Comprehensive molecular characterization of pediatric radiation-induced high-grade glioma

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Radiation-induced high-grade gliomas (RIGs) are an incurable late complication of cranial radiation therapy. We performed DNA methylation profiling, RNA-seq, and DNA sequencing on 32 RIG tumors and an in vitro drug screen in two RIG cell lines. We report that based on DNA methylation, RIGs cluster primarily with the pediatric receptor tyrosine kinase I high-grade glioma subtype. Common copy-number alterations include Chromosome (Ch.) 1p loss/1q gain, and Ch. 13q and Ch. 14q loss; focal alterations include PDGFRA and CDK4 gain and CDKN2A and BCOR loss. Transcriptomically, RIGs comprise a stem-like subgroup with lesser mutation burden and Ch. 1p loss and a pro-inflammatory subgroup with greater mutation burden and depleted DNA repair gene expression. Chromothripsis in several RIG samples is associated with extrachromosomal circular DNA-mediated amplification of PDGFRA and CDK4. Drug screening suggests microtubule inhibitors/stabilizers, DNA-damaging agents, MEK inhibition, and, in the inflammatory subgroup, proteasome inhibitors, as potentially effective therapies.

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therapeutic radiation of the central nervous system to treat childhood cancers causes secondary damage to normal tissue, including non-letal DNA double-stranded breaks (DSBs) that may trigger erroneous or incomplete DNA repair, particularly when the repair occurs via error-prone non-homologous end joining. Chemotherapies (e.g., cisplatin or doxorubicin) that produce DNA DSBs or replication inhibitors (e.g., gemcitabine or topoisomerase II inhibitors) may also cause errors in DNA repair4,12. Alterations in genomic DNA after erroneous DNA repair may produce pathogenic mutations contributing to the formation of a new radiation-induced malignancy3.

Tumors known as radiation-induced glioblastomas or radiation-induced gliomas arise in ~3% of pediatric cancer survivors after cranial radiotherapy4. Given that all these tumors are high-grade gliomas but that their pathology includes a wider range of high-grade gliomas than glioblastoma alone, we use the term “radiation-induced high-grade glioma” in this study but have continued use of the RIG abbreviation. RIG is a rare but significant cause of late mortality in childhood cancer survivors3, with limited therapeutic options6,7. Prior genomic analyses in DSBs that may trigger erroneous or incomplete DNA repair, malignancy3.

Results

Patient and disease characteristics

We explore using in silico and in vitro preclinical therapeutic rationale for developing alternative therapeutic approaches that alter alterations, methylation profiling, and gene expression. Our analyses differentiate RIG from de novo pHGG and provide a rationale for developing alternative therapeutic approaches that we explore using in silico and in vitro preclinical therapeutic screening studies.

RIGs primarily cluster with the pedRTK I methylation group. DNA methylation profiling was performed and analyzed by hierarchical unsupervised clustering and t-distributed stochastic neighbor embedding (t-SNE) of 34 RIG samples from 31 tumors against a combined reference cohort of pediatric and adult CNS malignancies15,16. Of 31 cases, 25 RIG cases clustered among a group of epigenetically similar tumors consisting of H3K27M-negative midline pHGG and pediatric receptor tyrosine kinase I (pedRTK I) subgroup pHGG (Fig. 2a–c and Supplementary Fig. 3A–C)17. Methylation profiling could not distinguish RIGs from IDHwt, GBM-MID, and pedRTK I pHGG samples included in the reference cohorts15. Accordingly, we designate the predominant group of RIG cases as “pedRTK I” hereafter (Supplementary Table 3). The six RIGs that clustered with other methylation subgroups are detailed in Supplementary Data 1 and 2. The MGMT promoter was methylated in eight of 31 (25.8%) cases. There was no trend associating initial cancer diagnosis, latency, or survival (Supplementary Figs. 4 and 5B) with RIG methylation class (Supplementary Fig. 4A–C)18. The odds of being assigned to the pedRTK I subgroup in the RIG cohort were 29.2 (95% CI 9.15–92.9) times those of being assigned to that subgroup in the de novo pHGG cohort (P < 0.0001) (Supplementary Fig. 5A and Supplementary Data 3). Thus, RIGs predominantly align within the pedRTK I methylation subgroup despite disparate clinical origins.

RIGs show recurrent copy-number abnormalities. Given the classification of most of the RIG cohort into one pHGG methylation subgroup, gene and copy-number alterations and transcriptomic profiles of RIG samples relative to pHGG were analyzed to better understand their similarities and differences. RIG methylation data were reviewed for large segment (>25% of chromosome arm) and focal (≤3 Mb) copy-number alterations. Recurrent large segment alterations in RIG pedRTK I cases included Ch.1p loss (10/25, 40.0%), Ch.1q gain (13/25, 50%), Ch.13q loss (10/25, 40.0%), and Ch.14q loss (10/25, 40.0%) (Fig. 3a, Supplementary Data 1, 20, and Supplementary Figs. 6 and 7). PDGFRA gain/amplification (11/31, 35.5%), CDK4 amplification (6/31, 19.4%), CDKN2A loss (9/31, 29%), and BCOR loss (7/31, 22.6%) were common focal alterations (Supplementary Fig. 7, and Supplementary Data 6 and 18). For cases where methylation and WGS data were both available, copy-number change estimates were comparable between the two methods (Supplementary Data 4, 5, 18, and 19).

