H3F3A K27M Mutation in Pediatric CNS Tumors

A Marker for Diffuse High-Grade Astrocytomas

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Abstract

Brain tumors are one of the most common childhood malignancies. Diffuse high-grade gliomas represent approximately 10% of pediatric brain tumors. Exon sequencing has identified a mutation in K27M of the histone H3.3 *gene (*H3F3A *K27M and G34R/V) in about 20% of pediatric glioblastomas, but it remains to be seen whether these mutations can be considered specific for pediatric diffuse high-grade astrocytomas or also occur in other pediatric brain tumors. We performed a pyrosequencing-based analysis for the identification of* H3F3A *codon 27 and codon 34 mutations in 338 pediatric brain tumors. The K27M mutation occurred in 35 of 129 glioblastomas (27.1%) and in 5 of 28 (17.9%) anaplastic astrocytomas. None of the other tumor entities showed* H3F3A *K27M mutation. Because* H3F3A *K27M mutations occur exclusively in pediatric diffuse high-grade astrocytomas, analysis of codon 27 mutational status could be useful in the differential diagnosis of these neoplasms.*

Tumors of the central nervous system (CNS) are one of the most common childhood malignancies and a leading cause of cancer-related morbidity and mortality.[1] Although diffuse high-grade gliomas (HGGs) are the most common CNS tumors in adults, they represent only approximately 10% of all pediatric CNS tumors. The overall prognosis of glioblastomas (GBMs) in children seems slightly better than in adults, with a higher percentage of long-term survivors; nevertheless, prognosis remains poor in general despite aggressive treatment with surgery, chemotherapy, and radiotherapy.[2] In contrast to adult diffuse high-grade astrocytomas, in which key genetic alterations (such as *IDH1/2* mutation and *EGFR* amplification) have been identified, molecular alterations in pediatric cases have not been fully elucidated. Although alterations of the p53/MDM2/p14 pathway are detectable at a similar frequency compared with adults, pediatric GBMs show frequent gains of chromosome 1q and only rarely gains of chromosome 7 and 10q losses.[3] The most frequent focal amplifications also differ, with *PDGFRA* and *EGFR* predominating in childhood and adult populations, respectively.[3]

Histones are basic nuclear proteins responsible for the nucleosome structure within eukaryotic cells. Among the 5 classes of histones, some are expressed only during the S phase, whereas others are replication independent and referred to as replacement histones, which are expressed in quiescent or terminally differentiated cells. H3.3 is a replacement histone subclass that is encoded by 2 distinct genes, *H3.3A* (*H3F3A*) and *H3.3B*.[4-6] Presently, pediatric cases of GBM are being investigated by genomic sequencing, leading to interesting new findings. Exon sequencing has identified a K27M driver mutation in *H3F3A* in 20% of pediatric cases of GBM but not in adult glioblastomas.[7-9] Diffuse intrinsic pontine

gliomas (World Health Organization [WHO] grades II-IV) have also been shown to carry *H3F3A* K27M mutations.[10] Finally, mutations at codon 34 of *H3F3A* (either G34R or G34V) have been reported in pediatric GBMs.[7] Although the glycine at codon 34 (G34) is not subjected to posttranslational modifications, it is in close proximity to a lysine at position 36 (K36), whose methylation status is involved in transcriptional elongation.[6,7]

The *H3F3A* K27M mutation is more frequent than G34V/R mutations in pediatric diffuse high-grade astrocytomas[7-9] and could therefore be used as a potential diagnostic marker for the identification of these tumors analogous to the use of *IDH1/2* mutations in the diagnosis of the adult diffuse gliomas. However, it is still unclear whether these mutations, particularly the K27M mutation, can be considered as specific for pediatric diffuse high-grade astrocytomas or also occur in other pediatric brain tumors. To answer this question, we performed a pyrosequencing-based analysis for *H3F3A* K27M mutation in 338 pediatric brain tumors.

