Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma


Glioblastoma multiforme (GBM) is a lethal brain tumour in adults and children. However, DNA copy number and gene expression signatures indicate differences between adult and paediatric cases1–4. To explore the genetic events underlying this distinction, we sequenced the exomes of 48 paediatric GBM samples. Somatic mutations in the H3.3-ATRX-DAXX chromatin remodelling pathway were identified in 44% of tumours (21/48). Recurrent mutations in H3F3A, which encodes the replication-independent histone 3 variant H3.3, were observed in 31% of tumours, and led to amino acid substitutions at two critical positions within the histone tail (K27M, G34R/G34V) involved in key regulatory post-translational modifications. Mutations in ATRX (α-thalassaemia/mental retardation syndrome X-linked)2 and DAXX (death-domain associated protein), encoding two subunits of a chromatin remodelling complex required for H3.3 incorporation at pericentric heterochromatin and telomeres6,7, were identified in 31% of samples overall, and in 100% of tumours harbouring a G34R or G34V H3.3 mutation. Somatic TP53 mutations were identified in 54% of all cases, and in 86% of samples with H3F3A and/or ATRX mutations. Screening of a large cohort of gliomas of various grades and histologies (n = 784) showed H3F3A mutations to be specific to GBM and highly prevalent in children and young adults. Furthermore, the presence of H3F3A-ATRX-DAXX/TP53 mutations was strongly associated with alternative lengthening of telomeres and specific gene expression profiles. This is, to our knowledge, the first report to highlight recurrent mutations in a regulatory histone in humans, and our data suggest that defects of the chromatin architecture underlie paediatric and young adult GBM pathogenesis.

Brain tumours are currently the leading cause of cancer-related mortality and morbidity in children. Glioblastoma multiforme (GBM) is a highly aggressive brain tumour and the first cancer to be comprehensively profiled by The Cancer Genome Atlas (TCGA) consortium. Whereas GBM is less common in the paediatric setting than in adults, affected children show dismal outcomes similar to adult patients, and the vast majority will die within a few years of diagnosis despite aggressive therapeutic approaches. Tumours arise de novo (primary GBM) and are morphologically indistinguishable from their adult counterparts. A number of comprehensive studies have identified transcriptome-based subgroups and indicator mutations in adult GBM, and have thus enabled its molecular sub-classification8–11. In contrast, although we and others have demonstrated the presence of distinct molecular subsets of childhood GBM and described different genetic alterations compared to adult cases, the paediatric disease remains understood12–14. There is currently insufficient information to improve disease management, and because conventional treatments universally fail, there is a crucial need to identify relevant targets for the design of new therapeutic agents.

To decipher the molecular pathogenesis of paediatric GBM, we undertook a comprehensive mutation analysis in protein-coding genes by performing whole-exome sequencing (WES) on 48 well-characterized paediatric GBMs, including 6 patients for whom we had matched non-tumour (germline) DNA. Samples from the tumour core containing more than 90% neoplastic tissue were collected from patients aged between 3 and 20 years (Supplementary Table 1). Coding regions of the genome were enriched by capture with the Illumina TruSeq kit and sequenced on an Illumina HiSeq 2000.
2000 platform (Supplementary Methods). The median coverage per tumour ranged from 3 to 31, with a mean of 15 (Supplementary Table 3). This is much lower than the rate observed using Sanger sequencing in other solid tumours including adult GBM\(^1\), but somewhat higher than in another paediatric brain tumour, medulloblastoma\(^2\) (Supplementary Table 4). Relevant mutations (as defined below) were validated by Sanger sequencing.