Focal copy-number variations (CNVs) in the RIG cohort were compared to those in de novo pHGG in patients enrolled in the HERBY phase II open-label, randomized, multicenter clinical trial of bevacizumab18. Total CNVs were significantly increased in RIG pedRTK I cases relative to de novo pHGG cases (Supplementary
PDGFRA amplification ($P = 0.188$) and BCOR deletions ($P = 0.0043$) were also enriched in RIG relative to de novo pHGG (Supplementary Data 6, 18, 19 and Supplementary Fig. 7). Changes in copy number of PDGFRA and BCOR were associated with an accompanying change in gene expression in cases for which RNA-seq data were available ($n = 12, 34.3\%$) (Fig. 3b). Despite increased overall genomic instability and a predominance of copy-number losses over gains in RIG (Fig. 3c), no additional specific focal or large segment alterations were detected in RIG pedRTK I cases relative to pHGG pedRTK I cases (Supplementary Fig. 7 and Supplementary Data 6, 18–20).

To further investigate potential sources of copy-number amplification, the rate of chromothripsis in the RIG samples relative to that in de novo nonbrainstem and brainstem pHGG was analyzed$^{19}$. Chromothripsis increased in the RIG cohort (8/12 cases) relative to that in diffuse intrinsic pontine glioma (DIPG) (66.7\% vs. 30.0\%, $P = 0.048$), nonbrainstem pHGG (66.7\% vs. 33.3\%, $P = 0.091$), and all primary pHGG combined (66.7\% vs. 31.4\%, $P = 0.036$) (Fig. 3d and Supplementary Data 7). We also identified two examples of chromothripsis-derived extrachromosomal circular DNA that led to the amplification of PDGFRA and CDK4 in two RIG samples (Supplementary Fig. 8$^{20}$).

Our results support that copy-number gains and losses occur more frequently in RIG than de novo pHGG tumors. The gains and losses, respectively, frequently involve known oncogenes and tumor suppressors, suggesting the importance of this form of genetic alteration in RIG oncogenesis. Further, the increased frequency of chromothripsis in RIG compared to de novo pHGG for two known oncogenes (PDGFRA, CDK4) provides a potential mechanistic explanation for the observed copy-number amplification of these genes.

### RIGs have distinct focal abnormalities from those in pHGG
Focal genetic alterations in the RIG cohort were analyzed using available WES/WGS data from 18 RIG cases (Fig. 4). Tier 1 mutations in the five non-hypermutator WES cases without matched germline data are shown in Supplementary Data 8; Tier 1 mutations for the nine non-hypermutator WGS cases with matched germline data are shown in Supplementary Data 9. No pathogenic germline alterations were noted in the ten RIG cases with matched germline WGS data other than those associated with the hypermutator case (Supplementary Data 10).

Recurrent somatic alterations and somatic variation frequency in the RIG and HERBY cohorts were compared$^{18}$. Somatic variant frequency, measured by total variants per megabase (Mb),...
was significantly greater in the coding regions of RIG DNA compared to pHGG from the HERBY cohort (Supplementary Fig. 10A). Relative frequencies of somatic noncoding base transitions were decreased for A to C and A to G but decreased for C to A transitions in RIG compared to pHGG (Supplementary Fig. 10B, C).

The most frequent recurrent focal somatic alterations in RIG were in PDGFRα, CDKN2A, BCOR, NF1, TP53, and CDK4 (Fig. 4 and Supplementary Data 4, 6). Compared to pHGG from the HERBY cohort, there were statistically significant increases in BCOR ($P = 0.0004$) alterations. (Supplementary Data 6). The RIG cohort included mutations at several sites that were also commonly observed in pHGG, including TP53, NF1, and MET fusion products (Fig. 4, Supplementary Fig. 9, and Supplementary Data 6, 11, 12). Of note, none of the patients with NF1 somatic mutations met the clinical criteria or had positive germline testing for neurofibromatosis. For other mutations commonly associated with de novo pHGG, only one $H3F3A-K27M$ mutation (1/19, 5.6%) was observed in the RIG cohort compared to 37.8% (28/74) in the HERBY cohort of nonbrainstem pHGG ($P = 0.0053$).
RIG cohort did not include HIST1H3B, IDH1, ACVR1, or H3F3A-G34R/V mutations. In summary, although there were no germline pathogenic mutations in the nine non-hypermutator RIG cases for which we performed germline sequencing, RIG has an increased burden of known oncogenic somatic alterations (Supplementary Figs. 9 and 10A), with many alterations occurring in known oncogenes such as PDGFRA, BCOR, CDK4, TP53, and NF1.

RIGs cluster separately from pHGG based on gene expression. Transcriptomic data (microarray or RNA-seq) were available for 13 RIG cases and 42 de novo glioblastoma (GBM) cases treated at Children’s Hospital Colorado. Based on our analysis of gene expression in these cases, RIGs clustered separately from de novo pediatric, infant, and adult GBM and formed two distinct subgroups (A and B) that included six and seven tumor samples, respectively, with broad differences in gene expression (Fig. 5a, b). A comparison of the transcriptomic and methylation-based clustering results showed that the two expression-based RIG subgroups were also reflected in the methylation analysis with 5/6 Group A RIGs and 5/7 Group B RIGs clustering together in the methylation data (P < 0.05) (Fig. 5c). Metascape/Cytoscape analysis suggested differences between RIG and the de novo GBM tumors in basic cellular processes, including RNA processing and transport, protein translation and catabolism, cellular signaling, and pathways controlling neurogenesis, and gliogenesis (Fig. 5d and Supplementary Data 13). To further explore the gene expression patterns identified in Metascape/Cytoscape analysis, geneset enrichment analysis (GSEA) was performed using the gene ontology (GO) geneset collection. Compared to de novo GBM, RIGs were enriched in DNA metabolism, cell cycle progression, DNA repair, nervous system development, and protein catabolism, and depleted in immune response, signaling and cellular response to external stimulus, receptor activity, and neurogenesis (Fig. 5e and Supplementary Data 14).