Materials and Methods

Patients

Fresh-frozen or formalin-fixed paraffin-embedded (FFPE) tissue specimens from 338 pediatric brain tumors were analyzed. The series includes 129 GBMs (WHO grade IV), 28 anaplastic astrocytomas (AAs; WHO grade III), 6 anaplastic oligoastrocytomas (WHO grade III), 6 anaplastic (pilocytic) astrocytomas (APAs; WHO grade III), 15 pilocytic astrocytomas (PAs; WHO grade I), 5 pilomyxoid astrocytomas (WHO grade II), 12 desmoplastic infantile gangliogliomas/astrocytomas (DIGs/DIAs; WHO grade I), 2 oligodendrogliomas (WHO grade II), 4 oligoastrocytomas (WHO grade II), 6 diffuse astrocytomas (WHO grade II), 9 anaplastic ependymomas (WHO grade III), 8 ependymomas (WHO grade II), 19 medulloblastomas (MBs; WHO grade IV), 14 atypical teratoid/rhabdoid tumors (ATRTs; WHO grade IV), 26 primitive neuroectodermal tumors (PNETs) of the CNS (WHO grade IV), 12 ependymoblastomas (EPBLs; WHO grade IV), 10 gangliogliomas (GGs; WHO grade I), and 10 choroid plexus carcinomas (CPCs; WHO grade III). All tumors were diagnosed according to the WHO classification of tumors of the CNS[11] using standard histologic and immunohistochemical methods. No stereotactic biopsy specimen material was included in this study.

Pyrosequencing Analysis

Fragments chosen for DNA extraction were checked histologically (either frozen or permanent section) to ensure that they consisted of at least 80% tumor cells. Extraction of DNA from frozen tissue was carried out using proteinase K digestion followed by phenol/chloroform extraction described elsewhere.[12] DNA from FFPE tumor tissues was extracted using the QIAamp DNA Mini Tissue Kit (Qiagen GmbH, Düsseldorf, Germany) according to the manufacturer's instructions. We screened the hotspot codons 27 and 34 of the *H3F3A* gene for mutations using a pyrosequencing assay. For this purpose, single-stranded DNA templates were immobilized on streptavidin-coated Sepharose high-performance beads (GE Healthcare, Uppsala, Sweden) using the PSQ Vacuum Prep Tool and Vacuum Prep Worktable (Biotage, Uppsala, Sweden) according to the manufacturer's instructions and then incubated at 80°C for 2 minutes and allowed to anneal to a 0.4-mmol/L sequencing primer at room temperature. Pyrosequencing was performed using PyroGold Reagents (Biotage) on the Pyromark Q24 instrument (Biotage) according to the manufacturer's instructions. Controls in which the sequencing primer or template were omitted were used to detect background signal. Pyrogram outputs were analyzed by the PyroMark Q24 software (Biotage) using the allele quantification software to determine the percentage of mutant vs wild-type alleles according to percentage relative peak height. In this study, a 240–base pair fragment of exon 2 of *H3.3* containing the coding region was amplified in a polymerase chain reaction (PCR) using 25 ng genomic DNA as the template and the following primer set for *H3.3:* forward, 5'-TGTTTGTAGTTGCATATGGG-3', and reverse, 5'-biotin-TACAAGAGAGACTTTTGTCC-3'. PCR amplicons of *H3.3* containing both mutation hotspots (codons 27 and 34) were analyzed on 2% agarose gels and subjected to pyrosequencing reactions using the pyrosequencing primer *H3.3* (-Py-5'-CAAAAGCCGCTCGCA-3') with the following nucleotide dispensation order: GAT GAGTGCGCTCTACTCGAGCGTGTGA. As a negative control, DNA derived from normal brain tissue was used.

