Epigenetic events in medulloblastoma development

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Over the last decade, the analysis of genetic defects in primary tumors has been central to the identification of molecular events and biological pathways involved in the pathogenesis of medulloblastoma, the most common malignant brain tumor of childhood. Despite this, understanding of the molecular basis of the majority of cases remains poor. In recent years, the emerging field of epigenetics, which describes heritable alterations in gene expression that occur in the absence of DNA sequence changes, has forced a revision of the understanding of the mechanisms of gene disruption in cancer. Accumulating evidence indicates a significant involvement for epigenetic events in medulloblastoma development. Recent studies have identified a series of candidate tumor suppressor genes (for example, RASSF1A, CASP8, and HIC1) that are each specifically epigenetically inactivated in a large proportion (> 30%) of medulloblastomas by promoter hypermethylation, leading to the silencing of their gene expression. These findings shed new light on medulloblastoma and offer great potential for an improved understanding of its molecular pathology. The authors review the current understanding of epigenetic events in cancer and their contribution to medulloblastoma development. Their nature, origins, and functional role(s) in tumorigenesis are considered, and the authors assess the potential utility of these events as a basis for novel diagnostic and therapeutic approaches.

KEY WORDS • medulloblastoma • epigenetics • hypermethylation • tumor suppressor gene

MEDULLOBLASTOMA

Background and Clinical Challenges

Medulloblastoma is an invasive embryonal tumor of the cerebellum, and is the most common malignant brain tumor of childhood.25,32 Medulloblastomas comprise a group of clinically and histologically heterogeneous neoplasms and, overall, 5-year survival rates of 60 to 70% are achieved using current risk-adapted combination therapies. At present, the disease risk is defined using clinical disease features alone; however, the outlook for high-risk cases (infants < 3 years old, patients with metastatic disease at diagnosis) remains particularly poor (~ 25–40% survival), and variability in outcome is observed within each risk group. In addition, the radiotherapeutic components of current treatment strategies are associated with long-term intellectual and neuroendocrine side effects in a significant number of cases.26

The accurate identification of clinical risk thus remains a major goal in medulloblastoma, because more robust risk-stratification systems would facilitate the targeted use of adjuvant therapies (intensive regimens for aggressive tumors and reduced long-term side effects for patients with responsive tumors). In addition, the development of more effective therapeutic approaches for high-risk cases remains paramount. A detailed understanding of the molecular mechanisms that contribute to medulloblastoma development, and the relationship of these mechanisms to disease biology, pathology, and clinical characteristics therefore offers considerable potential for the identification of more robust markers of disease risk for improved therapeutic stratification. Moreover, a comprehensive functional understanding of how these mechanisms contribute to tumorigenesis could highlight critical molecular targets, against which novel therapeutic strategies could be developed.

Genetic Events in Medulloblastoma Development

Recent years have witnessed advances in our understanding of the genetic events that underlie medulloblastoma development. Molecular cytogenetic studies have described a number of common chromosomal alterations, including isochromosome 17q, referred to as i(17q); gain of chromosome 7; and losses affecting chromosomes 8, 9q, 10q, 11, 16q, and 17p, either in isolation or as i(17q), which each occur in more than 25% of cases. A series of consistently reported genetic aberrations have also been identified, which highlight key biological pathways in medulloblastoma tumorigenesis. These include the following: 1) constitutive activation of the Sonic hedgehog signaling pathway (by mutually exclusive mutations of the PTCH1, SUFU, and SMO genes, which affect ~ 25% of cases overall); 2) activation of the Wnt/Wingless signaling pathway (by CTNNB1, APC, or AXIN1 mutations, in 10 to 30% of cases); 3) defects in the TP53 tumor suppressor pathway (TP53 mutations, p14ARF deletions; ~ 20% of cases); and 4) amplification of the MYC family of oncogenes (MYC or MYCN, each in 5–15% of cases). Furthermore, functional modeling and clini-
cal analysis of these genetic defects both substantiate their role(s) in medulloblastoma tumorigenesis and suggest that their differential involvement may indicate divergent mechanisms of pathogenesis and clinical behavior.25–28,50,81

Identifying Further Molecular Events in Medulloblastoma Development: an Emerging Role for Epigenetic Mechanisms?

Despite recent progress, a genetic basis for the majority of medulloblastomas remains to be discovered; genetic defects identified to date only describe limited subsets of medulloblastomas, and specific target genes have not been identified for the majority of chromosomal defects observed. Moreover, emerging evidence suggests that the disruption of specific genes in tumor development can occur by alternative mechanisms to genetic events (for example, gene mutation or deletion). In particular, the epigenetic inactivation of tumor suppressor genes by promoter hypermethylation has become the focus of intense investigation in multiple tumor types. Studies conducted over the last 5 years have begun to define the epigenetic events in-
Epigenetic events in medulloblastoma

Epigenetic events in medulloblastoma. These investigations have led to the identification of common gene-specific events that can occur either independently or in conjunction with genetic defects to disrupt genes involved in disease pathogenesis. We review our current understanding of the contribution of these events to medulloblastoma. We consider the nature of these events and the experimental strategies that have been undertaken to establish their role(s) in disease pathogenesis. Finally, we discuss their potential for diagnostic, prognostic, and therapeutic application in this disease.