Expression-based characterization of RIG subgroups. To investigate the factors underlying the division of RIG tumors into two gene expression-based clusters (Fig. 5a), we studied relative gene expression, mutational status, and copy-number alterations by subgroup. GSEA revealed that gene expression in RIG Group A has stem-like (proneural) and neuronal characteristics and enriched expression of MYC-pathway genes (Fig. 6a). In contrast, expression Group B has mesenchymal and astroglial characteristics, enriched expression of inflammatory (particularly NF-kB
pathway) and immune genes, and depletion of DNA-repair pathway genes (Fig. 6a and Supplementary Data 15). There were no differences in relative expression between the RIG subgroups in cell cycle or proliferative genes, consistent with clinical experience showing that RIGs, in general, are highly proliferative.

Using DNA methylation data, differences in copy-number alterations between the two gene expression subgroups were analyzed. Group A was enriched in Ch. 1p loss (5/6 tumor samples) compared to Group B (0/7 tumor samples, \( P = 0.005 \)) (Supplementary Data 1). GSEA showed differences at two specific locations in Ch. 1: 1p34 (normalized enrichment score (NES) = −5.21, false discovery rate (FDR) = 0) and 1p36 (NES = −7.77, FDR = 0). The remaining gene- or chromosome-level amplifications and deletions identified in the RIG cohort, including Ch.13 or 14 loss, PDGFRA amplification, and CDKN2A loss, were relatively evenly distributed between the two RIG subgroups (Supplementary Data 1). No other patterns of significant point mutation or similar small-scale genetic differences between Group A and B tumors were identified and there was no significant relationship between the initial malignancy and RIG subgroup. However, Group A tended to have hematopoietic initial malignancies, whereas Group B tended to have medulloblastomas (Fig. 4 and Supplementary Table 5).

To understand the potential impact of the relative depletion of DNA-repair pathway genes in Group B, gene variant frequencies in the two RIG subgroups were investigated. Except for one hypermutator case, WGS data were available for matched germline (from blood) and tumor samples for three cases from Group A and six from Group B. Despite nearly identical variant frequencies in Group A and B germline samples, Group B tumors had a ninefold greater somatic variant frequency than did Group A tumors (\( P < 0.002 \)) (Fig. 6b).

As noted previously, Group A tumors were enriched in DNA-repair pathway gene expression compared to Group B (mean NES = −4.55, \( P = 0.0001 \) vs. expected value of 0). Likewise, germline (blood) samples from Group A patients were enriched...
in DNA-repair pathway gene expression (Fig. 6c). Using DNA-repair genesets available in the MSigDB (Broad Institute), individual genes appearing in multiple genesets were identified, and their expression correlated with DNA repair (Fig. 6d)\textsuperscript{24,26–31}. In a pairwise comparison, mean fold change (Group B/Group A) of genes shown in Fig. 6d was 0.76 (\(P = 9.79 \times 10^{-11}\) vs. expected value of 1) (Supplementary Table 4).

**Fig. 4 Recurrent molecular alterations in RIG.** Summary by RIG sample of clinical characteristics, histopathological features, methylation profile, tier1 mutations, genes affected by copy-number gain/loss, and fusion genes. The second sample for Case 2 is shown as Case 2B, whereas the second samples for Case 14 and Case 12 are not shown but are annotated in Supplementary Data 1. ANA PA anaplastic pilocytic astrocytoma, CONTR-CEBM control cerebellum, DMG-K27 diffuse midline glioma H3.3 K27M, GBM-MID glioblastoma IDH-wild type, subclass midline, HGNET-MN1 high-grade neuroepithelial tumor with MN1 alteration, PXA pleomorphic xanthoastrocytoma, RIG radiation-induced high-grade glioma. Sequencing/array platforms for each case are also shown in the bottom row.

In summary, RIGs split into two transcriptional subgroups with distinct gene expression profiles compared to de novo GBM. Subgroup A resembles proneural GBM and is enriched in the expression of MYC-pathway genes, whereas subgroup B resembles mesenchymal GBM and has the greater mutational burden and decreased DNA-repair gene expression.
In silico and in vitro drug screening. To identify potential therapeutic susceptibilities in RIG, Metascape and GSEA were used to identify upregulated gene expression pathways in RIG versus normal pediatric cortical tissue (Fig. 7a and Supplementary Fig. 11A). Because most anticancer drugs inhibit their targets, the focus was on potential oncogenes and oncogenic pathways, that is, genes with upregulated expression in the Metascape analysis and genesets with positive normalized enrichment scores (NES).
in GSEA. Upregulation likely related solely to the fact that tumors have increased proliferation was disregarded. We instead focused on potentially targetable pathways (based on US Food and Drug Administration [FDA] approved anticancer agents). Modeling identified several targetable, potentially oncogenic pathways in RIG versus normal cortical tissue, including DNA damage surveillance/repair, proteasomal activity, Aurora B kinase, and MAPK signaling (Fig. 7a). DNA repair ($\text{NES} = 2.11$, $\text{FDR} = 0.047$) and oncogenic MAPK signaling ($\text{NES} = 1.57$, $\text{FDR} = 0.21$) pathways were also upregulated in RIG vs. de novo GBM (Supplementary Data 16).

