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Unique genetic and epigenetic mechanisms driving signatures of paediatric diffuse high-grade glioma

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Abstract

Diffuse high-grade gliomas (HGGs) of childhood are a devastating spectrum of disease with no effective cures. The two-year survival for paediatric HGG ranges from 30%, for tumours arising in the cerebral cortex, to less than 10% for diffuse intrinsic pontine gliomas (DIPGs), which arise in the brainstem. Recent genome-wide studies provided abundant evidence that unique selective pressures drive HGG in children compared to adults, identifying novel oncogenic mutations connecting tumourigenesis and chromatin regulation as well as developmental signaling pathways. These new genetic findings provide insights into disease pathogenesis, and the challenges and opportunities for improving patient survival in these largely incurable childhood brain tumours.

Keywords

High-grade glioma; Diffuse Intrinsic Pontine Glioma; Paediatric; genome; histone H3; ACVR1; glioblastoma; development; brain tumour

Introduction

Gliomas represent one of the most common types of central nervous system tumour across all age groups¹. In children, low-grade tumours predominate, while diffuse high-grade gliomas are the most common in adults¹. Diffuse high-grade lesions in children, although rare (approximately 1/100,000)¹, retain the same dismal prognosis as those that arise in adults (median survival 12–15 months), particularly for those arising in the pons (median survival 9–12 months)². In addition to the supratentorial locations in which the vast majority of adult tumours occur, children frequently present with histologically similar WHO grade IV glioblastoma in midline structures including the thalamus (13%)³ and spinal cord (3%)⁴ as well as the cerebellum (5%)⁵ (Figure 1). A diverse spectrum of grade III lesions may also occur (Text Box 1). This review will focus on the diffuse HGGs of childhood, which

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predominantly encompass glioblastoma (grade IV), as well as anaplastic astrocytoma (grade III).

Text Box 1

Classification of Paediatric Diffuse High-Grade Gliomas

Diffusely infiltrating gliomas are a heterogeneous spectrum of disease in which tumour cells share morphological features with normal glial cells, and they are categorized as high-grade (grades III and IV) according to criteria established in the World Health Organization (WHO) classification of nervous system tumours¹³. **Grade III** disease is distinguished from lower grade tumours on the basis of architectural and cytological features, pleomorphism and mitotic activity. The subclassification of grade III tumours is based on the morphological similarities of the predominant tumour cell population to normal glial cell counterparts. The most common subtype is anaplastic astrocytoma, in which tumour cells resemble astrocytes, and at much lower frequency, anaplastic oligodendroglioma and anaplastic oligoastrocytoma, in which tumour cells resemble oligodendrocytes, or a mixture of both, respectively. It remains unclear whether these morphologies reflect the tumour cell of origin, or variation in differentiation of a transformed glial progenitor or more primitive neural progenitor cell. A number of additional rare variants also exist.

Grade IV diffuse glioma is also known as **glioblastoma**, which is the most malignant of the diffuse astrocytomas. They share the high mitotic activity, nuclear atypia and cellular pleomorphism of grade III tumours, but are further distinguished by the additional features of microvascular proliferation and/or regions of necrosis.

Diffuse Intrinsic Pontine Gliomas (DIPGs) arise predominantly in the ventral pons and occur almost exclusively in children. Most samples evaluated at autopsy are classified as glioblastoma (grade IV), although biopsy studies have shown that some DIPGs may appear to be low grade (grade II), or grade III, at least in the small regions evaluated^{93, 94}. DIPGs represent a distinct entity from true low-grade gliomas of the brainstem (WHO grade I), which often have a pronounced dorsal exophytic component, and frequently harbour *BRAF:KIAA1549* gene fusions which are a hallmark of low-grade pilocytic astrocytomas¹⁵.

While the majority of adult glioblastomas, termed primary glioblastoma, also appear to arise as *de novo* lesions, progression of grade II to grade III gliomas is common, and a subset of adult glioblastomas termed secondary glioblastoma arise through further malignant transformation of lower grade tumors. These progressive lesions are strongly associated with the presence of *IDH1* mutations, and likely represent a subset of high-grade gliomas occurring in older adolescents³⁹. Because grade II and grade III gliomas in adults represent a continuum of progressive disease sharing a number of mutations, these tumors are often grouped together in analyses of adult glioma genetics. In contrast, grade III diffuse gliomas of childhood, like glioblastomas, generally occur without clinical or histological evidence of malignant transformation of a lower-grade precursor lesion, and progression of low-grade to high-grade disease is rare⁵⁹. As detailed in this review, pediatric grade III diffuse gliomas are more similar to glioblastoma than to grade II

tumours in terms of clinical behaviour, gene expression signatures and genetic abnormalities. Therefore, in this review we have grouped grade III and grade IV pediatric diffuse gliomas together for analysis and discussion of pediatric diffuse HGG.

Approximately half of all malignant gliomas in children arise in the brainstem, most frequently within the ventral pons as diffuse intrinsic pontine glioma (DIPG)⁶. ‘Typical’ DIPG represent one of the most severe challenges in neuro-oncology as the anatomical location precludes surgical resection and makes adequate drug delivery challenging.⁶ Radiotherapy provides only a transitory response and the use of chemotherapy is ineffective^{2, 7}. Research has been historically hampered by the lack of available material for biological research; re-introduction of a safe stereotactic procedure as well as engagement with families to access samples at autopsy have helped to address this challenge^{8–10}(Text Box 2).

