The Open Pediatric Cancer Project

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In Brief

Highlights

Summary

Keywords

Introduction

The Open Pediatric Cancer (OpenPedCan) project at the Children's Hospital of Philadelphia is an open analysis effort in which we harmonize pediatric cancer data from multiple sources, perform downstream cancer analyses on these data, and provide them on PedcBioPortal and v2.1 of NCI's Pediatric Molecular Targets Platform (MTP). The OpenPedCan analyses currently include the following datasets, described more fully below:

- OpenPBTA
- TARGET
- Kids First Neuroblastoma (X01)
- Kids First PBTA (X01)

- Chordoma Foundation
- MI-ONCOSEQ Study
- DGD (CHOP P30 Panel)
- GTEx
- TCGA
- CPTAC PBTA
- CPTAC GBM
- HOPE proteomics

Open Pediatric Brain Tumor Atlas (OpenPBTA) In September of 2018, the <u>Children's Brain Tumor Network (CBTN)</u> released the <u>Pediatric Brain Tumor Atlas (PBTA)</u>, a genomic dataset (whole genome sequencing, whole exome sequencing, RNA sequencing, proteomic, and clinical data) for nearly 1,000 tumors, available from the <u>Gabriella Miller Kids First Portal</u>. In September of 2019, the Open Pediatric Brain Tumor Atlas (OpenPBTA) Project was launched. OpenPBTA was a global open science initiative to comprehensively define the molecular landscape of tumors of 943 patients from the CBTN and the PNOC003 DIPG clinical trial from the <u>Pediatric Pacific Neuro-oncology Consortium</u> through real-time, collaborative analyses and <u>collaborative manuscript writing</u> on GitHub, now published in <u>Cell Genomics</u>. Additional PBTA data has been, and will be continually added to, OpenPedCan.

Therapeutically Applicable Research to Generate Effective Treatments (TARGET) The Therapeutically Applicable Research to Generate Effective Treatments (TARGET) Initiative is an NCI-funded collection of disease-specific projects that seeks to identify the genomic changes of pediatric cancers. The overall goal is to collect genomic data to accelerate the development of more effective therapies. OpenPedCan analyses include the seven diseases present in the TARGET dataset: Acute Lymphoblastic Leukemia (ALL), Acute Myeloid Leukemia (AML), Clear cell sarcoma of the kidney, Neuroblastoma, Osteosarcoma, Rhabdoid tumor, and Wilm's Tumor.

Gabriella Miller Kids First (Neuroblastoma) and PBTA The Gabriella Miller Kids First Pediatric Research Program (Kids First) is a large-scale effort to accelerate research and gene discovery in pediatric cancers and structural birth defects. The program includes whole genome sequencing (WGS) from patients with pediatric cancers and structural birth defects and their families. OpenPedCan analyses include Neuroblastoma and PBTA data from the Kids First projects.

Chordoma Foundation [1] The Chordoma Foundation seeks to advance research and improve healthcare for patients diagnosed with chordoma.

MI-ONCOSEQ Study [2] These clinical sequencing data from the University of Michigan were donated to CBTN and added to the PBTA cohort.

DGD (CHOP P30 Panel) CHOP's <u>Division of Genome Diagnostics</u> has partnered with CCDI to add somatic panel sequencing data to OpenPedCan and the Molecular Targets Platform.

The Genotype-Tissue Expression (GTEX) GTEx project is an ongoing effort to build a comprehensive public data resource and tissue bank to study tissue-specific gene expression, regulation and their relationship with genetic variants. Samples were collected from 54 non-diseased tissue sites across nearly 1000 individuals, primarily for molecular assays including WGS, WXS, and RNA-Seq. OpenPedCan project includes 17,382 GTEx RNA-Seq samples from GTEx v8 release, which span across 31 GTEx groups in the v12 release.

The Cancer Genome Atlas Program (TCGA) TCGA is a landmark cancer genomics program that molecularly characterized over 20,000 primary cancer and matched normal samples spanning 33 cancer types. It is a joint effort between NCI and the National Human Genome Research Institute.

OpenPedCan project includes 10,414 TCGA RNA-Seq samples (716 normal and 9698 tumor) from <u>33</u> cancer types.

Clinical Proteomic Tumor Analysis Consortium (CPTAC) PBTA proteomics study The CPTAC pediatric pan-brain tumor study [3] contains 218 tumors profiled by proteogenomics and are included in OPC.

CPTAC adult GBM proteomics study This CPTAC adult GBM study [4] contains 99 tumors profiled by proteogenomics and are included in OPC.

