Published in final edited form as:
Semin Radiat Oncol. 2014 October ; 24(4): 240–247. doi:10.1016/j.semradonc.2014.06.003.

The genetic signatures of pediatric high-grade glioma: no longer a one-act play

Alexander K. Diaz[1,2] and **Suzanne J. Baker, Ph.D.**[1,2,#]

[1]Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN 38105

[2]Integrated Biomedical Sciences Program, University of Tennessee Health Science Center, Memphis, TN 38163

Abstract

Advances in understanding pediatric high-grade glioma (pHGG) genetics have revealed key differences between pediatric and adult high-grade gliomas (aHGGs), and have uncovered unique molecular drivers among subgroups within pHGG. The three core aHGG pathways, the receptor tyrosine kinase(RTK)/Ras/Phosphatidylinositide 3-kinase (PI3K), p53, and retinoblastoma (RB) networks, are also disrupted in pHGG, but they exhibit a different spectrum of effectors targeted by mutation. There are also similarities and differences in the genomic landscape of diffuse intrinsic pontine glioma (DIPG) and pediatric non-brainstem high-grade glioma (pNBS-HGG). In 2012, histone H3 mutations were identified in nearly 80% of DIPGs and ∼35% of pNBS-HGG. These were the first reports of histone mutations in human cancer, implicating novel biology in pediatric gliomagenesis. Additionally, DIPG and midline pNBS-HGG vary in the frequency and specific histone H3 amino acid substitution compared to pNBS-HGGs arising in the cerebral hemispheres, demonstrating a molecular difference among pHGG subgroups. The gene expression signatures as well as DNA methylation signatures of these tumors also carry distinctive signatures, reflecting a combination of the driving mutations and the developmental context from which they arise. These data collectively highlight unique selective pressures within the developing brainstem and solidify DIPG as a specific molecular and biological entity among pHGGs. Emerging studies continue to identify novel mutations that distinguish subgroups of pHGG. The molecular heterogeneity among pHGGs will undoubtedly have clinical implications moving forward. The discovery of unique oncogenic drivers is a critical first step in providing patients with appropriate, targeted therapies. Despite these insights, our vantage point has been largely limited to an in-depth analysis of protein coding sequences. Given the clear importance of histone mutations in pHGG, it will be interesting to see how aberrant epigenetic regulation contributes to tumorigenesis in the pediatric context. New mechanistic insights may allow for the identification of distinct vulnerabilities in this devastating spectrum of childhood tumors.

[#]To whom Correspondence should be addressed: Suzanne Baker; Suzanne.Baker@stjude.org.

No potential conflicts of interest were disclosed.

Opening remarks

The last several years mark a period of tremendous growth in our understanding of pediatric high-grade glioma (pHGG). Advances in genome-wide array-based and sequencing technologies, their precipitous drop in cost, and evaluation of increasingly larger cohorts, have all contributed to novel insights into the genetics of these devastating cancers, greatly extending earlier studies that evaluated candidate genes based on their involvement in adult high-grade glioma (aHGG). Our aim is to provide context to these studies and highlight their contribution to the current state of pHGG knowledge.

There are a number of biological features to suggest pediatric gliomas differ from those arising in adults. Most adult gliomas are high-grade supratentorial tumors. In contrast, the majority of childhood gliomas are low-grade, and both low-and-high-grade gliomas commonly arise within the posterior fossa, an area seldom affected in adults. Diffuse intrinsic pontine glioma (DIPG) is a brainstem HGG that occurs almost exclusively in children[1,2]. Additionally, the current standard of chemotherapeutic care for aHGG, temozolomide, has not been shown to improve long-term survival in pediatric trials[3-5]. Furthermore, malignant transformation, the process whereby a low-grade lesion progresses into a high-grade tumor, is a common event in adults but infrequent in children[6]. Genetic analyses have illuminated molecular differences driving pediatric and adult high-grade gliomagenesis.

The three core aHGG pathways show a different spectrum of alteration in pHGG

As the genomic landscape of aHGG came into view, it shaped initial work into the pediatric disease. The first pHGG studies focused primarily on investigating the involvement of high-frequency recurrent events found in adult tumors. For example, epidermal growth factor receptor (EGFR) is the most commonly altered receptor tyrosine kinase (RTK) in aHGG; with the corresponding gene locus undergoing amplification, intragenic deletion, or both in ∼50% of cases[7-9]. First identified in adult glioblastoma, EGFRvIII is the most common EGFR variant in aHGG and is formed by deletion of exons 2-7 resulting in a constitutively active kinase[10-12]. Accordingly, investigators early on sought to examine the degree of EGFR involvement in pediatric cases of HGG.