Text Box 2

Clinical sampling of diffuse intrinsic pontine glioma (DIPG)

The diagnosis of DIPG relies upon radiological and clinical parameters, and not on the basis of histopathological features because resection is not a viable option due to its location in the brainstem. Indeed in 1993, an influential report from the Childrens Cancer Group recommended against the use of exposing patients to risks from surgical procedures, resulting in a lack of tissue for research⁹⁵.

The opening of rapid autopsy protocols

To expand the altruistic donation of tumour samples, groups at St Jude Children’s Research Hospital in Memphis and the Hospital for Sick Children in Toronto began to offer the possibility of enrolling in a rapid autopsy protocol for the collection of tumour tissue *post-mortem*^{9, 10}. Such protocols provided sufficient quantities of un-degraded DNA and RNA for molecular profiling^{15, 26} and genome sequencing studies^{18, 21, 50} which have revolutionised our understanding of the biology of the disease. With appropriate clinical and bereavement counselling, participating in such a programme has proven to be an overwhelmingly affirming experience for these families, and has encouraged the expansion of such an approach worldwide⁹⁶. It will remain an important source of material for DIPG study, particularly as it affords whole-brain study, and provides a unique opportunity for direct assessment of intratumoural heterogeneity through multi-region sampling approaches.

Reintroduction of safe biopsy procedures

In the past decade, neurosurgical teams as part of a multidisciplinary paediatric neuro-oncology group in Paris started to offer image-guided stereotactic biopsies of DIPG, with sample collection for future biological study a key endpoint. With nearly 200 patients undergoing these procedures, there have been no surgery-related deaths and low, transient morbidity rates of less than 4%⁸ and these samples provide sufficient quantity and quality of tumour cell material for histological and molecular pathology analysis^{24, 97}, as well as comprehensive genome sequencing^{19, 20}. The sampling of

tumours prior to the start of radiotherapy importantly allows for these experiments to be carried out in a treatment-naïve context, without the possibility of confounding radiation-induced mutations. At a multidisciplinary consensus conference in 2011, the use of biopsy was formally recommended to ascertain biological characteristics to enhance understanding and targeting of treatments, especially in clinical trials⁹⁸. As novel subgroup-specific drug targets are identified, this will become a critically important component of finally improving outcome in this most challenging of paediatric brain tumours.

DIPGs occur almost exclusively in children, with a peak in incidence from ages 6–8 years, coinciding with an expansion of a Nestin⁺, Olig2⁺ neural precursor-like cell population in the ventral pons, proposed as the possible cell-of-origin¹¹. By contrast, supratentorial tumours predominantly occur during adolescence (median 11–12 years)^{3, 5}, though they may also be found in other age groups including infants (< 3 years). Tumours in the infant age group appear to respond considerably better to chemotherapeutic regimens and have improved clinical outcomes¹², hinting at a distinct biology.

Clinical trials in paediatric HGG historically relied on biological hypotheses and preclinical data derived from studies of adult GBM. High-grade gliomas represent a spectrum of diseases, with shared histopathological features among childhood and adult tumours¹³. In addition, the same gene expression subgroups initially identified in adult HGG are easily identified in paediatric HGGs, further revealing the clear relationship among these disease groups^{14–17}. However, recent molecular profiling data, largely generated through collaborative studies, has shown definitively that childhood tumours are biologically distinct from their adult counterparts and strongly suggest that there are unique molecular processes driving the evolution of HGGs in children. This review will discuss exciting new insights into the molecular pathogenesis of this disease, illuminating distinct genetic mutations among subgroups of childhood HGG arising in particular anatomic regions as well as among different age groups.

Genetic complexity of paediatric high-grade gliomas

The majority of paediatric HGGs have complex genomic signatures, with significant copy number alterations (CNAs), single nucleotide variants (SNVs) and structural variants (SVs)^{15, 16, 18–27}. Although the number of non-synonymous mutations is lower than in common adult cancers, including adult glioblastoma, HGGs have a greater mutation burden than a number of other paediatric cancers (Figure 2)^{18–21}. Structural variants ranging from simple rearrangements to extensive and complicated abnormalities caused by chromothripsis [G] are common in paediatric HGG^{18–21}, with approximately 50% of all cases showing expression of fusion genes as detected by RNA-seq²¹.

While most paediatric high-grade gliomas show an average of 5–6 large scale CNAs, as well as focal deletions or amplifications, there is a subset of cases across the paediatric age group with very stable genomes, and few to no detectable CNAs^{16, 23}. At least some of these cases are hypermutator tumours that arise in the context of germline mutations in mismatch repair genes. In these cases, the number of somatic SNVs may be more than 100-fold higher than

that found in 95% of paediatric HGGs²¹ (Figure 2). Germline mutations in the *TP53* and *NF1* tumour suppressors are known to cause inherited predisposition to high-grade gliomas²⁸, and a subset of paediatric HGGs arise in this context of increased genetic risk. However, the overall mutation burden and specific cooperating mutations in these HGGs are similar to those seen in sporadic cases²¹.