Initially, we focused on the distribution of somatic, non-silent protein-coding mutations in the six tumours with matched germline DNA. Four samples had recurrent heterozygous mutations in H3F3A, which encodes the replication-independent histone variant H3.3. Both mutations were single-nucleotide variants (SNVs), in two samples changing lysine 27 to methionine (K27M), and in two samples changing glycine 34 to arginine (G34R) (Fig. 1a and Supplementary Table 3). These mutations are particularly interesting because histone genes are highly conserved throughout eukaryotes (Fig. 1b), and to our knowledge no human disorders have specifically been associated with mutations in histones, including H3.3. Both mutations are at or very near positions in the amino-terminal tail of the protein that undergo important post-translational modifications associated with either transcriptional repression (K27) or activation (K36) (Fig. 1b). All four samples additionally harboured mutations in ATRX, which encodes a member of a transcription/chromatin remodelling complex required for the incorporation of H3.3 at pericentric heterochromatin and at telomeres, as well as at several transcription factor binding sites\(^7,14–17\).

We extended our WES analysis to 42 additional tumour samples and focused on ATRX and H3F3A, as well as DAXX (because the gene product heterodimerizes with ATRX and participates in H3.3 recruitment to DNA\(^8\)). A total of 15 samples had heterozygous H3.3 mutations (9 K27M, 5 G34R, 1 G34V) and 14 samples had a mutation in ATRX, including frameshift insertions/deletions (6 samples), gains of a stop codon (4 samples), and missense SNVs (4 samples). Nearly all of the ATRX mutations occurred either within the carboxy-terminal helicase domain or led to truncation of the protein upstream of this context of the common post-translational modifications of the H3.3 N-terminal tail, which regulates the histone code. H3.3 has 136 amino acids, and is highly conserved across species from mammals to plants, including the residues subject to mutation in paediatric GBM (see multiple alignment of amino acids 11 to 60). c, Schematic of the mutations observed in ATRX in the 48 WES samples. d, Schematic of the overlap between mutations affecting ATRX-DAXX, H3F3A and TP53. Eight samples had all three mutations.

**Figure 1** | Most frequent mutations in paediatric GBM. a, Most frequent somatic mutations in 48 paediatric glioblastoma tumours. Mutations identified in genes listed in this table were confirmed by Sanger sequencing, and were not present in dbSNP nor in the 1000 Genomes data set (October 2011), except for the TP53 SNP at R273, which is associated with cancer. Detailed description of the mutations in affected samples is provided in Supplementary Table 5. b, Three recurrent non-synchronous single nucleotide variants (SNVs) were observed in H3F3A. The K27M, G34R and G34V mutations are shown in the context of the common post-translational modifications of the H3.3 N-terminal tail, which regulates the histone code. H3.3 has 136 amino acids, and is highly conserved across species from mammals to plants, including the residues subject to mutation in paediatric GBM (see multiple alignment of amino acids 11 to 60). c, Schematic of the mutations observed in ATRX in the 48 WES samples. d, Schematic of the overlap between mutations affecting ATRX-DAXX, H3F3A and TP53. Eight samples had all three mutations.
domain (Fig. 1c). Mutations were accompanied by an absence of detectable ATRX protein by immunohistochemistry in samples for which paraffin material was available (Supplementary Fig. 1). Two samples had heterozygous DAXX mutations, simultaneously with an ATRX mutation in one sample (Fig. 1a and Supplementary Table 3). Overall, 21 of 48 samples (44%) had a mutation in at least one of these three genes. Notably, we also identified TP53 mutations in 26 samples (25 somatic, 1 germline in PGBM26), which overlapped significantly with samples that had ATRX, DAXX and/or H3F3A mutations (18/21 cases, 86%, Fig. 1d; P = 1.1 × 10⁻⁴, permutation test). A list of all mutations discovered by WES in selected genes associated with GBM is given in Supplementary Table 5.

H3F3A, ATRX or DAXX were not part of the 600 genes sequenced by The Cancer Genome Atlas (TCGA) glioblastoma project⁶,¹⁰,¹¹, and no H3F3A mutations were identified in 22 adult GBM samples sequenced previously¹¹. To investigate whether H3F3A mutations occur mainly in younger patients (median age 11 years) and G34R/H3.3 mutations occurred in older patients (median age 20 years, range 9–42) and in tumours of the cerebral hemispheres (Fig. 2b). Further comparison of our data set with adult GBM databases⁶,¹⁰,¹¹,¹⁸,¹⁹ indicated limited overlap in frequently mutated genes between paediatric GBM and any of the four previously described adult GBM subtypes¹⁸ (Fig. 2c, Supplementary Fig. 2 and Supplementary Table 6).

