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Abstract

Epigenetic alterations have been recognized as important mechanisms in neoplastic transformation, malignant progression of cancer, and response to therapy. Epigenetic modifications include DNA methylation and post-translational modifications of histone proteins that influence the chromatin structure. Moreover, with the identification of the RNA interference machinery, a new layer of gene regulation has been added to the definition. The coordinated interaction of these processes regulates gene expression activity. The disruption of these mechanisms of control is involved in a wide variety of pathologies, including but not restricted to cancer. Although epigenetic changes are somatically inheritable, they are reversible and hence may represent actionable targets for novel therapies.

Here we will discuss the current understanding of alterations in the epigenetic landscape that occur in the evolution of brain tumors and their potential impact on patient therapy.

Keywords

DNA methylation • Histone modification • MicroRNAs • Epigenetic gene silencing • Epigenetic therapy

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The Epigenetic Control

The term “epigenetics” (literally “upon” genetics) was coined by Conrad Waddington in the early 1940s. It was initially used to explain why genetic variations sometimes do not lead to phenotypic variations and how genes might interact with their environment to yield a phenotype [1]. Currently epigenetics is defined as the study of mitotically and/or meiotically heritable changes in gene expression that involve molecular and structural changes of DNA but do not alter the

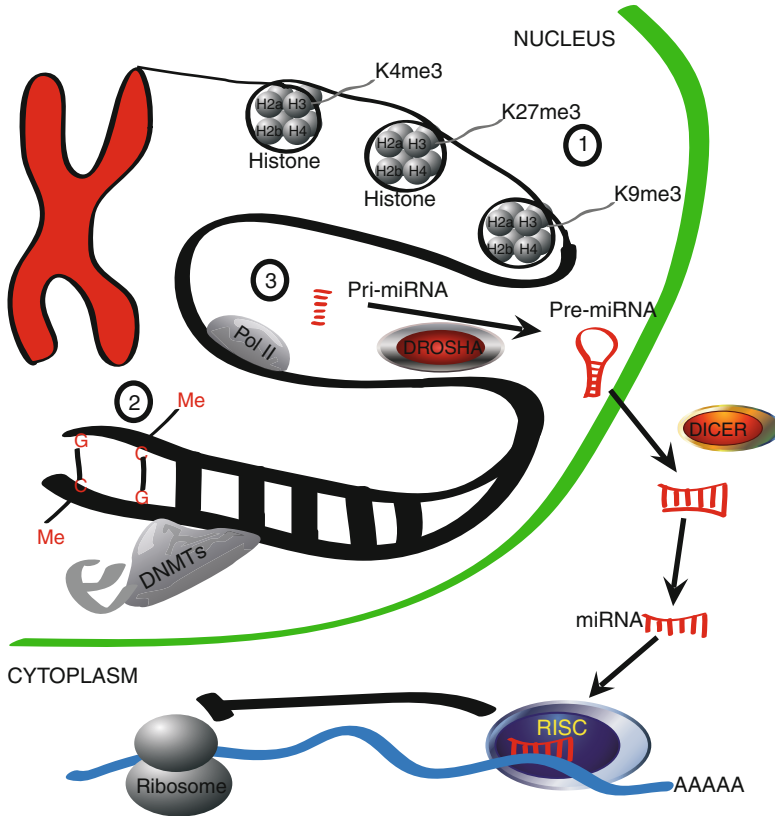


Fig. 2.1 Epigenetic regulation. Epigenetic regulation acts at different levels and with different molecular mechanisms like posttranslational modifications (PTMs) of the histone tails (1) DNA methylation of CpG dinucleotides (2) and microRNA expression (3) PTMs like tri-methylation (me3) of lysine number 4 (K4) of histone 3 (H3) (H3K4me3), H3K27me3, H3K9me3, etc. allow a more relaxed or compact chromatin status resulting in expression or repression of the genes under the modified histones. DNA methylation is catalyzed by DNMTs (DNA methyltransferases) and leads to gene silencing directly

by recruitment of methyl-CpG-binding domain (MBD) proteins that in turn recruit histone-modifying and chromatin-remodeling complexes to the methylated sites or indirectly by precluding the recruitment of DNA-binding proteins. MicroRNAs are transcribed from intragenic or intergenic regions by RNA polymerase II (PolII), and after several maturation steps mediated by the ribonucleases DROSHA and DICER, they are integrated in the RNA-induced silencing complex (RISC) that blocks translation of specific mRNAs

DNA sequence (Fig. 2.1) [2]. Epigenetic regulation ensures that the right genes are expressed at the right time to allow for cell-type-specific programs in development and differentiation, and adaptation to environmental cues that are not encoded in the DNA.

Epigenetic aberrations in cancer involve global DNA demethylation (hypomethylation) affecting intergenic regions, DNA repetitive sequences and gene bodies, and de novo methylation of CpG islands (hypermethylation) in promoter regions of tumor suppressor genes (Fig. 2.2). It has been

largely established that epigenetic silencing of key genes mediated by promoter methylation plays an important role in cancer [3]. In addition, dynamic regulation of the chromatin state is mediated by mechanisms such as covalent modifications of chromatin including histone acetylation, methylation, phosphorylation, and ATP-dependent chromatin remodeling. The latter is mediated by enzyme complexes using ATP-hydrolysis to slide away histones along the DNA, which may expose transcription factor binding sites and thus facilitate their association with

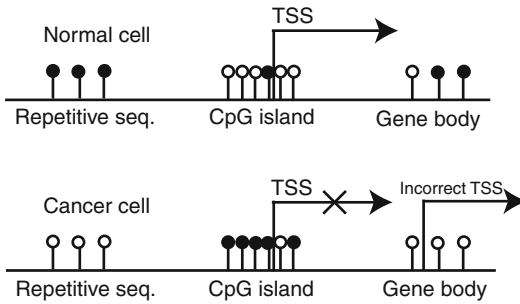


Fig. 2.2 Aberrant DNA methylation in cancer. In normal cells DNA methylation at CpG sites is mainly present in gene bodies and repetitive sequences and contributes to chromosomal stability. In cancer cells we observe global DNA demethylation prominent at repetitive sequences and specific hypermethylation of CpG islands of promoters that frequently affects suppressor genes. Aberrant hypomethylation of gene bodies may unblock alternative transcription start sites

regulatory sequences. The best-known chromatin remodelers of this type belong to the family of SWI/SNF complexes [4]. The three processes of DNA methylation, histone modification, and nucleosomal remodeling are intimately linked, and their alterations result in reprogramming of cancer-relevant genes (reviewed in [5]) (Fig. 2.1). Recent data in cancer biology emphasize the importance of epigenetic processes and illustrate that genetic and epigenetic phenomena cooperate at all stages of cancer development.