The genomic landscape of non-brainstem HGGs (NBS-HGGs) arising in children less than three years old is strikingly different, with significantly lower mutation burdens, in some cases showing only two non-silent mutations across the entire genome²¹ (Figure 2). Interestingly, NBS-HGG in this age group are also clinically distinct, showing a significantly greater survival compared to NBS-HGGs in older children¹². In a small cohort, 40% (4/10) of NBS-HGGs arising in this age group carried chimeric oncogenes involving fusions of 5' regions from different genes to sequences encoding the kinase domain from the neurotrophin receptor genes *NTRK1*, 2 or 3. Consistent with the extremely low number of additional mutations in infant NBS-HGGs, two *NTRK* fusions tested, *TPM3-NTRK1* and *BTBD1-NTRK3*, showed strong oncogenic activity, transforming *Tp53*-null astrocytes to drive rapid HGG formation *in vivo*²¹. Two of the *NTRK* fusion genes identified in paediatric HGG, the *ETV6-NTRK3* fusion and the *TPM3-NTRK1* fusion, were previously identified in other tumours. The *ETV6-NTRK3* fusion was identified in congenital fibrosarcomas and mesoblastic nephromas, leukemia, as well as adult secretory breast cancer, while the^{29–32} *TPM3-NTRK1* fusions are recurrent in papillary thyroid carcinoma³³. In brain tumours, *NTRK* fusions were not uniquely associated with infant NBS-HGG. They were also found at lower frequency in DIPG, NBS-HGG from older patients, as well as in paediatric low-grade glioma in the context of very few additional mutations, and in adult glioblastoma, where they were present with multiple additional mutations^{34–36}. Thus, the genomic complexity of paediatric HGG spans a wide range, with infant NBS-HGG and HGGs from patients with inherited mutations in mismatch repair genes at the extreme opposite ends of the spectrum (Figure 2). The paucity of mutations in infant tumours suggests that therapeutic targeting of *NTRK* fusions may be highly effective in the absence of a large number of other mutations. However, the genomic complexity of the majority of HGGs, including DIPGs, indicates that multiple genetic mechanisms generate numerous mutations providing the tumour with diverse potential pathways to therapeutic resistance.

Mutations in canonical cancer pathways

Paediatric diffuse HGGs show frequent genetic alterations targeting the same canonical cancer pathways that are deregulated by mutation in almost all adult glioblastomas; the receptor tyrosine kinase (RTK)/RAS/PI3K pathway, the TP53 pathway and the RB pathway^{37, 38}, however, the specific effectors most frequently targeted by mutation vary between childhood and adult tumours³⁹(Figure 3). Mutations of the catalytic and regulatory subunits of PI3K, encoded by *PIK3CA* and *PIK3RI*, respectively, are found in both paediatric and adult HGG, while biallelic inactivation of the *PTEN* tumour suppressor is common in adult, but rare in childhood HGGs. The hotspot *BRAF* V600E activating mutation is found in approximately 10% of paediatric cortical HGGs, lower frequencies in thalamic HGGs, and not detected in DIPG. V600E mutations were also found in more than 70% of pleomorphic xanthoastrocytomas, and approximately 50% of anaplastic

ganglioglioma, rare tumours found in both children and adults^{21, 22, 40–44}. Thus mutant BRAF may be a useful therapeutic target in a subset of childhood HGGs. *PDGFRA* is the most commonly mutated RTK in paediatric HGG, and EGFR mutations, which predominate in adult glioblastoma, are relatively rare^{15, 16, 21–27, 45}. Approximately 30% of paediatric HGGs across all brain regions carry focal amplification and/or mutation of *PDGFRA*, involving a unique spectrum of mutations compared to those found in adult glioblastoma^{22, 24, 45}. Amplification of *MET* and *IGF1R* are also found in paediatric HGG^{15, 16, 23–25}. Activating mutations in FGFR1 were identified in thalamic HGGs, but not in paediatric HGGs from the cortex or brainstem^{19, 21}. Although RTKs are actionable therapeutic targets with approved drugs to inhibit their activity, a survey of DIPGs showed that RTK amplification was extremely heterogeneous within tumours¹⁵, as has been shown in adult GBM^{46–48}. This genetic heterogeneity is likely to pose a very significant challenge to the design of personalized therapy to counteract specific mutations, as there may already be pre-existing resistant populations of tumour cells that lack the drug target.

TP53 mutations are found in approximately 55% of paediatric HGGs^{18–22}. There are also mutually exclusive mutations in 9–23% of DIPGs and midline HGGs in the *TP53* target gene *PPM1D*^{20, 21}, which plays a role downstream of *TP53* in DNA damage response. The RB pathway is targeted at a significantly lower frequency in paediatric HGGs than in adults, and shows striking differences in the genes involved based on tumour location. Homozygous deletions of *CDKN2A* are found in approximately 25% of non-brainstem-HGGs, but are extremely rare in DIPGs. Focal amplification of *CDK4*, *CDK6* or *Cyclin D1*, *D2*, or *D3*, the components of the cyclin/CDK complex that phosphorylate RB at the G1 checkpoint, are found in paediatric HGGs from all brain regions^{15, 23, 25}. Although loss of chromosome 13q is found in approximately one third of paediatric HGGs regardless of location, biallelic loss of function mutations in RB are extremely rare^{18–22}. Therefore both NBS-HGGs and DIPGs may be effectively targeted by CDK4/6 inhibitors. Indeed, preclinical studies with these agents showed some efficacy in a mouse model of DIPG⁴⁹. Thus, therapeutic approaches designed to counteract aberrant regulation of pathways that are of central importance in most common cancer types, are also highly relevant to childhood HGG. However, there are oncogenic mutations that are unique to paediatric HGG that must also be considered.