Project HOPE proteomics study Project HOPE is an adolescent and young adult high-grade glioma study (in preparation for publication) that contains 90 tumors profiled by proteogenomics and are included in OPC.

The OpenPedCan operates on a pull request model to accept contributions from community participants. The maintainers have set up continuous integration software via GitHub Actions to confirm the reproducibility of analyses within the project's Docker container.

The project maintainers include scientists from the <u>Center for Data-Driven Discovery in Biomedicine at the Children's Hospital of Philadelphia</u>. We invite researchers to join OpenPedCan to help rigorously characterize the genomic landscape of these diseases to enable more rapid discovery of additional mechanisms contributing to the pathogenesis of pediatric brain and spinal cord tumors and overall accelerate clinical translation on behalf of patients.

Results

Discussion

Acknowledgments

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Sangeeta Shukla	Data curation, Formal analysis, Investigation, Methodology, Software, Writing – Original draft, Writing – Review and editing
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Krutika S. Gaonkar	Data curation, Formal analysis, Investigation, Methodology, Software, Writing – Original draft, Writing - Review and editing
Run Jin	Data curation, Formal analysis, Visualization, Writing – Original draft, Writing - Review and editing

Author	Contributions
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Yuankun Zhu	Data curation, Formal analysis, Investigation, Methodology, Supervision
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Chuwei Zhong	Formal analysis
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Adam C. Resnick	Conceptualization, Funding acquisition, Resources, Supervision
Alvin Farrel	Supervision, Investigation, Visualization, Methodology, Funding acquisition
Deanne Taylor	Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Writing – Original draft, Writing - Review and editing
Jo Lynne Rokita^	Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Writing – Original draft, Writing - Review and editing

Declarations of Interest

Figure Titles and Legends

Table Titles and Legends

OPENPEDCAN METHODS

RESOURCE AVAILABILITY

Lead contact

Requests for access to OpenPedCan raw data and/or specimens may be directed to, and will be fulfilled by Jo Lynne Rokita (rokita@chop.edu).

Materials availability

This study did not create new, unique reagents.

Data and code availability

Within OpenPedCan (OPC), we harmonized, aggregated, and analyzed data from multiple sources. We harmonized data from the Therapeutically Applicable Research to Generate Effective Treatments (TARGET cohort) Initiative, an NCI-funded collection of disease-specific projects that seeks to identify the genomic changes of pediatric cancers [5]. We included already harmonized neuroblastoma samples from the Gabriella Miller Kids First (GMKF cohort) Pediatric Research Program, a large-scale effort to accelerate research and gene discovery in pediatric cancers and structural birth defects [6]. Additionally, we re-harmonized all samples from the Open Pediatric Brain Tumor Atlas (OpenPBTA, PBTA cohort), an open science initiative led by Alex's Lemonade Stand Foundation Childhood Cancer Data Lab and the Center for Data-Driven Discovery (D3B) at the Children's Hospital of Philadelphia (CHOP), which genomically characterized pediatric brain tumor data from the Children's Brain Tumor Network (CBTN), and the Pacific Pediatric Neuro-oncology Consortium (PNOC) [7,8]. Building on the work of OpenPBTA, OPC contains additional data from the following studies:

- PBTA X01 [9]
- Chordoma Foundation [1]
- MI-ONCOSEQ Study [2], donated to CBTN by the University of Michigan, to the PBTA cohort
- Children's Hospital of Philadelphia (CHOP) P30 Panel data generated by CHOP's Division of Genomic Diagnostics (DGD cohort) which includes targeted DNA and fusion panel data [7].
- National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) PBTA proteomics study [3]
- CPTAC adult GBM proteomics study [4]
- Project HOPE adolescent and young adult high-grade glioma proteomics study (in preparation for publication)

In addition to pediatric cancer data, OpenPedCan contains adult data from large science consortia as references. For normal gene expression, GTEx [10] was used, and for comparison with adult cancers, The Cancer Genome Atlas (TCGA) [11] was included.