DNA Methylation

DNA methylation is the most intensively studied regulatory mechanism involved in the epigenetic control. It occurs predominantly on cytosine residues in CpG dinucleotides. So far, three enzymes that catalyze DNA methylation have been described: the DNA methyltransferases DNMT1, DNMT3a, and DNMT3b. All of them use the substrate *S*-adenosyl-L-methionine as source of methyl groups. DNMT1 preferentially methylates hemi-methylated DNA and is responsible for maintenance of the methylation patterns during DNA replication. DNMT3a and DNMT3b act on unmethylated DNA substrates and are responsible for *de novo* methylation [6, 7]. CpG dinucleotides are not evenly distributed across the human

genome but are concentrated in short CpG-rich DNA stretches called “CpG islands.” They are preferentially located at the 5′ end of genes and are present in about 60 % of human gene promoters [8] or reside in regions of large repetitive genomic sequences [9, 10]. DNA methylation of repetitive sequences has been proposed as a mechanism to prevent chromosomal instability by suppressing events such as homologous recombination [11], while gene body methylation is thought to prevent uncontrolled transcription initiation (reviewed by Portela et al. [5]). DNA hypermethylation of CpG islands located in the promoter regions has been associated with loss of expression (Fig. 2.2). Epigenetic gene silencing following CpG island methylation is mediated through recruitment of methyl-CpG-binding domain (MBD) proteins that in turn recruit histone-modifying and chromatin-remodeling complexes to the methylated sites [12, 13] or indirectly by precluding the recruitment of DNA-binding proteins from their target sites [14]. Normally, most CpG islands remain unmodified during development and in differentiated tissues [15]. However, there are some exceptions like the CpG island methylation occurring during X-chromosome inactivation and those for imprinted genes [9]. Recent findings also suggest that extensive DNA methylation changes caused by differentiation take place at CpG island “shores,” regions of comparatively low CpG density close to CpG islands [16–18].

Although CpG methylation is the most studied epigenetic modification, it is not the only one that can occur at the DNA level. Recently other regulatory chemical modifications have been described like the methylation at non-CpG sites like CHG and CHH (where H is A, C, or T) or the 5-hydroxymethylcytosine (5-hmC). Methylated CHG and CHH have been found in stem cells and seem to be enriched in gene bodies directly correlated with gene expression, while they are depleted in protein binding sites and enhancers [19]. The levels of non-CpG methylation decrease during differentiation and are restored in induced pluripotent stem cells (iPS), suggesting a key role in the maintenance of pluripotency [19, 20]. The function of 5-hmC is not yet understood and poorly studied at the moment especially because this

modification cannot be easily distinguished technically from the classic 5-methylcytosine [21].

Detection Methods for Methylated DNA

In the last decade, the study of DNA methylation has become essential for the understanding of regulatory processes in biology, and more recently aberrantly methylated genes have been identified as biomarkers in cancer with clinical applications. This has led to the development of many methods for its detection using various technical strategies that are associated with different resolution. Choice of technology depends on the purpose, ranging from diagnostic tests for individual genes for patient selection to genome-wide methylation profiling allowing for an unbiased comprehensive view of DNA methylation.

One of the most common methods to differentiate between methylated and unmethylated CpG sites uses a bisulfite treatment. This step converts unmethylated cytosine—but not 5-methylcytosine—in the DNA to uracil [22] that after amplification by polymerase chain reaction (PCR) is replaced by thymidine. Subsequently, the altered sequence can be identified by any technology allowing sequence-specific readouts that differentiates between cytosine and thymidine (Fig. 2.3). A popular method is methylation-specific PCR (MSP) that uses distinct sets of primers, and each set is designed to bind either only to completely methylated or unmethylated sequences, respectively [23]. Each primer typically interrogates a series of three to five CpGs. Quantitative versions of MSP, QMSP, allow definition of cutoff, standardization, and high-throughput analysis [24, 25]. Other quantitative/semiquantitative methods comprise methylation-specific pyrosequencing and methylation-specific clone sequencing [26]. For genome-wide analysis of bisulfite-treated DNA, high-density bead chip arrays are available (e.g., Infinium 450 K Methylation-Bead Chip, Illumina) for high-throughput analysis, while deep sequencing technology (MethylC-seq) allows for unbiased evaluation of the methylome [27].