Methylation of DNA and Epigenetic Transcriptional Silencing

Epigenetic events can be defined as those that effect heritable changes in gene expression without a change in the DNA sequence. In mammals, the best understood epigenetic modification of DNA is postreplicative methylation of the Cs position of cytosine residues in CpG dinucleotides. This is intimately related to a series of posttranslational epigenetic modifications of histones, and together these two mechanisms interact to cause the remodeling of chromatin into a transcriptionally repressive structure, which affects changes in gene expression by altering the accessibility of DNA to the cell’s transcriptional machinery (Fig. 1). The human genome is generally depleted in the dinucleotide CpG; however, approximately 60% of genes contain CpG islands, regions of DNA with a high G+C content and a high frequency of CpG dinucleotides relative to the bulk genome (Fig. 2). These CpG islands are usually located near upstream promoter regions and/or transcriptional start sites of genes. In normal adult somatic cells these promoter-associated CpG islands are generally unmethylated and their associated genes are transcriptionally active (exceptions are discussed later in this review), whereas the majority of CpG dinucleotides elsewhere in the genome (for example, in parasitic and repetitive DNA elements) are generally methylated. Briefly, DNA methylation patterns are established and maintained by a family of DNA methyltransferases, and their interplay with histone modifications and chromatin remodeling through a cascade of proteins, including methyl-CpG binding proteins, histone acetylases, and acetyltransferases (Fig. 1). Mechanisms underlying the establishment and maintenance of DNA methylation patterns, histone modifications, and transcriptional repression have been reviewed in detail elsewhere.

Epigenetic Events and Human Disease

The establishment and maintenance of appropriate DNA methylation patterns is essential in normal mammalian development and function, and plays a critical role in the regulation of a range of normal cellular processes, including embryonic growth and development, X chromosome inactivation, and genomic imprinting. Methylation of DNA has also been proposed as an important mechanism in the regulation of genomic stability, including the silencing of retroviruses and transposable elements in the genome, and plays a role in the regulation of tissue-specific patterns of transcription. In line with such fundamental biological roles, aberrations in DNA methylation patterns and processes have been associated with a range of human diseases, including imprinting disorders. Examples include: Beckwith–Wiedemann, Prader–Willi, and Angelman syndromes, repeat-instability diseases (such as fragile X syndrome), and cancer.

Epigenetic Events in Tumor Development

A large body of evidence now implicates a major role for epigenetic mechanisms in cancer development. Patterns of DNA methylation become disrupted in cancer at two major levels. 1) The genome of the cancer cell generally becomes hypomethylated relative to normal cells, contributing to the genomic instability frequently observed in malignancy. 2) Hypermethylation events arise that affect specific genes and lead to their epigenetic inactivation, and hence play more specific functional roles in cancer pathogenesis. These represent the most widely investigated epigenetic events in cancer, and typically occur somatically at CpG islands, which are normally unmethylated, resulting in an altered chromatin structure and transcriptional silencing (as described earlier; Fig. 1).

Hypermethylation is critically involved in the epigenetic inactivation of tumor suppressor genes, which normally function to suppress the neoplastic phenotype. The list of tumor suppressor genes silenced by hypermethylation has been steadily growing. The first example reported was of hypermethylation of the RB1 gene in sporadic retinoblastomas, in 1993, today the M. D. Anderson Cancer Center at the University of Texas maintains a database (http://www.mdanderson.org/departments/methylation), in which are listed more than 60 genes that have been reported to be hypermethylated in human cancers (although it is noteworthy that this list is not fully comprehensive, and more genes continue to be described).

Aberrant DNA hypermethylation events affecting tumor suppressor genes have been described for most tumor types, and characterize genes involved across the spectrum of tumor development processes, including cell cycle control, metastasis, angiogenesis, apoptosis, cell signaling, and DNA repair. Importantly, hypermethylation events can occur either biallelically (that is, affecting both alleles), or in conjunction with genetic events (for example, mutation or deletion), to deliver the two hits necessary to inactivate both copies of a tumor suppressor gene, and thus promote tumor development.

The initiating events that cause somatic methylation of CpG islands in cancers are not fully understood. Recent studies have proposed that de novo methylation can initiate from normally methylated CpG residues at the peripheries of a CpG island, or as “seeds” of methylation affecting individual CpG sites within an island, which then spread to encompass the entire CpG island, accompanied by associated histone modifications, transcriptional silencing, a growth advantage, and subsequent clonal selection. Gene-specific hypomethylation events, which lead to increased gene expression, have also been reported in cancer and are discussed in later sections.

Identification of Hypermethylated Genes in Medulloblastoma

Initial clues that patterns of DNA methylation are disrupted at the whole-genome level during medulloblasto-
Pathogenesis were provided by studies in which methylation-sensitive restriction enzymes and two-dimensional gel electrophoresis (called RLGS) were used. Restriction enzymes recognize and cut defined nucleotide sequences within the genome. A subset of these enzymes is inhibited by methylation of a CpG residue within their recognition sites and thus allows the distinction of methylated and unmethylated CpG residues within a CpG island. In RLGS, digestion of total genomic DNA with a combination of methylation-sensitive and other restriction enzymes, followed by two-dimensional gel electrophoresis, produces an electropherogram that displays approximately 2500 different sequences containing CpG residues. The pattern of these spots can be compared between medulloblastoma and normal cerebellar tissue, providing an insight into the methylation changes occurring at the global level. This analysis has led researchers to propose that up to 6% of CpG islands showed evidence of an altered methylation status in medulloblastoma. Researchers have proceeded to look for specific genes affected by DNA methylation in medulloblastoma. To date, the majority of genes analyzed have been assessed for evidence of epigenetic inactivation on a “candidate” gene basis. Individual genes have been selected for analysis either because they are known to be inactivated by genetic mechanisms in medulloblastoma, or because they are genes that show evidence of epigenetic inactivation in other cancers.