Merged summary files for the latest release of OpenPedCan are openly accessible in <u>CAVATICA</u> or via download-data.sh script in the https://github.com/PediatricOpenTargets/OpenPedCan-analysis repository. Cancer group summary data from release v11 are visible within the NCl's pediatric https://github.com/PediatricOpenTargets/OpenPedCan-analysis repository. Cancer group summary data from release v11 are visible within the NCl's pediatric https://github.com/PediatricOpenTargets/OpenPedCan-analysis repository. Cancer group summary data from release v11 are visible within the NCl's pediatric https://github.com/PediatricOpenTargets/OpenPedCan-analysis

OpenPedCan analysis modules were developed within OpenPBTA [8], modified based on OpenPBTA, or newly created and can be found within the following publicly available repositories. OpenPBTA module analyses can be found at https://github.com/AlexsLemonade/OpenPBTA-analysis. OpenPedCan module analyses can be found at

https://github.com/PediatricOpenTargets/OpenPedCan-analysis. OpenPedCan api code can be found at https://github.com/PediatricOpenTargets/OpenPedCan-api.

All original code was developed within the following modules in the OpenPedCan analyses repository as listed below. Links to the modules are available here, and within each module is a detailed README that describes the purpose and intended usage of the scripts, along with pointers to the results from the data those scripts process.

List of OpenPedCan Analyses Modules
<u>chromosomal-instability</u>
<u>cnv-frequencies</u>
<u>collapse-rnaseq</u>
<u>compare-gistic</u>
copy_number_consensus_call
<u>create-subset-files</u>
data-pre-release-qc
<u>efo-mondo-mapping</u>
<u>filter-mtp-tables</u>
focal-cn-file-preparation
<u>fusion-frequencies</u>
<u>fusion-summary</u>
<u>fusion filtering</u>
gene-set-enrichment-analysis
gene match
<u>immune-deconv</u>
<u>independent-samples</u>
long-format-table-utils
methylation-preprocessing
methylation-summary
molecular-subtyping-ATRT
molecular-subtyping-CRANIO
molecular-subtyping-EPN
molecular-subtyping-EWS

List of OpenPedCan Analyses Modules
molecular-subtyping-HGG
molecular-subtyping-LGAT
molecular-subtyping-MB
molecular-subtyping-NBL
molecular-subtyping-chordoma
molecular-subtyping-embryonal
molecular-subtyping-integrate
molecular-subtyping-neurocytoma
molecular-subtyping-pathology
<u>mtp-annotations</u>
<u>mtp-tables-qc-checks</u>
mutational-signatures
pedcbio-cnv-prepare
pedcbio-sample-name
pedot-table-column-display-order-name
<u>rna-seq-expression-summary-stats</u>
rnaseq-batch-correct
<u>run-gistic</u>
<u>snv-frequencies</u>
<u>tmb-calculation</u>
tp53 nf1 score
<u>tumor-gtex-plots</u>
<u>tumor-normal-differential-expression</u>

Software versions are documented in **Table XX**.

Data releases

We maintained a data release folder on Amazon S3, downloadable directly from S3 or our open-access CAVATICA project, with merged files for each analysis (See data and code availability section). As we produced new results that we expected to be used across multiple analyses, or identified data issues, we created new data releases in a versioned manner.

METHOD DETAILS

Nucleic acids extraction and library preparation

For the PBTA X01 cohort, libraries were prepped using the Illumina TruSeq Strand-Specific Protocol to pull out poly-adenylated transcripts.

cDNA Library Construction Total RNA was quantified using the Quant-iT™ RiboGreen® RNA Assay Kit and normalized to 5ng/ul. Following plating, 2 uL of ERCC controls (using a 1:1000 dilution) were spiked into each sample. An aliquot of 325 ng for each sample was transferred into library preparation. The resultant 400bp cDNA went through dual-indexed library preparation: 'A' base addition, adapter ligation using P7 adapters, and PCR enrichment using P5 adapters. After enrichment, the libraries were quantified using Quant-iT PicoGreen (1:200 dilution). Samples were normalized to 5 ng/uL. The sample set was pooled and quantified using the KAPA Library Quantification Kit for Illumina Sequencing Platforms.

miRNA Extraction and Library Preparation Total RNA for CBTN samples was extracted as described in OpenPBTA [8] and prepared according to the HTG Edge Seq protocol for the extracted RNA miRNA Whole transcriptome assay (WTA). 15ng of RNA were mixed in 25ul of lysis buffer, which were then loaded onto a 96-well plate. Human Fetal Brain Total RNA (Takara Bio USA, #636526) and Human Brain Total RNA (Ambion, Inc., Austin, TX, USA) were used as controls. The plate was loaded into the HTG EdgeSeq processor along with the miRNA WTA assay reagent pack. Samples were processed for 18-20 hours, then were barcoded and amplified using a unique forward and reverse primer combination. PCR settings used for barcoding and amplification were 95C for 4 min, 16 cycles of (95C for 15 sec, 56C for 45 sec, 68C for 45 sec), and 68C for 10 min. Barcoded and amplified samples were cleaned using AMPure magnetic beads (Ampure XP,Cat# A63881). Libraries were quantified using the KAPA Biosystem assay qPCR kit (Kapa Biosystems Cat#KK4824) and CT values were used to determine the pM concentration of each library.