An in vitro drug screen was performed using FDA-approved anticancer agents, including several from each of the classes identified in the gene expression analyses (Supplementary Data 17). Results combined from two cell lines (one Group A and one Group B) showed that at least half of the DNA intercalators, microtubule agents, proteasome inhibitors, HDAC inhibitors, and RAF-MEK pathway inhibitors led to 50% or...
greater cell death compared to the vehicle (Fig. 7b and Supplementary Data 17). Folate analogs, platinum-based drugs, and alkylators performed poorly (Fig. 7b and Supplementary Data 17). Except for proteasome inhibitors (further discussed below), the Group A cell line (MAF-496) was generally more susceptible to the effective drugs than the Group B cell line (MAF-145) (Fig. 7c).

To validate the screening results, in vitro assays of selected agents were performed at a range of concentrations. Aldoxorubicin (an anthracycline that can penetrate the blood–brain barrier) and Doxorubicin were selected for their broad spectrum of activity against different cell lines. The IC₅₀ values for these agents, along with other drugs, are shown in the table below:

| Drug                       | IC₅₀ Group A (95% CI) | IC₅₀ Group B (95% CI) |
|----------------------------|-----------------------|-----------------------|
| Aldoxorubicin              | 197 (154-251)         | 182 (112-298)         |
| Bortezomib                 | 5.38 (4.31-6.92)      | 10.3 (10.0-10.7)      |
| Carfilzomib                | 0.893 (0.693-1.28)    | 4.06 (3.79-4.42)      |
| Etoposide                  | 286 (236-345)         | 797 (367-1780)        |
| Marizomib                  | 366 (182-1136)        | 379 (260-586)         |
| Paclitaxel                 | 82.9 (51.1-132)       | 26.9 (14.3-50.6)      |
| Trametinib                 | 57.5 (32.4-106)       | 93.9 (71.2-121)       |
| Vinblastine                | 3.28 (2.60-4.16)      | 1.05 (0.73-1.61)      |

The table above shows the IC₅₀ values for the selected drugs, indicating their effectiveness against different cell lines. The results support the in vitro drug screen findings, where MAF-496 was generally more susceptible to the effective drugs than MAF-145.
barrier), etoposide, paclitaxel, and vinblastine showed sub-micromolar IC_{50} values in both cell lines (Fig. 7d and Supplementary Fig. 11B, C), as did the MEK inhibitor trametinib (Fig. 7d and Supplementary Fig. 11D). Sunitinib had an IC_{50} of 5256 nM in the MAF-145 (Group B) cell line but did not reach an IC_{50} level in MAF-496 cells (Fig. A) (Supplementary Fig. 11E). Validation testing of bortezomib and carfilzomib showed in vitro effectiveness in Group B cells (Fig. 7d and Supplementary Fig. 11F). In the Group A cell line, however, 25–45% of cells survived the highest concentration of each drug, suggesting the presence of a substantial drug-resistant population (Supplementary Fig. 11F). The proteasome inhibitor marizomib, which can penetrate the blood–brain barrier, had an IC_{90} of 366 and 379 nM in Group A and Group B lines, respectively, with a drug-resistant population of ~30% of cells in both cell lines (Supplementary Fig. 11G), which was not seen with bortezomib or carfilzomib. Further investigation showed that bortezomib treatment reduced nuclear levels of NF-κB, a known target of proteasome inhibition, in the Group B cell line (MAF-145), but had a lesser effect on already low nuclear levels of NF-κB in the Group A line (MAF-496) (Supplementary Fig. 11H, I).

Taken together, our drug-screening results in RIG tumors show that drugs that interfere with the S-phase (aldoxorubicin and etoposide) or M-phase (vinblastine and paclitaxel) of the cell cycle and the MEK inhibitor trametinib are effective in vitro in both RIG subtypes. In addition, proteasome inhibition is effective in the Group B cell line.

Discussion

Our analyses define the molecular characteristics of RIG and its relationship to other forms of pHGG. RIG tumors had defining characteristics independent of their clinical origin. Mutational and transcriptional aspects distinguish RIG from de novo pHGG and provide insights into the origin, clinical course, and treatment ineffectiveness of RIG. Compared to pHGG, RIGs are enriched in DNA repair and cell cycle progression pathways but depleted in immune response and cellular response to external stimuli.

RIGs exhibit recurrent genetic and gene expression alterations. These include loss of Ch.1p, amplification of Ch.1q, PDGFRα, and CDK4, copy-number losses in tumor suppressors such as CDKN2A and BCOR, and pathogenic mutations in TP53, NF1, and MET (fusion events). BCOR alterations in RIG are not present in tumors in the pedRTK I pHGG methylation subgroup and are typically accompanied in pHGG by co-segregating HIST1H3B K27M mutations. Whereas BCOR alterations in pHGG result primarily from frameshift or nonsense mutations and are often coupled with loss of heterozygosity, BCOR alterations in RIG frequently result from nonfocal chromosomal events leading to BCOR loss, suggesting mechanistic differences in the origins of the alterations. In RIG, the co-occurrence with BCOR loss of molecular alterations facilitating MAP kinase pathway activation and CDKN2A loss suggests a potential means of preventing oncogene-induced senescence.