### Alterations in DNA Methylation in Medulloblastoma Tumors and Cell Lines

At least 16 published studies have now analyzed 24 genes for evidence of CpG island methylation in medulloblastoma. The methods used for the detection of DNA methylation in these tumors are illustrated and reviewed in Fig. 3, and their key features are summarized in Table 1. Of the 24 genes analyzed, 16 have shown evidence of CpG island methylation in a percentage of primary medulloblastomas in at least one study (p16 inhibitor, p14α, TP73, TP53, RB1, RASSF1A, HIC1, EDNRB, CASP8, DAPK, CDH1, THBS1, TIMP3, GSTP1, GSTP2, GSTP3, GSTP4, GSTP5, GSTP6). The plots showing identification of promoter-associated CpG islands in the human genome are illustrated and reviewed in Fig. 2. The plots show the observed vs expected CpG residues, the percentage GC content, and the putative CpG islands.
These data are summarized in Table 2. The RASSF1A, CASP8, and HIC1 genes consistently show methylation in a high percentage (>60%) of cases, whereas methylcytosine residues (CH3) remain unaffected. The frequencies of methylation show variation between individual studies. This is a likely consequence of two key factors; first, small cohorts were analyzed in many studies (that is, <20 primary tumors), and second, different methods were used, which can influence rates of detection (Table 1). In particular, methylation-specific PCR, one of the most commonly used methods of detection, can exhibit variable detection rates depending on the precise experimental conditions used.17 Despite this, consistent evidence of methylation has been found for four of the genes (RASSF1A, CASP8, HIC1, and p16(INK4A)), which have been analyzed using multiple methods across three or more independent studies, thus providing strong corroborative evidence for their methylation in medulloblastoma. Four additional genes (MGMT, CDH1, TIMP3, and GSTP1) have exhibited consistent evidence of hypermethylation in two independent studies. The remaining genes have been the

Fig. 3. Schema showing methods used for the assessment of DNA methylation status in medulloblastoma. A: Sodium bisulfite modification of DNA forms the basis of most commonly used epigenetic analysis methods. Treatment of single-stranded DNA with sodium bisulfite selectively deaminates cytosine residues (C), converting them to uracils (U), whereas methylcytosine residues (CH3) remain unaffected. B: The modified DNA sequence can then be analyzed for evidence of sequence variations by using a range of PCR amplification-based methods. The most widely used techniques include those depicted in panels i, ii, and iii. In bisulfite DNA sequencing (i), converted DNA is amplified using PCR with the aid of primers that do not differentiate between methylated and unmethylated sequences. The PCR products are then either sequenced directly or ligated into a plasmid vector for cloning and subsequent sequencing. Only methylated cytosine residues are retained as cytosines in the resulting sequence, whereas unmethylated cytosines are converted to uracils and appear as thymidine residues (T). In combined bisulfite restriction analysis (COBRA [ii]), DNA is amplified using PCR as per (i), and is then subjected to digestion by using sequence-specific restriction enzymes whose DNA recognition site(s) contain CpG residues. In the example shown, the TCGA recognition site of restriction enzyme Taq1 is retained in the methylated (unaltered) DNA sequence (Meth DNA) but is lost in the unmethylated DNA sequence (Unmethyl DNA) as a result of the bisulfite treatment. The Taq1-digested PCR products are then resolved by agarose gel electrophoresis. In methylation-specific PCR (MSP [iii]), the primers used specifically recognize and amplify either methylated (M) or unmethylated (U) target DNA sequences. The DNA is amplified in parallel with the two primer sets and the PCR products are then analyzed using agarose gel electrophoresis.
Furthermore, genetic evidence indicates that medulloblastoma cell lines are generally derived from more aggressive tumors and do not represent the full spectrum of disease subtypes observed clinically. It is therefore critical that, in addition to cell lines, methylation events are assessed in representative tumor cohorts to determine accurately the extent of their involvement in the clinical disease.

Establishing Tumor Specificity: Origins of DNA Methylation in Medulloblastoma. Although promoter-associated CpG islands are usually unmethylated in adult tissues, several significant precedents exist for genes that are transcriptionally silenced by DNA methylation in normal tissues. These include genes silenced by imprinting (a developmental mechanism that regulates gene dosage), and results in the expression of a gene from only one of the two parental chromosomes, with the other copy silenced by DNA methylation. More than 80 imprinted genes have been described to date in the human genome. Similarly, a set of testis-specific genes exist (for example, members of the MAGE [melanoma-associated antigen] and GAGE [G-antigen] gene families) that are epigenetically silenced by methylation in all somatic tissues.

Several genes have also been described that exhibit tissue-specific, methylation-controlled expression (that is, they are methylated and silenced in some somatic tissue types and not others). Examples include SERPINB5, MCJ (DNAJDI), and TH. The transcriptional inactivity of one copy of the X chromosome in females (X inactivation) is associated with widespread methylation of the CpG islands of its genes. Finally, evidence has been reported that supports the acquisition of methylation of certain genes in somatic tissues in an age-dependent manner. Considering these precedents, it is important to establish the relationship between any patterns of methylation detected in tumors and those observed in the normal tissue from which they have arisen, and also whether they represent tumor-specific events. Such investigations should ideally be performed using matched normal control tissues that are developmentally and histologically relevant to the tumor type of interest. Nevertheless, the selection of appropriate controls for medulloblastoma tumors poses particular problems. Although strong evidence supports the development of a subset of medulloblastomas that contain defects in the Sonic hedgehog signaling pathway, from the external granular layer of the developing cerebellum (< 30%), the histogenesis of the majority of medulloblastomas, and hence their most relevant control cell populations, are less well defined.