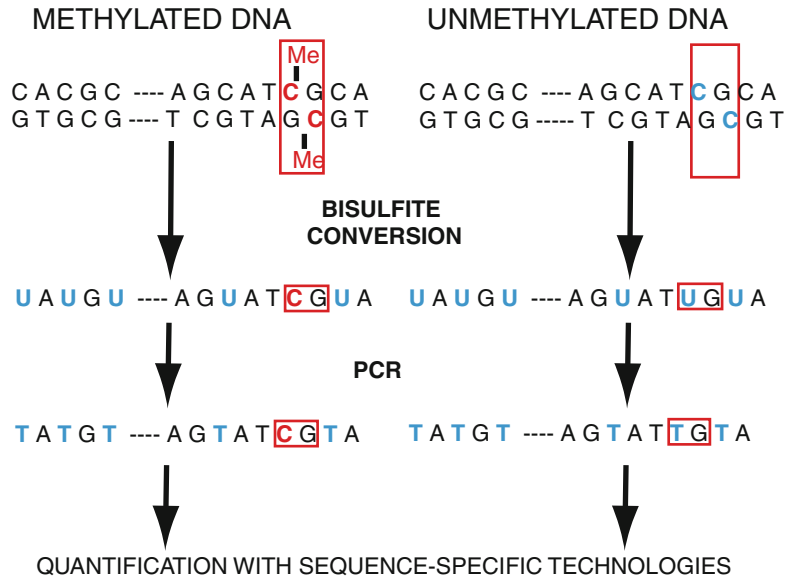
Methods not depending on bisulfite conversion for differentiating methylated from unmethylated CpGs take advantage of methylation-sensitive restriction endonucleases that recognize and cleave sequence-specific either methylated or unmethylated CpGs only, followed by amplification for detection and quantification of characteristic restriction fragments. Other methods enrich methylated DNA fragments using antibodies against methylated CpGs (MeDIP) or affinity columns loaded with recombinant peptides derived from DNA methylation-binding proteins, such as the methyl-CpG-binding protein 2 (MeCP2). These enriched methylated DNA fragments are then used as input for detection methods such as deep sequencing or DNA microarrays that allow quantification of captured methylated DNA fragments [28–30].

The detailed comparison of the different technologies is beyond the scope of this chapter. Comparison of different technologies used to determine the methylation status of marker genes such as *MGMT* has been reviewed in Weller et al. [31], and the assessment of different technologies for unbiased genome-wide DNA methylation analysis has been published recently [28–30].

Posttranslational Modification of Chromatin

The eukaryotic genome is packaged into chromatin, a highly ordered structure that contains DNA, RNA, histones, and other chromosomal proteins. Chromatin was originally classified into two domains, euchromatin and heterochromatin, based on the density of staining of the nucleic acid in micrographs [32, 33]. The definition of these domains has since been expanded. Euchromatin is gene-rich, transcriptionally active, hyperacetylated, and hypomethylated chromatin. Conversely, heterochromatin is gene-poor, transcriptionally inactive, hypoacetylated, and hypermethylated chromatin [32–34]. The basic unit of chromatin is the nucleosome, which is composed of two copies of the histones H2A, H2B, H3, and H4 wrapped with 146 base pairs of DNA [33, 35]. The ability of chromatin to condense can be regulated in

Fig. 2.3 Discrimination between methylated and unmethylated CpGs using bisulfite conversion. Methylated cytosines (5-methylcytosines) are resistant to bisulfite conversion, whereas unmethylated cytosines are converted into uracil. Sequence-specific technologies allow then sequence-specific discrimination and quantification of methylated and unmethylated CpGs, respectively



part by posttranslational modification (PTM) of the N-terminal tails of the histones which include acetylation, methylation, phosphorylation, sumoylation, poly(ADP)-ribosylation, and ubiquitination. These modifications regulate key cellular processes such as transcription, replication, and repair. So far over 60 different modifications on histones have been described [34] defining the so-called “histone code” that refers to the patterns of modifications where different combinations of histone modifications designate or regulate specific cellular processes and events [36–38]. Active genes have been associated with particular modifications also called active histone marks, e.g., tri-methylation of lysine 4 (H3K4me3) and acetylation of lysine 9 (H3K9ac). In contrast histone marks for inactive genes may comprise H3K9me2, H3K9me3, H3K27me2, and H3K27me3. However, many active and inactive genes have overlapping patterns of histone modifications. In fact bivalent histone marks are a hallmark of embryonic stem cells that is thought to keep the genes in a “transcription-ready” state and may predispose important regulatory genes to inactivation by aberrant DNA hypermethylation that results in heritable gene silencing during malignant transformation and tumor progression [39]. For almost each modification, enzymes exist which either lay down the appropriate mark or

remove it. Histone acetyltransferases (HATs) and histone methyltransferases (HMTs) add acetyl and methyl groups, respectively, whereas histone deacetylases (HDACs) and histone demethylases (HDMs) remove them [40, 41]. These histone-modifying enzymes interact with each other as well as other DNA regulatory mechanisms to tightly link chromatin state and transcription. Although there is an intimate relationship between DNA methylation and PTM of histones, the former is considered to be relatively stable, while PTMs of histones are more dynamic, balanced by the activities of the histone-modifying enzymes removing or adding respective modifications. In cancer cells this equilibrium is disturbed by deregulated expression of HMTs and HDMs and overexpression of HDACs. Deregulated expression of histone-modifying enzymes makes them potential targets for therapy to normalizing their equilibrium.

Like DNA methylation the study of the post-transcriptional modification of chromatin led to the development of several methods of analysis. Most of them are based on immunoprecipitation of the chromatin cross-linked to DNA using specific antibodies against the different PTM of the chromatin. Coprecipitated DNA is subsequently analyzed and quantified by PCR (ChIP-PCR), on DNA chips (ChIP on CHIP), or by

genome-wide deep sequencing (ChIP-seq) to identify and quantify the chromatin status at loci of interest.