Connecting chromatin regulation and cancer

Genome-wide sequencing efforts of paediatric glioblastoma and DIPG identified recurrent and hotspot mutations in *H3F3A* and *HIST1H3B*, which encode the histone H3.3 variants H3.3 and H3.1 respectively^{22, 50}. This unexpected finding was the first example of a human disease driven by direct mutation of histones^{51, 52}. Moreover, as these mutations are not found in adult HGG, this data provided compelling evidence that distinct molecular processes underlie paediatric and adult HGG.

Histone H3 isoforms H3.1 and H3.2 are synthesized during the S phase of the cell cycle to package newly replicated DNA, while H3.3 is synthesized throughout the cell cycle and used to replace histones that are lost during processes that disrupt nucleosomes^{53, 54}. Histone H3.1 is encoded by 10 genes encoding identical proteins, though to date, almost all histone H3.1 mutations are found in *HIST1H3B* with two rare examples of *HIST1H3C*

K27M in DIPG^{18, 19}. *HIST1H3B* mutant DIPG furthermore appear to represent a distinct subgroup, with a younger age at diagnosis (4–5 years) and longer survival time (median = 15 months), though with no improvement in eventual clinical outcome^{18–21}(Figure 4).

Histone H3 mutations occur at two residues – the lysine at position 27 (lysine-to-methionine, K27M) and the glycine at position 34 (glycine-to-arginine or –valine, G34R/V). Strikingly, the mutations have a distinct anatomical segregation, illustrating a previously unrecognized highly specific selection pressure evident in different cell types during brain development, and providing strong evidence that the molecular pathogenesis of DIPG was significantly different from tumours arising in the cerebral hemispheres.

K27M mutations were observed in 78% of DIPGs, as well as other tumours of midline structures including thalamus (40%), and spinal cord, with the majority of these mutations in *H3F3A* encoding histone H3.3, and up to 25% in histone H3.1^{18, 20–22, 50, 55, 56}.

By contrast, G34R/V mutations are exclusively in *H3F3A*, and predominantly restricted to tumours of the cerebral hemispheres (15%)^{17, 19, 21, 22, 50, 55–57}. The different mutations also have a distinct age of diagnosis (median 6–7 years, K27M; median 13–14 years G34R/V) and survival (median = 12 months, K27M; median = 24 months G34R/V)^{17–21, 55, 56}, in part reflecting the onset and survival associated with HGGs from cortical versus brainstem and midline locations. Importantly, G34R/V mutations are found in HGG patients beyond the paediatric age group, extending through patients diagnosed in early adulthood up to approximately 30–40 years of age, indicating that molecular diagnostics spanning traditional paediatric and adult neuro-oncology services will be vital in the common management of these patients as one disease entity⁵⁶. Similarly, although rare in the adult population, 88% of thalamic tumours arising in patients between the ages of 21–49 years old have K27M mutations⁵⁸.

Paediatric HGGs generally appear to arise as *de novo* lesions without evidence of lower grade precursor disease⁵⁹, therefore it is not clear whether histone H3 mutations arise as early or late events in tumorigenesis. However, the mutations clearly provide a strong selective advantage, because in most cases next generation sequencing shows that the *H3F3A* mutation appears to be heterozygous in 100% of cells, indicating clonal selection without significant intratumoral heterogeneity^{18–22, 50, 55}. Furthermore, histone H3 K27M mutations have been identified at similar high frequency in both untreated biopsies and autopsy specimens after exposure to radiotherapy and other therapies, as well as histological low-grade and high grade regions of DIPGs^{18–21, 50, 55}.

Structural consequences of histone mutations in paediatric HGG

Both amino acids targeted by recurrent mutations reside in the unstructured N-terminal tail of histone H3. Post-translational modification of histone tails represents a key regulatory mechanism by which cells exert fine-control of gene transcription. These processes appear particularly important in spatio-temporal control of gene expression during normal development⁶⁰, stem cell maintenance⁶¹ and differentiation⁶².

The regulation of histone tails is generated through diverse combinations of different post-translational modifications including methylation, acetylation, or ubiquitination of lysine residues, as well as phosphorylation of serine or threonine residues, and methylation of arginine residues. The enzymes that catalyze the addition or removal of these moieties are known as chromatin ‘writers’ or ‘erasers’, respectively. The resulting complex patterns create a histone code in which effector proteins, or ‘readers’, are recruited to the chromatin by specific binding to particular histone modifications, resulting in localization of functional complexes that impact transcriptional regulation⁶³. Chromatin compaction is also affected by post-translational modifications of histone tails as well as through the action of other chromatin remodeling proteins which may act to change nucleosome position and conformational structure. All of these different levels of chromatin regulation are increasingly understood to play critical roles in oncogenesis, with numerous recurrent mutations observed in chromatin writers, erasers, readers and remodelers in a diverse series of human cancers⁶⁴. Owing to the ubiquity of cellular histone expression and the high degree of redundancy of genes encoding these proteins, it was assumed that these genes were unlikely to be mutational targets themselves⁵². Remarkably, high grade gliomas in children shifted this paradigm.