Studies of medulloblastoma to date have typically used a variety of normal postmortem brain tissues from unrelated individuals as controls for these purposes; however, only a subset of these have included normal nonneoplastic cerebellar tissues representing a range of ages and developmental stages (Table 2). From these studies, three genes that are methylated in medulloblastomas also show evidence of methylation in nonneoplastic brain tissues; these are CASP8, HIC1, and EDNRB. The patterns of methylation observed for these genes are markedly different. Methylation observed for these genes are markedly different.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS: high-resolution mapping</td>
<td>semiquantitative, allows analysis of whole</td>
<td>time consuming &amp; relatively expensive</td>
</tr>
<tr>
<td>of methylation status</td>
<td>genome for long stretches of sequence</td>
<td></td>
</tr>
<tr>
<td>across large stretches (~100–500 bp) of bisulphite-converted DNA</td>
<td>containing multiple CpG residues</td>
<td></td>
</tr>
<tr>
<td>MSP: PCR reactions designed to</td>
<td>rapid &amp; highly sensitive</td>
<td></td>
</tr>
<tr>
<td>specifically amplify either</td>
<td>detection highly dependent on assay conditions, not quantitative, assesses methylation status of only a few CpG residues, cannot determine differences in methylation pattern between different CpG sites contained in primer recognition sequences</td>
<td></td>
</tr>
<tr>
<td>DNA after bisulphite conversion</td>
<td>only assesses methylation status of CpG residues at specific DNA restriction sites, therefore usually only assesses a limited number of CpG residues</td>
<td></td>
</tr>
<tr>
<td>COBRA: PCR products from bisulphite-treated DNA are digested with a restriction enzyme specific to methylated or unmethylated DNA sequences</td>
<td>quantitative, relatively straightforward</td>
<td></td>
</tr>
<tr>
<td>MSRA: digestion of genomic DNA with methylation-sensitive restriction enzymes followed by detection with Southern blot or PCR amplification</td>
<td>avoids bisulphite treatment, can be quantitative</td>
<td>requires large amounts of DNA, lengthy procedure, only assesses methylation status of CpG residues at specific DNA restriction sites</td>
</tr>
</tbody>
</table>

* BS = bisulfite DNA sequencing; COBRA = combined bisulfite and restriction analysis; MSP = methylation-sensitive PCR; MSRA = methylation-sensitive restriction analysis.
tic cerebellar tissues, indicating that it is probably reflective of a normal level of tissue-specific methylation, and not a tumor-related event.

Methylation patterns observed for \textit{CASP8} and \textit{HIC1}, however, are more complex. For both genes, a background level of partial methylation is observed in normal brain and cerebellar biopsy samples, with higher levels of methylation observed in a proportion of medulloblastomas, which is indicative of an enhanced tumor-related methylation state.

The remaining medulloblastoma-methylated genes that have been investigated in control tissues fall into two broad groups. First, \textit{RASSF1A} is frequently completely methylated in primary medulloblastomas but shows no evidence of methylation in a range of nonneoplastic cerebellar biopsy samples, indicating that its methylation is tumor specific. Second, there is a group of genes that displays lower frequencies of methylation in primary tumors (typically \(\leq 20\%\); \textit{p14^{ARF}}, \textit{p16^{INK4A}}, \textit{MGMT}, \textit{CDH1}, \textit{TIMP-3}, \textit{DAPK}, \textit{GSTP1}, \textit{TP53}, \textit{TP73}, \textit{RB1}, and \textit{THBS1}). Although these genes were unmethylated when examined in limited numbers of control tissues, investigations have not been sufficiently extensive (in number or tissue type) to indicate

<table>
<thead>
<tr>
<th>Gene</th>
<th>MB Cell Lines</th>
<th>Primary MBs</th>
<th>Nonneo Brain Samples</th>
<th>Method</th>
<th>Assoc w/ Silencing</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{CASP8}</td>
<td>4 of 5 (80)</td>
<td>36 of 40 (90)</td>
<td>MSP</td>
<td>22</td>
<td></td>
<td></td>
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<tr>
<td>\textit{p16^{INK4A}}</td>
<td>2 of 3 (67)</td>
<td>1 of 1 (100)</td>
<td>BS/MSP</td>
<td>yes‡</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>\textit{HIC1}</td>
<td>11 of 11 (100)</td>
<td>5 of 5 (100)</td>
<td>MSP</td>
<td>yes‡</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td>\textit{CDH1}</td>
<td>1 of 1 (100)</td>
<td>0 of 3 (0)</td>
<td>0 of 2 (0)</td>
<td>MSP</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>\textit{RASSF1A}</td>
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<td>1 of 11 (9)</td>
<td>0 of 2 (0)</td>
<td>MSP</td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>\textit{CDH1}</td>
<td>1 of 1 (100)</td>
<td>0 of 1 (0)</td>
<td>0 of 2 (0)</td>
<td>MSP</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>\textit{TIMP-3}</td>
<td>5 of 5 (100)</td>
<td>5 of 5 (100)</td>
<td>MSP</td>
<td>yes‡</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>\textit{DAPK}</td>
<td>0 of 3 (0)</td>
<td>0 of 16 (0)</td>
<td>0 of 2 (0)</td>
<td>MSP</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>\textit{GSTP1}</td>
<td>0 of 3 (0)</td>
<td>0 of 16 (0)</td>
<td>0 of 2 (0)</td>
<td>MSP</td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>\textit{p14^{ARF}}</td>
<td>4 of 11 (36)</td>
<td>2 of 11 (18)</td>
<td>0 of 2 (0)</td>
<td>MSP</td>
<td></td>
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<tr>
<td>\textit{TP53}</td>
<td>0 of 11 (0)</td>
<td>0 of 11 (0)</td>
<td>0 of 2 (0)</td>
<td>MSP</td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>\textit{TP73}</td>
<td>0 of 11 (0)</td>
<td>0 of 11 (0)</td>
<td>0 of 2 (0)</td>
<td>MSP</td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>\textit{EDNRB}</td>
<td>9 of 11 (82)</td>
<td>26 of 44 (59)</td>
<td>3 of 5 (60)§</td>
<td>BS/COBRA</td>
<td>yes‡</td>
<td>53</td>
</tr>
<tr>
<td>\textit{MCJ}</td>
<td>3 of 9 (33)</td>
<td>2 of 28 (7)</td>
<td>0 of 11 (0)</td>
<td>MSP</td>
<td></td>
<td>54</td>
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<tr>
<td>\textit{RB1}</td>
<td>1 of 11 (9)</td>
<td>0 of 11 (0)</td>
<td>0 of 2 (0)</td>
<td>MSP</td>
<td></td>
<td>34</td>
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<tr>
<td>\textit{THBS1}</td>
<td>3 of 11 (27)</td>
<td>3 of 11 (27)</td>
<td>0 of 2 (0)</td>
<td>MSP</td>
<td></td>
<td>34</td>
</tr>
</tbody>
</table>