Data generation

PBTA X01 Illumina Sequencing Pooled libraries were normalized to 2nM and denatured using 0.1 N NaOH prior to sequencing. Flowcell cluster amplification and sequencing were performed according to the manufacturer's protocols using the NovaSeq 6000. Each run was a 151bp paired-end with an eight-base index barcode read. Data was analyzed using the Broad Picard Pipeline which includes demultiplexing and data aggregation.

PBTA miRNA Sequencing Libraries were pooled, denatured, and loaded onto sequencing cartridge. Libraries were sequenced using an Illumina Nextseq 500 per manufacturer guidelines. FASTQ files were generated from raw sequencing data using Illumina BaseSpace and analyzed with the HTG EdgeSeq Parser software v5.4.0.7543 to generate an excel file containing quantification of 2083 miRNAs per sample. Any sample that did not pass the quality control set by the HTG REVEAL software version 2.0.1 (Tuscon, AR, USA) was excluded from the analysis.

DNA WGS Alignment

Please refer to the OpenPBTA manuscript for details [8].

Quality Control of Sequencing Data

Please refer to the OpenPBTA manuscript for details [8]. We also ran somalier relate [12] to identify potential mismatched samples. We required that at least 20M total reads with 50% of RNA-Seq reads mapped to the human reference for samples to be included in analysis. We required at least 20X coverage for tumor DNA samples to be included in this analysis.

SNP calling for B-allele Frequency (BAF) generation

Please refer to the OpenPBTA manuscript for details [8].

Somatic Mutation and INDEL Calling

For matched tumor/normal samples, we used the same mutation calling methods as described in OpenPBTA manuscript for details [8]. For tumor only samples, we ran Mutect2 from GATK v4.2.2.0 using the following workflow.

VCF annotation and MAF creation

Somatic variants were annotated by the Ensembl Variant Effect Predictor (VEP v105) [13]. From tumor only variant calls, we removed variants with $alt_depth == 0$ or $t_depth < 4$.

Consensus SNV Calling (tumor/normal only)

We adopted the consensus SNV calling method described in OpenPBTA manuscript with adjustment [8]. For SNV calling, we combined four consensus SNV calling algorithms: Strelka2[14], Mutect2[15], Lancet[16], and VarDict[doi? 10.1093/nar/gkw227].

Strelka2 outputs multi-nucleotide polymorphisms (MNPs) as consecutive single-nucleotide polymorphisms. In order preserve MNPs, we gather MNP calls from the other caller inputs, and search for evidence supporting these consecutive SNP calls as MNP candidates. Once found, the Strelka2 SNP calls supporting a MNP are converted to a single MNP call. This is done to preserve the predicted gene model as accurately as possible in our consensus calls. Consensus SNV from all four callers were collected and by default, calls that were detected in at least two calling algorithms or marked with "HotSpotAllele" were retained.

For all SNVs, potential non-hotspot germline variants were removed if they had a normal depth <= 7 and gnomAD allele frequency > 0.001. Final results were saved in MAF format.

Somatic Copy Number Variant (CNV) Calling

We called copy number variants for tumor/normal samples using Control-FREEC [17,18] and CNVkit [19] as described in the OpenPBTA manuscript [8]. We used GATK [20] to call CNVs for matched tumor/normal WGS samples when there were at least 30 male and 30 female normals from the same sequencing platform available for panel of normal creation. For tumor only samples, we used Control-FREEC with the following modifications. Instead of the b-allele frequency germline input file, we used the dbSNP_v153_ucsc-compatible.converted.vt.decomp.norm.common_snps.vcf.gz dbSNP common snps file and to avoid hard-to-call regions, utilized the hg38_canonical_150.mappability mappability file. Both are also linked in the public Kids First references CAVATICA project. The Control-FREEC tumor only workflow can be found here.

