrays, different sequencing approaches or mass spectrometry (MassARRAY EpiTyper assay, not depicted) allow to distinguish cytosines converted to uracil from unaltered cytosines. Chromatin immunoprecipation (ChIP) is frequently used to investigate specific histone modifications. Therefore, histones are stably cross-linked to DNA by formaldehyde, *in vivo*. After cell lysis, DNA is fragmented by sonification. Antibodies against the modified histone of interest are added, bind to the modified histone and are afterwards pulled down by, for example, protein A/G coated agarose beads (protein A/G is capable of binding the Fc fragments of the specific antibodies). Thereafter, DNA protein cross-links are reversed, and the DNA that was bound to the modified histone is purified for subsequent analyses. PCR and real-time PCR focus on specific DNA regions, whereas microarray based methods (ChIP-on-chip) or next generation sequencing methods (ChIP-seq) enable whole genome coverage. Histones are symbolized by green circles; two different histone modifications are symbolized by a purple star and a yellow hexagon, respectively; DNA-protein cross-linking is indicated by a red lock; the grey circle represents an agarose bead with protein A/G coating.

Table 1. Summary of commonly used methods for the detection of methylated DNA.

	Bisulfite conversion	Methylation-sensitive restriction enzymes	Immunoprecipitation or affinity-binding of methylated DNA
Locus-specific DNA methylation analyses	MSP MethyLight COBRA Sanger-Sequencing Pyrosequencing	Digest followed by PCR or Southern Blot MS-MLPA	Immunoprecipitation or affinity-binding followed by PCR
Genome-wide DNA methylation analyses	NGS	RLGS DMH MCA NGS	MeDIP MIRA NGS

COBRA = combined bisulfite restriction analysis; DMH = differential methylation hybridization; MS-MLPA = methylation-specific multiplex ligation-dependent probe amplification; MCA = methylated CpG island amplification; MeDIP = methylated DNA immunoprecipitation; MIRA = methylated CpG island recovery assay; NGS = next-generation sequencing; MSP = methylation-specific PCR; RLGS = restriction landmark genomic scanning.

DNA extracted from FFPE tissue but provides data only for the specific restriction enzyme recognition site.

The gold standard for mapping of methylated CpG sites is sequencing of sodium bisulfite-modified DNA (44) either by chain termination sequencing ("Sanger sequencing") or pyrosequencing, which is a sequencing-by-synthesis method (21, 128). Both sequencing methods rely on the sodium bisulfite conversion of genomic DNA and PCR amplification of the region of interest using primers specific for bisulfite-converted DNA but without covering CpG sites. Both methods generate a complete picture of the methylation pattern of the region of interest. In addition, pyrosequencing allows for the quantification of multiple CpG methylation sites in one sequencing run. In contrast to conventional sequencing, however, only short sequences can be read efficiently, therefore limiting the number of CpG sites that may be analyzed in one read.

The EpiTYPER™ assay (Sequenom, San Diego, CA, USA) involves PCR amplification from bisulfite-converted DNA, followed by *in vitro* transcription into RNA from the reverse strand and base-specific cleavage of the transcribed RNAs. The cleavage products result in distinct fragments for methylated and non-methylated DNA that are detected by MALDI-TOF mass spectrometry (MassARRAY™ system; Sequenom). This method allows for the rapid, sensitive and quantitative detection of methylation patterns of multiple CpG-containing amplicons of up to 600 base pairs and is ideally suited for the screening of defined gene sets in larger sample numbers. However, it requires access to a specialized mass spectrometry system that is not available at all places.

Several nonbisulfite methods for the detection of methylated cytosines in specific DNA sequences rely on the digestion of DNA with methylation-sensitive enzymes followed by different detection methods of the restriction fragments, for example, by PCR or Southern blot analysis. Another method often used for the analysis of methylated cytosines is based on multiplex ligation-dependent probe amplification (MLPA) in combination with methylation-sensitive restriction enzymes (methylation-specific MLPA, MS-MLPA). In MS-MLPA the ligation of the MLPA probe oligonucleotides is combined with digestion of the genomic DNA-probe hybrid complex by methylation-sensitive restriction enzymes (102). This method is also being used for the semiquantitative detection of *MGMT* promoter hypermethylation in the routine diagnostic assessment of gliomas (61).

Methods used for large-scale DNA methylation analysis

One of the earliest methods used for genome-wide, non-microarray-based DNA methylation analysis is restriction landmark genomic scanning (RLGS) (52). RLGS is a two-dimensional DNA gel electrophoresis technique that allows for the quantification of gene copy number and methylation status at the same time. A drawback of the RLGS method is its limited sensitivity for the detection of methylated CpG sites.

Another method based on the use of methylation-sensitive restriction enzymes is differential methylation hybridization (DMH) that allows for the determination of the methylation levels of a large number of CpG-rich loci at the same time (57). DMH is an array-based method employing CpG island fragments gridded on high-density arrays. The genomic DNA of interest is digested with methylation-sensitive enzymes, and the digestion products are used as templates for PCR after linker ligation. The amplification products are labeled and used as probes that are hybridized to the CpG island arrays. Neither further cloning nor sequencing of the fragments of interest is necessary. A major disadvantage of the methods using restriction enzymes is the reliance on specific restriction enzyme-cutting sites and incomplete digestion that can result in false-positive results. The methylated CpG island amplification (MCA) method addresses the problem of false-positive results generated by RLGS or DMH by using methylation-sensitive and insensitive isoschizomeres followed by adaptor ligation and PCR amplification (129). Thereby, methylated CpG-rich sequences are preferentially amplified, labeled and hybridized to microarrays. This method generates less frequently false-positive results than DMH, but the genome coverage is still limited.

