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Radiation-induced DNA damage cooperates with heterozygosity of TP53 and PTEN to generate high grade gliomas

Pavlina K. Todorova¹, Eliot Fletcher-Sananikone¹, Bipasha Mukherjee¹, Rahul Kollipara², Vamsidhara Vemireddy³, Xian-Jin Xie⁴, Peter M. Guida⁵, Michael D. Story¹, Kimmo Hatanpaa⁶, Aryn A. Habib^{3,7}, Ralf Kittler², Robert Bachoo³, Robert Hromas⁸, John R. Floyd⁹, and Sandeep Burma^{1,9,*}

¹Department of Radiation Oncology, University of Texas Southwestern Medical Center, Dallas, TX

²Eugene McDermott Center for Human Growth and Development, University of Texas Southwestern Medical Center, Dallas, TX

³Department of Neurology and Neurotherapeutics, University of Texas Southwestern Medical Center, Dallas, TX

⁴College of Dentistry and College of Public Health, University of Iowa, Iowa City, IA

⁵Biology Department, Brookhaven National Laboratory, Upton, NY

⁶Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX

⁷Veterans Affairs North Texas Health Care System, Dallas, TX

⁸Department of Medicine, University of Texas Health, San Antonio, TX

⁹Department of Neurosurgery, University of Texas Health, San Antonio, TX

Abstract

Glioblastomas (GBM) are lethal brain tumors which are treated with conventional radiation (X-rays and gamma rays) or particle radiation (protons and carbon ions). Paradoxically, radiation is also a risk factor for GBM development, raising the possibility that radiotherapy of brain tumors could promote tumor recurrence or trigger secondary gliomas. In this study, we determined whether tumor suppressor losses commonly displayed by GBM patients confer susceptibility to radiation-induced glioma. Mice with Nestin-Cre-driven deletions of Trp53 and Pten alleles were intracranially irradiated with X-rays or charged particles of increasing atomic number and linear energy transfer (LET). Mice with loss of one allele each of Trp53 and Pten did not develop spontaneous gliomas but were highly-susceptible to radiation-induced gliomagenesis. Tumor development frequency after exposure to high-LET particle radiation was significantly higher compared to X-rays, in accordance with the irreparability of DNA double-strand breaks (DSB) induced by high-LET radiation. All resultant gliomas, regardless of radiation quality, presented histopathological features of grade IV lesions and harbored populations of cancer stem-like cells

*To whom correspondence should be addressed: Division of Molecular Radiation Biology, Department of Radiation Oncology, University of Texas Southwestern Medical Center, 2201 Inwood Road, NC7.208, Dallas, TX 75390, Phone: (214) 648-7440; Sandeep.Burma@UTSouthwestern.edu.

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with tumor-propagating properties. Furthermore, all tumors displayed concomitant loss of heterozygosity of Trp53 and Pten along with frequent amplification of the Met receptor tyrosine kinase, which conferred a stem cell phenotype to tumor cells. Our results demonstrate that radiation-induced DSB cooperate with pre-existing tumor suppressor losses to generate high grade gliomas. Moreover, our mouse model can be used for studies on radiation-induced development of GBM and therapeutic strategies.

Keywords

glioblastoma; ionizing radiation; particle radiation; TP53; PTEN; MET

Introduction

Glioblastomas (GBM) are the most aggressive tumors of the adult central nervous system (CNS) [1, 2]. Although the median age at clinical presentation is 64 years, these tumors also occur in children, making them a leading cause of cancer-associated deaths in both adult and pediatric patients [3, 4]. GBM patients are subjected to an aggressive therapy regimen comprising de-bulking surgery, radiotherapy (50–60 Gy), and chemotherapy with temozolomide [1]. Unfortunately, populations of glioma stem-like cells and infiltrating tumor cells can survive radio-chemotherapy, acquire additional mutations, and give rise to therapy-resistant recurrent GBM [5–9]. Thus, tumor control is always transient, and recurrence leads to mortality, usually within 14 months of diagnosis.