MicroRNAs

MicroRNAs are endogenously expressed short noncoding RNAs, 18–25 nucleotides in length, that repress protein translation through binding to target mRNAs [42]. More than 1,000 human microRNAs have been discovered to date, and recent studies have estimated that they are responsible for the regulation of up to one-third of all human genes [43]. MicroRNAs are mostly transcribed from intragenic or intergenic regions by RNA polymerase II into primary transcripts called pri-microRNAs [44, 45]. The primary transcripts undergo further processing usually by a ribonuclease named DROSHA resulting in a hairpin intermediate of about 70–100 nucleotides, called pre-microRNA [46, 47]. The pre-microRNA is then transported out of the nucleus to the cytoplasm by exportin 5 [48]. In the cytoplasm, the pre-microRNA is processed by another ribonuclease, DICER, into a mature double-stranded microRNA [49, 50]. After strand separation, the guide strand or mature microRNA is incorporated into an RNA-induced silencing complex (RISC), whereas the passenger strand is degraded [50–53] (Fig. 2.1). RISC comprises also argonaute proteins that have a crucial role in microRNA biogenesis, maturation, and miRNA effector functions [51–53]. The mature guide strand is important for target recognition and for the incorporation of specific target mRNAs into RISC [50–53]. The specificity of microRNA targeting is defined by Watson–Crick complementarities between positions 2 and 8 from the 5' miRNA (also known as the seed), with the 3' untranslated region (UTR) of their target mRNAs [53]. When microRNA and its target mRNA sequence show perfect complementarities, the RISC induces mRNA degradation. Should an imperfect microRNA–mRNA target pairing occur, translation into a protein is blocked [53] (Fig. 2.1). Analyzing the complementarities between

microRNA and mRNA has revealed that each microRNA can potentially target multiple mRNAs [50, 54–56], while a single mRNA can be targeted by several different microRNAs [54, 55]. Many of these predictions have been validated experimentally, suggesting that microRNAs might cooperate with each other to regulate gene expression [56]. Similar to promoter methylation of genes, expression of these regulatory RNAs may also be silenced by aberrant CpG methylation.

Besides the canonical mechanisms of microRNA gene regulation, other “noncanonical” microRNA-mediated mechanisms of mRNA expression modulation are emerging [50, 57–61]. Some microRNAs have been shown to bind to the open reading frame or to the 5' UTR of the target genes, and, in some cases, they have been shown to activate rather than to inhibit gene expression [57, 58]. Moreover, some studies have recently reported that microRNAs can also regulate gene expression at the transcriptional level by binding directly to the DNA [50, 59–61].

Epigenetic Deregulation in Cancer

The cancer epigenome is characterized by extensive aberrations at any level of epigenetic control. The integrity of epigenetic regulation including maintenance of appropriate patterns of histone modifications, DNA methylation, and microRNA expression is not only crucial for normal development and differentiation but is also intimately associated with tumor initiation and progression [62]. It is also becoming clear that epigenetic deregulation may precede classical transforming events like mutations in cancer-relevant genes and genomic instability [63]. Disruption of the epigenetic machineries, either by mutation, deletion, or altered expression of any of their components, contributes to epigenetic deregulation. Aberrant promoter methylation of genes may complement mutation or deletion of the second allele, as postulated by the two-hit model for inactivation of tumor suppressor genes, or even provide both hits by methylation of both alleles [18, 64, 65]. Identification

of new “epimutations” is rapidly increasing with the availability of more performing technologies. Similar to genetic alterations, tumor-type-specific patterns of epigenetic alterations are observed [66]. The current challenge is to differentiate drivers from passenger alterations and identify those that are actionable for future treatment approaches or select the ones already drug-gable by available therapies [67] and develop respective biomarkers for patient selection.

Epigenetic Deregulation in Glioma

Silencing by Promoter Methylation of O6-Methylguanine-DNA Methyltransferase Gene (MGMT)

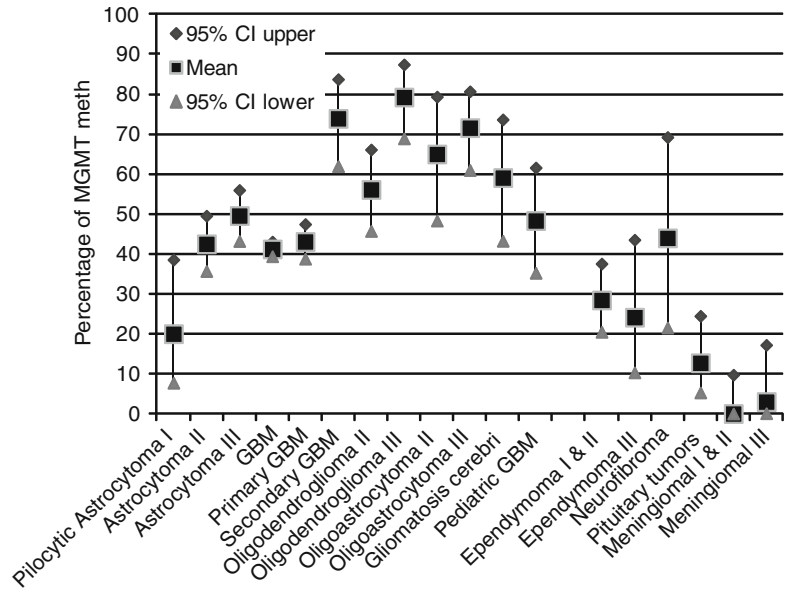
In glioma probably the best-known epigenetic alteration is promoter hypermethylation of the repair gene that encodes the O6-methylguanine-DNA methyltransferase (MGMT) that has become the first epigenetic biomarker in this disease [31, 68]. MGMT rapidly reverses alkylation (including methylation) at the O6 position of guanine by transferring the alkyl group to the active site of the enzyme, in a suicide reaction [69], hence annihilating the therapeutic effect of alkylating agents such as temozolomide. Consequently, epigenetic inactivation of the *MGMT* gene by promoter methylation renders tumor cells more sensitive to alkylating agents. The clinical relevance of epigenetic silencing of the *MGMT* promoter for benefit from alkylating agent therapy was shown in a randomized trial for newly diagnosed glioblastoma (GBM) [70–72]. Patients whose tumors contained a methylated *MGMT* promoter had a clear survival benefit from the addition of the alkylating agent temozolomide (TMZ) to standard radiotherapy (RT) with a median overall survival (OS) of 23.4 months as compared to 12.6 months in patients with an unmethylated *MGMT*, while in the radiotherapy arm, OS was 15.3 months in the *MGMT* methylated and 11.8 in the unmethylated patients, respectively [70]. A predictive effect of *MGMT* methylation for benefit from TMZ is suggested, however, the result for OS is confounded

by the fact that TMZ was given to 60 % of the patients in the RT arm at relapse. The predictive effect is supported by the data from progression-free survival (PFS). Patients with a methylated *MGMT* had a median PFS of 10.3 months, as compared with 5.9 months for patients who received radiotherapy alone. In contrast, patients with an unmethylated *MGMT* did not show such a benefit from the addition of TMZ with a PFS of 5.3 months, as compared with 4.4 months for patients who were treated with radiotherapy alone [68].