The third basic method for DNA methylation analysis is the enrichment of methylated genomic sequences by affinity-binding of methylated sequences to 5mC antibodies (methylated DNA immunoprecipitation; MeDIP) (141) or to columns that contain methyl-CpG-binding domain proteins, like MBD2 (methylated CpG island recovery assay; MIRA) (109). The enriched methylated sequences can be used as templates either for locus-specific amplification or for genome-wide methylation analysis using microarrays. MeDIP and MIRA allow for the rapid identification of multiple CpG sites in the genomic DNA of interest. However,

they depend on the availability of specific and efficiently working antibodies and methyl-CpG-binding domain proteins, and, like the other microarray-based, genome-wide DNA methylation detection methods, the genome coverage is still limited because the entire human genome is not represented on the microarrays. This problem is circumvented by the use of high-throughput genomic sequencing approaches. On next-generation sequencing platforms, bisulfite converted DNA and methylated DNA enriched by using methylation-sensitive restriction enzymes, as well as affinity binding of methylated sequences to 5mC antibodies or to methylated DNA binding proteins can be used to assess millions of DNA fragments, thus allowing for detection of DNA methylation across the entire genome, including interspersed repeat sequences that are inaccessible when using microarrays (42).

Methods to distinguish 5mC from 5-hydroxymethylcytosine

The discovery of 5-hydroxymethylcytosine, which is highly abundant in the human brain (70), raised the question of how to specifically identify 5-hydroxymethylcytosine and how to distinguish it from 5mC. Techniques based on bisulfite conversion of DNA are incapable of distinguishing 5mC from 5-hydroxymethylcytosine as both bases are not altered by sodium bisulfite and are recognized as "cytosine" in downstream detection methods. Methylation-sensitive restriction enzymes are also strongly inhibited by 5-hydroxymethylcytosine, while techniques based on immunoprecipitation with antibodies against methylcytosine or methyl-CpG-binding domain proteins are specific for 5mC and do not detect 5-hydroxymethylcytosine (63). Recently, specific antibodies against 5-hydroxymethylcytosine have become available as well as assays that enable the relative quantitation of 5mC and 5-hydroxymethylcytosine by the addition of glucose to the hydroxyl group of 5-hydroxymethylcytosine via an enzymatic reaction utilizing T4 β-glycosyltransferase, thereby converting a cleavable *MspI* site to a noncleavable site.

Detection of histone modifications

The gold standard for the detection of post-translational modifications of histone proteins is mass spectrometry (34). It is the most accurate method for identifying histone modifications but requires a high degree of technical expertise and is difficult to apply genome-wide. More commonly, the chromatin immunoprecipitation assay (ChIP-assay) (90) is used for linking histone modifications to particular DNA sequences. Chromatin immunoprecipitation is performed with antibodies against specific histone modifications, for example, trimethylation of histone H3 at lysine 4 (H3K4me3), a mark associated with active chromatin (19). The immunoprecipitated DNA is then typically analyzed by PCR with specific primers to investigate a candidate gene region of interest. To identify histone modifications at a genome-wide level, the immunoprecipitated DNA can be hybridized to microarrays (ChIP-on-chip) (113) or can be applied to massively parallel sequencing techniques (ChIP-seq) (10).

FREQUENT ALTERATIONS OF CpG METHYLATION AND HISTONE MODIFICATION IN CANCER CELLS

Cancer cells often show a variety of epigenetic aberrations including altered DNA methylation, as well as changes in histone

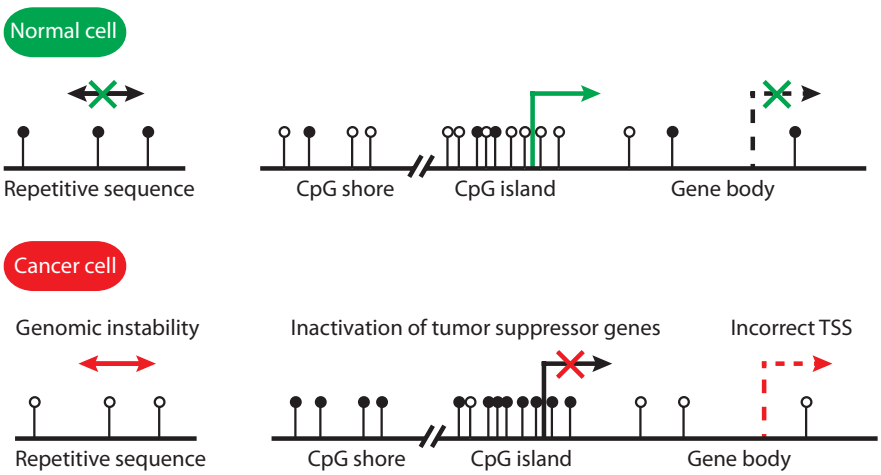

Figure 2. Common epigenetic alterations in cancer. Epigenetic changes are observed in many cancer types. Hypermethylation of gene promoters decreases the expression of proteins or non-coding RNAs that have antiproliferative or proapoptotic effects, or mediate DNA repair, or inhibit invasion or angiogenesis. In some cases, hypomethylation of the promoters of proto-oncogenes was demonstrated (not depicted). In certain cancers, for example, colorectal carcinomas, differential methylation was found mainly in so-called CpG island shores that reside upstream of promoter-associated CpG islands. As an example, CpG island shore hypermethylation is depicted resulting in reduced transcription. Hypomethylation of the gene body may unblock alternative transcription start sites. Moreover, hypomethylation of repetitive sequences is associated with genomic instability. The cancer-associated changes in DNA methylation pattern are accompanied by further "epimutations", such as histone modifications that alter the chromatin structure (not depicted). Methyl groups are symbolized by black circles; green and red crosses symbolize the block of gene expression or transposition; TSS: transcriptional start site.