Although the genomic changes occurring in GBM have been studied extensively [10–12], the etiology of the disease remains poorly understood. Ionizing radiation (IR), which is administered to a majority of glioma patients, is paradoxically the only known risk factor for GBM development [13, 14]. The Life Span Study of survivors of the Hiroshima and Nagasaki atomic bombings demonstrates that survivors have a clear radiation-related excess risk of cancer (including brain) throughout life, which becomes statistically significant at doses above 0.1 Gy [15]. In accordance with the Life Span Study, even low doses of radiation (<0.1 Gy), commonly delivered for medical diagnostics, increase the relative risk of de novo brain tumors [13, 14]. Moreover, patients undergoing CNS irradiation (50–60 Gy) for non-GBM-related malignancies present with radiation-induced gliomas (RIG) later in life [13, 16–18]. Clinical reports describing such secondary RIGs suggest that the disease progression may be worse than in sporadic GBM [16, 19–21]. The link between tumor aggressiveness and exposure to IR is further evident from recurrent gliomas, inevitably arising after GBM radiotherapy, which are more therapy-resistant compared to radiotherapy-naïve tumors [22]. Conventional radiation treatment volumes for primary GBM include a 2–3 cm margin around the tumor, and a majority of recurrences occur at or near this margin [23]. This raises the possibility that radiation could select for therapy-resistant cancer cells or perhaps even transform premalignant clones within the tumor margin. Taken together, these studies clearly show that radiation exposure can trigger primary gliomas, while radiotherapy could potentially promote GBM recurrence and aggressiveness. Thus, understanding the mechanisms underlying RIG development and progression is important

both for prevention of radiation-induced gliomas and long-term tumor control after radiotherapy.

The nature of radiation-induced damage is dependent on ionization of the target tissue, which, in turn, is dictated by the amount and distribution of energy deposited per unit distance traveled, a quality referred to as the linear-energy transfer (LET). IR can be fundamentally divided into two categories: a) electromagnetic radiation (X-rays, gamma rays) which have low LET, and b) particle radiation (accelerated ions) which typically have high LET [24]. Charged particles, unlike electromagnetic radiation, can directly collide with the DNA double-helix causing clustered double-strand breaks (DSBs) that are poorly repaired and trigger genomic instability [25, 26]. Charged particles, especially hydrogen ions (protons) and carbon ions, are being increasingly used for cancer therapy including that of glioblastoma due to better dose distribution and higher relative biological effectiveness [24, 27, 28]. However, while the cancer risks related to electromagnetic radiation are well-documented [16], the adverse effects of particle radiation are poorly understood because of its relatively recent adoption as a treatment modality [29]. Moreover, accelerated nuclei of naturally abundant elements like silicon and iron comprise Galactic Cosmic Radiation (GCR), the environmental radiation present in space [30, 31]. Since current spacecraft shielding materials cannot protect from these nuclei, exposure to charged particles poses an ill-defined cancer risk to astronauts on long-duration missions [30]. As charged particles potentially carry a greater risk of cancer development, and because of their increasing use in the treatment of a host of cancers, experimental model systems are needed to fully understand how low- versus high-LET radiation triggers malignancies, especially therapy-resistant cancers like GBM.

Comprehensive genomic and gene expression studies have revealed that all GBMs carry alterations in the TP53, RB1, and PTEN tumor suppressor pathways [10, 11]. We previously established that susceptibility to radiation-induced gliomagenesis in the absence of oncogene activation is conferred by *p14^{ARF}*, *p16^{INK4A}* and *p15^{INK4B}* losses [32]. In GBMs, deletion of *p14^{ARF}* and *p16^{INK4A}* occurs in a somewhat mutually-exclusive manner with deletions/mutations of *TP53* [33, 34]. In this study, we examined cooperativity between low doses of IR (0.5 Gy) and loss of *Trp53* and/or *Pten* in GBM development using mice with brain-targeted deletions of these tumor suppressor genes (TSGs). We find that loss of one allele of *Trp53* and *Pten* each is sufficient to render these mice highly-susceptible to radiation-induced gliomagenesis in an LET-dependent manner. The radiogenic gliomas phenocopy human grade IV disease, have a prominent cancer stem cell phenotype, and exhibit frequent amplification of the Met receptor tyrosine kinase (RTK) in combination with loss of heterozygosity (LOH) of *Trp53* and *Pten*. Our results show that radiation-induced DSBs can cooperate with pre-existing TSG losses to generate high grade gliomas, and provide a valuable mouse model that can be used to understand mechanisms underlying radiation-induced GBM development and recurrence.

Materials and Methods

Irradiations

Particle beams of hydrogen, carbon, silicon, and iron were provided by the National Aeronautics and Space Administration (NASA) Space Radiation Laboratory at Brookhaven National Laboratory (BNL), as described [26, 35]. Particle energies and LET values are shown in Figure 2A. X-ray irradiations were carried out using an X-RAD 320 device (Precision X-ray, North Branford, CT).