Subsequent to this trial, the *MGMT* methylation status has been evaluated in many studies, revealing that frequencies are specific to the glioma subtype and malignancy grade, ranging from 40 % in GBM to over 80 % in anaplastic oligoastrocytoma (WHO grade III), while *MGMT* promoter methylation in pilocytic astrocytoma and most non-glial brain tumors is infrequent (Fig. 2.4) [31, 73–83].

Surprisingly, the methylation status of the *MGMT* promoter has only a prognostic as opposed to a predictive effect in anaplastic gliomas. In two studies, it was shown that the prognostic significance of *MGMT* promoter methylation was similar in the RT as compared to the chemoradiotherapy arm [84, 85]. The underlying reason for this puzzling result became more clear when it was shown that in contrast to GBM, in anaplastic glioma (WHO grade III), *MGMT* methylation is associated with good prognostic factors such as 1p/19q co-deletions and mutations of the isocitrate dehydrogenase 1 gene (IDH1) [84]. Furthermore, it is of note that 80 % of GBMs have loss of one copy of chromosome 10 which combined with *MGMT* promoter methylation that is located on 10q26 leads to complete loss of MGMT function. This is not the case in anaplastic glioma. They do not frequently exhibit loss of chromosome 10; hence, *MGMT* promoter methylation may affect both or only one allele, resulting either in complete loss or just reduced “gene dosage,” respectively. Taken together, the impact of *MGMT* methylation on response to alkylating agent therapy needs to be established for each tumor type.

Fig. 2.4 Frequency of methylated *MGMT*. Meta-analysis of *MGMT* methylation frequencies compiled from the literature (updated from Weller et al. [31] and [73–83]). Mean values and confidence intervals (CI) of the *MGMT* methylation frequencies are given for different brain tumors. Large confidence intervals usually mean few and small series published



Epigenetic Deregulation of Cancer-Relevant Pathways in Gliomas

Besides inactivation of DNA repair as exemplified by *MGMT* silencing, DNA methylation analyses have revealed that silencing of negative regulators of mitogenic pathways or activators of apoptosis is common in cancer showing tumor type-specific patterns. In GBM the WNT pathway may be activated through promoter methylation of negative regulators such as the WNT inhibitory factor 1, the family of secreted frizzled-related proteins (sFRPs), dickkopf (DKK), and naked (NKDs) [86, 87]. Another example is the ras pathway that in a subset of GBM is deregulated by silencing of the negative regulators Ras association (RalGDS/AF-6) domain family members RASSF1A and RASSF10 [88, 89]. RASSF1 is methylated in many tumor types and is thought to contribute to ras signaling [90]. Examples of genes and respective affected pathways are given in Table 2.1 [86–89, 91–98].

Glioma CPG Island Methylator Phenotype (G-CIMP)

Improvement of technology in the last few years allows comprehensive analysis of genome-wide

DNA methylation on high-throughput platforms. Large-scale analysis in GBM on aberrant DNA methylation at CpG sites has unraveled a plethora of genes that are affected. A project of the “The Cancer Genome Atlas” (TCGA) has classified GBM into three distinct DNA methylation GBM subgroups [93]. A striking pattern with highly concordant DNA methylation was identified in 8 % of GBM, indicative of a glioma CpG island methylator phenotype (G-CIMP) [93] (Fig. 2.5) [93, 99, 100]. Patients with G-CIMP tumors are younger at the time of diagnosis and experience significantly improved outcome. G-CIMP tumors constitute a subgroup of the proneural subtype as defined by the Verhaak gene expression-based classification of GBM [101]. Furthermore, G-CIMP is highly correlated with *IDH1* gene mutations. Hence, G-CIMP is also associated with secondary GBM, arising from lower-grade glioma [102]. In grade II and grade III glioma, G-CIMP was also commonly identified with a strong association with *IDH1/2* mutations, suggesting an early event in the evolution of these tumors [91, 93, 103] (Table 2.1). In anaplastic glioma, G-CIMP has also been reported as good prognostic factor [103]. These observations further support the hypothesis that primary glioblastoma with low frequencies of *IDH1* mutations and G-CIMP

Table 2.1 Methylated genes and affected pathways in gliomas (without MGMT)

Tumor type	Pathway affected	Gene	% methylation	Ref.
<i>Glioblastoma</i>				
	RAS pathway	RASSF1A	47	[88]
		RASSF10	65	[89]
	WNT pathway	WIF1	26	[86]
		SFRP1	80	[87]
		SFRP2	77	[87]
		SFRP4	50	[87]
		SFRP5	20	[87]
		NKD1	0	[87]
		NKD2	81	[87]
		DKK1	0	[87]
		DKK3	0	[87]
		FZD9	72	[91]
	Metalloproteinase	MMP14	75	[91]
		MMP2	97	[91]
	PI3K pathway	PTEN	9	[92]
	G-CIMP		8	[93]
	Others			
		HOXA9	50	[91]
		HOXA11	75	[91]
		TMS1/ASC	21	[94]
	HIC1	100	[95]	
<i>Secondary glioblastoma</i>				
	RAS pathway	RASSF1A		[88]
		RASSF10	69	[89]
	WNT pathway	SFRP1	5	[87]
		SFRP2	12	[87]
		SFRP4	0	[87]
		SFRP5	40	[87]
		NKD1	0	[87]
		NKD2	13	[87]
		DKK1	83	[87]
		DKK3	100	[87]
	PI3K pathway	PTEN	82	[92]
	G-CIMP		75	[93]

(continued)

Table 2.1 (continued)