methylation and acetylation (117) (summarized in Figure 2). Interestingly, such epigenetic changes may already be present in early tumor stages before characteristic mutations of tumor suppressor genes or proto-oncogenes are detectable (39, 43). In general, malignant tumor cells typically demonstrate genome-wide hypomethylation, site-specific CpG island hypermethylation (117), as well as differential methylation of CpG island shores (59). DNA hypomethylation of repetitive elements, retrotransposons and introns has been linked to increased genomic instability (32), whereas focal hypermethylation of CpG island in promoter regions may cause transcriptional silencing of tumor suppressor genes and other genes (117). Promoter hypomethylation, on the other hand, may activate the transcription of proto-oncogenes (140). Moreover, the histone code, that is, the pattern of post-translational modifications of core histones, is commonly altered in cancer cells. For instance, levels of H4K20me3 and H4K16ac are often reduced at hypomethylated DNA repetitive sequences in cancer (43).

EPIGENETIC ALTERATIONS IN GLIOMA—TOWARD UNRAVELING THE GLIOMA EPIGENOME

Glioma-associated histone modifications

Several studies have provided evidence for a deregulation of genes encoding proteins involved in the regulation of histone modifications in gliomas. In particular, large-scale sequencing of glioblastoma samples detected several mutations in genes encoding HDAC (*HDAC2*, *HDAC9*), histone demethylases (*JMJD1A JMJD1B*), histone methyltransferases (*SET7, SETDB2, MLL, MLL3, MLL4*) and the methyl-CpG-binding domain protein 1 (*MBD1*) (106). In medulloblastoma, mutations in the histone-lysine N-methyltransferase genes *MLL2* or *MLL3* were recently identified in 16% of the cases (107). The nuclear receptor SET domain containing protein-1 (*NSD1*) gene, which encodes a histone methyltransferase involved in chromatin regulation, has been shown to undergo CpG island methylation and subsequent transcriptional downregulation in neuroblastomas and glioblastomas, thereby demonstrating an interesting crosstalk between different layers of epigenetic regulation, that is, the epigenetic inactivation of NSD1 by DNA methylation leads to reduced methylation of the histone lysine residues H4-K20 and H3-K36, respectively (11). Other authors reported that transcript levels of various histone deacetylases (HDAC5-11) are decreased in glioblastoma when compared with low-grade astrocytoma and normal brain tissue, a finding that was associated with higher levels of histone H3 acetylation in glioblastoma (84). Immunohistochemical analysis of glioma tissue microarrays showed strong nuclear expression of HDAC1, HDAC2 and the nuclear receptor corepressor 2 (NCOR2) in glioma cells, whereas NCOR1 and HDAC3 were sparsely detected in the cytoplasm and nuclei of tumor cells (18). HDAC3 expression inversely correlated with tumor grade and increased HDAC3 expression was a marker of better prognosis. On the other hand, expression of HDAC1 and HDAC2 increased during tumor recurrence and progression (18). Another immunohistochemical study investigated the expression of various histone H3 and H4 modifications in tumor tissue samples of 230 glioma patients and identified prognostically distinct patient subgroups based on the expression of different sets of histone modifications (80).

In addition to these global aberrations at the histone level, individual histone modifications and their downstream effects in gliomas have been studied. For example, the candidate tumor suppressor gene *RRP22* was shown to be transcriptionally repressed in gliomas by 5′-CpG island hypermethylation and/or heterochromatinization as evidenced by increased levels of H3K9me3 and decreased pan-Ac-H3 bound to this gene (119). HDAC4 was reported to suppress expression of the cell cycle regulator $p21^{waf1/Cip1}$ in cancer cells by reducing histone H3 acetylation at the Sp1/Sp3-binding site-rich proximal promoter of the *CDKN1A* gene (94). Silencing of HDAC4 induced $p21^{WAF1/Cip1}$ and reduced tumor growth of glioblastoma cells, suggesting HDAC4 inhibition as a possible therapeutic target (94). Other authors identified a mechanism by which the oncogenic PI3K-AKT pathway, which is frequently altered in malignant gliomas, upregulates the expression of HOXA9 through histone modifications, which could possibly be initiated by AKT-induced EZH2 histone methyltransferase activity (24). HOXA9 is a pro-proliferative, antiapoptotic transcription factor and associated with poor prognosis in gliomas (24). Despite these interesting studies, current knowledge on the functional roles of histone modifications in gliomas is still limited, and the relevant set of genes epigenetically regulated by post-translational histone changes remains to be characterized. However, a growing number of studies provides evidence that drugs resulting in global modifications of the histone code, in particular HDAC inhibitors, may have therapeutic effects on glioma growth by reducing proliferation and inducing differentiation (see later discussion).