\* Additional genes analyzed and found to be unmethylated in medulloblastoma include \textit{APC} (0/16 tumors), \textit{CDH1} (0/16 tumors), \textit{DLC-1} (0/21 tumors), \textit{p15^{INK4B}} (0/11 medulloblastoma cell lines and 0/20 tumors), \textit{RAR} (0/16 tumors), \textit{TSCL1} (0/11 medulloblastoma cell lines), and \textit{VHL} (0/20 medulloblastomas). (See references 36 [for \textit{APC}, \textit{CDH1}, and \textit{RAR} genes], 62 [\textit{DLC-1}], 29 [\textit{p15^{INK4B}} and \textit{VHL}], and 53 [\textit{p15^{INK4B}}, \textit{RIZ1}, and \textit{TSCL1}]). Abbreviations: assoc = association; MB = medulloblastoma; Nonneo = nonneoplastic; PNET = primitive neuroectodermal tumor; Ref = reference.

† Brackets denote methylation more intense than normal brain in the percentage of cases indicated.

‡ Transcriptional silencing and gene reactivation after 5-AzaCdR treatment in cell lines.

§ Includes cerebellar tissue.

\| Cohort included supratentorial PNET samples.

** Expression levels correlate with methylation status in cell lines.

†† Expression correlates with methylation status in primary tumors.
whether their methylation in medulloblastoma is tumor specific.

These studies together identify three genes (RASSF1A, HIC1, and CASP8) that show evidence of differential methylation between medulloblastoma tumors and control cerebellar tissues, which is indicative of tumor-specific hypermethylation. Furthermore, the identification of normal tissue-specific methylation events in the nonneoplastic cerebellum highlights the importance of including relevant somatic tissue controls in medulloblastoma methylation studies. Age-matched normal cerebellar material currently represents the best available human control for such experiments; however, the origins of the apparent tumor-specific methylation observed in medulloblastomas remain elusive. Although functional evidence is accumulating to support bona fide tumor suppressor roles for these genes, and hence a direct role for their methylation in tumorigenesis (see later sections), the mechanisms underlying de novo methylation in tumor development are unclear (see earlier sections). The possibility also remains that the methylation patterns observed in medulloblastomas could reflect their histogenesis and clonal expansion from specific subpopulations of cells in the normal or developing cerebellum, and any cell-specific methylation patterns contained therein. Specific patterns of global DNA methylation and demethylation have also been described in early embryogenesis, which may relate to patterns of methylation observed in tumors. Clearly, further investigations in primary tumors and developing tissues in both humans and model organisms are now required to determine the relationships between normal methylation events in cerebellar development, de novo methylation events, and their roles in tumor histogenesis and establishment of the methylation patterns observed in medulloblastomas.

Establishing Epigenetic Inactivation: Relationships to Transcriptional Silencing. Once evidence of DNA methylation has been established for a given gene in cell lines and tumors, it is important next to determine whether this methylation leads to epigenetic inactivation (that is, transcriptional silencing) of the corresponding gene transcript. This is not always the case; methylation of certain CpG islands downstream from the promoter in mammalian genes have been shown not to block transcription. Correlation of the messenger RNA levels in cell lines and primary tumors with the presence/absence of methylation provides evidence for epigenetic inactivation; however, such an approach is often difficult in primary tumors because the presence of contaminating normal tissues may obscure the results. More direct evidence comes from the treatment of cultured cell lines with demethylating agents such as 5-AzaCdr, a nucleotide analog that inhibits cytosine methylation, causing reactivation of genes transcriptionally silenced by DNA methylation. Ideally, messenger RNA transcripts should be absent before treatment in cell lines in which there is evidence of DNA methylation (that is, lines showing evidence of transcriptional silencing) and be restored following treatment, with such a result providing strong evidence that any observed transcriptional silencing is methylation dependent. An illustrative example of such an analysis is shown in Fig. 4. For RASSF1A, HIC1, and CASP8, consistent evidence of a relationship between DNA methylation and epigenetic inactivation has been observed across multiple studies in medulloblastoma. Additionally, similar relationships have been reported for p16 INK4A and MCI in medulloblastoma in individual studies (see Table 2 and the studies cited therein). Nevertheless, the remaining studies of other genes in medulloblastoma have reported CpG island methylation without establishing its relationship to transcriptional silencing in medulloblastomas or cell lines (Table 2), although equivalent relationships have been reported for many of these genes in other tumor types. A number of assays of alternative markers of epigenetic inactivation are also available (for example, chromatin immunoprecipitation assays for evidence of DNA methylation-associated histone modification and chromatin remodeling), although these have not been used yet in reported studies of DNA methylation in medulloblastoma.