Animal studies

All animal studies were performed under protocols approved by the Institutional Animal Care and Use Committees of The University of Texas Southwestern Medical Center and BNL. Mice were obtained from the Mouse Cancer Model Consortium. Cohorts of 4–8 weeks old mixed gender, mixed strain mice (FVB/NJ-C57BL6 mixed background) with brain-specific Nestin-Cre (^{NesCre}) driven LoxP-excision of *p53* (exons 2–10) and *Pten* (exon 5) alleles [36, 37] were irradiated with a single intracranial dose of 0.5 Gy. Mice were monitored daily up to 15 months post-IR for neurological symptoms (lack of clasp reflex, ataxia, and seizures) and for additional symptoms such as hydrocephalus, lethargy, or weight loss. Moribund mice were euthanized, and brains were examined for evidence of tumors by serial sectioning and hematoxylin and eosin (H&E) staining. Tumor classifications were assigned by the study neuropathologist (K.H.) based on the World Health Organization (WHO) criteria [38]. To rule out occult lesions, all brains from asymptomatic mice were similarly screened at the conclusion of the experiment.

Glioma ex-vivo cultures

Brains of tumor-bearing mice were micro-dissected and glioma tissue was mechanically and enzymatically disaggregated for the generation of ex-vivo cultures. The cultures were plated as single cells in serum-free DMEM/F-12 1:1 media (Life Technologies) supplemented with B27 without Vitamin A (Life Technologies), 10 ng/mL EGF (Peprotech), and 10 ng/mL basic FGF (Peprotech), and grown as tumor neurospheres. Every 3–4 days, the cells were dissociated by triturating with Accutase (Sigma-Aldrich) and sub-cultured, as described previously [39]. All cell lines were routinely mycoplasma tested using the MycoAlert mycoplasma detection kit (Lonza). For serum-induced differentiation studies, the neurosphere cultures were dissociated into single cells and the growth medium was switched to Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Hyclone). The cells were grown under these conditions for 7–10 days and assessed for expression of differentiation markers. For sphere formation assays, cells were dissociated and plated at single-cell density in Nunclon Sphera Surface ultra-low adhesion 96-well plates (Thermo Fisher) in neurosphere medium. Sphere numbers per 96-well plate were quantified after 12 days. Primary astrocytes were isolated from 3-day old pups and cultured in DMEM supplemented with 10% FBS.

Subcutaneous and intracranial injections

Subcutaneous and intracranial tumors were generated as previously described [39]. For subcutaneous tumors, cells (as indicated) were suspended in 100 μ L of equal volumes of phosphate buffered saline (Fisher Scientific) and Matrigel (Corning), and injected into the flanks of 6-week old Nu/Nu nude mice (Charles River Laboratories International, Stock#88). Tumors were measured with digital calipers (Fisher Scientific), and tumor volumes were calculated (volume = length $\times$ width $\times$ height $\times$ 0.5). For intracranial stereotactic injections, 500 cells were suspended in PBS (3 μ L) and delivered into the right corpus striatum of the brains of 6-week-old syngeneic mice (FVB/NJ-C57BL6 mixed background). The mice were monitored daily for neurological deficits and euthanized when they became moribund.

Immunohistochemistry and immunofluorescence

Following cardiac perfusion with cold phosphate buffered saline (Fisher Scientific), brain and tumor tissues were dissected and fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned at 5 μ m thickness. Tumor tissue sections were deparaffinized, rehydrated and stained using standard immunohistochemistry and immunofluorescence protocols, as described before [26, 32, 39]. The following antibodies were used: phospho-Erk (Thr202/Tyr204), Ki67/Mib-1, Met, phospho-Met (Tyr1234/1235), Pten, phospho-S6 ribosomal protein (Ser235/236) (Cell Signaling Technology), Gfap (Biocare Medical), Olig2, Sox2 (Abcam), Tuj1 (Sigma), 53BP1 (Santa Cruz Biotechnology), horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories), and Alexa488/568-conjugated secondary antibodies (Thermo Fisher). For immunofluorescence, 4',6-diamidino-2-phenylindole counterstain (DAPI, Vector Labs) was used to visualize nuclei. Ki67 indices were quantified by manually counting at least 500 nuclei in the area of highest staining, as described [40]. All immunohistochemistry images were acquired on a Keyence BZ-X800 microscope using the Keyence BZ-X software (Keyence). All immunofluorescence images were acquired on a Zeiss Axio Imager 2 microscope using the Zen Imaging software (Carl Zeiss).