Alterations of DNA methylation in gliomas

Global DNA hypomethylation and focal hypermethylation of CpG-rich promoter-associated sequences are common findings in malignant gliomas (86). Hypomethylation has been detected at Sat2 pericentromeric DNA, the subtelomeric repeat sequence D4Z4 and interspersed Alu elements (17). Moreover, hypomethylation was associated with copy number changes of the adjacent euchromatin and induced the reactivation of the cancer-testis antigen MAGEA1, indicating that global hypomethylation may promote glioma growth by increasing genomic instability and the disinhibition of proto-oncogenes (17). Interestingly, global DNA hypomethylation, as evidenced by reduced levels of *LINE-1* methylation, has been associated with less favorable outcome in glioma patients (103). Another recent study found that overexpression of DNMT1 and DNMT3B in glioma was mediated by histone modification and promoter hypomethylation, respectively, and that inhibition of these DNA-methyltransferases resulted in re-expression of tumor suppressor genes (108).

The list of genes reported as being epigenetically silenced in gliomas by aberrant promoter methylation is steadily growing and includes genes involved in a variety of cellular processes linked to glioma development and progression. For example, focused methylation analyses of candidate genes in primary glioma tissues revealed promoter methylation of cell cycle regulatory genes, for example, *RB1* (98), $CDKN2A/p16^{INK4a}$, $p14^{ARF}$ and $CDKN2B/p15^{INK4b}$ (25, 97, 146); apoptosis-regulating genes, for example, *PYCARD* (121); DNA repair genes, for example, *CHK2* (139) and *MGMT* (see later discussion); migration and invasion related genes, for example, *TIMP3* (35), *CDH1* (30), *PCDH-gamma-A11* (137) and *SOCS3* (79), as well as various established or putative

tumor suppressor genes, such as *PTEN* (6), *RASSF1A* (56), *DIRAS3* (114), *EMP3* (1), *CITED4* (127), *BLU* (83), *NDRG2* (126), *CTMP* (68), *ECRG4* (48) and different Wnt pathway regulatory genes (49). Accelerated by recent large-scale DNA methylation analyses, many more genes have been identified as being aberrantly methylated in gliomas (74, 101), with certain types of gliomas showing concurrent hypermethylation of multiple genes. This phenomenon has been referred to as glioma-CpG island methylator phenotype (*G-CIMP*) (101), analogous to the CIMP observed in certain epithelial cancers, in particular colorectal carcinomas (60). However, promoter hypermethylation was found to be inconsistently associated with transcriptional downregulation (74, 101), and the individual roles of the hypermethylated genes in promoting glioma growth remain to be clarified.

Patterns and frequency of promoter hypermethylation depend on glioma type and grade

Evidence is increasing that not only the patterns of genetic aberrations but also the epigenetic profiles in gliomas differ between different tumor types and malignancy grades. For example, secondary glioblastomas were shown to have a higher frequency of promoter methylation of $p14^{ARF}$, $p16^{INK4a}$ (97), *RB1* (98), *MGMT* (96), *TIMP3* (99), *EMP3* (73), and the Wnt inhibitor *DKK1* (49) when compared with primary glioblastomas. On the other hand, promoter hypermethylation and transcriptional downregulation of *NDRG2* (126), *SFRP1*, *SFRP2*, and *NKD2* (49) is detectable in at least 40% of primary glioblastomas but only in low fractions of secondary glioblastomas.

Oligodendrogliomas often show aberrant promoter methylation of multiple genes (2, 146), which has recently been found to correspond to the G-CIMP phenomenon (101). In fact, many of the genes found to be commonly hypermethylated in oligodendroglial tumors are also hypermethylated in diffuse astrocytic gliomas and secondary glioblastomas but not in primary glioblastomas. Thus, gliomas may be stratified into two major groups according to their epigenomic profiles, that is, one group consisting mostly of diffuse gliomas and secondary glioblastomas with concurrent hypermethylation of multiple gene promoters, which largely overlaps with the group of *IDH1* mutant gliomas, and another group consisting mostly of primary glioblastomas with global DNA hypomethylation and less frequent promoter hypermethylation, which largely overlaps with *IDH1* wild-type gliomas (74, 101). Within the group of diffuse gliomas with multiple hypermethylated genes, tumors with or without 1p/19q deletion have been reported to display evidence for distinct methylation profiles (74). Similarly, a study investigating the methylation status at CpG islands associated with 15 distinct gene loci in 139 gliomas demonstrated characteristic methylation profiles in different glioma subtypes, with pilocytic astrocytomas rarely showing hypermethylation at the investigated loci (131). Interestingly, global methylation patterns of individual gliomas seem to remain strikingly stable upon tumor progression and recurrence (74, 101). This fact may indicate that altered DNA methylation is usually an early event in glioma development. In line with these findings, the *MGMT* promoter methylation status remains stable between primary and recurrent tumors in the vast majority of glioblastoma patients (40).