Tumor type	Pathway affected	Gene	% methylation	Ref.
<i>Anaplastic astrocytoma</i>				
	RAS pathway	RASSF1A	64	[88]
		RASSF10	80	[89]
	WNT pathway	SFRP1	10	[87]
		SFRP2	0	[87]
		SFRP4	25	[87]
		SFRP5	20	[87]
		NKD1	0	[87]
		NKD2	0	[87]
		DKK1	17	[87]
	PI3K pathway	PTEN	68	[92]
	G-CIMP		52	[93]
	Others	HIC1	100	[95]
<i>Diffuse astrocytoma</i>				
	RAS pathway	RASSF1A	75	[88]
		RASSF10	60	[89]
	WNT pathway	SFRP1	5	[87]
		SFRP2	10	[87]
		SFRP4	25	[87]
		SFRP5	20	[87]
		NKD1	0	[87]
		NKD2	6	[87]
		DKK	0	[87]
	Cell proliferation	TES		[96]
	PI3K pathway	PTEN	43	[92]
	G-CIMP		80	[93]
		TET2	14	[97]
	Others	CDH1	65	[98]
		HIC1	100	[95]
<i>Pilocytic astrocytoma</i>				
	RAS pathway	RASSF1A	20	[88]
		RASSF10	0	[89]

have a different pathogenetic/epigenetic origin than secondary glioblastoma and should be classified separately.

The correlation of the neomorphic IDH1/2 mutants with a DNA methylator phenotype was also observed in acute myeloid leukemia (AML). This provided an important mechanistic link, together with the fact that *IDH1/2* mutations in leukemia were exclusive with tet oncogene family member 2 (*TET2*) mutations. The oncometabolite D-2-hydroxy glutarate (D-2HG) produced by neomorphic IDH mutants accumulates to high concentrations in the tumor tissues and has been shown to be a competitive inhibitor of α -KG-

dependent dioxygenases, hence reducing the activities of the families of histone demethylases and TET 5-methylcytosine hydroxylases, including TET2. This leads to a genome-wide increase of DNA methylation (5-methylcytosine) and reduction of 5-hydroxymethylcytosine [104, 105]. Consequently suggesting a functional link between IDH1/2 mutations and the development of a methylator phenotype—metabolism meets epigenetics! Evidence for this functional link was provided recently by introducing an *IDH1* mutant into primary human astrocytes that leads to extensive hypermethylation reminiscent of patterns identified in G-CIMP-positive low-grade

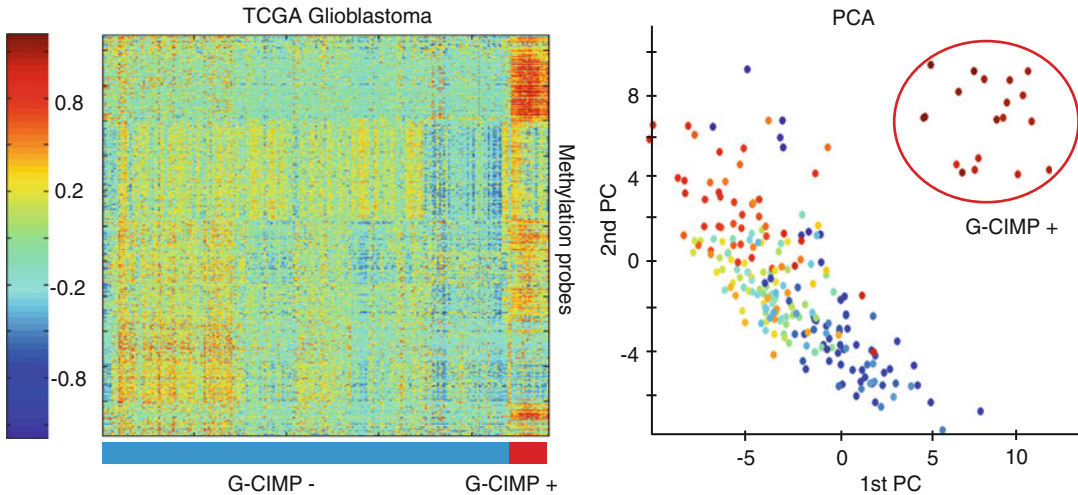


Fig. 2.5 Glioma CpG island methylator phenotype (*G-CIMP*). The heatmap shows a panel of 241 GBM clustered according to the 2,285 most variable Infinium DNA methylation probes. The structure of the heatmap identifies several GBM methylation subtypes, of which one group exhibits a highly coordinated gene methylation pattern that has been denominated as G-CIMP. The striking difference of

these G-CIMP-associated GBM from the G-CIMP-negative GBM is well reflected in the principal component analysis (PCA) on the right side. The DNA methylation data has been downloaded from The Cancer Genome Atlas database [93], subjected to a variation filter ($SD > 0.19$) and clustered using coupled two-way clustering (CTWC) [99, 100]. Red, high methylation; blue, low methylation

glioma, hence establishing *IDH* mutations as the molecular bases of CIMP in glioma [106].

Posttranslational Modifications of Histones

Epigenetic alterations in glial tumors frequently involve proteins controlling the PTM of histones. In particular the enhancer of zeste human homolog 2 gene (*EZH2*), which is the catalytic component of the polycomb repressive complexes 2 (PRC2) and PRC3, has been demonstrated to play an important role in gliomas. It is involved in setting the H3K27me3 marks and also links different layers of epigenetic control. Indeed, *EZH2* may indirectly control DNA methylation through providing a platform for recruiting DNA methyltransferases [107]. *EZH2* is overexpressed in most astrocytic and oligodendroglial tumors and even more highly expressed in GBM [108]. Nevertheless, proteins belonging to PRCs are not the only histone modifiers altered in gliomas. Expression of some histone deacetylases (HDAC) has been reported to be altered in GBM especially class II and IV HDACs [109].