Consensus CNV Calling (WGS samples only)

We adopted the consensus CNV calling described in OpenPBTA manuscript [8] with minor adjustments. For each caller and sample with WGS performed, we called CNVs based on consensus among Control-FREEC [17,18], CNVkit [19], and GATK [doi? 10.1101/gr.107524.110]. Sample and consensus caller files with more than 2,500 CNVs were removed to de-noise and increase data quality, based on cutoffs used in GISTIC [21]. For each sample, we included the following regions in the final consensus set: 1) regions with reciprocal overlap of 50% or more between at least two of the callers; 2) smaller CNV regions in which more than 90% of regions were covered by another caller. For GATK, if a panel of normal was not able to be created (required 30 male and 30 female with the same sequencing platform), consensus was run for that tumor using Control-FREEC, CNVkit, and MantaSV. We defined copy number as NA for any regions that had a neutral call for the samples included in the

consensus file. We merged CNV regions within 10,000 bp of each other with the same direction of gain or loss into single region.

Any CNVs that overlapped 50% or more with immunoglobulin, telomeric, centromeric, segment duplicated regions, or that were shorter than 3000 bp were filtered out. The CNVKit calls for WXS samples were appended to the consensus CNV file.

Somatic Structural Variant Calling (WGS samples only)

Please refer to the OpenPBTA manuscript for details [8].

Methylation Analysis

Methylation array preprocessing

We preprocessed raw Illumina 450K and EPIC 850K Infinium Human Methylation Bead Array intensities using the array preprocessing methods implemented in the minfi Bioconductor package [22]. We utilized either preprocessFunnorm when an array dataset had both tumor and normal samples or multiple OpenPedcan-defined cancer_groups and preprocessQuantile when an array dataset had only tumor samples from a single OpenPedcan-defined cancer_group to estimate usable methylation measurements (beta-values and m-values) and copy number (cn-values). Some Illumina Infinium array probes targeting CpG loci contain single-nucleotide polymorphisms (SNPs) near or within the probe [23], which could affect DNA methylation measurements [24]. As the minfi preprocessing workflow recommends, we dropped probes containing common SNPs in dbSNP (minor allele frequency > 1%) at the CpG interrogation or the single nucleotide extensions.

Details of methylation array preprocessing are available in the <u>OpenPedCan methylation-preprocessing module</u>.

Methylation beta-values summaries

We comprehensively summarized gene-level and isoform-level metrics for the methylation betavalues estimated by array preprocessing to provide insight into the variations in overall genomic DNA methylation levels observed across different pediatric tumors by computing CpG probe-level summary metrics in each cancer group within a cohort, including 1) beta-values quantiles, 2) gene expression (TPM) and methylation (beta-values) correlation, 3) TPM median expression, and 4) transcript representation - a proxy for percent isoform expression in a gene. In addition, each CpG probe was annotated with a gene feature to identify the genomic regions likely involved in regulating gene expression.

Details of the analysis are available in the OpenPedCan methylation-summary module.

Methylation sample classification

The Clinical Methylation Unit Laboratory of Pathology at the National Cancer Institute Center for Cancer Research ran the DKFZ brain classifier version 12.6, a comprehensive DNA methylation-based classification of CNS tumors across all entities and age groups [25] and/or the Bethesda Brain tumor classifier v2.0 (NIH_v2) and the combo reporter pipeline v2.0 on docker container trust1/bethesda:latest. Unprocessed IDAT-files from the Children's Brain Tumor Network (CBTN)

Infinium Human Methylation EPIC (850k) BeadChip arrays were used as input and the