Immunoblotting

Whole-cell SDS extracts were prepared and assayed by western blotting, as described previously [41]. The following antibodies were used: Actin (Sigma-Aldrich), Akt, phospho-Akt (Ser473), Met, phospho-Met (Tyr1234/1235), p53, phospho-p53 (Ser15), Pten (Cell Signaling Technology), Sox2 (Abcam), V5 (Thermo Fisher), and horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad).

PCR and Quantitative real-time PCR

Genomic DNA was extracted from flash-frozen kidney, pancreas, brain, and glioma tissues using the DNeasy Blood and Tissue Kit (Qiagen). Polymerase Chain Reactions (PCR) were performed using the DreamTaq Green PCR Master Mix (2X) (Thermo Fisher). Each sample (250 ng total DNA) was amplified with primers specific to the wild-type (WT) and Lox-P-flanked (f) variants of *p53* and *Pten*. Total RNA was extracted from flash-frozen tissue or cultured cells using the RNeasy Mini Kit (Qiagen). Reverse Transcription Polymerase Chain

Reactions (RT-PCR) were performed using the Superscript III 1st Strand Kit (Thermo Fisher), and Quantitative Polymerase Chain Reaction (qPCR) was performed with a Bio-Rad CFX 96 Touch Real-Time PCR Detection system (Bio-Rad) using the SYBR Green detection system (Applied Biosystems). All samples were amplified in triplicate. Relative expression of each gene was normalized to 18s mRNA. All primers are listed in Supplementary Table 1.

Array comparative genomic hybridization

Genomic DNA was extracted from flash-frozen normal brain or glioma tissue from NesCre^{p53+/f};Pten^{+/f} animals using the DNeasy Blood and Tissue Kit (Qiagen). Tumor and control DNA samples were hybridized to Sure Print G3 Mouse CGH Microarray 1 × 1M (Agilent); the probes were annotated against the NCBI Build 37 (USSC mm9). Hybridization was automated using an Agilent Hybridization SureHyb chamber kit and gasket slide kit (Agilent), and the arrays were scanned at 2 µm resolution using a fluorescence scanner (Tecan). Feature extraction of raw signals was performed using the ImaGene 9.0 program. Raw signals were imported in Partek (Partek Inc.), and raw copy number calls from Partek were used as input for cghFlasso R package. cghFlasso uses fused lasso regression method to smoothen the raw calls and defines hotspots within the data. This CNV data was merged into segments using circular binary segmentation (CBS) algorithm. Regions of the genome that were significantly amplified or deleted, and the likely gene targets within those regions were identified by the GISTIC2.0 module (q value < 0.05 and G-score > 1.5 cutoffs) [42]. The raw CN calls were visualized using circos (v. 0.69.3). Array comparative genomic hybridization data has been deposited with the NCBI GEO (accession number GSE130677).

Lentiviral transduction

Human MET cDNA was obtained from Addgene (gift from Joan Brugge; Addgene, Cambridge, MA, USA; plasmid no. 17493) and sequentially cloned into pLenti6.3/V5-DEST using the Gateway Cloning system (Thermo Fisher). Virus production was carried out in 293T cells (ATCC) using ViraPower Lentiviral Packaging Mix (Thermo Fisher), as specified by the manufacturer. Transfection by Lipofectamine 2000 (Thermo Fisher) was performed according to the manufacturer's protocol. The lentiviral supernatant was harvested at 48h post transfection and concentrated using LentiX Concentrator (Clontech). Target cells were transduced with viral particles at a multiplicity of infection (MOI) of 10 with 4 µg/ml polybrene (Santa Cruz Biotechnology). Inoculated cells were maintained under selection with 4 µg/ml blasticidin (Thermo Fisher) and screened for expression by Western blotting. Where indicated, cells expressing V5-tagged MET were treated with 50 ng/mL HGF (Peprotech).

Statistical Analysis

Kaplan-Meier functions were used to illustrate glioma-free survival profile, and logrank test was used to compare survival functions between groups. Glioma frequencies were compared using a chi-square test. Sphere formation assays were analyzed by unpaired, two-tailed t tests with Welch correction. The p21 and Mdm2 induction assays were analyzed by t-tests. A p-value 0.05 was considered to be statistically significant. Significance was indicated as

follows: * p-value 0.05, ** p-value 0.005, *** p-value 0.0005. All statistical analyses were performed with GraphPad Prism Version 7.

Results

Trp53 and Pten are critical barriers to radiation-induced glioma development.