A number of studies found that *EGFR* alteration was less frequent in pHGG[13-19], although gene amplification and EGFRvIII expression were detected in some pHGG[20-23]. Through genome-wide studies, *PDGFRA*, which encodes PDGFRα was identified as the most commonly targeted RTK in both DIPG and pNBS-HGG. Alterations in the gene itself include amplification, mutation, or both[15-19,23-27]. Experimentally, overexpression of wild type (WT) or mutant PDGFRα conferred a growth advantage to astrocytes, an effect that was diminished by introduction of the ATP-competitive inhibitors crenolanib or dasatinib[27]. PDGFRα mutants drive glioma formation *in vivo*[27,28], with murine-derived HGGs recapitulating critical features of the human disease such as histopathologic characteristics and expression profiles[27]. In an effort to target PDGFR therapeutically, pediatric trials using dasatinib, crenolanib or imatinib have been launched[29-31]. Unfortunately, the benefit derived

from selective RTK inhibitors may be marginal at best. pHGGs show evidence of intratumoral heterogeneity, with some cells co-amplifying multiple RTK genes or discrete cell populations within the same tumor amplifying different genes; suggesting that resistant populations are likely to be present even before treatment with targeted agents[15,16,26].

Both PDGFRA and EGFR are part of the RTK/Ras/Phosphatidylinositide 3-kinase (PI3K) signaling cascade, which is altered in nearly 90% of aHGGs. Additionally, ∼80-90% of adult tumors show evidence of retinoblastoma (RB) and p53 pathway dysregulation[7-9,32]. For this reason, many of the first genetic pHGG studies focused on these same networks (Figure 1).

In adults the most commonly targeted components of the RTK/Ras/PI3K axis downstream of RTKs include activation of PI3K itself, or loss of function of PTEN, the main negative regulator of PI3K signaling, or NF1, a negative regulator of Ras-mediated signaling[7-9]. Activation of PI3K signaling caused by mutations of *PIK3CA*, encoding the catalytic p110α subunit of PI3K, or *PIK3R1*, encoding the regulatory subunit of PI3K, are usually present in mutually exclusive patterns, occurring in approximately 20% of aHGGs and a similar frequency of pHGG, including DIPG [9,25,33-39]. The *PTEN* tumor suppressor is located on chromosome 10q. It remains unclear whether all tumors with loss of chromosome 10q are targeting PTEN loss of function when a wild-type *PTEN* allele is still retained. However, there are examples in experimental systems where *PTEN* haploinsufficiency contributes to tumorigenesis. Loss of heterozygosity of chromosome 10q, with or without concurrent PTEN mutation is very frequent in adult glioblastoma, with 10q LOH in approximately 80% and *PTEN* mutation in 25-40%, while the frequency is significantly lower in pHGGs, with 10q LOH in approximately 30% and *PTEN* mutation in less than 5-15% [9,13,15-19,23-26,35,36,40-42].

RB pathway dysregulation is common in both pNBS-HGGs and DIPG (Figure 1). The *CDKN2A* locus codes for two tumor suppressors, p16INK4a and ARF[43]. Notably, homozygous deletion of *CDKN2A/B* appears to be almost exclusive to pNBS tumors and largely absent in DIPG[15-19,23,25,26]. In contrast, amplification of *CDK4/6* or *CCND1/2/3* is found in approximately 30% of DIPG[16,19,24]. *CDK4/6* code for cyclin D-dependent kinases that phosphorylate the retinoblastoma protein (pRb), facilitating G1/S cell cycle progression. To become active, these kinases must bind to cyclin D family members (encoded by *CCND1/2/3*), which themselves confer substrate specificity[43]. Therapeutic inhibition of this cyclin/CDK complex, using PD-0332991, a highly-selective non-ATP competitive CDK4/6 inhibitor, significantly increased survival in a murine model of DIPG, both as a single agent or following irradiation[44].

TP53 mutations occur in up to 35% of pNBS-HGGs (range, 18-35%) and appear to be more common in DIPGs (40-50% of cases). When including alterations to other components of the pathway, such as MDM2 and/or ARF, those frequencies can reach 83% for both brainstem and NBS tumors[14,25,32,35,40-42,45-47].