Functional consequences of histone mutations in paediatric HGG

The unique functional consequences associated with each of the mutations observed in paediatric HGG are just beginning to be elucidated. The K27M mutation occurs at a specific lysine methylation/acetylation site, and as such was immediately presumed to have profound consequences on the control of gene expression associated with this key residue. Trimethylated lysine 27 interacts with the polycomb repressive complex (PRC2) to repress transcription of target genes, a process seemingly key in stem cell regulation and development⁶⁵. K27M (and to a lesser extent K27I) represents the only amino acid substitution at this residue which can ablate this trimethylation⁶⁶, suggestive of why this specific mutation is selected for in pHGG. What is more extraordinary is that although the mutant histone appears to represent only 3.6–17.6% of the total H3 pool in the cell⁶⁶, loss of H3K27me3 is near-absolute, indicative of a trans-dominant negative effect across all three isoforms of the wild-type H3 protein^{66–69}. This has been shown to result in a generalized hypomethylation and upregulation of gene expression⁶⁸, exemplifying the complex interactions between genetic and epigenetic mechanisms of gene transcription control. In addition, the predicted de-repression of PRC2 target genes is clearly observed in K27M mutant tumours, likely influencing diverse pro-tumourigenic processes^{66, 68}. Of note, there remains a small proportion of trimethylated marks present at H3K27, which then appear to bind distinct target genes not associated with wild-type cells, although further studies may be required to elucidate whether transcripts are consistently dysregulated by this mechanism^{68, 69}.

This differential binding of mutated H3.3 variants may be even more pronounced in cortical tumours harboring G34R/V mutations. The mutated residue is not itself a known target of post-translational modification, however it is adjacent to the lysine at position 36, trimethylation of which is associated with activation of gene transcription as well as effects on alternative splicing and DNA repair⁷⁰. The glycine-to-arginine G34 substitution has been

shown to diminish trimethylation at the K36 position *in vitro*, however only on the mutant allele⁶⁶. Thus, the G34 mutations do not exert the same dominant effect over the entire cellular pool of histone H3 that is observed with K27M mutations.

In a single cell line model of G34V mutant paediatric glioblastoma, the mutant protein was found to bind at a distinct set of target genes associated with cortical development and stem cell maintenance¹⁷. Strikingly, the most significantly differentially bound and expressed gene was *MYCN*, a potent oncogene in diverse paediatric cancers shown to transform neural stem cells derived from the developing cortex and to produce glioblastomas in mice⁷¹. This upregulation of *MYCN* was also seen in primary paediatric tumours with G34R/V mutations compared to *H3F3A* wild-type, with the exception of *MYCN* amplified cases, which are mutually exclusive with histone mutations¹⁷. While therapeutic approaches to directly target H3.3 mutations remain elusive, numerous strategies have emerged to destabilise the MYCN protein⁷². Thus MYCN may be of immediate translational relevance in children with these tumours. It remains unclear how the glycine-to-arginine substitutions differ mechanistically compared with the glycine-to-valine. Arginine may itself be a target of post-translational modification⁷³, hinting at key functional differences, however a lack of G34 model systems has hampered our understanding.

Recently, alternate histone H3 mutations were found in two rare childhood bone tumours. Giant cell tumours of bone harbor G34W or G34L mutations in *H3F3A* in 92% cases, indicating that different amino acid substitutions for G34 may be oncogenic in different tumour types. In addition, 95% of chondroblastomas have K36M mutations in *H3F3B*⁷⁴, which encodes the histone variant H3.3. Intriguingly, the substitution of methionine for lysine results in a dominant loss of all trimethylation of lysine 36, thus showing a similar function to the K27M mutation, with both mutations showing specificity for dominantly decreasing trimethylation of the lysine in the same position as the mutation. It remains to be seen whether additional tumour types harbor similar histone mutations, and whether there is a specific commonality yet to be elucidated between bone and brain development.

Developmental origins of pHGG subtypes

Paediatric HGGs with histone H3 mutations were associated with distinct signatures of genome-wide DNA methylation⁵⁶. These methylation signatures as well as the profound and differential gene expression signatures associated with histone H3 mutations also provide clues to the developmental origins of the tumours in which they are found. Comparison of gene expression signatures between DIPGs and non-brainstem HGGs showed a significant differential expression of transcription factors associated with hindbrain patterning¹⁵. Comparing maps of histone mutation-specific expression signatures from paediatric HGG to published datasets of human brain developmental expression signatures showed that signatures for the various pHGG subtypes have distinct developmental origins, mimicking what has been described in other paediatric brain tumours such as ependymoma⁷⁵ and medulloblastoma⁷⁶. G34R/V signatures are highly enriched in the ganglionic eminences that are present in the late embryonic and early fetal stages of development and which are the regions from which cortical interneurons arise, as well as a rich pool of neuroprogenitors¹⁷. By contrast, K27M signatures overlap with hindbrain developmental structures at later fetal

timepoints, and likely point to a different cell of origin^{17, 56}. Of note, the gene expression signatures of K27M mutant tumours from the pons and thalamus show strong similarities^{21, 24}, suggestive that these tumours share a common origin and ought to be considered together clinically – supported by the dismal clinical outcome for a significant proportion of thalamic GBM³.