A mechanistic link between IDH1 mutation and the altered epigenome of gliomas

Noushmehr and colleagues performed an unsupervised hierarchical clustering of the glioblastoma samples included in The Cancer Genome Atlas project based upon methylation profiles (101). They found a small cluster of glioblastomas (8.8%) that was characterized by concerted hypermethylation of many gene loci (G-CIMP). Further analyses revealed that G-CIMP was common in secondary and recurrent glioblastomas but also in diffuse gliomas, in particular oligodendroglial tumors (53/56 cases). G-CIMP was associated with proneural gene expression profiles (135) in glioblastomas and with prolonged survival (101). Among the differentially hypermethylated genes within the proneural G-CIMP-positive tumors, the expression of several genes, for example, *FABP5, PDPN, CHI3L1* and *LGALS3*, was also downregulated (101), while high expression of these four genes was associated with poor prognosis (22). It has been speculated that patients with G-CIMP gliomas may benefit from therapeutic modification of DNA methylation (20).

Interestingly, G-CIMP was strongly associated with mutation of the *IDH1* gene, which represents an early and common event in the pathogenesis of diffuse astrocytic and oligodendroglial tumors, as well as secondary glioblastomas, but are rare in primary glioblastomas and all other types of brain tumors (8, 106, 115, 150). In the series of Noushmehr *et al*, one of four secondary glioblastomas was G-CIMP negative. This particular tumor did not carry an *IDH1* mutation. Among the investigated primary (*de novo*) glioblastomas, the majority of tumors lacked *IDH1* mutations and were G-CIMP negative (101). Two independent studies confirmed that a hypermethylation phenotype was common in diffuse gliomas, including secondary glioblastomas, and tightly associated with *IDH1* mutation (20, 74).

Recent studies provided a mechanistic explanation for the striking association between mutation of IDH1 or IDH2, that is, enzymes that take part in the tricarboxylic acid cycle, and the observed large-scale epigenetic changes in glioma and acute myeloid leukemia cells (Figure 3). The $NADP^+$-dependent isocitrate dehydrogenase 1 normally catalyzes the oxidative decarboxylation of isocitrate to α-KG (synonym: 2-oxoglutarate) (111). *IDH1* mutations, however, reduce IDH1 activity as compared with the wild-type enzyme, which results in reduced α-KG levels (153). Moreover, the mutant IDH1 enzyme gains a neomorphic enzymatic activity and catalyses the NADPH-dependent reduction of α-KG to R(-)-2-hydroxyglutarate (2-HG). Therefore, *IDH1* mutant tumors, including gliomas and acute myeloic leukemias, demonstrate elevated levels of the neometabolite 2-HG (27, 41). In addition, *IDH1* and *IDH2* mutations decrease NADPH production (150), probably predisposing cells to oxidative stress (111). Profiling of more than 200 metabolites revealed altered levels of amino acids, glutathione metabolites, choline derivatives and tricarboxylic acid cycle intermediates in cells expressing mutant IDH1 (112), which demonstrates an extensive impact of *IDH1* mutations on the cellular metabolome.

Importantly, recent studies revealed that 2-HG competitively inhibits the activity of various α-KG-dependent dioxygenases, including histone demethylases and the TET family of 5mC hydroxylases (41, 149). Xu and collaborators showed that ectopic expression of *IDH1* R132H in U-87 cells decreased α-KG levels by 60% and increased 2-HG levels more than 20-fold resulting

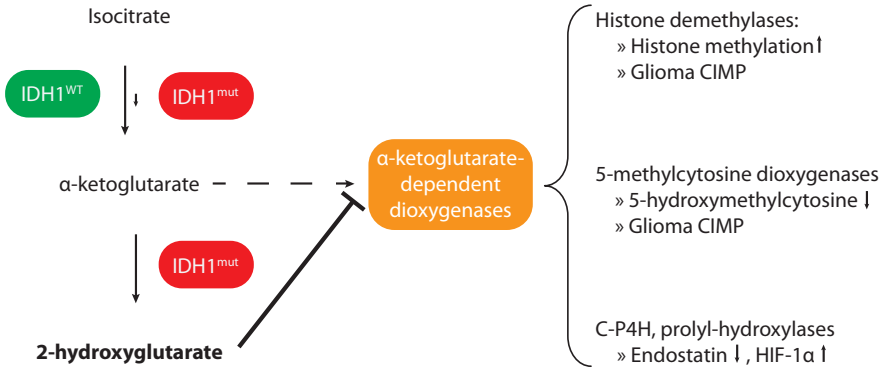

Figure 3. Impact of IDH1 mutation on epigenetic control. Isocitrate dehydrogenase 1 (IDH1) catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG), which is coupled to the reduction of $NADP^+$ to NADPH + H^+. IDH1 (eg, R132H mutation) results in a neomorphic IDH1 activity that catalyzes the NADPH consuming reduction of α-KG to 2-hydroxyglutarate (2-HG). Thereby, α-KG levels are reduced, and 2-HG levels are strongly increased in IDH1 mutant tumor cells. 2-HG is a competitive inhibitor of a large number of α-KG dependent dioxygenases, including histone demethylases and 5-methylcytosine hydroxylases of the TET protein family. Thereby, IDH1 mutation leads to altered methylation at different histone residues and decreased levels of 5-hydroxymethylcytosine. Together, both epigenetic alterations are supposed to result in widespread changes in DNA methylation and the histone code, namely hypermethylation of multiple promoter-associated CpG islands (G-CIMP) and more compact chromatin. In addition, 2-HG produced by mutant IDH1 also inhibits collagen-prolyl-4-hydroxylase (C-P4H) and prolyl-hydroxylases, which may lead to tumor promotion by stabilization of HIF-1α.