From the above data we can conclude that although the 3 main signaling pathways affected in aHGG are also affected in pHGG, pediatric and adult tumors differ with regard to the most frequently mutated effectors.

Copy number imbalances and gene expression profiling

Despite some common copy number imbalances such as 13q and 14q loss in approximately one third of HGG regardless of age or location, adult and pHGGs also exhibit a unique constellation of gains and losses that distinguish one from the other, and the same can be said for DIPGs and pNBS-HGGs[15-19,23-26]. This suggests that unique combinations of genetic drivers underlie adult and pediatric tumorigenesis, and among childhood HGG, DIPG and pNBS-HGG tumorigenesis.

Transcriptional analysis of tumors supports similar conclusions. Clustering of gene expression signatures from aHGGs identifies three to four major gene expression subgroups, with the most robust distinction between proneural and mesenchymal subgroups[48-51]. Unlike the WNT and sonic hedgehog subgroups of medulloblastoma[52], the mutations associated with particular HGG subgroups are much less consistent. These same subgroups were identified in pHGGs by unsupervised comparisons, showing a clear relationship in the gene expression signatures of gliomas among different age groups and locations. However, supervised comparisons revealed expression signatures that distinguished adult from pediatric tumors, and within childhood glioma, DIPGs from pNBS-HGGs[16,17,19,24,53,54].

Because biopsy on DIPG patients is not routinely performed in the US, most research material is acquired at autopsy from irradiated patients. The vast majority of these samples are designated WHO grade IV[55]. In contrast, clinicians in France regularly performed pre-treatment biopsies, and although most samples were HGGs, some were classified as low-grade[56], raising the possibility that there may be a low-grade to high-grade malignant transformation in the genesis of DIPG. Importantly, there is a high degree of similarity in the copy number imbalances and expression signatures from DIPGs collected as biopsy samples prior to treatment and those collected at autopsy, which were treated by radiation in most cases, with or without chemotherapy [16,24]. Furthermore, expression signatures of pediatric brainstem low-grade gliomas (LGGs) are much more closely related to non-brainstem LGG and not DIPGs, emphasizing different etiology underlying genesis of low-grade and high-grade gliomas arising in the brainstem[16].

Histones make their mark

The work discussed above established the concept that oncogenic events driving pediatric HGG were different from those arising in adults. This appreciation, however, was not fully cemented until early 2012, with the discovery of recurrent histone mutations in pHGG (Figure 2). As the first reports of histone mutations in human cancer, these mutations implicated novel mechanisms in pHGG tumor biology that are not found to play a significant role in the adult disease.

Whole-genome sequencing of 7 DIPGs and matched germline DNA, as part of the St. Jude Children's Research Hospital-Washington University Pediatric Cancer Genome Project,

identified somatic mutations in *H3F3A* leading to a p.K27M substitution in histone H3.3 in 4 of 7 cases. A fifth case contained an analogous mutation in *HIST1H3B* yielding a p.K27M alteration in histone H3.1. Subsequent targeted sequencing of all 16 genes encoding histone H3 in a larger cohort of pHGG found p.K27M somatic mutations in 78% of DIPGs; 60% were mutations in *H3F3A* and 18% in *HIST1H3B*[57]. Similar frequency of histone H3 mutation was found in an independent cohort; however, the proportion of *H3F3A* and *HIST1H3B* mutations varied between the groups, likely due to patient age, with HIST1H3B mutations arising in younger children[57,58]. In pNBS-HGG, *H3F3A* and *HIST1H3B* p.K27M substitutions were found in 19% and 3% of cases, respectively. Additionally, 14% of pNBS-HGGs harbored somatic mutations in *H3F3A* leading to p.G34R substitution, whereas no such alteration was identified in any DIPG. Most of the DIPG samples evaluated were collected at autopsy. However, of 8 DIPG samples collected from patients who had not received adjuvant therapy, 7 contained p.K27M substitutions. Hence, histone H3 alterations were not necessarily secondary to therapy[57].