Somatic mutations in ATRX and DAXX have recently been reported in a large proportion (43%) of pancreatic neuroendocrine tumours (PanNETs), a rare form of pancreatic cancer with a 10-year overall survival of ~40%, and no reported association with TP53 or H3F3A mutations¹⁹. A follow-up study found ATRX mutations in a series of

![Figure 2](image)

**Figure 2** | Mutations in H3F3A, ATRX and DAXX distinguish paediatric from adult GBM. **a**. H3F3A mutations in a set of 784 gliomas from all ages and grades. H3F3A mutations are exclusive to high-grade tumours and the vast majority occur in glioblastoma (GBM) and in the paediatric setting. A, diffuse astrocytoma grade II; AA, anaplastic astrocytoma; AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma; O, oligodendroglioma; OA, oligoastrocytoma; PA, pilocytic astrocytoma. **b**. H3.3 mutations are specific to paediatric and young adult glioblastoma (GBM). K27M-H3.3 mutations occur mainly in younger patients (median age 11 years) and G34R/V-H3.3 mutations occur in older children and young adults (median age 20 years). No H3.3 mutations were identified in older patients with GBM. **c**. Comparison of the most frequently mutated genes in paediatric and adult GBM shows that H3F3A, ATRX and DAXX mutations are largely specific to paediatric disease. Except for similarities in the mutation rate for TP53 and PDGFRA with the previously identified proneural adult GBM subgroup, the rate and type of genes mutated were distinct between paediatric and adult GBM whatever the molecular subgroup²⁶ (Supplementary Fig. 2). Data for adult GBM regarding other genes included in the table was compiled from refs 11 and 18. **d**. ATRX and DAXX immunohistochemical staining of a paediatric GBM tissue microarray (TMA) comprising 124 samples. View of the TMA slide and an example of a negative and of a positive core at high magnification to show specific nuclear staining (or lack thereof) for DAXX and ATRX. No gender bias for ATRX loss was observed. Overall survival and progression-free survival were similar in patients with and without loss of ATRX and/or DAXX (data not shown). **e**. Differential association of K27M and G34R/V H3F3A mutations with ATRX mutations. G34R/V-H3.3 mutations were always associated with ATRX mutations (two-sided Fisher’s exact test, P = 0.0016), whereas a non-significant overlap was observed for K27M.
cancers, including GBM, where ATRX (but not DAXX) mutations were identified in 3/21 paediatric GBMs (14%) and 8/122 adult GBMs (7%) to evaluate further the prevalence of ATRX and DAXX mutations in paediatric GBM, we performed immunostaining for these proteins on a well-characterized tissue microarray (TMA) with samples from 124 paediatric GBM patients. Lack of immunoreactivity for ATRX was seen in 35% of cases (40/113 scored, 22 females and 18 males) and for DAXX in 6% (7/124 scored) (Fig. 2d and Supplementary Fig. 1). Overall, 37% of samples had lost nuclear expression of either factor, corroborating our WES findings. Strikingly, ATRX-DAXX mutations (as assessed by direct sequencing or loss of protein expression) were found in 100% of G34-H3.3 mutant cases in the larger cohort of GBMs (13/13) where sufficient material was available (P = 1.4 × 10⁻⁸ permutation test). The overlap of ATRX mutations with K27M-H3.3-mutated samples was not significant in either the exome data set (3/9 samples, P = 0.58) or the full set of GBM screened (5/13, P = 0.40) (Fig. 2e).

The histone code—post-translational modifications of specific histone residues—regulates virtually all processes that act on or depend on DNA, including replication and repair, regulation of gene expression, and maintenance of centromeres and telomeres. Accordingly, although recurrent histone mutations have not previously been reported in cancer, mutations in genes affecting histone post-translational modifications are increasingly described. H3.3 is a universal, replication-independent histone predominantly incorporated into transcription sites and telomeric regions, and associated with active and open chromatin (reviewed in ref. 23). This role is conserved in the single histone H3 present in yeast, indicating its importance throughout evolution. It functions as a neutral replacement histone, but also participates in the epigenetic transmission of active chromatin states and is associated with chromatin assembly factors in large-scale replication-independent chromatin remodelling events.