in an increase of different histone methylations (H3K4me, H3K27me2, H3K4me3, H3K9me3, H3K79me2). Other authors also observed altered histone methylation in gliomas with *IDH1* mutation (20). Moreover, 2-hydroxyglutarate was shown to inhibit the activity of 5mC hydroxylases of the TET family, in particular TET2, and thereby lower the levels of 5-hydroxymethylcytosine in *IDH1* mutant leukemias and gliomas (41, 149). In hematopoietic malignancies, *TET2* mutations were mutually exclusive with *IDH1* mutations and similarly linked to a DNA hypermethylation phenotype, which fueled the hypothesis that reduced DNA demethylation because lowered 5-hydroxymethylcytosine levels eventually results in hypermethylation of multiple CpG islands (41). In low-grade gliomas lacking *IDH1* and *IDH2* mutation, only a small proportion of tumors showed *TET2* promoter hypermethylation, while no *TET2* mutations were detected (67). Taken together, the finding that 2-HG, which is aberrantly produced by mutant IDH1 and IDH2 proteins, inhibits α-KG-dependent enzymes involved in the regulation of DNA, and histone methylation provides a mechanistic link between mutations in either of these genes, altered glioma cell metabolism and widespread epigenetic changes, in particular, aberrant methylation of multiple genes and global histone modifications.

Are there genetic changes other than IDH1 mutation that may alter the glioma epigenome?

Several recent studies have identified mutations in different genes directly involved in the regulation of DNA methylation and chromatin remodeling. In myeloid malignancies, for example, somatic mutations in different epigenetic regulators have been identified, including the *de novo* methyltransferase gene *DNMT3A*, the 5mC hydroxylase gene *TET2*, as well as the polycomb protein and histone methylase gene *EZH2* in subsets of acute and chronic myeloid leukemias [for review, see (38)]. Interestingly, these mutations appear to occur mutually exclusive to each other and to mutations in *IDH1* or *IDH2*.

Additional potential targets for cancer-associated mutations leading to widespread epigenetic changes include adenosine triphosphate (ATP)-dependent chromatin remodeling enzymes (69, 145), histone variants (125), histone chaperones (5) and histone readers (152). In fact, recent large-scale sequencing studies have identified frequent mutations in various chromatin remodeling genes in different types of cancer. For example, mutations in *UTX*, *MLL-MLL3*, *CREBBP-EP300*, *NCOR1*, *ARID1A* and *CHD6* have been detected in the majority of transitional cell carcinomas of the bladder (50). In renal cell carcinomas, 41% of the cases carried mutations in the switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex gene *PBRM1*, with mutations in the histone demethylase genes *UTX* and *JARID1C* or the histone methylase *SETD2* being each restricted to smaller subsets of cases (134). Among non-Hodgkin lymphomas, 32% of diffuse large B-cell lymphomas and 89% of follicular lymphomas were found to carry somatic mutations in the histone methyltransferase gene *MLL2*, while 10%–15% of these lymphomas had mutations in *MEF2B*, a calcium-regulated gene involved in histone acetylation (93). Moreover, somatic mutations in the chromatin remodeling enzyme genes *ATRX* and *DAXX* are frequent in pancreatic neuroendocrine tumors and have been associated with altered telomeres and increased genomic instability (62). The histone variant macroH2A suppresses melanoma progression *in vitro* and *in vivo* (65). ASF1B was identified as a histone chaperone isoform that is nescessary for proliferation and whose mRNA overexpression is associated with outcome in breast cancer patients (23). Aberrant expression of the histone reader TRIM24, which binds unmethylated H3K4, H3K23ac and estrogen receptor to induce estrogen-dependent genes, also correlates with the survival of breast cancer patients (130).

Among pediatric malignant brain tumors, mutations in the ATP-dependent SWI/SNF chromatin-remodeling complex gene *SMARCB1* (*INI1*) and, less commonly, *SMARCA4* (*BRG1*) are characteristic features of atypical teratoid rhabdoid tumors (51). In addition, pediatric medulloblastomas carry inactivating mutations in the histone methyltransferase genes *MLL2* or *MLL3* in 16% of the

cases (107). In contrast, little is known about alterations of any of these genes in gliomas to date. Nevertheless, as these alterations appear to be so common in various types of cancers, it may be possible that genetic mutations or epigenetic changes of any of these genes, or in yet other epigenetic regulators, are involved in glioma pathogenesis, for example, in those gliomas lacking *IDH1* or *IDH2* mutation but demonstrating widespread epigenomic alterations.

CLINICAL RELEVANCE OF EPIGENETIC MARKERS IN GLIOMAS

The paradigm of *MGMT* promoter methylation in malignant gliomas

The impressive progress in unraveling epigenetic alterations in cancer has not only greatly advanced our understanding of tumor pathogenesis but has already achieved clinical relevance by providing valuable molecular biomarkers, as well as promising novel therapeutic targets. In particular, the promoter methylation status of the *MGMT* gene has become an important prognostic and predictive biomarker in the diagnostic assessment of patients with high-grade malignant gliomas [for recent reviews see (116, 142)]. Based on a subpopulation of glioblastoma patients from the EORTC/NCIC 22981/26981 trial (122), Hegi and colleagues found a significantly better survival of patients treated with radiotherapy combined with concurrent and adjuvant temozolomide when their tumors demonstrated a hypermethylated *MGMT* promoter (54). In contrast, patients with *MGMT*-unmethylated glioblastomas did not appear to derive a significant survival benefit from the addition of temozolomide to radiotherapy, suggesting that the *MGMT* promoter methylation status serves as a predictive marker for response to alkylating chemotherapy in glioblastoma patients. Mechanistically, the predictive function of *MGMT* promoter methylation is explained by the fact that MGMT functions as a repair enzyme that counteracts the DNA damage induced by alkylating chemotherapeutic agents. These drugs cause an alkylation on the O6 position of guanine that conveys most of the cytotoxic effects (46). Tumor cells with high MGMT protein levels can repair this therapy-induced damage and, thereby, poorly respond to the treatment. In turn, *MGMT* promoter methylation, as seen in about 40% of primary glioblastomas, leads to low or absent MGMT expression and, thereby, inability to counteract the therapy-induced DNA damage and consequently better therapy response.