Results

Our series of 163 diffuse pediatric HGGs included 77 male and 86 female patients. The mean age at surgery was 9.48 years (age range, newborn to 18 years). Among these 163 HGGs, we found 40 cases harboring an *H3F3A* K27M mutation (24.5%). GBM showed a K27M mutation in 35 cases (27.1%; 15 males, 20 females) (specificity, 87.5%; sensitivity, 27.1%), whereas 5 of 28 (17.9%; 3 males, 2 females) AAs presented the same mutation. With regard to the classification of a tumor to the group of diffuse malignant astrocytic tumors (ie, GBM and AA), the sensitivity was 25.5%, but the specificity was 100%. Among the 6 anaplastic oligoastrocytomas, no *H3F3A* mutation could be detected. The G34R mutation (not shown) was observed in

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only 7 of 129 of the pediatric GBMs (5.4%), as well as in 1 case of anaplastic oligoastrocytoma, but not in AA. Our study included 3 cell lines from patients with pediatric GBM (SF 188, CHLA-200, and UKB-pGBM1). They contained exclusively wild-type alleles. All other tumor entities investigated in this study showed no evidence of an *H3F3A* K27M mutation. The results are summarized in **Table 1**. Exemplary pyrograms are shown **Figure 1**.

Table 1
Central Nervous System Tumors and Cell Lines Investigated for *H3F3A* K27M Mutation Status

Tumor Entity	No. of Cases	K27M Mutations	%
Glial tumors			
Diffuse			
Glioblastoma multiforme, WHO grade IV	129	35	27.1
Anaplastic astrocytomas, WHO grade III	28	5	17.9
Anaplastic oligoastrocytomas, WHO grade III	6	0	0
Diffuse astrocytomas, WHO grade II	6	0	0
Oligodendrogliomas, WHO grade II	2	0	0
Oligoastrocytomas, WHO grade II	4	0	0
Nondiffuse			
Pilocytic astrocytomas, WHO grade I	15	0	0
Pilomyxoid astrocytomas, WHO grade II	5	0	0
Anaplastic (pilocytic) astrocytomas, WHO grade III	6	0	0
Gangliogliomas, WHO grade I	10	0	0
Desmoplastic infantile gangliogliomas/astrocytomas, WHO grade I	12	0	0
Ependymal tumors	17	0	0
Ependymomas, WHO grade II	8	0	0
Anaplastic ependymomas, WHO grade III	9	0	0
Embryonal tumors			
Medulloblastomas, WHO grade IV	19	0	0
Atypical teratoid/rhabdoid tumors, WHO grade IV	14	0	0
Ependymoblastomas, WHO grade IV	12	0	0
CNS-PNET, WHO grade IV	26	0	0
Choroid plexus tumors			
Choroid plexus carcinomas, WHO grade III	10	0	0
GBM cell lines derived from pediatric patients			
SF188, CHLA-200, UKB-pGBM1	3	0	0

CNS-PNET, primitive neuroectodermal tumor of the central nervous system; GBM, glioblastoma; WHO, World Health Organization.