Summary. The assessment of candidate tumor suppressor genes for evidence of promoter hypermethylation has demonstrated that epigenetic events are a significant feature of medulloblastoma development, and has highlighted a series of genes that are epigenetically inactivated in medulloblastomas. Most notably, epigenetic inactivation of
Epigenetic events in medulloblastoma

**RASSF1A, HIC1, and CASP8** represent the most prevalent gene-specific events described in medulloblastoma to date. Each gene shows strong and consistent evidence of tumor-related CpG island hypermethylation in a significant proportion (>30%) of medulloblastomas, across several independent studies using a range of methodologies, which is associated with their epigenic inactivation by transcriptional silencing. In studies in which quantitative methylation detection methods have been used, the complete methylation observed for **RASSF1A** in the majority of primary medulloblastomas is consistent with a role as an early event in tumorigenesis. For most other genes, however, analysis has been restricted to a preliminary examination of methylation by methylation-specific PCR in small tumor cohorts in single studies. From the accumulated data, promoter-associated CpG island methylation in medulloblastoma appears to be restricted to specific genes, and medulloblastomas do not show evidence of concordant methylation of multiple genes, the so-called CpG island methylator phenotype reported in colorectal carcinomas and some other cancers.

**Epigenetically Inactivated Genes in Medulloblastoma: Evidence for Roles in Tumorigenesis**

Genes hypermethylated in medulloblastomas encode proteins that play a role in a diverse range of functions, including roles in cell cycle control (**TP53**, **TP73**, **RB1**, **p14ARF**, and **p16INK4A**), microtubule stabilization and the regulation of mitosis (**RASSF1A**), regulation of transcription (**HIC1**), apoptosis (**CASP8**, **DAPK**), cell adhesion (**CDH1**), regulation of the extracellular matrix (**TIMP3**), DNA repair (**MGMT**), and response to chemotherapeutic agents (**MCJ**). Although **RASSF1A**, **CASP8**, and **HIC1** represent the most frequently epigenetically inactivated genes in medulloblastoma, and are strong candidates for tumor suppressor genes in its pathogenesis, none of these genes has been investigated in detail at the functional level in this disease. Nonetheless, studies in other tumor types are beginning to shed light on the potential mechanism(s) whereby loss of these genes may contribute to tumorigenesis at the genetic and functional levels.

**RASSF1A**. The **RASSF1A** gene is located at 3p21.3 and was originally identified as a candidate tumor suppressor gene in lung cancer, where it is inactivated by a combination of genetic and epigenetic mechanisms, usually involving deletion of one allele and transcriptional silencing by hypermethylation of the other. Mutations in **RASSF1A** are rare, although its transcriptional silencing by hypermethylation, in combination with 3p21.3 deletion, is commonly observed in a range of solid tumor types, including breast, renal, and nasopharyngeal carcinomas. Inactivation of **RASSF1A** can also occur in tumor types in which 3p21.3 deletions are not involved, and medulloblastoma provides an important example of a tumor type in which **RASSF1A** inactivation occurs by biallelic hypermethylation, with no involvement of genetic mutation or deletion. In addition to genetic and epigenetic data consistent with the inactivation of both **RASSF1A** alleles in primary tumors, several independent lines of evidence support a tumor suppressor role for the **RASSF1A** protein. Reintroduction of **RASSF1A** into **RASSF1A**-silenced cell lines from a range of tumor types suppresses tumor cell growth in vitro and tumor formation in vivo. Conversely, depletion of **RASSF1A** in a cell line with a functioning **RASSF1A** gene has been shown to accelerate mitotic progression. Recent publications have described a number of potential functions for **RASSF1A** that are consistent with multifaceted roles in tumor suppression. These include roles in microtubule stabilization, apoptosis, and cell cycle arrest mediated through both the G1/S phase and G2/M phase mitotic checkpoints. Finally, homozygous disruption of **Rassf1a** in mice causes increased susceptibility to spontaneous tumors, including lung adenoma, lymphoma, and breast adenocarcinoma.

**CASP8**. The **CASP8** gene is located at 2q33-q34 and encodes Caspase-8, an initiator caspase (cysteine-aspartyl-protease) involved in death receptor-mediated apoptosis, and apoptosis triggered by other stimuli such as exposure to chemotherapeutic agents. Apoptosis (programmed cell death) is a crucial regulator of tissue homeostasis, and the abrogation of apoptotic pathways is a frequent event in tumor development. A significant proportion of medulloblastomas shows evidence of complete methylation of **CASP8**, although genetic loss of the 2q33-q34 region is rare, suggesting a primary role for epigenetic mechanisms in its inactivation in medulloblastoma. The **CASP8** gene thus represents a candidate medulloblastoma tumor suppressor gene, and inactivation of **CASP8** by CpG island methylation may play a role in abrogation of apoptotic pathways during medulloblastoma development. Nevertheless, little mechanistic evidence currently exists to substantiate any such functional roles in this disease.