The anatomical distribution within the cortex of diffuse HGGs arising during the transition from late adolescence through young adulthood also suggests a potential difference in tumor susceptibility associated with developmental context. Here *IDH1*-mutant gliomas, strongly associated with adult secondary glioblastomas and the lower-grade diffuse gliomas from which they arise, more frequently occur within the frontal lobe, while the mutually exclusive population of histone H3 G34-mutant tumors are more commonly found in the other lobes of the cerebral cortex^{22, 39, 56}.

Mutations in other chromatin regulators in pHGG

In addition to the specific mutations in the genes encoding the histone variants, other chromatin regulators may also be targeted in paediatric HGG. The histone chaperones *ATRX* or *DAXX* are mutated in 15–25% of tumours, co-segregating with G34R/V mutations^{19, 21, 22}. These proteins are involved in the loading of histone H3.3 to the heterochromatic regions at the telomeres⁷⁷, and the concurrent *H3F3A* and *ATRX/DAXX* mutant tumours are strongly associated with a telomerase-independent alternative lengthening of telomeres (ALT) phenotype in a proportion of paediatric HGG²². In adults, mutations in *ATRX* and the telomerase reverse transcriptase (*TERT*) promoter are mutually exclusive and represent alternative mechanisms to lengthen telomeres. Although *TERT* promoter mutations are found in 83% of adult glioblastomas, these mutations are found in less than 3% of paediatric diffuse HGGs^{21, 78}. In addition, the specific methyltransferase responsible for depositing the methyl marks at the K36 residue, *SETD2*, is also found mutated in 5–15% of paediatric HGG^{19, 21, 57}. Whether this mutation confers the same phenotype as the G34 mutations is not clear, however unlike *H3F3A*, these mutations are not mutually exclusive with those in *IDH1/2*⁵⁷, more frequently found in clinical secondary young adult (40–50yrs) GBM⁷⁹. Strikingly, mutations in writers or erasers of all modified methylated lysines of the histone H3 tail were found except those that impact K27^{18–21}. Thus, in paediatric HGG, disrupted regulation of K27 modification is mediated directly through mutation of histone H3.

Further non-recurrent mutations are found in diverse chromatin/reader writers such as members of the myeloid/lymphoid or mixed-lineage leukemia (*MLL*), lysine (K)-specific demethylase (*KDM*) and chromodomain helicase DNA (*CHD*) families^{18–21}, in common with other tumour types including primary adult GBM⁸⁰. The complex interplay of these mutations with the mutant histones is not yet clear, nor is the most efficacious way to target the histone mutations themselves clinically. A concurrent upregulation of acetylation at K27 in association with the loss of trimethylation seen in K27M cells⁶⁶ points towards the use of inhibitors of histone acetyltransferases, whilst differential loading of residual H3K27me3 may suggest a role for specific inhibitors of the histone demethylases *JMJ3D/UTX*⁸¹. Targeting the downstream consequences of these mutations may also prove to be useful,

such as MYCN in G34R/V tumours, and specific K27M targets such as CDK6 and PDGFRA^{68, 69}, also dysregulated by gene amplification^{16, 23, 25, 26}, pointing to their potential importance in paediatric gliomagenesis.

ACVR1/ALK2 mutations; a selective role for BMP signaling in DIPGs

The identification of recurrent mutations in the BMP receptor ACVR1/ALK2 in 46/195 (24%) DIPGs^{18–21}, but not in more than 100 non-brainstem paediatric high-grade gliomas^{19, 21, 22} provides another clear example of unique selective pressures driving DIPGs. *ACVR1* mutant DIPGs have an earlier age of diagnosis and longer overall survival time,^{20, 21} occur more frequently in females, and frequently also contain histone H3.1 K27M mutations (80%). Given the apparently equivalent consequence of K27M mutations in H3.1 or H3.3 to decrease total cellular levels of H3K27me3, the significant co-occurrence of *ACVR1* and *HIST1H3B* mutations may reflect a specific developmental setting. Surprisingly, some of the same somatic *ACVR1* mutations identified in DIPG are found as heterozygous germline mutations causing the congenital malformation syndrome fibrodysplasia ossificans progressiva (FOP), a disease characterized by the formation of heterotopic ossifications in soft tissues at sites of inflammation^{82, 83}. FOP is not associated with cancer predisposition, despite the presence of germline mutations that are identical to those found in DIPG. This strongly suggests that *ACVR1* mutations do not have a potent tumour initiating effect in DIPG, but rather provide a selective advantage when they are combined with other mutations.

ACVR1 is a type I BMP receptor, which is activated in response to extracellular BMP, and in turn phosphorylates and activates downstream growth promoting genes through SMAD transcription factors (Figure 5). Mutations in *ACVR1* associated with DIPG encode amino acid substitutions in the ATP binding pocket of the kinase domain, or in the adjacent glycine/serine-rich inhibitory domain, and would be predicted to result in a shift of the enzyme to the active conformation⁸⁴. Indeed, mutations of these residues induce a weak gain of function, as determined by increased phosphorylation of the downstream substrates SMAD1/5/8^{18–21}. As in the wild-type setting, interaction of mutant ACVR1 with type II BMP receptors is still required for active signaling⁸⁵.

