Online Resource (Electronic Supplementary Material) Captions

Online Resource 1 Pediatric Brain Tumor Tissue Protein Expression Profiles

Principal component analysis (PCA) of tumor protein profiles (fold change values of protein expression in tumor compared to average expression across all normal brain tissue) demonstrates independent clustering of DIPG tumor specimens (navy blue) from other tumor types

Online Resource 2 Pediatric Brain Tumor Tissue Protein Expression Profiles

Protein expression profiles of brain tumor tissue specimens (n=31), reported as fold change in expression for each tumor vs. average expression across all normal tissue specimens

Online Resource 3 Pairwise DIPG Tissue Protein Expression Profiles

Tissue protein expression profiles of DIPG patients (n=10), reported as fold change in expression for each tumor vs. normal specimen pair

Online Resource 4 Comparative Analysis of DIPG Protein Expression Profiles

Filtered ANOVA results (p-value<0.05 and FC $\leq$-2 or $\geq$2) comparing protein expression profiles of DIPGs by molecular subgroup (pairwise tumor vs. normal fold change values), reported as fold change expression values in Subgroup 1 vs. Subgroup 2

Online Resource 5 Comparison of DIPG CSF, Fresh Frozen, and Fresh Froze Paraffin Embedded (FFPE) Tissue Proteomes

Listing of detected proteins (Swiss-Prot IDs) in DIPG CSF, Fresh Frozen and FFPE Tissue, with overlap between datasets

Online Resource 6 Spectral Representation of TLN1, EF2 and CLU

Tissue protein profiles of specimens from Patient IDs 6 and 7 were inspected for the presence of spectra representing TLN1, EF2 and CLU. Each spectra representing the parent ion (MS) was further searched to confirm the presence of identified peptide in the amino acid sequence (MS/MS). Each spectra represents one positively identified peptide of a given protein as identified by b and y ions listed in accompanying tables

Online Resource 7 Pediatric Brain Tumor Tissue mRNA Expression Profiles

mRNA expression profiles of brain tumor tissue specimens (n=28), reported as fold change in expression for each tumor vs. average expression across all normal tissue specimens (ANOVA, *p*-value Dx <0.05 and FC $\leq$-2 or $\geq$2)

Online Resource 8 Unsupervised clustering of Tumor mRNA profiles

Unsupervised hierarchical clustering of tumor tissue mRNA expression profiles (fold change values of mRNA expression in tumor compared to average expression across all normal brain tissue specimens) identified differential expression of 1,249 genes. DIPGs cluster independently from other tumor types but closest to supratentorial astrocytomas (ANOVA, *p*-value<0.05 and FC$\leq$-2 or $\geq$2): (*) H3.3 K27M, (**) H3.1 K27M, ( ) H3 wild type

Online resource 9 Unsupervised hierarchical clustering of high-grade astrocytoma mRNA expression values (tumor vs. average normal FC values: DIPG n=13, Yellow; pediatric supratentorial GBM n=4, Green) demonstrate unique profiles by tumor location and Histone 3 K27M mutation status (H3.1 Red, H3.3 Orange, H3 wild type Gray)

Online Resource 10 Pairwise DIPG Tissue mRNA Expression Profiles

mRNA expression profiles of DIPG tissue (n=8), reported as fold change in expression for each tumor vs. normal specimen pair

Online Resource 11 Comparative Analysis of DIPG mRNA Expression Profiles

Comparison of mRNA expression profiles (pairwise tumor vs. normal fold change values) by molecular subgroups revealed through unsupervised clustering, reported as fold change expression values in Subgroup 1 vs. Subgroup 2

Online Resource 12 Unsupervised clustering of DIPG mRNA profiles

Unsupervised hierarchical clustering of DIPG tissue mRNA expression profiles revealed two distinct DIPG subgroups, Subgroup 1 (red) and Subgroup 2 (blue), with differential expression of 158 genes (ANOVA, *p*- value<0.05, FC$\leq$-2 or $\geq$2): (*) H3.3 K27M, (**) H3.1 K27M, ( ) H3 wild type

Online Resource 13 Role of TLN1 and CLU in the Canonical Actin Cytoskeletal Signaling Pathway