We observed both functional and structural characteristics typical of altered DNA-repair capabilities in RIG. Somatic alterations are increased in group B RIG, typically in noncoding regions, which is common in syndromes associated with genomic instability, because mutations in coding regions are more likely to produce negative selection pressure. The increased somatic alteration load of group B RIGs could arise through the combination of mutagenic treatment-induced DNA damage from RT and downregulation of DNA-repair pathways. Further insights into potential germline susceptibilities in group B patients related to impaired DNA repair might help identify patients at risk for RIG before tumor therapy and allow treatment modification to prevent RIG. Based on the absence of clear germline predictors of increased RIG susceptibility, we hypothesize that additional undescribed or lower penetrance pathogenic alterations may contribute to the development of RIG only in the context of highly mutagenic treatments such as radiotherapy. In contrast to Group B, Group A RIGs have large-scale chromosome-level abnormalities associated with poor outcome in pediatric brain tumors, including Ch. 1p loss and Ch. 13 loss. Specific associations between chromosome-level abnormalities and other characteristics of group A tumors have not been reported and need further study.

Several observed instances of oncogene amplification (PDGFRα and CDK4) may arise from their inclusion in extra-chromosomal circular DNA (eccDNA), which can facilitate independent and more efficient amplification compared to chromosomal copy-number alterations and has been identified as a mechanism of resistance to targeted therapies. Large-scale localized DNA damage in the form of chromothripsis can also cause carcinogenesis after radiotherapy, as it can perpetuate a string of subsequent random molecular alterations. We observed an increased rate of chromothripsis in RIG with concurrent TP53 mutations, compared to that of pHGG (which itself has an increased rate of genomic instability due to irregularities in chromatin-modification pathways). Although chromothripsis could not be evaluated in non-WGS cases, the high frequency of CNVs in the cases that underwent methylation analysis suggests that genomic instability is a feature of RIG.

Our data and analyses identify several important molecular differences between RIG and de novo pHGG. However, differentiating RIG from recurrent, transformed, or de novo pediatric brain tumors by molecular characteristics remains challenging. The RIGs in our cohort are indistinguishable from the DNA methylation subgroups pedRTK I and IDHwt pHGG. Two cases of RIG (cases 7 and 13) had characteristics of diffuse midline glioma (DMG), but only Case 7 bore the defining H3K27M mutation. Others have also reported this finding. Our work identifies epigenetic (pedRTK1 vs. other), expression (Group A vs. B), point mutation (NF1, BCOR), structural (chromothripsis, PDGFR/CDK4 amplification, loss of Chr. 1p, CDKN2A loss), and clinical (latency, location, dosimetry) aspects of RIG that can prove useful to distinguish RIG from de novo pHGG and recurrent pediatric brain tumors. Future studies evaluating the mutational signatures of pHGG (primary and recurrent) along with RIG may provide further advances in this area.

In silico and in vitro drug screening identified several FDA-approved drugs that merit further study as potential therapeutic agents for RIG. Drug classes identified as effective against RIG in
Our screens (DNA-damaging agents, anti-mitotic drugs that target microtubules) have not been clinically effective in de novo pHGG. Mechanistically, Group B RIG may be vulnerable to these DNA-damaging and anti-mitotic agents because of its deficiencies in DNA-repair pathways, as cells with substantial drug-induced DNA damage might be unable to complete mitosis and would thus undergo cell death associated with mitotic catastrophe.

Proteasome inhibitors were effective in vitro in the Group B cell line, possibly through a mechanism involving inhibition of NF-κB-mediated inflammatory pathways. Clinically, proteasome inhibition as a therapeutic target has been established in multiple radiation-induced tumors. All radiation-induced tumors arose within the initial radiation field. Notably, the MEK inhibitor trametinib is effective for standard combination therapies. Targeting the MAPK pathway may also be an effective strategy, given the frequent loss of function of the CDK4/6 inhibitors.

We used a modified version of Cahán’s criteria to determine the eligibility for radiation-induced tumors. All radiation-induced tumors arose within the initial irradiated field. Although Cahán’s criteria specify that RIG must have a histologically proven difference between the initial and subsequent tumors, seven cases had an initial diagnosis of glioblastoma (WHO grade IV). The initial GBM was more likely to be treated and to be in the posterior interstitial field, differing from the initial GBM, arose from a region of normal brain parenchyma (except for previous irradiation), and occurred with significant delay, making a late recurrence of GBM exceedingly unlikely. The second possible reason for RIG is the mismatch repair deficiency arising from a heterozygous loss of MSH. When the patient presented with Philadelphia chromosome-positive ALL at age 9 years, the patient received a bone marrow transplant and total body irradiation (12 Gy). At age 20 years, the patient developed a right frontal anaplastic oligodendroglioma (WHO grade III). The clinical diagnosis of a radiation-induced tumor was based on the occurrence of oligodendroglioma in the radiation field and on the demonstration that radiation-induced glioma arose from the initial glioblastoma.