An activated BMP signaling cascade regulates diverse developmental processes in different tissues. Expression of ACVR1 mutations found in DIPG disrupts normal dorsoventral patterning, and causes an increased development of ventral structures at the expense of dorsal structures in zebrafish embryos, phenocopying the effects of activated BMP signaling^{21, 86}. Overexpression of BMP4 in the brains of transgenic mice induced astrocyte differentiation at the expense of oligodendrocyte formation⁸⁷. Consistent with overactive BMP signaling decreasing the pool of oligodendrocytes, central nervous system (CNS) demyelination was detected in a mouse knock-in model carrying the *Acvr1* R206H mutation found in both FOP and DIPG, and white matter lesions perhaps associated with demyelination were found in a small group of FOP patients evaluated for CNS abnormalities⁸⁸. In adult glioblastoma cancer stem cells, BMP signaling induced differentiation in one study, and proliferation in another, depending on epigenetic silencing of BMP receptor 1B^{89, 90}. BMP signaling induces differentiation signals and inhibits

tumourigenesis of medulloblastoma, a paediatric brain tumour arising from granule neuron progenitors⁹¹. Somatic activating mutations in *ACVR1* presumably function as oncogenes in DIPG, likely conferring different downstream cellular responses in this specific context, consistent with different outcomes of BMP signaling during brain development. Indeed, *ACVR1* mutations were significantly associated with tumours from younger children, suggesting a developmental context for this oncogenic signal even within DIPG.

Conclusions

Overall, paediatric HGGs share histological and clinical features with adult HGGs, as well as mutations in common cancer pathways. However, the recent insights from genome-wide sequencing efforts show that there are unique selective pressures driving tumourigenesis in the developing brain, which vary according to age, but also according to anatomic location within the same age group. There is significant interpatient heterogeneity in the genomic abnormalities between these subgroups as well as within subgroups. Given the similarities in dismal clinical outcome, it appears that there are multiple genetic and epigenetic mechanisms that drive diffuse HGG, with different mutations converging on shared pathways as has been observed in the genomic landscapes of many types of cancer⁹². The selection of unique mutations in specific subgroups likely reflects the context-dependent regulation of signaling that varies with developmental setting. These distinctions in disease pathogenesis likely indicate that very different therapeutic approaches will be needed to effectively counteract the underlying genetic mutations. The extremely high frequency of histone H3 mutations in DIPG revealed a surprising unifying molecular mechanism driving the disease, and therefore offers hope that if these mutations could be targeted, that effective treatment for this devastating disease could be possible. While there have been exciting advances in understanding some functional consequences of histone H3 mutations, how these mutations contribute to oncogenesis, and the basis for their regional selectivity remain unclear. Similarly, understanding the oncogenic output of other context-specific mutations such as aberrant BMP signaling in DIPGs is critical. It remains to be determined how therapeutic targeting of paediatric HGG-specific mutations may alter activity of other signalling pathways in tumor cells or impact non-tumor tissues. Although a paucity of *in vitro* and *in vivo* models remains a major limitation in translating these new findings into patient benefit, knowledge of these mutations present an opportunity for new therapeutic development as well as novel insights into connections between chromatin regulation, BMP signaling and tumourigenesis.

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Glossary

Pons	The portion of the brainstem between the midbrain and the medulla oblongata
Supratentorial	brain regions above the tentorium cerebelli, which separates the cerebellum from the occipital and temporal lobes of the cerebral cortex
Stereotactic	a system of three-dimensional coordinates used in surgery to locate a precise position
Chromothripsis	local chromosome shattering in which complex re-arrangements with multiple inter-connecting breakpoints correspond to genomic segments of oscillating copy number states
Ganglionic Eminence	a transient structure in the embryonic telencephalon that guides tangential cell migration
Exophytic	growing outward beyond the surface from which it originates
Pilocytic astrocytoma	a low-grade (grade I) astrocytoma that is the most common primary brain tumour of childhood

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Key points

- Diffuse high-grade gliomas carry a dismal prognosis in both children and adults, however genome-wide molecular analyses have shown that the disease pathogenesis differs significantly between these age groups.
- There are at least several distinct subgroups of paediatric diffuse high-grade glioma based on clinical features and recurrent mutations.
- Diffuse intrinsic pontine gliomas (DIPGs) arise in the brainstem, occur almost exclusively in children, and are incurable.
- Aberrant epigenetic regulation plays an important role in paediatric high-grade gliomas, with hotspot pK27M histone H3 mutations found in nearly 80% of diffuse intrinsic pontine gliomas (DIPGs), and alternative pG34R/V mutations found in paediatric high-grade gliomas of the cerebral hemispheres.
- Recurrent mutations of the BMP receptor *ACVR1/ALK2* are restricted to the youngest DIPG patients, highlighting critical connections between development and gliomagenesis.
- HGGs in children less than three years of age contain very few genomic abnormalities, recurrent gene fusions, and a better outcome than HGGs in older children.
- An improved understanding of the oncogenic mutations driving paediatric diffuse HGG has identified new potential therapeutic targets, and shown that different strategies will be needed to combat this disease in children and adults.