Functional pathways analysis of DIPG tissue protein profiles revealed Actin Cytoskeletal Signaling as the top pathway of interaction. Dense interaction with validated tumor tissue proteins Clusterin (CLU) and Talin-1 (TLN1) is observed. Protein expression levels are depicted as up- (red) or down- (green) regulated fold change values (tumor vs. normal)

Online Resource 14 Differential Expression of Genes involved in Myc and Hh Pathways Identified through Functional Pathway Analysis of DIPG Subgroups

Functional pathway analysis of gene expression profiles from DIPG and normal tissue implicated differential expression of genes related to Myc and Hh signaling pathways between DIPG Subgroup 1 and 2. Gene expression fold changes are represented as average of pairwise tumor vs. normal expression values across specimens in a given subgroup, listed in parentheses following each gene

Online Resource 15 Functional Analysis of Molecular Profiles Reveals Differential expression of Myc and Hh Signaling Pathways between DIPG Subgroups

Top Panel Comparative functional analysis of DIPG tumor tissue molecular profiles revealed differential activity of regulatory molecules Myc (top) and GLI1 (Hh pathway molecule, bottom) between Subgroup 1 and 2. Gene expression in respective molecular networks depicted as fold change values (pairwise tumor vs. normal) in a Myc (PID 3) and Hh patient (PID 13). Heat map representation of mRNA dysregulation observed in Myc (top) and Hh (bottom) networks are depicted.

Bottom Panel Hedgehog (Hh) pathway analysis depicts mRNA dysregulation in a Hh patient (PID 13). mRNA expression levels are depicted as up- (red) or down- (green) regulated fold change values (tumor vs. normal)

Online Resource 16 Sanger Sequencing of *H3F3A* and *HIST1H3B* Mutation in DIPG Specimens

Sanger sequencing chromatogram demonstrating *H3F3A* and *HIST1H3B* mutation encoding pLys27Met (K27M) substitution in DIPG tumor tissue. (*) indicates A→T substitution in mutant specimens

Online Resource 17 Supervised hierarchical clustering of DIPG protein expression profiles (n=14, tumor vs. average normal FC values) revealed significantly 112 differentially expressed proteins by Histone 3 K27M mutation status (ANOVA, *p*-value<0.05 and FC≤-2 or ≥2, H3.1: Red, H3.3: Orange, H3 wild type: Gray)

Online Resource 18 Principle component representation of high-grade astrocytoma mRNA expression profiles (DIPG n=13: yellow, pediatric supratentorial GBM n=3: green, tumor vs. average normal FC values) representing 2,274 significantly differentially expressed molecules detected on supervised analysis by tumor location and demonstrating unsupervised profile clustering by Histone 3 K27M mutation status (ANOVA, *p*-value<0.05 and FC≤-2 or ≥2, H3.1 Red, H3.3 Orange, H3 wild type Gray)

Online Resource 19 Differential Patterns of mRNA Expression in DIPG tissue based on H3.3 K27M Mutation Status

Top Panel Supervised clustering of pairwise mRNA profiles by H3.3 mutation status, demonstrating 345 differentally expressed genes between mutant (n=5) and wild type (n=4) patients (ANOVA, *p* value <0.05, FC≥-2 or ≤2). H3.1 mutation denoted by (*)

Bottom Panel Pathways analysis of dysregulated genes identified in analysis above (Top Panel). mRNA expression levels are provided in parentheses as up- (red) and down- (green) regulated fold change values (mutant vs. wild type)

Online Resource 20 Functional Analysis of Gene Dysregulation Based on Histone H3.3 K27M Status

Top networks of molecular interaction and biological function were identified via functional pathway analysis of differentially expressed genes in DIPG by H3.3 K27M status. mRNA expression levels are provided in parentheses as fold change values (pairwise tumor vs. normal)

Online Resource 21 Immunohistochemical Staining of Adult GBM Tissue for ATRX

Adult GBM tissue was stained as a positive control for ATRX expression. Scale bar = 100μm)

Online Resource 22 DIPG Tissue DNA Methylation Profiles Expression Profiles

DNA methylation profiles of DIPG patients (n=9), reported as fold change in methylation in tumor vs. normal tissue (ANOVA, *p*-value with FDR$\leq$0.05 and FC $\leq$-3 or $\geq$3)