Collaborating groups in Canada and Germany performed whole-exome sequencing of 48 pNBS-HGGs and identified p.K27M, p.G34R, and p.G34V alterations in 19%, 10%, and 2% of cases, respectively, with all changes affecting *H3F3A* encoding the histone H3.3 variant. Additional targeted sequencing of the *H3F3A* locus in over 700 gliomas of various grades and patient ages revealed these mutations to be exclusive to high-grade tumors and significantly enriched in pediatric cases. Of the 11 adult cases harboring *H3F3A* mutation, all were missense substitutions of G34, and the majority was identified in young adults aged 20-30 years old. The specific histone mutation was associated with anatomical location. p.K27M mutations occured in tumors involving midline structures (such as the brainstem, cerebellum, and thalamus); while p.G34R/V mutations occurred in non-midline supratentorial lesions[35,59] (Figure 3).

Nucleosomes are the basic unit of chromatin, in which DNA is wrapped around a nucleosome core comprised of a histone octamer with two copies each of histones H2A, H2B, H3, and H4 (Figure 2). *H3F3A* and *HIST1H3B* code for the two histone H3 variant isoforms H3.3 and H3.1, respectively. There are three isoforms of histone H3. Histone H3.1 and H3.2 are encoded by 10 and 3 separate genes, respectively, and are synthesized during S phase of the cell cycle to package newly replicated DNA. Histone H3.3 is synthesized throughout the cell cycle and selectively incorporated into promoter regions of active genes, and through interactions with ATRX and DAXX, into pericentromeric heterochromatin and subtelomeric regions[60]. Notably, recurrent *ATRX* loss of function mutations were found in approximately one quarter of pHGG, which were also associated with alternative lengthening of telomeres (ALT). All tumors with *H3F3A* p.G34R/V mutation carried concomitant *ATRX* mutations, suggesting synergy between the two mutations[35].

All identified histone mutations occur in the N-terminal tails of histones, unstructured regions that undergo extensive post-translational modification (PTM). These modifications in turn facilitate recruitment of effector proteins that regulate transcriptionally active or silent chromatin states[61].

Understanding mutant histone gain-of-function

All histone H3 mutations in pHGG were heterozygous, and in any individual tumor, only one of 16 genes encoding histone H3 was mutated. This pattern clearly indicates a dominant gain-of-function effect.

Lysine 27 on histone H3 (H3K27) is a residue that can be acetylated or mono-, di-, or trimethylated (H3K27me3). Although mutant histone H3.1/3.3 make up a minority of the total cellular histone H3 pool[62], p.K27M mutations led to loss of total H3K27me2/3 of the entire cellular H3 pool, most of which is wild-type[62-65]. This dominant negative effect appears to be caused by inhibition of the H3K27 methylase EZH2 due to interaction with p.K27M mutant histone H3[62,63]. Globally, both p.K27M and p.G34R/V tumors exhibit DNA hypomethylation[59,63]. In an unsupervised comparison of genome-wide DNA methylation signatures, tumors with p.K27M and tumors with p.G34R/V form independent clusters based on histone mutation status. Many genes with differentially methylated promoters showed differential gene expression that was associated with the anatomic origin of the tumors, suggesting that both DNA methylation and gene expression may be a result of the origin of the tumor that may be further influenced by histone mutations[59,63,64,66].

The dominant mechanism of action is less apparent for mutations affecting G34. While there is no clear dominant effect on modification of the nearby H3K36, there appears to be an altered genome-wide distribution of H3K36me3 binding. Interestingly, one of the most upregulated genes in such tumors is *MYCN*, a well-known oncogene[66].

Additional genetic associations with pHGG subgroups

Genome-wide sequencing approaches revealed that 20-32% of DIPGs harbored somatic missense mutations in *ACVR1*, also known as *ALK2*[47,67-69] which encodes a receptor serine/threonine kinase mediating bone morphogenetic protein (BMP)-induced signal transduction[70]. These mutations frequently co-occur with histone H3.1 p.K27M substitutions. Both alterations tend to occur in younger DIPG patients, and were not found in pHGG arising outside the brainstem[47,67-69]. Thus, these mutations further clarify molecular subgroups within DIPG. Surprisingly, some of the somatic mutations found in DIPG were the same as previously reported *ACVR1* germline mutations in fibrodysplasia ossificans progressiva (FOP), a disease characterized by heterotopic bone formation exacerbated by inflammation that is not associated with cancer predisposition[71].