**HIC1**. The **HIC1** gene is located at 17p13.3, a region frequently deleted in human cancer. Abnormalities of chromosome 17 are the ones most commonly observed in cytogenetic studies of medulloblastoma. Overall, losses of 17p are observed in 30 to 50% of cases, and frequently encompass a common region of deletion at 17p13.3, implicating the probable location of a medulloblastoma tumor suppressor gene at this locus. Although the situation is complicated by the level of background methylation observed for **HIC1** in the normal cerebellum (see previous sections), complete methylation of **HIC1** is observed in a significant number of medulloblastomas. The **HIC1** gene is hypermethylated and transcriptionally silent in several other types of human cancer (for example, breast cancer, cervical cancer, and retinoblastoma) and, although mutations in **HIC1** are very rare, its inactivation appears to adhere to a classic two-hit model, arising either through biallelic epigenetic mechanisms or epigenetic mechanisms together with chromosomal deletions. The **HIC1** gene encodes a zinc finger transcription factor that is proposed to function as a transcriptional repressor. The consensus binding sequence for **HIC1** has been established, but no specific target genes have yet been identified. Mice with one disrupted copy of **Hic1** develop a range of malignant tumors, including a predominance of epithelial cancers in males and lymphomas and sarcomas in females. Further work has shown that the spectrum of tumors that develop in mice is influenced by the loss of other tumor suppressor genes; heterozygous disruption of **Hic1** and **p53** cooperate to cause predisposition to breast and ovarian carcinomas and metastatic osteosarcomas, with epigenetic inactivation of the wild-type **Hic1** allele also observed in these tumors, consistent with a tumor suppressor role and two-hit inac-
Accumulated genetic, epigenetic, and functional evidence thus implicates \textit{HIC1} as an epigenetically inactivated tumor suppressor gene, and as a strong candidate for medulloblastoma tumor suppressor gene at 17p13.3. Further investigations are now required to clarify the relative contributions of normal tissue-specific methylation, de novo methylation, and 17p deletion to \textit{HIC1} loss in medulloblastoma, and also to clarify any functional role it may play in medulloblastoma pathogenesis.

\textbf{Mechanisms of Tumor Suppressor Gene Inactivation: Implications for Gene Discovery Strategies in Medulloblastoma}

Knudson’s$^{48}$ two-hit model of tumor suppressor gene inactivation states that both copies of a tumor suppressor gene should be inactivated to promote tumor development, with evidence of inactivation by genetic mutation (usually nonsense or truncating mutations) considered the hallmark of a tumor suppressor gene.\textsuperscript{55} Nevertheless, studies conducted in multiple tumor types highlight a new generation of tumor suppressor genes that redefine Knudson’s hypothesis to include epigenetic inactivation as one or both of the two hits required for tumor suppressor gene inactivation.\textsuperscript{91}

Data for candidate tumor suppressor genes investigated in medulloblastoma appear consistent with this revised hypothesis. Studies indicate that genetic and epigenetic events can work either in combination or in isolation to inactivate tumor suppressor genes in this disease. Of the methylated genes reported in medulloblastoma, \textit{p16\textsuperscript{INK4A}} is also homozygously deleted in a small percentage (10–15\%) of cases.\textsuperscript{4} Similarly, \textit{HIC1} is frequently methylated and is located at 17p13.3 (a common region of deletion in medulloblastoma), although \textit{HIC1} coding mutations are rare.\textsuperscript{72,88} suggesting that both genetic and epigenetic events contribute to the inactivation of these two genes in medulloblastoma. Nevertheless, the involvement of \textit{RASSF1A} and \textit{CASP8} in medulloblastoma development was not suspected prior to epigenetic studies, because neither gene is located in a chromosomal region exhibiting frequent loss in tumors. Indeed, loss of \textit{RASSF1A} occurs entirely by biallelic methylation in medulloblastoma, with no involvement of genetic mutations or deletion.\textsuperscript{59} Therefore, epigenetic studies have the power to implicate genes involved in medulloblastoma pathogenesis that are not identified in genetic studies. The role of promoter hypermethylation in the inactivation of tumor suppressor genes that are mutated in medulloblastoma is less clear. Only a limited number of these genes has been investigated epigenetically; methylation of \textit{p14\textsuperscript{ARF}} has been reported as well as its homozygous deletion in other cases,\textsuperscript{28,50} but methylation is not commonly observed for the \textit{TP53} or \textit{APC} tumor suppressor genes,\textsuperscript{54,84} suggesting that these genes are inactivated solely by genetic mechanisms. The contribution of epigenetic events to the inactivation of other mutated medulloblastoma tumor suppressor genes (for example, \textit{PITCH}, \textit{SUFU}, and \textit{AXIN1}) has not been investigated.

Candidate approaches to the identification of epigenetically inactivated genes have been successful in identifying gene-specific events associated with the development of a significant proportion of medulloblastoma cases, and strongly suggest the involvement of further hypermethylated genes. Nevertheless, only a modest number of genes have been analyzed so far for evidence of methylation in this way, and such potential approaches are by definition limited. More expansive genome-wide approaches must now be considered to aid the identification of additional epigenetically regulated genes in medulloblastoma. Such approaches include RLGS (discussed earlier), or the use of expression arrays to identify methylation-dependent candidate tumor suppressor genes, which are upregulated following demethylation treatments or inhibition of histone deacetylases.\textsuperscript{80,86} Similarly, CpG island arrays have been developed that allow the investigation of changes in the methylation status of thousands of CpG islands in a single experiment.\textsuperscript{30}

Finally, the recent elucidation of additional epigenetic events that may contribute to tumor development provides important precedents that merit investigation to establish any role(s) in medulloblastoma. These include gene-specific hypomethylation events such as loss of methylation (or imprinting) of normally somatically methylated (or imprinted) growth-promoting genes, which could lead to the activation of normally silent alleles and a growth advantage (for example, \textit{S100A4} in colorectal cancer or \textit{IGF2} in multiple cancer types).\textsuperscript{16,57,84} Moreover, micro-RNAs are a recently discovered family of short nonprotein-coding RNA species that negatively epigenetically regulate the expression of genes involved in the control of development, proliferation, and apoptosis, and whose aberrant expression appears to contribute to cancer initiation and progression.\textsuperscript{15}