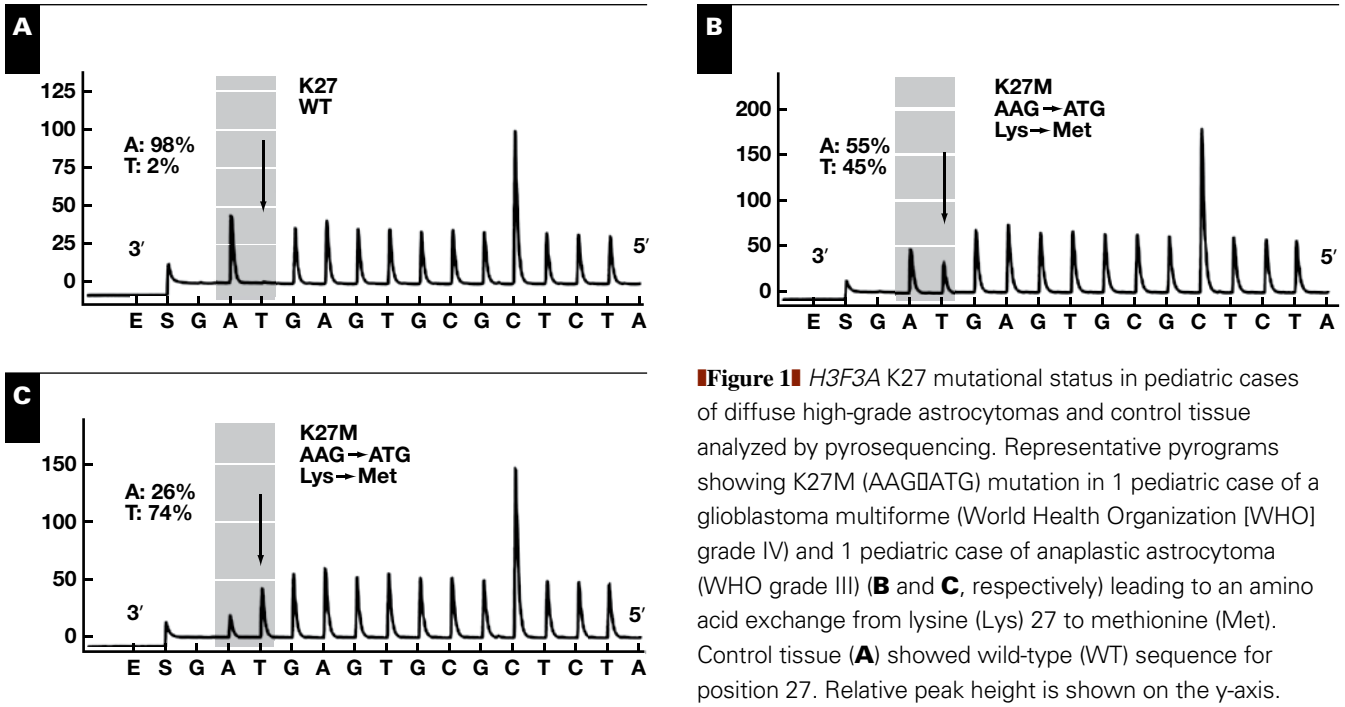

Figure 1 *H3F3A* K27 mutational status in pediatric cases of diffuse high-grade astrocytomas and control tissue analyzed by pyrosequencing. Representative pyrograms showing K27M (AAG→ATG) mutation in 1 pediatric case of a glioblastoma multiforme (World Health Organization [WHO] grade IV) and 1 pediatric case of anaplastic astrocytoma (WHO grade III) (**B** and **C**, respectively) leading to an amino acid exchange from lysine (Lys) 27 to methionine (Met). Control tissue (**A**) showed wild-type (WT) sequence for position 27. Relative peak height is shown on the y-axis.

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Discussion

Histones are eukaryotic nuclear proteins that play an important role in the regulation of DNA replication, transcription, and storage by changing the nucleosome structure depending on their posttranslational modifications. Among the 5 classes of histones, *H3.3* is a replacement histone subclass that is encoded by 2 different genes, *H3.3A* (*H3F3A*) and *H3.3B*.[5] *H3.3* is incorporated into chromatin independently of the cell replication cycle.[5] Both *H3F3A* mutations found in GBMs are located in a region undergoing posttranslational modifications.[7,9] In particular, the amino acid exchange from lysine to methionine at position 27 (K27M) prevents posttranslational methylation, associated with polycomb-mediated gene repression[6] and K27 acetylation, present at active promoters and enhancers.[6] Because of its significant frequency in pediatric diffuse high-grade astrocytomas, the *H3F3A* K27M mutation could be potentially used as a molecular diagnostic marker for these tumors. Molecular markers are emerging as an important tool in routine diagnostic neuropathology, notably in pediatric tumors such as ATRT (*INI-1* mutation) and in PA (*KIAA1549-BRAF* fusions and *BRAF V600E* mutation). For high-grade gliomas, *IDH-1* mutations represent the best-known diagnostic markers,[13,14] but because of the low *IDH-1* mutation rate in pediatric glial tumors,[15] they unfortunately have only a limited utility in this age group. In contrast to *IDH-1* mutations, mutations in *H3F3A* have been frequently observed in pediatric astrocytomas (pediatric GBMs and pediatric diffuse intrinsic pontine gliomas).[7-10] In GBM, we found an overall mutation rate of 27.1%, which is in line with the frequency described in other studies.[7,9] With regard to the classification of a tumor to the group of diffuse malignant astrocytic tumors (ie, GBM and AA), the sensitivity was 25.5%, but the specificity was 100%. Because this group of tumors is treated according to the same protocols in pediatric oncology, the secure assignment to this group is important. Although *H3F3A* K27M mutations occur only in one fourth of these tumors, we did not find this mutation in any other pediatric brain tumor, including low-grade gliomas and nondiffuse high-grade tumors. Because the *H3F3A* K27M mutation is significantly more frequent than the G34R/V mutation (ratio of 6:1 in our series) in pediatric diffuse high-grade astrocytomas, the former is suitable as a molecular marker for this tumor group. In addition, G34R/V mutations seem to be less specific in pediatric tumors because they also occur in CNS-PNET.[16]