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following information was compiled into the histologies.tsv file: dkfz_v12_methylation_subclass (predicted methylation subtype), dkfz_v12_methylation_subclass_score (classification score), dkfz_v12_methylation_mgmt_status (MGMT methylation status), dkfz_v12_methylation_mgmt_estimated (estimated MGMT methylation fraction), NIH_v2_methylation_Superfamily, NIH_v2_methylation_Superfamily_mean_score, NIH_v2_methylation_Superfamily_Consistency_score, NIH_v2_methylation_Class_mean_score, NIH_v2_methylation_Class_mean_score, NIH_v2_methylation_Class_consistency_score, NIH_v2_methylation_Superfamily_match, and NIH_v2_methylation_Class_match.
```

Gene Expression

The tumor-normal-differential-expression module performs differential expression analyses for all sets of Disease (cancer_group) and Dataset (cohort) across all genes found in the gene-expression-rsem-tpm-collapsed.rds table. The purpose of this analysis is to highlight the correlation and understand the variability in gene expression in different cancer conditions across different histological tissues. For OpenPedCan v12 data release, this module performs expression analysis over 102 cancer groups across 52 histological tissues for all 54,346 genes found in the dataset. This analysis was performed on the Children's Hospital of Philadelphia HPC and was configured to use 96G of RAM per CPU, with one task (one iteration of expression analysis for each set of tissue and cancer group) per CPU (total 102x52=5304 CPUs) using the R/DESeq2 package. Please refer to script run-tumor-normal-differential-expression.sh in the module for additional details on Slurm processing configuration. The same analysis can also be performed on CAVATICA, but requires further optimization. The module describes the steps for CAVATICA set up, and scripts to publish an application on the portal. The required data files are also available publicly on CAVATICA under the Open Access. Refer to the module for detailed description and scripts.

Abundance Estimation

Among the data sources used for OpenPedCan, GTEx and TCGA used GENCODE versions v26 and v36, respectively. Moreover, the gene symbols used in these different GENCODE versions also varied. Therefore, the gene symbols had to be harmonized for compatibility to map unique gene identifiers to their gene symbols. ENSG IDs from each data source were pulled and mapped to the GTF/GFF3 file from GENCODE v39 to extract unique gene symbols and remove duplicates. Additionally, the gene expression matrices had some instances where multiple Ensembl gene identifiers mapped to the same gene symbol. This was dealt with by filtering the expression matrix to only genes with [FPKM/TPM] > 0 and then selecting the instance of the gene symbol with the maximum mean [FPKM/TPM/Expected_count] value across samples. This enabled many downstream modules that require RNA-seq data have gene symbols as unique gene identifiers. Refer to collapse-rnaseq module for scripts and details.

Gene Expression Summary Statistics

We generated RNA-Seq gene expression (TPM) summary statistics for independent tumor samples from the combined OpenPedCan gene expression matrices, including cancers from pediatric cohorts (PBTA, GMKF, and TARGET) and adult cancers from the TCGA cohort. We grouped selected samples into two groups containing samples from a cancer group in either each cohort or all cohorts, and calculated TPM means, standard deviations, gene-wise z-scores, group-wise z-scores, and ranks for each group as described in the OpenPedCan rna-seq-expression-summary-

stats module in detail. The resulting gene-wise and group-wise summary statistics tables were annotated with EFO and MONDO disease codes associated with the cancer groups.

Gene fusion detection

CPTAC PBTA, CPTAC GBM, and HOPE proteogenomics

The following methods are the general proteomics approaches used for the CPTAC PBTA [3], CPTAC GBM [4], and HOPE (pre-publication, correspondence with Dr. Pei Wang) studies. For specific descriptions of sample preparation, mass spectrometry instrumentation and approaches, and data generation, processing, or analysis please refer to the relevant publications.

TMT-11 Labeling and Phosphopeptide Enrichment

Proteome and phosphoproteome analysis of brain cancer samples in the CPTAC PBTA (pediatric), CPTAC GBM (adult), and HOPE (adolescent and young adult, AYA) cohort studies were structured as TMT11-plex experiments. Tumor samples were digested with LysC and trypsin. Digested peptides were labeled with TMT11-plex reagent and prepared for phosphopeptide enrichment. For each dataset, a common reference sample was compiled from representative samples within the cohort. Phosphopeptides were enriched using Immobilized Metal Affinity Chromatography (IMAC) with Fe3+NTA-agarose bead kits.

Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Analysis

To reduce sample complexity, peptide samples were separated by high pH reversed phase HPLC fractionation. For CPTAC PBTA a total of 96 fractions were consolidated into 12 final fractions for LC-MS/MS analysis. For CPTAC GBM and HOPE cohorts a total of 96 fractions were consolidated into 24 fractions. For CPTAC PBTA, global proteome mass spectrometry analyses were performed on an Orbitrap Fusion Tribrid Mass Spectrometer and phosphoproteome analyses were performed on an Orbitrap Fusion Lumos Tribrid Mass Spectrometer. For CPTAC GBM and HOPE studies, mass spectrometry analysis was performed using an Orbitrap Fusion Lumos Mass Spectrometer.