Moreover, large-scale sequencing analysis in GBMs uncovered mutations in many genes encoding proteins involved in epigenetic regulation, including histone deacetylases HDAC2 and HDAC9, histone demethylases JMJD1A and JMJD1B, histone methyltransferases SET7, SETD7, MLL, and MLL4, and methyl-CpG-binding domain protein 1 (MBD1), although they have not been confirmed yet as drivers of glioma genesis [110].

Aberrant Expression of MicroRNAs

Many studies have shown that glial tumors are also characterized by strong alterations in microRNA content. One of the best characterized alterations in GBM is represented by the miR-21. Identified targets of miR-21 are *TP53*, *TGF β* , the mitochondrial apoptotic pathway, and probably the tumor suppressor gene *PTEN* [111]. Its expression levels have been correlated with overall and disease-free survival and suggested to be a biomarker for chemoresistance in other types of cancer including leukemia and pancreatic and lung cancer [112, 113]. A growing number of other microRNAs

have been recently linked with gliomagenesis; striking examples are miR-10b [114] and miR-196 glioma involved in glioma progression [115]. In particular miR-10b is often upregulated in both low-grade and high-grade glioma and seems to downregulate *BCL2L1/BIM*, *TFAP2C/AP-2γ*, *CDKN1A/p21*, and *CDKN2A/p16* that normally protect cells from uncontrolled growth. Furthermore, the use of high-throughput technologies has allowed identification of expression signatures of microRNAs that characterize GBM subtypes or exert a prognostic value for survival in GBM [116, 117]. Again using TCGA data, microRNA expression profiles yielded biologically meaningful subclassification of GBM. Five subclasses were proposed that relate to developmental patterns, of which three overlap substantially with three of the four subclasses defined by the Verhaak gene expression classification [101]. The “oligoneural” microRNA profile was associated with “proneural,” the “radial glial” with “classical,” and the “astrocytic” with “mesenchymal” gene expression-defined classification [116].

Epigenetic Deregulation in Other Brain Tumors

Ependymal Tumors

We know little about the mechanisms involved in initiation, maintenance, or progression of ependymal tumors. This is in part due to the heterogeneity and the low incidence of these tumors. Most of the epigenetic studies on these tumors have used a candidate gene approach with genes mostly selected looking at their methylation status in other brain tumor types [118]. Despite the limited information available, a number of aberrantly methylated genes have been identified. The most commonly methylated gene in ependymomas seems to be *RASSF1A* with a reported incidence of 86 % [118, 119]. Another gene commonly methylated in ependymomas is *HIC1* with an incidence of 83 % that has been associated with a non-spinal localization [120]. Finally, Rousseau and colleagues have shown promoter hypermethylation of *CDKN2A*, *CDKN2B* [118,

121]. A non-exhaustive list of methylated genes and their pathways identified in different brain tumors is available in Table 2.2 [88, 118–136]

Pineal Tumors

The first gene identified in sporadic pituitary tumors affected by promoter methylation has been *CDKN2A* [122, 137]. Subsequent studies have described methylation-mediated gene silencing in multiple other genes including *RBI*, fibroblast growth factor receptor 2 (*FGFR2*), death-associated protein kinase (*DAPK*), and galectin 3 [122]. High-throughput technologies enormously boosted the discoveries in the field that led to the observation that *MEG3a* and *GADD45γ* are frequently inactivated in pituitary tumors by promoter hypermethylation [122, 126, 138] (Table 2.2). Finally, alterations involving PTM of histones have also been described for pituitary tumors. The MLL-p27(Kip1) pathway, for instance, is often downregulated in pituitary adenomas [139].

Medulloblastoma

Among the embryonal brain tumors, medulloblastoma (MB) is probably the most studied at both genetic and epigenetic level. Epigenetic inactivation of specific genes by DNA methylation has been found for *HIC1*, *RASSF1A*, and *CASP8* [88, 127–129]. More recently also *SFRP1*, *SFRP2*, and *SFRP3* have been found to be methylated in primary MB [133] (Table 2.2). BMI1, a component of the polycomb repressive complex (PRC) 1 is also significantly upregulated in medulloblastoma. Recently BMI1 expression has been associated with poor survival [140, 141]. Furthermore, recent evidence suggests that microRNAs play an important role in medulloblastoma. The miR-124 has been one of the first microRNAs identified as important in medulloblastoma. It is able to modulate the cell cycle, and its expression is significantly decreased in medulloblastoma [142]. Moreover, microRNA expression profiles from medulloblastoma overexpressing

Table 2.2 Methylated genes and pathways in selected non-glial brain tumors

Tumor type	Pathway affected	Gene	% methylation	Refs.
<i>Ependymoma</i>				
	RAS pathway	RASSF1A	86	[118, 119]
	Cell cycle regulation	CDKN2A (p16/ARF)	21	[118, 121]
		CDKN2B	32	[118, 121]
	Others	HIC1	83	[120]
<i>Pituitary tumors</i>				
	Cell cycle regulators	RB1	26	[122, 123]
		FGFR2	45	[122, 124]
		INK4a (p16)	34	[125]
		GADD45 γ	58	[126]
	Apoptosis	CASP-8	54	[125]
		THBS1	43	[125]
<i>Medulloblastoma</i>				
	RAS pathway	RASSF1A	100	[88, 127–131]
	Apoptosis	CASP8	62	[132]
	WNT pathway	SFRP1	24	[133]
		SFRP2	4	[133]
		SFRP3	16	[133]
	Others	HIC1	85	[134]
<i>Meningioma WHO II</i>				
	RAS pathway	RASSF1A	64	[135]
	Metalloproteinase	TIMP3	67	[136]
	Others	TP73	82	[135]
<i>Meningioma WHO III</i>				
	RAS pathway	RASSF1A	43	[135]
	Metalloproteinase	TIMP3	22	[136]
	Others	TP73	71	[135]

either Her2 or c-Myc allowed the identification of specific microRNA signatures in each group of medulloblastoma. Expression of miR-10b, miR-135a, miR-135b, miR-125b, miR-153, and miR-199b was altered in Her2-overexpressing tumors, whereas c-Myc-overexpressing medulloblastomas had expression changes in miR-181b, miR-128a, and miR-128b [143]. Finally, the miR-17–92 cluster has been found to functionally collaborate with the sonic hedgehog pathway in medulloblastoma development [144].