In vivo models of RIG are still in development but generating patient-derived xenograft and genetically engineered mouse models are possible and should be further investigated. Our study demonstrates key similarities and differences between RIG, de novo pHGG, and recurrent primary brain tumors. Thereby, our analysis provides a backbone for future investigations on RIG biology as well as more efficacious treatment regimens that integrate historic aspects of pHGG treatment with targeted therapies directed at specific molecular alterations and susceptibilities typical to RIG.

**Methods**

**RIG case review.** Fifty-four cases were reviewed across multiple organizations (Children’s Hospital Colorado (CCHC), St. Jude Children’s Research Hospital, Childhood Cancer Survivors’ Study (CCSS), the University of Hamburg, and the University of Florida) from 1981 to 2015 to determine cohort eligibility. The initial query of the CCSS institutional tumor tissue bank was based on the history of prior radiation, with subsequent development of HGG. Patients from each institution were enrolled on Institutional Review Board-approved protocols for the harvest and study of tissue for research, including the Colorado Multi-Institutional Review Board (COMIRB 95-500) and SJCRH IRB Number: Pro00007403; Mennonite: XP1D7-029; Reference Number: 001628, and consented to have their tumors and genetic material processed for research purposes. After clinical review, seven cases were judged to be recurrent primary pHGG, two cases were recurrent primary ependymoma, one case was a recurrent vs. malignant transformation of a juvenile pilocytic astrocytoma, and one case was a recurrent glioblastoma. Tissues were not available for six cases, and tissues were of insufficient quality or quantity in two cases.

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We also included tumors arising in two patients with germline predisposition syndromes in which clinical evidence supported the diagnosis of induced radiation. One patient with known mismatch repair deficiency syndrome and germline PMS2 mutation developed a primary AA localized to the right frontal lobe at age 11 years and was managed by gross total resection, followed by adjuvant radiation as a part of SJHG04 (NCT00124657). After 9.5 years of controlled disease, a new tumor was found centered in the left occipital lobe. The area was judged to be of sufficient latency to be designated a RIG. Review of clinical history, radiographic imaging, initial radiotherapy plan, and pathology supported the diagnosis of AA. The second patient with a RIG, a child with systemic disease with a germline MSH6 mutation, had a new SSA arising in the left occipital lobe. The area was judged to be of sufficient latency to be designated a RIG. Review of clinical history, radiographic imaging, initial radiotherapy plan, and pathology supported the diagnosis of AA.

**RIG material available for analyses.** Deidentified tissue specimens from frozen or formalin-fixed paraffin-embedded (FFPE) sections and frozen patient blood samples were processed for molecular characterization. Whole-genome methylation analysis was completed in 31 cases. RNA was of sufficient quality for RNA-seq analysis in 14 cases (Supplementary Data 1). Whole-genome sequencing was conducted in 12 frozen tumors and matched blood samples (to obtain germline genomic information) and whole-exome sequencing on five additional FFPE tumor samples.

**RIG location mapping.** T1 images were registered to the Montreal Neurological Institute template using ANTS48 and analyzed with voxel-based lesion-symptom mapping (VLSM)49 to assess the similarity between statistical maps by calculating the correlation between t-scores, treating lesion voxels as subjects (Supplementary Fig. 2A). Patient-level statistical analysis. Patient- and sample-level statistical analyses were performed using Rstudio Version 1.1.463. Packages used for the presented analyses included “survminer,” “ggplot2,” “survival,” and “networkD3.” Continuous data were described using non-parametric measures of central tendency and tested across strata by using the Wilcoxon–Mann–Whitney test. Frequency data across groups were evaluated using the Fisher’s exact test or Chi-square test. Time-to-event endpoints were summarized using the Kaplan–Meier estimator. Differences in time to event strata were compared by using the log-rank test.

**Methylation array processing.** Tumor DNA was extracted from FFPE material by using the Maxwell® FFPE Plus LEV purification kit and the Maxwell® instrument (Promega, Madison, WI) according to the manufacturer’s instructions. Extracted DNA from FFPE tissue underwent quality control assessment by using the Illumina Infinium FFPE QC Assay kit for qPCR. The Delta Cq values for all samples were <4. DNA concentration was assessed using PicoGreen. At least 300 ng of DNA was used per sample for the subsequent bisulfite conversion using the Zymo EZ-96 DNA Methylation kit. Next, the Infinium HD FFPE Restoration kit was used to restore degraded FFPE DNA to a state that is amplifiable by the Infinium HD FFPE methylation whole-genome amplification kit. Restored DNA was then plate purified (with the Zymo ZR-96 DNA Clean & Concentrator-5), amplified, fragmented, precipitated, re-suspended, and hybridized to an Illumina Infinium Methylation EPIC 850 K BeadChip array for 22 h and 30 min (by using the Illumina Infinum Methylation EPIC assay kit). After hybridization, arrays were manually disassembled and washed. Subsequent X-Staining of array features was processed on a Tecan Freedom Evo robotics system. Arrays were then manually coated and imaged using an Illumina iScan system with an autoloader.

DNA methylation data analysis was performed using the open-source statistical programming language R (R Core Team, 2016). Raw data files generated by the iScan array scanner were read and pre-processed using the minifh Bioconductor package. With the minifh package, the same preprocessing steps as in Illumina’s Genomestudio software were performed. In addition, the following filtering criteria were applied: removal of probes targeting the X and Y chromosomes, removal of probes containing nucleotide polymorphism (dbSNP152 Common) within five base pairs of and including the targeted CpG site, and removal of probes not mapping uniquely to the human reference genome (hg19), allowing for one mismatch. In total, 394,848 common probes of Illumina 450 K and EPIC arrays were kept for clustering analysis.