Protein Identification

The CPTAC PBTA spectra data were analyzed with MSFragger version 20190628 [26] searching against a CPTAC harmonized RefSeq-based sequence database containing 41,457 proteins mapped to the human reference genome (GRCh38/hg38) obtained via the UCSC Table Browser on June 29, 2018, with the addition of 13 proteins encoded in the human mitochondrial genome, 264 common laboratory contaminant proteins, and an equal number of decoy sequences. The CPTAC GBM and HOPE spectra data were analyzed with MS-GF+ v9881 [27,28,29] searching against the RefSeq human protein sequence database downloaded on June 29, 2018 (hg38; 41,734 proteins), combined with 264 contaminants, and a decoy database composed of the forward and reversed protein sequences.

Protein Quantification and Data Analysis

Relative protein (gene) abundance was calculated as the ratio of sample abundance to reference abundance using the summed reporter ion intensities from peptides mapped to the respective gene. For phosphoproteomic datasets, data were not summarized by protein but left at the phosphopeptide level. Global normalization was performed on the gene-level abundance matrix (log2 ratio) for global proteomic and on the site-level abundance matrix (log2 ratio) for phosphoproteomic data. The median, log2 relative protein or peptide abundance for each sample was calculated and used to normalize each sample to achieve a common median of 0. To identify TMT outliers, inter-TMT t-tests

were performed for each individual protein or phosphopeptide. Batch effects were checked using the log2 relative protein or phosphopeptide abundance and corrected using the Combat algorithm [30]. Imputation was performed after batch effect correction for proteins or phosphopeptides with a missing rate < 50%. For the phosphopeptide datasets, 440 markers associated with cold-regulated ischemia genes were filtered and removed.

QUANTIFICATION AND STATISTICAL ANALYSIS

Focal Copy Number Calling (focal-cn-file-preparation analysis module)

Please refer to the OpenPBTA manuscript for details on assignment of copy number status values to CNV segments, cytobands, and genes [8]. We applied criteria to resolve instances of multiple conflicting status calls for the same gene and sample, which are described in detail in the <u>focal-cn-file-preparation</u> module. Briefly, we prioritized 1) non-neutral status calls, 2) calls made from dominant segments with respect to gene overlap, and 3) amplification and deep deletion status calls over gain and loss calls, respectively, when selecting a dominant status call per gene and sample. These methods resolved >99% of duplicated gene-level status calls.

Gene Set Variation Analysis (gene-set-enrichment-analysis analysis module)

Please refer to the OpenPBTA manuscript for details [8].

Fusion prioritization (fusion_filtering analysis module)

The <u>fusion_filtering</u> module filters artifacts and annotates fusion calls, with prioritization for oncogenic fusions, for the fusion calls from STAR-Fusion and Arriba. After artifact filtering, fusions were prioritized and annotated as "putative oncogenic fusions" when at least one gene was a known kinase, oncogene, tumor suppressor, curated transcription factor, on the COSMIC Cancer Gene Census List, or observed in TCGA. Fusions were retained in this module if they were called by both callers, recurrent or specific to a cancer group, or annotated as a putative oncogenic fusion. Please refer to the module linked above for more detailed documentation and scripts.

Splicing quantification

To detect alternative splicing events, we utilized rMATS turbo (v. 4.1.0) with Ensembl/GENCODE v39 GFF annotations using the <u>Kids First RNA-Seq workflow</u>. We used _-variable-read-length and _ t paired options and applied an additional filter to include only splicing events with total junction read counts greater than 10.

Mutational Signatures (mutational-signatures analysis module)

Tumor Mutation Burden [TMB] (tmb-calculation analysis module)

Recent clinical studies have associated high TMB with improved patient response rates and survival benefit from immune checkpoint inhibitors [doi.org/10.1002/gcc.22733?].

The <u>Tumor Mutation Burden (TMB)</u> <u>tmb-calculation</u> module was adapted from the <u>snv-callers module</u> of the OpenPBTA project [8]. Here, we use mutations in the <u>snv-consensus-plus-hotspots.maf.tsv.gz</u> file which is generated using <u>Kids First DRC Consensus Calling Workflow</u> and is included in the OpenPedCan data download. The consensus MAF contains SNVs or MNVs called in at least 2 of the 4 callers (Mutect2, Strelka2, Lancet, and Vardict) plus hotspot