Online Resource 23 Pairwise DIPG Tissue DNA Methylation Profiles

DNA methylation profiles of DIPG patients (n=9), reported as fold change in methylation for each tumor vs. normal specimen pair

Online Resource 24 Comparative Analysis of DIPG DNA Methylation Profiles by Molecular Subgroup

Filtered ANOVA results (*p*-value with FDR <0.05 and FC $\leq$-2 or $\geq$2) of DNA methylation profiles (pairwise tumor vs. normal fold change values) compared by DIPG subgroup, reported as fold change methylation values in Subgroup 1 vs. Subgroup 2

Online Resource 25 Patterns of DNA Methylation Supervised by DIPG Subgroups

Supervised comparison of genome-wide methylation profiles between DIPG Subgroup 1 and 2 revealed 786 differentially methylated loci. Top canonical pathways (top section) and networks of molecular interactions (bottom section) were identified, with differentially methylated genes between the two subgroups. Fold changes are listed in parentheses following each gene

Online Resource 26 Differential DNA Methylation of Genes Involved in Myc and Hh Pathways Between DIPG Subgroups

A total of 29 differentially methylated genes pathways between DIPG subgroups 1 and 2 were identified through unsupervised analysis of methylation status of 486 CpG loci related to Myc and Hh signaling. DNA methylation (CpG) sites for each gene are presented along with gene (column 3) and protein (column 4) expression values. For methylation analysis, we used average pairwise tumor vs. normal fold change values in Subgroup 1 relative to Subgroup 2; for gene and protein expressions, we used average pairwise tumor vs. normal fold change values by subgroup. Fold changes are listed in parentheses following each gene

Online Resource 27 Comparative Analysis of Tissue DNA Methylation Profiles by H3.3 Mutation Status

Filtered ANOVA results (p-value<0.05 and FC$\leq$-3 or $\geq$3) of DIPG DNA methylation profiles compared by H3.3 K27M mutation status, reported as fold change methylation values in H3.3 mutant vs. wild type patients

Online Resource 28 Differential Patterns of DNA Methylation in DIPG Tissue by H3.3 K27M Mutation Status

Supervised analysis of DNA methylation profiles based on H3.3 K27M mutation status (n=9 pairs, pairwise tumor vs. normal fold change values) revealed 645 differentially methylated loci (ANOVA, p-value <0.05 and FC $\leq$-3 or $\geq$3). Genome wide mapping of differences in DNA methylation patterns between H3.3 K27M mutants and wild type tissue specimens is depicted. X-axis=Chromosomes 1-Y. Y-axis=Fold change in DNA methylation of identified gene across all related CpG sites in mutant relative to wild type tissue

Online Resource 29 Comparative Analysis of Pairwise DIPG DNA Methylation Profiles by H3.3 Mutation Status

Filtered ANOVA results (p-value<0.05 and FC$\leq$-3 or $\geq$3) of pairwise DIPG DNA methylation profiles (pairwise tumor vs. normal fold change values) compared by H3.3 K27Mmutation status, reported as fold change methylation values in H3.3 mutant vs. wild type patients

Online Resource 30 Supervised Clustering of Methylation in DIPG tissue based on *H3F3A* Mutation Status

Top Panel Supervised analysis of DNA methylation profiles based on H3.3 mutation status revealed 645 differentially methylated loci. The heat map depicted represents the 212 loci with similar methylation pattern in all specimens based on H3.3 mutation status. H3.1 Mutant denoted by (*)

Bottom Panel Functional analysis of loci hypomethylated in all H3.3 mutants mapped to cell death inhibition (see **Online Resource 31)**. DNA methylation, listed in parentheses as fold change values (pairwise tumor vs. normal) in mutant specimens relative to wild type specimens, and *p*-value are provided for each molecule

Online Resource 31 Patterns of DNA Methylation in DIPG Supervised by H3.3 K27M Mutation Status

Pathway analysis of supervised H3.3 mutation status data identified top biological functions and molecular networks of interaction. Fold changes are listed in parentheses following each gene