In anaplastic gliomas, *MGMT* promoter methylation has been linked to more favorable outcome not only in patients treated with temozolomide but also in patients treated with radiotherapy only (133, 144). One potential explanation for this surprising observation may again be related to the frequent presence of G-CIMP in anaplastic gliomas. In addition to *MGMT* promoter methylation, these tumors may in fact carry epigenetically silenced genes whose gene products normally confer radiotherapy resistance. Further studies addressing this hypothesis by using genome-wide methylation analyses are needed to identify and characterize such candidate genes.

Diagnostic testing for *MGMT* promoter methylation has already become a standard in clinical trials involving patients with high-grade gliomas. In fact, several trials are ongoing that specifically recruit only patients with either *MGMT* promoter methylated or unmethylated glioblastomas. Concerning the routine diagnostic setting, however, *MGMT* testing has not yet become a standard of care, mainly because of the still limited therapeutic alternatives that can be offered to glioblastoma patients. Nevertheless, it is foreseeable that clinical *MGMT* testing will gain increasing importance also in the routine diagnostic assessment of malignant glioma patients.

Various methods are being used to detect *MGMT* promoter methylation in clinical specimens, including a number of the techniques discussed above, such as MSP analysis (54, 55), methylation-specific pyrosequencing (91), the COBRA method (91), as well as techniques that do not require bisulfite conversion of DNA, such as methylation-quantification of endonuclease-resistant DNA (Methyl-QESD) (14) and MS-MLPA (61). The use of various methods and thresholds for the detection of *MGMT* promoter methylation, as well as the testings of variable numbers and locations of CpG sites within the promoter region, may give rise to a substantial degree of interlaboratory variation in testing results. Thus, centralized *MGMT* methylation testing is required in clinical trials. Moreover, application of *MGMT* promoter methylation testing in the routine diagnostic setting should be subjected to standardization, for example, by the definition of standard operating procedures and the implementation of interlaboratory quality control measures.

Role of other epigenetic markers in gliomas

A number of studies have reported on individual genes whose epigenetic silencing by promoter methylation was found to correlate with clinical outcome of glioma patients. Moreover, recent data even suggest the global DNA methylation status determined by *LINE1* methylation analysis as a powerful prognostic marker (103). However, none of these molecular alterations has as yet been promoted to the level of a clinically relevant marker that would be comparable with the *MGMT* status. In fact, many of the other reported prognostically relevant genes are preferentially hypermethylated in 1p/19q-deleted and/or *IDH1* mutant gliomas, thus, are likely members of the large group of genes concurrently hypermethylated in G-CIMP-positive gliomas (see above). In these tumors, the prognostic impact of each individual hypermethylated gene is difficult to assess. Moreover, it is not entirely clear whether the clinically less aggressive course of *IDH1* mutant and G-CIMP-positive gliomas is primarily because of the widespread epigenomic alterations or possibly related to other, yet to be characterized metabolic effect of the *IDH1* mutation. Interestingly, both oligodendroglial and diffuse astrocytic gliomas share frequent *IDH1* mutation, while the clinical outcome in anaplastic glioma patients appears to be significantly better in patients with oligodendroglial tumors (144). This suggests that genetic and/or epigenetic aberrations in addition to *IDH1* mutation/G-CIMP are of prognostic relevance in diffuse gliomas, for example, the 1p/19q status [for review, see (115)]. In this respect, the functional and prognostic roles of the recently identified candidate genes on 1p and 19q that are frequently mutated in oligodendrogliomas, namely *FUBP1* and *CIC* (13), remain to be investigated, in particular as to how they may synergize with *IDH1* mutation and G-CIMP in oligodendrogliomas.

Interestingly, glioma-associated epigenetic DNA modifications can not only be demonstrated in resected tumor tissue specimens but may also be detectable in tumor DNA circulating in body fluids, such as serum and cerebrospinal fluid (CSF). Several studies reported on the diagnostic utility of detecting promoter methyla-

tion of various genes, including *MGMT, CDKN2A, THBS1, TIMP3* and *RASFF1* in these body fluids (7, 75, 81). These studies found a high concordance between methylation of these genes detected in serum or CSF in relation to the corresponding tumor tissue, suggesting a potential role of serum-based DNA methylation profiling in the diagnostic and prognostic assessment of glioma patients.