mutations if called in 1 of the 4 callers. We calculated TMB for tumor samples sequenced with either WGS or WXS. Briefly, we split the SNV consensus MAF into SNVs and multinucleotide variants (MNVs). We split the MNV subset into SNV calls, merged those back with the SNVs subset, and then removed sample-specific redundant calls. The resulting merged and non-redundant SNV consensus calls were used as input for the TMB calculation. We tallied only nonsynonymous variants with classifications of high/moderate consequence ("Missense_Mutation", "Frame_Shift_Del", "In_Frame_Ins", "Frame_Shift_Ins", "Splice_Site", "Nonsense_Mutation", "In_Frame_Del", "Nonstop_Mutation", and "Translation_Start_Site") for the numerator.

All mutation TMB For WGS samples, we calculated the size of the genome covered as the intersection of Strelka2 and Mutect2's effectively surveyed areas, regions common to all variant callers, and used this as the denominator. WGS_all_mutations_TMB = (total # mutations in consensus MAF) / intersection_strelka_mutect_vardict_genome_size For WXS samples, we used the size of the WXS bed region file as the denominator. WXS_all_mutations_TMB = (total # mutations in consensus MAF)) / wxs_genome_size

Coding only TMB We generated coding only TMB from the consensus MAF as well. We calculated the intersection for Strelka2 and Mutect2 surveyed regions using the coding sequence ranges in the GENCODE v39 gtf supplied in the OpenPedCan data download. We removed SNVs outside of these coding sequences prior to implementing the TMB calculation below: WGS_coding_only_TMB = (total # coding mutations in consensus MAF) / intersection_wgs_strelka_mutect_vardict_CDS_genome_size For WXS samples, we intersected each WXS bed region file with the GENCODE v39 coding sequence, sum only variants within this region for the numerator, and calculate the size of this region as the denominator. WXS_coding_only_TMB = (total # coding mutations in consensus MAF) / intersection_wxs_CDS_genome_size

Finally, we include an option (nonsynfilter_focr) to use specific nonsynonymous mutation variant classifications recommended from the <a href="https://example.com/memory.com/me

Clinical Data Harmonization

WHO Classification of Disease Types

Molecular Subtyping

Here, we build upon the molecular subtyping performed in OpenPBTA [8].

High-grade gliomas.

A new high-grade glioma entity called infant-type hemispheric gliomas (IHGs), characterized by distinct gene fusions enriched in receptor tyrosine kinase (RTK) genes including *ALK*, *NTRK1/2/3*, *ROS1* or *MET*, was identified in 2021 [doi? 10.1038/s41467-019-12187-5]. To identify IHG tumors, first, tumors which were classified as "IHG" by the DKFZ methylation classifier or diagnosed as "infant type hemispheric glioma" from pathology_free_text_diagnosis were selected [25]. Then, the corresponding tumor RNA-seq data were utilized to seek the evidence for RTK gene fusion. Based on the specific RTK gene fusion present in the samples, IHGs were further classified as "IHG, ALK-altered", "IHG, NTRK-altered", "IHG, ROS1-altered", or "IHG, MET-altered". If no fusion was observed, the samples were identified as "IHG, To be classified".

Atypical teratoid rhabdoid tumors.

Atypical teratoid rhabdoid tumors (ATRT) tumors were categorized into three subtypes: "ATRT, MYC", "ATRT, SHH", and "ATRT, TYR" [31]. In OpenPedCan, the molecular subtyping of ATRT was based solely on the DNA methylation data. Briefly, ATRT samples with a high confidence DKFZ methylation subclass score (>= 0.8) were selected and subtypes were assigned based on the DKFZ methylation subclass [doi10.1038/nature26000?]. Samples with low confidence DKFZ methylation subclass scores (< 0.8) were identified as "ATRT, To be classified".

Neuroblastoma tumors.

Neuroblastoma (NBL) tumors with a pathology diagnosis of neuroblastoma, ganglioneuroblastoma, or ganglioneuroma were subtyped based on their MYCN copy number status as either "NBL, MYCN amplified" or "NBL, MYCN non-amplified". If pathology_free_text_diagnosis was "NBL, MYCN non-amplified" and the genetic data suggested MYCN amplification, the samples were subtyped as "NBL, MYCN amplified". On the other hand, if pathology_free_text_diagnosis was "NBL, MYCN amplified" and the genetic data suggested MYCN non-amplification, the RNA-Seq gene expression level of MYCN was used as a prediction indicator. In those cases, samples with MYCN gene expression above or below the cutoff (TPM >= 140.83 based on visual inspection of MYCN CNV status) were subtyped as "NBL, MYCN amplified" and "NBL, MYCN non-amplified", respectively. MYCN gene expression was also used to subtype samples without DNA sequencing data. If a sample did not fit none of these situations, it was denoted as "NBL, To be classified".

Integration of brain tumor methylation classifications

TP53 Alteration Annotation (tp53_nf1_score analysis module)

Please refer to the OpenPBTA manuscript for details [8].

Prediction of participants' genetic sex

Please refer to the OpenPBTA manuscript for details [8].

Selection of independent samples (independent-samples analysis module)

For analyses that require all input biospecimens to be independent, we use the OpenPedCan-analysis <u>independent-samples</u> module to select only one biospecimen from each input participant. For each input participant of an analysis, the independent biospecimen is selected based on the analysis-specific filters and preferences for the biospecimen metadata, such as experimental strategy, cancer group, and tumor descriptor.

Supplemental Information Titles and Legends

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