\textbf{Prognostic and Therapeutic Potential}

The exploitation of molecular characteristics of the disease for diagnostic and therapeutic benefit offers significant promise for improving the outlook for patients with medulloblastoma. A number of the genetic features detected in medulloblastomas show significant associations with pathological features of the disease (for example, \textit{MYC} or \textit{MYCN} amplification and the large cell/anaplastic pathological subtype).\textsuperscript{21,50} and independent markers of both favorable (\textit{CTNNB1} status) and adverse (loss of chromosome 17p, \textit{MYC/MYCN} amplification) prognoses based on cohorts studied in clinical trials have been reported.\textsuperscript{27,50} Of the most frequently methylated genes identified in medulloblastoma, \textit{HIC1} methylation levels in excess of those observed in the normal brain have been reported to be independently predictive of poor overall survival.\textsuperscript{72} Similarly, tumor-specific methylation of \textit{CASP8} appears to be specific to medulloblastomas with classic and large cell/anaplastic histopathological features, and weak or low expression of the \textit{CASP8} protein in medulloblastomas has been reported as an independent indicator of poor survival.\textsuperscript{53,65} In contrast, \textit{RASSF1A} hypermethylation occurs in the majority of medulloblastomas (\textasciitilde{} 90\%) and describes tumors of all clinical and histological subtypes; it thus lacks real potential as a correlate of differential clinicopathological behavior.\textsuperscript{53,55}

Initial data therefore suggest that the investigation of epigenetic events could help identify markers of differential clinicopathological disease behavior in medulloblastoma for use in refined diagnostic and prognostic schemes. Along with the identification of additional markers for evaluation, the next phase in the development of these markers should therefore involve confirmatory studies, followed by prospective testing in large, uniformly treated cohorts of pa-
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tients. Other molecular, clinical, and pathological indices should also be evaluated to establish any applications as independent markers for use in routine practice.

The discovery of epigenetically inactivated genes in medulloblastoma raises the possibility of new therapies for this tumor. Unlike genetic inactivation, methylation-mediated transcriptional silencing is reversible by demethylating agents such as 5-AzaCdr, which is used clinically in the treatment of myelodysplasia and acute leukemia and has become the first drug to be approved by the US Food and Drug Administration for the treatment of myelodysplastic syndromes. Histone deacetylase inhibitors such as trichostatin A are also currently being investigated in clinical trials. Conceivably, treatment with demethylating agents could reactivate epigenetically silenced tumor suppressor genes involved in processes such as mitosis and the cell cycle (such as RASSF1A, TP53, p14ARF, and p16INK4A) and slow down tumor growth. Similarly, the reactivation of genes such as CASP8, DAPK, and MCI may sensitize tumors to other chemotherapeutic agents. Nevertheless, demethylating agents and histone deacetylase inhibitors lack specificity and have the potential to reactivate a wide range of epigenetically silenced genes, including both normally methylated and tumor-specific genes. In recent array-based experiments, treatment of cancer cells with demethylating agents and histone deacetylase inhibitors resulted in the methylation-dependent deregulation of hundreds of genes, including those predicted to be both growth promoting and growth inhibitory. It therefore seems unlikely that demethylating drugs or histone deacetylase inhibitors act through the targeted reactivation of cancer-specific genes, but more likely promote cell death through mechanisms that include the widespread deregulated expression of multiple gene transcripts.

Gene-specific epigenetic events detected in medulloblastoma also merit exploration as potential therapeutic targets. Our current understanding of the normal cellular functions of the major epigenetically inactivated genes (RASSF1A, HIC1, and CASP8) and the consequences of their disruption in tumor development varies greatly among genes (see previous sections). The specific restoration of tumor suppressor gene function does not seem realistic. Nevertheless, given their extensive hypermethylation in medulloblastoma and major adult tumor types, along with a growing understanding of their biological roles, HIC1 and RASSF1A-dependent functional pathways could represent potential future targets for exploitation in cancer, once the dependence of tumor growth on their inactivation has been established.

CONCLUSIONS

Over the last decade, the identification of genes disrupted by genetic mutation in medulloblastoma has provided critical insight into the molecular pathways underlying its pathogenesis; however identification of a molecular basis for the majority of medulloblastomas remains elusive. Recent reports describing gene-specific epigenetic events in these tumors have begun to further expand and inform our understanding of this disease, and of the genes disrupted in its development. Among these, RASSF1A, CASP8, and HIC1 have all been identified as candidate tumor suppressor genes that are epigenetically inactivated by hypermethylation and transcriptional silencing in a large proportion of cases. Indeed, these represent the most common gene-specific events identified to date in medulloblastoma. These events display apparent tumor specificity, although the identification of normal somatic methylation events in the normal nonneoplastic cerebellum highlights the requirement for appropriate comparative controls in methylation studies and investigations of the origins of methylation in tumors and their relationship to histogenesis of the disease. Concordant with tumor-specific roles, data from other tumor systems are consistent with tumor-suppressive functions for the RASSF1A and HIC1 protein products, and initial clinical studies support a role for epigenetic events as markers of differential clinical and pathological disease behavior in medulloblastoma.

Epigenetic events represent common molecular features of medulloblastoma. Their investigation has provided significant new insight into the molecular basis of this disease, and has led to the identification of genes that may play an important role in its pathogenesis. Nevertheless, our current understanding of the epigenetic basis of medulloblastoma is limited to the examination of a modest series of candidate genes. Based on current precedents, additional epigenetically regulated genes are likely to exist in medulloblastoma. More expansive genome-wide investigations are now required to identify these, and to establish with more certainty the functional roles and contributions of epigenetic events to medulloblastoma development, along with assessments of the clinicopathological relevance and potential for therapeutic exploitation of these events.

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References

function accentuates the role of Hic1 function accentuates the role of \( p53 \) in tumorogenesis. Cancer Cell 6:387–398, 2004


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