RNA-seq analysis allowed the identification of fusion genes that were generated by genomic rearrangements. Expression of fusion genes was common in pHGG, including DIPG, although most of the fusion genes were not recurrent. Strikingly, chimeric genes fusing N-terminal sequences from a number of different genes to the kinase domain of the neurotrophic tyrosine receptor kinase (NTRK) family members were recurrent gene fusions, found in 40% of infant NBS-HGGs, and at much lower frequency in pHGG overall[47]. The prognosis for pNBS-HGG in children younger than three is significantly better than for older children[72]. The NTRK fusion genes may provide a useful new therapeutic target for this patient population. NTRK fusions were also identified in adult glioblastoma as well as pLGG, but do not appear to be as enriched as in infant NBS-HGG[73-75].

Closing remarks

Advances in microarrays and next-generation sequencing technologies have provided unprecedented insight into pHGG biology. But this insight is only a starting point. Researchers and clinicians must now endeavor to not only understand how this unique mutation spectrum contributes to tumorigenesis, but also more importantly, seek to exploit these genetic defects therapeutically. Recent discoveries should inform the generation of improved pre-clinical models that more faithfully resemble the pediatric disease, to be utilized for mechanistic studies as well as pre-clinical testing of new therapies.

We are grounded in knowing that our understanding of the protein coding genome far surpasses that of the remaining 98% of DNA sequence, which potentially hosts a vast network of regulatory elements. Furthermore, we are just beginning to piece together the role of epigenetics in normal and neoplastic contexts. The high frequency of histone mutations in pHGG strongly suggests that epigenetic dysregulation plays a major role in tumors arising within the pediatric setting. We must also recognize that our analyses yield snapshots of an ever-changing process. The genetics and epigenetics of tumors are dynamic, and the portrait of each cancer landscape evolves as a function of any selective pressure introduced, therapy included[76].

The above studies, both large-and-small scale, genome-wide and targeted, have given today's researchers and clinicians a better understanding of pHGG than ever before. The biologies of DIPG, pNBS-HGG, and infant NBS-HGG are distinctly different, and these differences in turn require specific clinical consideration in terms of appropriate intervention. With a two-year survival rate of less than 20%, the prognosis for pHGG remains unacceptably poor[77]. However, there is a palpable excitement within the community that meaningful change is within reach.

Acknowledgments

We thank Dr. Zoltan Patay for the MRI images used in Figure 3. SJB is supported by NIH grant P01 CA096832 and ALSAC of St Jude Children's Research Hospital.