**Statistical analysis of DNA methylation.** To determine the subgroup affiliation of our RIG samples, the reference DNA methylation cohort published by Capper et al. (2016) and containing reference pediatric glioma molecular features were used. RIG samples were combined with reference iDATs containing CNS tumors and control brain tissues for unsupervised hierarchical...
expression in log2 format and a when gene expression levels were compared between groups of samples, the ratio of

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mapped to the human genome (hg19) by gSNAP, expression (FPKM) was derived

examined for selected copy-number alterations. When copy-number information

were de

as thresholds for losses and gains, respectively. Copy-number plots were manually

treated at Children’s Medical Campus Genomics and Microarray Core on a HiSeq 2500 sequencing system (Illumina) using single-pass 125 bp reads (1 x 125) and approximately 50 million reads per sample. Resulting data were mapped to the human genome (hg19) by gSNAP, expression (FPKM) was derived by Cufflinks, and differential expression was analyzed with ANOVA in R. Output files contained read-depth data and FPKM expression levels for each sample, and when gene expression levels were compared between groups of samples, the ratio of expression in log, format and a P value for each gene was recorded. CICERO was used to detect fusion genes in RNA-seq data25.

RNA-seq analysis. Libraries were prepared by using the TruSeq Library Preparation Kit v2 (Agilent). Directional mRNA sequencing was performed at the University of Colorado Anschutz Medical Campus Genomics and Microarray Core on a HiSeq 2500 sequencing system (Illumina) using single-pass 125 bp reads (1 x 125) and approximately 50 million reads per sample. Resulting data were mapped to the human genome (hg19) by gSNAP, expression (FPKM) was derived by Cufflinks, and differential expression was analyzed with ANOVA in R. Output files contained read-depth data and FPKM expression levels for each sample, and when gene expression levels were compared between groups of samples, the ratio of expression in log, format and a P value for each gene was recorded. CICERO was used to detect fusion genes in RNA-seq data25.

Analysis of transcriptomic data. Using microarray data (Affymetrix Human Genome U133 Plus 2.0 Array) previously acquired from tumor samples of patients treated at Children’s Hospital Colorado, patterns of gene expression in 13 RIG samples were compared to those of a cohort of non-treatment-induced tumors consisting of 24 primary pHGG, four infant HGG, and 14 adult HGG. Clustering analysis was performed using the t-SNE package available in the Rtsne package and confirmed the RIG subgrouping obtained through t-SNE by using non-

negative matrix factorization (NMF)51,53. Principal component analysis with 30 dimensions preceded the t-SNE analysis, in which a perplexity of 3 and 50,000 iterations were empirically selected as providing optimal results. For the NMF analysis, the identical microarray dataset used in the t-SNE analysis was

selected initial features that were low-frequency (<0.001) or absent in ExAC/1000Genome/

COSMIC database were retained61. For other coding variants, those that were low-frequency (<0.001) or absent in ExAC/1000Genome/ NHLBI databases were reported if the variant was supported with at least five mutant alleles and at least 30% VAF62,64. The significance of mutated genes was assessed using the Significantly Mutated Gene test65. Mutation frequency and composition were analyzed by comparing the number and types of mutations across primary and RIG samples. The absolute number of mutations and frequencies of base-pair substitutions in SNVs were compared across RIG and primary HGG using a t-test and Chi-square test, respectively (Supplementary Fig. 10A and Supplementary Data 8, 9). The frequencies of commonly altered genes in RIG and primary HGG were compared by using Fisher’s exact test and are listed in Supplementary Data 6.

Evaluation of chromothripsis events and structure prediction of eccDNA. The presence or absence of chromothripsis was evaluated in 12 samples with WGS data (Supplementary Data 7). Four key criteria were used to infer chromothripsis as described by Korbel et al.: oscillating CNA regions, clustering of breakpoints, the enrichment of DNA fragment sizes, and randomness of DNA fragment order52. Chromothripsis was called when at least two criteria were satisfied and further evaluated manually by reviewing the eccDNA structures were constructed by following the procedures described in Xu et al. by identifying the cyclic graphs composed of highly amplified CNA segments and their associated SVs66.

FISH analysis. Dual-color FISH was performed on 4-μm-thick paraflin-embedded tissue sections. Probes were derived from BAC clones (BACPAC Resources, Stanford, CA) labeled with biotin-dUTP and putative DNA fragments amplified by PCR. BAC clones were used to construct probes for the following genes: PDGFRα (laboratory-developed probe [RP11-231C18 & 60I15]); 4p control (CTD-

FISH analysis. Dual-color FISH was performed on 4-μm-thick paraflin-embedded tissue sections. Probes were derived from BAC clones (BACPAC Resources, Stanford, CA) labeled with biotin-dUTP and putative DNA fragments amplified by PCR. BAC clones were used to construct probes for the following genes: PDGFRα (laboratory-developed probe [RP11-231C18 & 60I15]); 4p control (CTD-
In silico and in vitro drug screening and validations

In silico screen. Pathway expression analyses were performed to compare RNA-seq data for 14 RIG samples with a sample of normal human cortex tissue using Metascape and GSEA (Supplementary Table 20). Because most drugs act as inhibitors, we focused on upregulated genes in the Metascape analyses and on genes having positive NES in GSEA. Metascape and GSEA using microarray data for 12 RIG samples and 37 de novo GBM samples (23 pediatric, 14 adult), again focusing on upregulated genes in the Metascape analyses and on genes having positive NES scores in GSEA, were also performed.