CONCLUSIONS AND PERSPECTIVES

During the last decades, a large number of epigenetic changes have been discovered in gliomas that contribute to the inactivation of tumor suppressor genes and increase genomic instability of these tumors. With *MGMT* promoter methylation, one "epimutation" has reached clinical significance as a molecular biomarker of predictive and prognostic relevance in malignant gliomas. Moreover, the identification of a mechanistic link between *IDH1* or *IDH2* mutation and widespread epigenetic alterations manifesting as G-CIMP via the inhibition of α-KG-dependent epigenetic regulators represents a major step forward in the understanding of glioma pathogenesis. The recent technical advancements concerning genome-wide sequencing and large-scale epigenetic profiling methods will certainly provide important new insights into the complex interplay between genetic and epigenetic alterations in these tumors, thereby leading to a deeper understanding of the genomes and epigenomes of various glioma subtypes and malignancy grades.

Perspectives concerning "epigenetic therapy" of gliomas

More detailed knowledge of glioma-associated mutations and epimutations will also be necessary to further characterize those genes and pathways that essentially drive glioma growth and thus represent promising novel targets for pathogenesis-based therapeutic approaches. In contrast to genetic alterations, pharmacological repair of epigenetic changes may be easier to accomplish, although targeted reversal of specific epimutations without globally changing the epigenetic landscape is still challenging. To date, "epigenetic therapies" mainly consist in a rather broadly acting inhibition of the epigenetic machinery, in particular, inhibition of DNA methyltransferases and HDACs. However, the arsenal of epigenetic drugs is constantly growing, and the issue of more precisely modulating distinct epigenetic players is intensively being investigated [for review, see (16, 117)]. Concerning glioma treatment, preclinical data suggested efficacy of HDAC inhibition in experimental glioma models when given as single therapy (36, 151) or in combination with radiotherapy (33). Preliminary clinical results suggested low toxicity and moderate activity of treatment with HDAC inhibitors in pediatric malignant glioma patients (88), as well as adult patients with recurrent glioblastoma (45). In addition, prolonged survival has been noted in a subset of glioblastoma patients of the EORTC/NCIC trial who had been treated with valproic acid, an antiepileptic drug that has HDAC inhibitory activity (143). Several other trials evaluating HDAC inhibitors in the treatment of malignant glioma patients are ongoing to evaluate potential synergistic effects in combination with radiotherapy and various types of chemotherapy (see http://www.clinicaltrials.gov/).

Another aspect of potential clinical relevance concerns the effects of cytotoxic therapy, in particular, alkylating chemotherapy, on the glioma epigenome and its potential role in acquired therapy resistance. While the *MGMT* promoter methylation status usually remains stable between primary and recurrent glioblastomas after radiochemotherapy (40), experimental studies revealed that temozolomide and carmustine may cause large-scale heterochromatin reorganization in glioma cells by decreasing global levels of histone H3 acetylation and increasing levels of histone H3 trimethylated on lysine 9 (105). In fact, these epigenetic effects of DNA alkylators may be counteracted by concurrent treatment with HDAC inhibitors, thereby possibly providing one explanation for a synergistic role of both treatment approaches.

Glioma epigenetics—role of noncoding RNAs

Finally, it has to be acknowledged that an additional level of complexity in cancer epigenetics is emerging by recent insights into the role of noncoding RNAs as modifiers of DNA methylation and histone modification. This includes a variety of distinct types of noncoding RNAs, as well as different molecular mechanisms, such as double-stranded RNA (dsRNA)-mediated activation of gene expression associated with histone demethylation, endogenous antisense RNA-mediated CpG demethylation, putatively miRNA-mediated paramutation, piwi-RNA-mediated *de novo* DNA methylation of transposons, endogenous antisense RNA-mediated imprinting, long noncoding RNA- and xiRNA-mediated X inactivation, and other mechanisms [for review, see (154)]. Interestingly, mammalian microRNAs (miRNAs) that usually regulate gene expression post-transcriptionally by binding to target mRNAs in their 3′-UTR also may directly affect transcriptional gene silencing. For example, *miR-320a* is encoded on the minus strand within the promoter region of *POLR3D*. By inducing the association of AGO1, the polycomb group member EZH2, and H3K27me3 with the *POLR3D* promoter, *miR-320a* is able to directly repress the transcription of *POLR3D* (66). Moreover, miRNAs may change the epigenome of tumor cells by inhibiting DNA methyltransferases and other epigenetic regulators, as exemplified by the function of the *miR-29* family in the post-transcriptional regulation of *DNMT3A* expression. Overexpression of *miR-29* in lung cancer cell lines restored normal patterns of DNA methylation, induced re-expression of methylation-silenced tumor suppressor genes, and inhibited tumorigenicity (37). In gliomas, *miR-128* has been shown to regulate the chromatin-modifying protein Bmi-1, thereby increasing H3K27 trimethylation in addition to Akt phosphorylation and upregulation of $p21^{waf1}$ (47). On the other hand, expression of many miRNAs appears to be epigenetically modified by aberrant DNA methylation in cancer cells. A recent meta-analysis revealed a total of 122 miRNAs that were reported to be epigenetically regulated in 23 different cancer types (72). Hypermethylated miRNAs often are located in or have associated 5′-CpG islands and appear to cluster in certain genomic regions mapping to chromosome arms 1q, 7q, 11q, 14q and 19q, respectively (72).

Taken together, there is still a long way to go to unravel the glioma epigenome in its entire complexity. However, the tools are available to comprehensively characterize the various levels of epigenetic aberrations in these tumors, and novel discoveries are to be expected in the near future in this rapidly advancing field of molecular neuro-oncology. These will hopefully result in the identification of additional clinically useful biomarkers and promising novel targets for innovative epigenetic therapy approaches.

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