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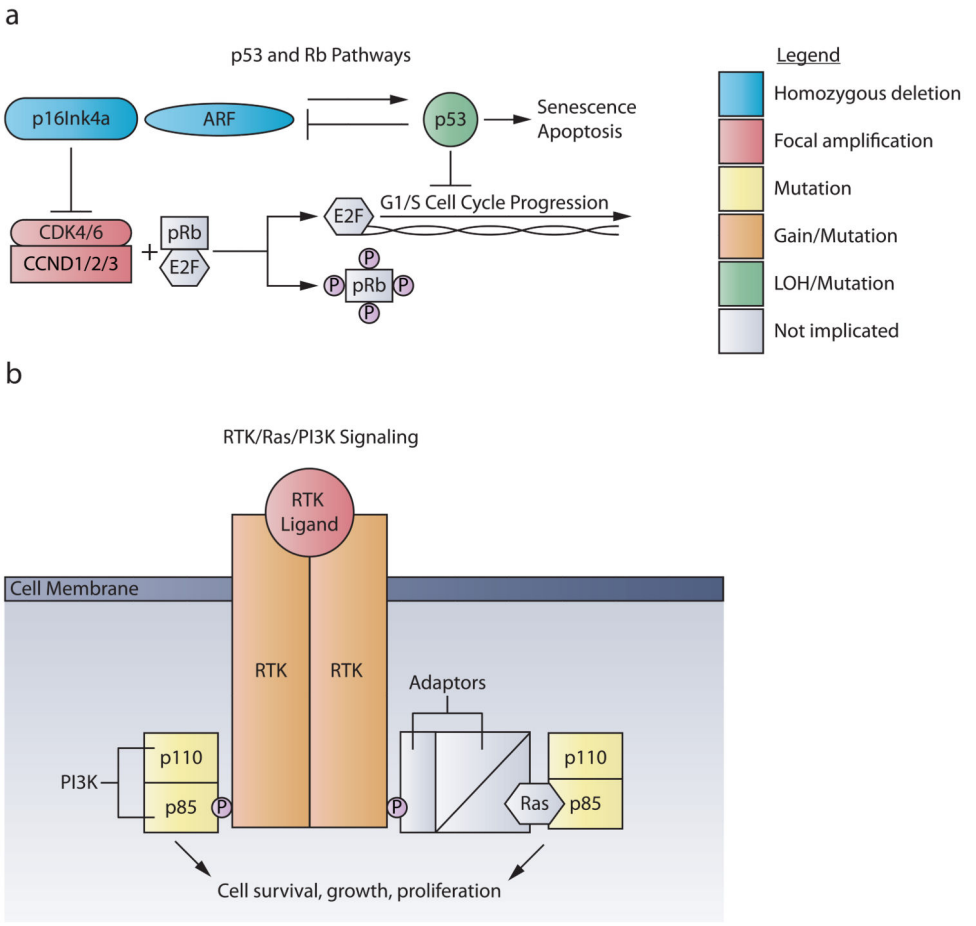
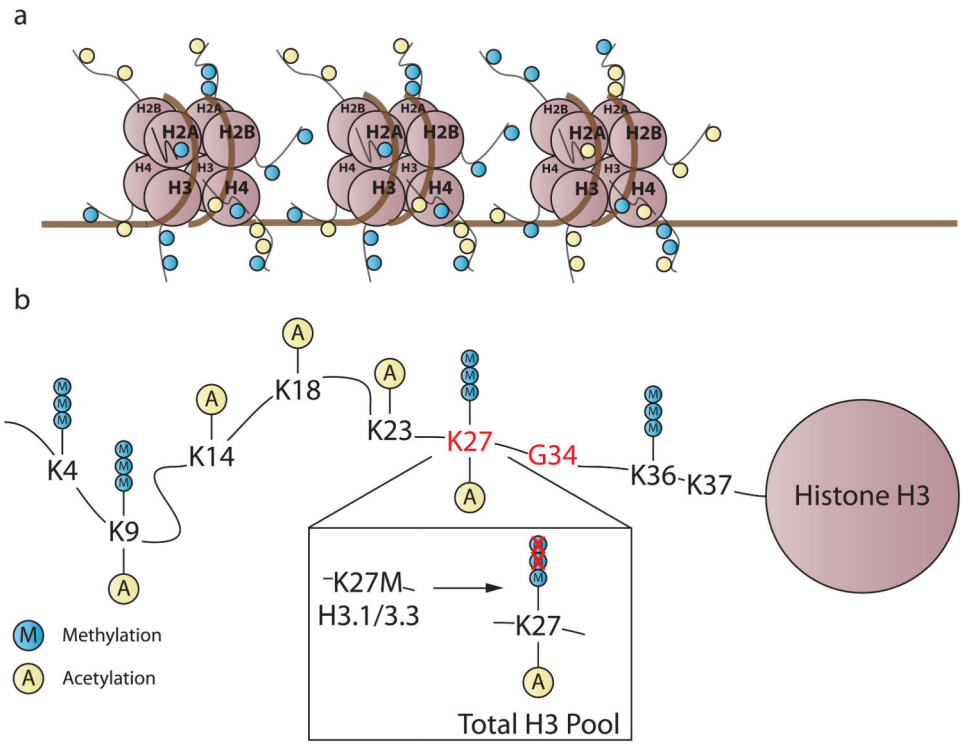


Figure 1. The p53/RB and RTK/Ras/PI3K pathways are dysregulated in pHGG

a. **The p53 and RB pathways regulate G1 cell cycle checkpoints**. Mitogenic signaling activates the cyclin D-dependent kinases CKD4 or CKD6, coupled with cyclin D family members (CCND1/2/3). This complex phosphorylates pRB, releasing E2F and promoting transcription of genes responsible for G1/S cell cycle progression. Gene amplifications of *CDK4*, *CDK6*, or any of the three *Cyclin D* family members are found in pHGG, with greater frequency in DIPG. The tumor suppressor locus *CDKN2A* encodes two different proteins through translation of two different reading frames, p16INK4A and p19ARF. P16INK4A inhibits the activity of the cyclin D-dependent kinases CKD4 and CKD6. Oncogenic signals, DNA damage, or induction of P19ARF induce p53, leading to cell cycle arrest, apoptosis or senescence. Homozygous deletions of *CDKN2A* occur almost exclusively in NBS-HGGs; whereas *TP53* mutations are common in both pNBS-HGG and DIPG.