The non-random recurrence of the exact same mutation in different tumours, and the absence of truncating mutations, indicate that K27M-H3.3 and G34R/V H3.3 have specific gene expression profiles. Clustering was based on the top 100 genes by standard deviation from autosomal genes detected as present in >10% of samples (see also Supplementary Fig. 3). b, Genes involved in development and differentiation show H3.3 mutation-specific expression patterns. Expression levels of developmental-related genes including DLX2, SFRP2, FZD7 and MYT1 are distinct among H3.3-K27 mutant and H3.3-G34 mutants following gene expression profiling (see also Supplementary Table 7).

Figure 3 | H3F3A mutation variants show distinct expression profiles and are associated with alternative lengthening of telomeres. a, Unsupervised hierarchical clustering of differentially expressed genes in 27 of the 48 GBM samples analysed by whole-exome sequencing shows that samples with K27M and G34R/V H3.3 have specific gene expression profiles. Clustering was based on the top 100 genes by standard deviation from autosomal genes detected as present in >10% of samples (see also Supplementary Fig. 3). b, Genes involved in development and differentiation show H3.3 mutation-specific expression patterns. Expression levels of developmental-related genes including DLX2, SFRP2, FZD7 and MYT1 are distinct among H3.3-K27 mutant and H3.3-G34 mutants following gene expression profiling (see also Supplementary Table 7). c, Alternate lengthening of telomere (ALT) is associated with the presence of mutant H3F3A/ATRX in a tissue microarray (TMA) comprising 124 paediatric GBM samples. We assessed ALT using telomere-specific FISH (shown here and in Supplementary Fig. 4) on the paediatric TMA we investigated for ATRX expression (Fig. 2d) and using telomere-specific Southern blotting of high molecular weight genomic DNA (data not shown). Fisher’s exact test was used to identify any association relationship. Representative images of ALT-positive and -negative staining of a paediatric GBM tissue microarray and a control brain are provided.
H3F3A mutations are most probably gain-of-function events. Lysine 27 is a critical residue of histone 3 and its variants, and methylation at this position (H3K27me), which may be mimicked by the terminal CH3 of methionine substituted at this residue, is commonly associated with transcriptional repression24. In contrast, H3K36 methylation or acetylation typically promotes gene transcription15,25,26. Thus, although their morphological phenotype is very similar (K27M and G34R/V mutant tumours are histologically indistinguishable), the two H3.3 variants are expected to act through a different set of genes. This indeed seems to be the case when looking at expression profiles of GBMs harbouring these two mutations. Unsupervised hierarchical clustering of gene expression from 27 of the WES cohort samples for which sufficient RNA was available revealed a clear separation in the expression of K27M versus G34R/V mutant samples (Supplementary Fig. 3). Further analysis of just those samples harbouring an H3F3A mutation additionally showed a clear distinction in the expression pattern of these two variants (Fig. 3a and Supplementary Table 7). Among these differentially expressed genes were several linked to brain development that showed a clear mutation-specific expression pattern when comparing both between K27 and G34 mutants and with H3.3 wild-type GBMs, including DLX2, SFRP2, FZD7 and MYT1 (Fig. 3b). We also identified increased levels of H3K36 trimethylation in cells carrying the G34V-H3.3 mutation in one sample for which we had available material (PGBM14) compared to other cells, potentially supporting this hypothesis (Supplementary Fig. 5).

ATRX loss, frequently observed in this study, has recently been shown to be associated with alternative lengthening of telomeres (ALT) in PanNETs and GBMs20. We performed telomere-specific fluorescence in situ hybridization (FISH) on the samples with K27M or G34R/V mutations identified by WES for which we had slides available (Supplementary Fig. 4) and on the paediatric GBM TMA (Fig. 3c). These experiments showed that ALT is strongly correlated with ATRX loss (37/47 samples with ALT showed ATRX loss, P < 0.001). However, some samples with nuclear ATRX staining still showed ALT, indicating that additional defects may also account for elongated telomeres in GBM. The presence of ALT was best explained by the simultaneous presence of ATRX/H3F3A/TP53 mutations (P = 0.0002, Fisher’s exact test). Tumours without ATRX/H3F3A/TP53 mutations almost invariably showed shorter telomeres than are observed with ALT, as seen in telomerase-positive gliomas27.