Meningeal Tumors

Like for ependymal tumors, little is known about epigenetic alterations in meningeal tumors. What seems to be clear is that *MGMT* is not methylated

in this tumor type [82]. Nevertheless, some epigenetic alterations have been observed. *RASSF1A*, *TIMP3*, and *TP73*, for instance, are frequently methylated in meningiomas (Table 2.2). Moreover, downregulation of miR-200a in meningioma seems to promote growth by reducing E-cadherin and activating the WNT/beta-catenin signaling pathway [145].

Epigenetic Treatments

Epigenetic therapies have already been FDA approved for leukemia and comprise DNA demethylating agents and HDAC inhibitors, and combinations thereof have been tested in clinical trials (see review by Kelly et al. [146]). In glioblastoma HDAC inhibitors have entered clinical trials (see

<http://clinicaltrials.gov/>), while demethylating agents have not been considered. This is likely due to the fact that the methylated *MGMT* promoter sensitizes the tumors to alkylating agents and that the alkylating agent TMZ is part of the current standard of care for GBM [70] (see respective paragraph above). Furthermore, TMZ-containing treatment schedules are tested or are already used for most other glioma subtypes. Demethylating agents such as 5-Aza-cytidine or 5-Aza-2'-deoxycytidine lock DNMT enzymes on to the DNA, thereby inhibiting further DNA methylation. Consequently, demethylating agents require cell division for activity, hence targeting rapidly dividing cells. Due to their unspecific mechanism, demethylating agents may lead to reexpression not only of tumor suppressor genes but also of oncogenes. Furthermore, the treatment may induce expression of alternative transcripts due to demethylation of gene bodies and further accentuate hypomethylation of repetitive sequences leading to increased genomic instability.

The HDAC inhibitor vorinostat (SAHA) has shown modest benefit as single agent in a phase II trial for recurrent GBM [147]. Analysis of respective tumor tissues for histone acetylation and RNA expression profiles indicated that the tested dose schedule affected targeted pathways. At present, vorinostat is tested in phase I/II trials for recurrent GBM in combination with various drugs or in newly diagnosed GBM in combination with standard chemoradiotherapy. Combination therapies with vorinostat are also tested in embryonal tumors of the CNS. In contrast, the HDAC inhibitor romidepsin was reported as ineffective in a phase I/II study for patients with recurrent GBM as single agent at the standard dose and schedule [148]. Interestingly, the treatment of GBM patients with valproic acid as antiepileptic drug has shown a survival advantage in combined chemoradiotherapy [149]. Valproic acid is considered to have weak HDAC inhibitor properties and is currently tested in a phase 2 trial for newly diagnosed GBM in combination with standard chemoradiotherapy (NCT00302159). Other HDAC inhibitors (entinostat, panobinostat phenylbutyrate) are in clinical evaluation for recurrent high-grade glioma

or refractory pediatric brain tumors and neuroblastoma. Drugs attempting to interfere with histone methylation that are expected to deplete PRC2 components are in preclinical testing: They comprise drugs like SL11144 that inhibits lysine (K)-specific demethylase 1A (KDM1A) and DZNep, an inhibitor of S-adenosylhomocysteine hydrolase [146]. Targeting of DNA–histone H1 complexes with a 131-iodine conjugated monoclonal antibody (Cotara) delivered by convection-enhanced delivery is under investigation in a phase II study for recurrent GBM [150].

Outlook

New concepts suggest that resistance to therapy may be partly mediated by epigenetic changes, based on the observation that acquired drug resistance was associated with alterations in the chromatin structure [151]. Indeed, treatment with HDAC inhibitors resensitized the drug-resistant cells, hence, providing evidence that development of drug resistance may be reversible in nature. This would explain the clinical observation of re-treatment response of tumors after “drug holidays.” Consequently, new drug schemes are suggested adding concomitant HDAC inhibitors to therapies to prevent or at least delay acquisition of epigenetically mediated treatment resistance. Respective trials are ongoing.

Targeting of aberrantly overexpressed microRNAs as a therapeutic option has become technically feasible using locked nucleic acid (LNA)-modified phosphorothioate oligonucleotide technology that renders them more stable. First phase II trials using this technology are performed in hepatitis C infection [152, 153]. The question if microRNAs are actionable in brain tumors remains to be determined and tested preclinically.

The hypothesis that the neomorphic mutants of IDH1/2 by means of inhibition of DNA demethylases through production of high concentrations of the oncometabolite D-2HG are the underlying cause of G-CIMP makes them a prominent drug target. It remains to be seen if

inhibition of the neomorphic function of IDH1/2 mutants is sufficient to reverse the methylator phenotype in these tumors. It is not known if the IDH mutants are required for maintenance of the tumors. Efforts aim at developing the oncometabolite D-2HG as biomarker detectable by magnetic resonance spectroscopy that would provide a noninvasive diagnostic tool to identify IDH1/2 mutant gliomas [154]. In contrast, the detection of the oncometabolite in the serum of patients afflicted with IDH1/2 mutant gliomas has been reported not to be successful [155].

Finally, mining epigenomics in cancer, as uncovered by large-scale analyses of DNA methylation profiles and chromatin structure, has just started. Insights into the molecular mechanisms and pathways affected by epigenetic cancer-related changes will provide new targets. The challenge will be to identify changes with the quality of drivers versus passengers and to find actionable targets. Most interestingly, some of these epigenetic alterations can be converted into the “Achilles heel” of the affected tumors upon treatment with certain classes of anticancer agents. These may include DNA repair pathways as we have shown previously for GBM with a methylated *MGMT* gene that particularly benefit from treatment with the alkylating agent temozolomide.

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