Figure 1. Brain regions and selective mutations distinguish subgroups of paediatric HGG
 High-grade gliomas in children frequently arise in the midline as DIPG in the pons, or involving the thalamus, or much less frequently, the cerebellum or spinal cord. Childhood HGGs also arise in the cerebral cortex, the region where the majority of HGGs in adults originate. Mutation frequencies indicate differences in selective pressures between different regions of developing brain. For example, histone H3.3 G34R/V mutations and *BRAF* V600E mutations are predominantly found in cortical tumours, histone H3 K27M mutations are found in all midline locations with highest frequency in DIPG, *ACVR1* mutations are restricted to DIPG, and *FGFR1* mutations are found in thalamic HGGs.

Figure 2. Mutational burden of paediatric HGG

Somatic non-synonymous coding mutations per sample are plotted on a log scale for a variety of paediatric and adult cancer types, and ordered by increasing median (red bar)^{20, 21, 30, 34, 35}. Infant HGG (shaded orange) have the lowest number of somatic mutations per sample other than paediatric low grade glioma, with a median of 2. Paediatric HGG (pink) and DIPG (red) have very similar profiles, with a median of 15 non-synonymous coding mutations per sample for both, considerably lower than for adult GBM (61 mutations per sample, $p < 0.001$ for both pHGG and DIPG, Mann-Whitney U test). There was no significant difference between pre-treatment DIPG samples taken at biopsy and post-treatment samples at autopsy. Untreated paediatric HGG classed as ‘hypermutator’ (dark red) harboured the highest mutational burden of all, with a median of 6,810 (range 262–18,369) non-synonymous coding mutations per sample. AML: acute myeloid leukaemia. CML: chronic lymphocytic leukaemia. LGG: low grade glioma.

Figure 3. Recurrent mutations activate PI3K and MAPK signaling pathways in paediatric HGG Growth factor binding to receptor tyrosine kinases (RTKs) activates downstream signaling through the PI3 kinase and MAP kinase signaling pathways⁹⁹. PI3 kinase alpha is comprised of the p85 regulatory subunit and the p110α catalytic subunit, encoded by *PIK3R1* and *PIK3CA*, respectively. PI3K phosphorylates the signaling lipid PIP2 to generate PIP3, leading to subsequent activation of downstream cascades, including the serine/threonine kinase AKT. The PTEN tumour suppressor is a protein and lipid phosphatase that directly reverses the action of PI3K. Growth factor binding to RTKs can activate both RAS and PI3K through different adapter proteins, or through direct interaction with PI3K, depending on the specific receptor. RAS is a small G-protein which is activated when in the GTP-bound state. The NF1 tumour suppressor is a GTP-ase activating protein that negatively regulates RAS activity. RAS participates in PI3K pathway signaling as well as downstream signaling through the serine/threonine kinase BRAF to additional downstream targets in the MAP kinase pathway. The frequency of mutations for the most recurrently mutated components of these pathways in paediatric high-grade glioma are indicated^{18–22}. Some mutations show different frequencies in cortical (Cort) versus thalamic/midline HGGs or DIPGs. Of note, *PTEN* and *EGFR* mutations, which are frequent in adults, are not highly recurrent in childhood HGGs. mut: mutated, amp: amplified

Figure 4. Histone and chromatin modifier mutations in paediatric high-grade and diffuse intrinsic pontine glioma

(a) Specific recurrent mutations in the genes encoding the histone variants H3.3 (*H3F3A*) and H3.1 (*HIST1H3B* and *HIST1H3C*). K27M mutations in H3.3 and H3.1 result in a global reduction of H3K27me₃, leading to derepression of targets of the polycomb repressive complex 2 (PRC2). H3.3 K27M mutations are found in nearly two-thirds of DIPG, half of thalamic HGG, and other midline tumours. H3.1 K27M mutations are found in over 20% of DIPG and 5% of midline HGGs, and are strongly associated with younger patient age^{18–21}. H3.3 G34R/V mutations are found in 15% paediatric HGG and are restricted to cerebral hemispheric tumours of adolescents and young adults, and confer differential distribution of the activating H3K36me₃ mark. (b) Mutations in other chromatin modifiers. Numerous writers (top) and erasers (bottom) of histone H3 lysine marks are mutated in paediatric HGG and DIPG, with an apparent exception of lysine 27. Lysines in the H3 tail that are modified by trimethylation (me marks) or acetylation (ac) are indicated. In addition, H3 chaperones such as ATRX or DAXX are mutated in 15–20% of supratentorial tumours, along with a variety of other chromatin remodellers in tumours of all locations^{20–22}.

Figure 5. ACVR1 mutations in diffuse intrinsic pontine glioma

(a) Cartoon showing somatic mutations in the glycine/serine-rich domain inhibitory domain (GS) and kinase domains of ACVR1 in 46/195 (24%) DIPG^{18–21} samples. No mutations were identified in the extracellular (ECD) or transmembrane (TM) domains. All residues targeted are common to those observed in the germlines of fibrodysplasia ossificans progressiva (FOP) patients, with the same amino acid substitutions except for R258G and G328V, which are to date unique to DIPG. (b) BMP signalling pathway. Upon ligand binding, a type II receptor such as BMPRII heterodimerises with and phosphorylates ACVR1, a serine threonine kinase, which in turn phosphorylates the SMADs 1/5/8 transcription factors, causing binding to SMAD4, translocation to the nucleus, and transcription of target genes such as *ID1* and *ID2*. The *ACVR1* mutations in DIPG and FOP enhance the kinase function and/or disrupt binding of the negative regulator FKBP12 to ACVR1, conferring activation of this pathway.