Genetic stability was also assessed through evaluating DNA copy number aberrations (CNAs) in 31 of the 48 tumours using Illumina SNP arrays containing ~2.5 million oligonucleotides (Supplementary Tables 1, 8, 9). Loss of heterozygosity (whole chromosome changes, broad and focal heterozygous deletions, Supplementary Table 9) was common in paediatric GBM samples, as we have previously reported4, and the focal gains and losses we identified in our study showed a high degree of overlap with other published paediatric data sets21. The number of CNAs per tumour was higher in samples with H3F3A/ATRX-DAXX/TP53 mutations (Supplementary Fig. 6).

Recurrent point mutations in IDH1 (mainly R132H) are gain of function mutations commonly identified in secondary GBM and the lower-grade tumours from which they develop (86–98% of these astrocytomas), and typically occur in younger adults11,18. Strikingly, IDH1 and H3F3A mutations were mutually exclusive in our sequencing cohort (P = 1.6 × 10^-4). Neomorphic enzyme activity resulting from IDH1 mutation leads to the production of high quantities of the onco-metabolite 2-hydroxylglutarate (2-HG)29. Increased 2-HG inhibits histone demethylases, specifically inducing increased methylation of both H3K27 and H3K3629,30. The two residues affected directly (K27) or indirectly (K36) by the mutations in H3F3A uncovered in this study. Furthermore, overlap of H3F3A and TP53 mutations in children with GBM (all of the G34R/V and 82% of K27M mutants also harbour TP53 mutations) mirrors the large overlap of IDH1 mutations with TP53 mutations in the prooncneural adult GBM sub-group18. Thus, mutations which directly (H3F3A), or indirectly (IDH1) affect the methylation of H3.3 K27 or H3.3 K36, in combination with TP53 mutations, characterize the pathogenesis of paediatric and young adult GBM.

Our data indicate a central role of H3.3/ATRX-DAXX perturbation in paediatric GBM. Mutant H3.3 recruitment would occur across the genome and induce abnormal patterns of chromatin remodelling to yield distinct gene expression profiles for the K27 and G34 mutations. Additional loss of ATRX may act to reduce H3.3 incorporation at a subset of genes important in oncogenesis, preventing mutant H3.3 from altering their transcription. ATRX loss will also impair H3.3 loading at telomeres and disrupt their heterochromatic state, facilitating alternative lengthening of telomeres (ALT). Our findings provide an intriguing example of the interplay of genetic and epigenetic events in driving cancer, indicate a new mechanism through which these epigenetic alterations are brought about (mutation of key residues in a regulatory histone), and provide a rationale for targeting the chromatin remodelling machinery in this deadly paediatric cancer.

METHODS SUMMARY

All samples were obtained with informed consent after approval of the Institutional Review Board of the respective hospitals they were treated in and were independently reviewed by senior paediatric neuropathologists (S.A., A.K.) according to the World Health Organization guidelines. Standard manufacturer protocols were used to perform target capture with the Illumina TruSeq exome enrichment kit and sequencing of 100 bp paired end reads on Illumina HiSeq. We generated approximately 10 gigabases of sequence for each subject such that >90% of the coding bases of the exome defined by the consensus coding sequence (CCDS) project were covered by at least 10 reads. We removed adaptor sequences and quality trimmed reads using the Fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and then used a custom script to ensure that only read pairs with both mates present were subsequently used. A complete description of the materials and methods is provided in the Supplementary Information.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Sample characteristics and pathological review. All samples were obtained with informed consent after approval of the Institutional Review Board of the respective hospitals they were treated in and were independently reviewed by senior paediatric neuropathologists (S.A., A.K.) according to the WHO guidelines. Forty-eight paediatric grade IV astrocytomas (glioblastoma GBM) patients between the age of 3 and 20 years were included in the study. Clinical characteristics of patients are summarized in Supplementary Table 1. Samples were taken at the time of the first surgery, before further treatment as needed. Tissues were obtained from the London/Ontario Tumour Bank of the Pediatric Cooperative Health Tissue Network, the Montreal Children’s Hospital and from collaborators in Hungary and Germany. Seven hundred and eighty-four glioma samples from all grades and histological diagnoses across the entire age range in this study were obtained from collaborators across Europe and North America.