Although previous studies have shown the absence of *H3F3A* mutations in adult HGGs and in extracerebral pediatric solid tumors (such as rhabdomyosarcoma, osteosarcoma, and neuroblastoma),[9] only a limited number of the more common pediatric CNS tumors, such as PAs, MBs, and ependymomas, have been analyzed for the presence of *H3F3A* mutations.[7,9] We did not find any mutations in low-grade gliomas (DIG, DIA, PA, GG), embryonal tumors (MB, ATRT, CNS-PNET, EPBL), (anaplastic) ependymomas, or CPCs. Our analysis thus confirms that *H3F3A* K27M mutations are restricted to pediatric diffuse high-grade astrocytomas.

From a diagnostic point of view, the absence of *H3F3A* K27M mutations in PAs, APAs, and CNS-PNETs is noteworthy.

Histologically, APAs are defined by the presence of hypercellularity, moderate to severe cytologic atypia, and brisk mitotic activity with or without necrosis. Nevertheless, they have a better prognosis than diffuse astrocytomas and GBM. At the molecular level, APAs do not show the typical molecular features of GBM (ie, *PDGFRA* or *EGFR*) amplifications but frequently present *PI3K/AKT* pathway alterations.[17] Based on practical experience, the distinction between GBM and APA, as well as in some cases between high-grade astrocytoma and PA, may be difficult in small tumor biopsy specimens. Because these entities also share similar neuroradiological features, reliable molecular markers useful in this differential diagnosis are particularly welcome. The combined analysis of the presence of *KIAA1549-BRAF* fusion and *H3F3A* K27M mutation in pediatric cases of ambiguous histology should be helpful in distinguishing PA and APA from GBM. However, *KIAA1549-BRAF* fusions also have been found in single adult GBM,[18] but they have been not shown in pediatric cases of GBM.

By routine histopathologic diagnostics, CNS-PNETs are also difficult to distinguish from diffuse high-grade gliomas and often represent an "exclusion diagnosis" based on the identification of a noncerebellar embryonal neoplasm with MB-like histology, frequently associated with expression of neuronal markers.[19] However, a definitive distinction between a CNS-PNET and a GBM remains sometimes impossible: in such cases, the final diagnosis depends on the expertise of the pathologist or relies on additional institution-defined criteria, which may vary from laboratory to laboratory.[20] Unfortunately, definitive molecular markers for CNS-PNET, in contrast to other embryonal tumors (such as *INI-1* alterations in ATRTs or chromosome 19q13.14 amplification in EPBLs), are not available. In this view, the presence of an *H3F3A* K27M mutation in a histologically undifferentiated tumor reliably excludes the diagnosis of a CNS-PNET.

In conclusion, *H3F3A* K27M mutation occurs exclusively in diffuse high-grade pediatric astrocytomas, and therefore analysis of *H3F3A* codon 27 could provide significant help in the differential diagnosis of these neoplasms.

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