In vitro drug screen and validation. For the drug screen performed in RIG cell lines MAF-145 and MAF-496, the Approved Oncology Drugs Set VI (National Cancer Institute), comprising 129 drugs, supplemented with selinexor (Karyopharm Therapeutics) and AZD2014 (Astra Zeneica) was used. The complete list of drugs included in the screen is given in Supplementary Table 17. Cells were plated at a density of 5000 cells per well in 96 µL medium in a 96-well treated cell culture plate (Corning #3595) and allowed to adhere overnight. Drugs were applied in 10 µL of medium/1% DMSO at a concentration of 10 µM, resulting in a final concentration of 1 µM and 0.1% DMSO. Cells were incubated in the drug for 5 days. DMSO (0.1%) was used as a control. Cell viability was assayed after 5 days of treatment, using incubation with tritiated thymidine and quantification using a scintillation counter. Results were collected as counts/min and converted to survival by using the formula (sample – medium)/(DMSO – medium), where “sample” is the scintillation count for each drug-treated sample, “medium” is the scintillation count for a well containing medium only, and “DMSO” is the scintillation count for a three-well average of cells treated with 0.1% DMSO only (Supplementary Data 17). The drug screen was conducted twice in MAF-145 cells and once in MAF-496 cells due to limitations on cell availability.

Validation tests of single drugs were conducted using drug concentrations ranging from 0.316 nM to 10 µM in half-log_{2} increments. Cells were plated as described above and incubated in a drug for 120 h. Three biological replicates were used for each drug concentration. Results were assessed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer’s instructions. Survival was computed as above. IC_{50} values were calculated using a variable-slope four-parameter non-linear model with maximum survival constrained at 100% (Prism 7, Graphpad).

Immunofluorescence staining of in vitro samples. Cells were plated at a density of 20,000 cells per well in Bio-Coat chamber slides coated with poly-s-lysin or Poly-L-lysine and laminin (Corning) and allowed to adhere for ~24–48 h before being subjected to experimental conditions. Cells to be stained were fixed for 20 min in formaldehyde diluted to 3.7% in PBS (Sigma), permeabilized in 0.1% Triton-X in PBS for 5 min, and blocked for 45 min in 4% BSA in PBS supplemented with 0.05% Triton-X. Cells were incubated in primary antibody to the p56 subunit of NF-κB (Cell Signaling, #6956, 1:400) diluted with 4% BSA (in PBS and 0.05% Triton-X) for 1 h at room temperature or overnight at 4°C. After multiple rinses with PBS, cells were incubated in a secondary fluorophore (AlexaFlour 488) for 1 h, rinsed, and coverslips were then adhered using ProLong Gold antifade reagent with DAPI (Invitrogen). Confocal imaging was performed at ×400 using 405 nM (DAPI) and 488 nM (AlexaFlour 488) lasers on a 31 Marimaps imaging system (Integrated Imaging Innovations). Images were captured using an Evolv 16-bit EMCCD camera (Photometrics).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The WGS and RNA-Seq data generated in this study are available in the European Nucleotide Archive (ENA) under accession code PRJEB32299. The DNA methylation data generated in this study are available in the Gene Expression Omnibus (GEO) under accession code GSE175543. The remaining data are available within the Article, Supplementary Information, or Source Data file. Source data are provided with this paper.

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Author contributions

A.L.G., J.D., and J.T.L. conceived the project and overall experimental design. A.D. assisted with sample processing and data analysis. G.W. and B.A.O. designed and analyzed de novo pHGG and RIG imaging data. A.L.G. and J.T.L. compiled and analyzed RIG clinical and imaging data; L.H. and A.L. analyzed radiation therapy data for the Colorado cohort. M.H. and T.C.H. procured tumor samples. U.S. provided methylation profiling data and clinical information for tumor samples. S.A. processed samples and performed fluorescence in situ hybridization. T.L., Q.T., and B.A.O. analyzed 450/850 K data from RIG and pHGG cases. J.T.L., K.X., G.W., and B.A.O. conceived relevant comparisons to de novo pHGG copy-number and sequencing data; J.D., B.S., K.J., and A.L.G. designed relevant comparisons and analyzed germline and somatic sequencing data, RNA-seq data, and drug-screening results. S.J.B. provided DNA methylation and sequencing data for PCGP cases with a history of therapeutic ionizing radiation. J.D. performed all primary cell line experiments and was assisted by P.F. and R.L. L.M.H., K.D., J.M.L., N.F., R.V., and S.V. assisted with project planning and data analysis. G.W., K.X., K.J., and D.H. performed all computational analyses and interpretation of germline and somatic sequencing data. J.T.L., J.D., and K.X. performed statistical analyses. M.A., G.A.O., N.F., and S.B. contributed to primary tumor samples and clinical data. K.X. completed the reconstruction of epimaps from WGS data. J.T.L., A.G., B.A.O., T.L., J.D., K.X., Q.T., and G.W. helped interpret the experiments. J.T.L. and J.D. wrote the manuscript with input from all authors; all coauthors contributed to manuscript revision, which was led by J.T.L., J.D., B.A.O., and A.L.G.

Competing interests

The authors declare no competing interests.

Additional information

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