Alignment and variant calling for whole-exome sequencing. We followed standard manufacturer protocols to perform target capture with the Illumina TruSeq exome enrichment kit and sequencing of 100 bp paired end reads on Illumina HiSeq. We generated approximately 10 Gb of sequence for each subject sequenced at our centre.

Somatic mutation identification for whole-exome sequencing. A variant called in a tumour was considered to be a candidate somatic mutation if the matched normal sample had at least 10 reads covering this position and had zero variant reads, and the variant was not reported in dbSNP131 or the 1000 Genomes data set (October 2011). For the resulting 117 candidate somatic mutations, we manually examined the alignment of each to check for sequencing artefacts and alignment errors. Fifteen variants were easily identified as sequence-specific error artefacts commonly seen shortly downstream of GGC sequences on Illumina sequences. Once genes of interest were identified (H3F3A, ATRX, DAXX, TP53, NF1), we examined positions in these genes in the 34 tumour samples where less than 20% of the reads supported the variant. This identified only two additional variants, both in sample PGBM19 where there were low read counts for frameshift insertions in both ATRX (6/32 reads) and DAXX (8/47 reads).

Immunohistochemistry and immunoblotting. Formalin-fixed, paraffin-embedded sections of paediatric GBM and TMA (4 μm) were immunohistochemically stained for ATRX and DAXX proteins. Unstained sections were subjected to antigen retrieval in 10 mM citrate buffer (pH 6.0) for 10 min at sub-boiling temperatures. Individual slides were incubated overnight at 4 °C with rabbit anti-ATRX (1:750 dilution, Signa, catalogue no. HPA001906) or rabbit anti-DAXX (1:100 dilution, Signa, catalogue no. HPA008736) antibodies. After incubation with the primary antibody, secondary biotin-conjugated donkey anti-rabbit antibodies (Jackson) were applied for 30 min. After washing with PBS, slides were developed with diaminobenzidine (Dako) as the chromogen. All slides were counterstained using Harris haematoxylin. The criterion for positive staining was described previously. Immunohistochemistry staining on TMA was scored by three individuals independently, including a pathologist. To test the level of mono-, di- and trimethylated H3 at position K36, cell lysates from tumour cells were analysed by western blot. Antibodies against H3K36me3 (Abcam, catalogue no. ab9050), H3K36me2 (Abcam, catalogue no. ab9049), H3K36me1 (Abcam, catalogue no. ab9048) and H3.3 (Abcam, catalogue no. ab97968) were used, with conditions suggested by the manufacturer.

Gene expression profiling. Total RNA from frozen samples was hybridized to Affymetrix-HG-UI133 plus 2.0 gene chips (Affymetrix). Array quality assurance was determined using β-actin and GAPDH 3′/5′ ratio, as recommended by the manufacturer.

Genome-wide SNP array. DNA from 31 of the 48 paediatric GBM tumours analysed by whole-exome sequencing was hybridized to Illumina Human Omni 2.5M Single Nucleotide Polymorphism (SNP) arrays, according to the manufacturer’s protocol. Copy number alterations were analysed using Illumina GenomeStudio Data Analysis Software (Illumina) as previously described. Statistical analysis of Fisher’s exact test was performed using GraphPad Prism software.

Telomere specific fluorescence in situ hybridization (FISH). Telomere-specific FISH was done using a standard formalin-fixed paraffin-embedded FISH protocol (as described in ref. 20), using a FITC peptide nucleic acid telomere probe from Dako.