b. Mutations in the RTK/RAS/PI3K pathway transduce unregulated signals for cell proliferation, growth and survival. RTK signaling begins when growth factor ligand binding leads to receptor dimerization. In pediatric HGG, PDGFRα is the RTK most frequently targeted by amplification and/or mutation. Upon dimerization, RTKs trans-phosphorylate one another at tyrosine residues in their cytosolic tails. p85, the regulatory subunit of PI3K, can then either directly bind to these phosphor-tyrosine residues or connect to RTKs through adaptor molecules and Ras. PI3K is comprised of catalytic (p110) and regulatory (p85)

subunits, both of which are targeted by mutation, usually in a mutually exclusive pattern, in pHGG.



c

Gene	Histone	Mutation	Effect	DNA Methylation	Gene Expression
H3F3A	H3.3	p.K27M	Decreased total H3K27me2/3	Changed	Changed
HIST1H3B	H3.1	p.K27M	Decreased total H3K27me2/3	Changed	Changed
H3F3A	H3.3	p.G34R/V	No change in total H3K27me2/3	Changed	Changed

Figure 2. Hotspot histone mutations occur in nearly 80% of DIPGs and ~35% of NBS-HGGs

a. The basic unit of chromatin is the nucleosome; DNA wrapped around a histone octamer consisting of two copies of H2A, H2B, H3, and H4. The N-terminal tails of histones undergo post-translational modifications (PTMs), represented by yellow and blue circles, which in turn alter chromatin accessibility and recruitment of effector proteins, together influencing transcriptional permissiveness. This is accomplished because PTMs can 1) themselves alter the strength of DNA-histone interactions 2) facilitate recruitment of chromatin remodeling complexes or histone PTM-binding effector proteins.

b. p.K27M substitutions occur in histone H3.1 and H3.3; p.G34R/V substitutions occur in H3.3. Histone H3.1/3.3 p.K27M exerts a dominant effect, preventing the accumulation of H3K27me2/3 on the wild-type histone H3 expressed in the same cell. p.G34R/V mutations do not exert the same effect on H3K27me3, but p.K27M and p.G34R/V histone mutations are associated with distinct genome-wide DNA methylation and gene expression tumor signatures.

c. Table summarizing the functional consequences of histone mutations.

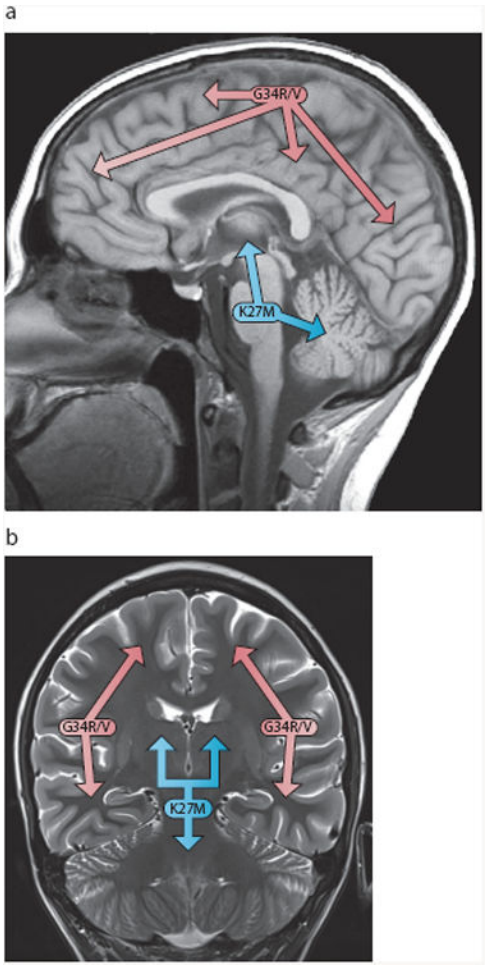


Figure 3. Histone mutations are strongly associated with anatomical location

Midsagittal (a) and coronal (b) MR images demonstrating that p.K27M mutations occur predominantly in tumors involving midline structures (such as the brainstem, cerebellum, and thalamus); while p.G34R/V mutations mainly occur in tumors arising in the cerebral hemispheres.