The high prevalence of TP53 and PTEN alterations in GBM patients indicates that these TSGs serve as primary barriers to sporadic glioma initiation and progression [11, 12]. To determine whether p53 and Pten are equally important in preventing radiation-induced gliomagenesis, we used transgenic mouse lines with conditional (Nestin-Cre-driven) deletion of one or both copies of *p53* and/or *Pten* in the brain [36, 37]. We used Fe ions (^{56}Fe ; 600 MeV/u) for induction of radiogenic gliomas as we previously showed that these ions induce complex DNA damage [26], and are highly transforming both in vitro [43] and in vivo [32]. Cohorts of Nestin-Cre positive ($^{\text{NesCre}}$) mice with cumulative losses of *p53* and *Pten* alleles were irradiated intracranially with 0.5 Gy of Fe ions (Fig. 1A). Deletion of floxed (f) alleles was confirmed by PCR analyses of representative mouse brains (Supp. Fig. 1A). The induction of DSBs by Fe ions was confirmed by staining for 53BP1 foci, a surrogate marker for DSBs [26], in select mouse brains at 1h post-IR exposure (Supp. Fig. 1B). Mice were monitored for up to 15 months post-IR exposure for neurological symptoms, and were euthanized when they became moribund. Although wild-type mice ($^{\text{NesCre}}\text{p53}^{+/+};\text{Pten}^{+/+}$) did not form brain tumors following irradiation, mono-allelic deletion of either tumor suppressor was sufficient to drive tumorigenesis subsequent to Fe ion exposure (8.1% and 2.5% in the $^{\text{NesCre}}\text{p53}^{+/f};\text{Pten}^{+/+}$ and $^{\text{NesCre}}\text{p53}^{+/+};\text{Pten}^{+/f}$ cohorts, respectively) (Fig. 1A). The frequency of radiogenic gliomas increased sharply to 27.5% upon mono-allelic deletion of both *p53* and *Pten* ($^{\text{NesCre}}\text{p53}^{+/f};\text{Pten}^{+/f}$) (Fig. 1A, B). Importantly, only one mouse out of a cohort of 40 mock-irradiated $^{\text{NesCre}}\text{p53}^{+/f};\text{Pten}^{+/f}$ mice developed a spontaneous glioma, making this genotype optimal for studying radiation-induced gliomagenesis. Taken together, these results indicate that p53 and Pten together constitute a critical barrier to radiation-induced GBM development.

The frequency of glioma development varies as a function of LET and DNA damage complexity.

In order to assess the effect of radiation quality on gliomagenesis, we compared glioma development in $^{\text{NesCre}}\text{p53}^{+/f};\text{Pten}^{+/f}$ mice in response to either X-rays (low LET) or H, C, Si or Fe ions (particles of increasing atomic number and LET) (Fig. 2A). We first irradiated mouse astrocytes in vitro to confirm that these ions of increasing LET were increasingly more deleterious with regard to DNA damage repair (Supp. Fig. 2 A) and cell killing (Supp. Fig. 2B). Next, we exposed cohorts of $^{\text{NesCre}}\text{p53}^{+/f};\text{Pten}^{+/f}$ mice to a single intracranial dose of 0.5 Gy of X-rays or H, C, Si, or Fe ions, and monitored these mice for glioma development. The induction of DSBs by these ions in vivo was confirmed by staining for 53BP1 foci in select mouse brains at 1 h post irradiation (Supp. Fig. 2C). We found that a low dose of electromagnetic radiation (X-rays) was capable of inducing gliomas in our mouse models (Fig. 2A). Further, we found that low-LET particle radiation (H) of clinically-relevant energy was similar to X-rays with regard to glioma induction (Fig. 2A, B). Interestingly, glioma frequencies increased with increasing LET with maximum tumor

development seen with silicon (LET=79 keV/ μ m) (Fig. 2B). These results indicate that while H may be equivalent to X-rays in terms of gliomagenesis, exposure to particle radiation of higher LETs may pose a much greater GBM risk.

Radiation-induced gliomas in mice are histopathologically similar to primary human GBM.

Grade IV gliomas (glioblastoma, GBM) are defined by the presence of mitotic figures, cellular atypia, pseudopalisading necrosis, and diffuse infiltration into the surrounding brain parenchyma [38]. Both low- and high-LET-induced mouse brain tumors were histopathologically indistinguishable from human grade IV disease. H&E staining of brain tumor sections revealed single unilateral masses with no distinct borders, oftentimes infiltrating through the corpus callosum or the subarachnoid space (Fig. 3A, Supp. Fig. 3A). Microscopically, the tumors showed pseudopalisading necrosis, profound nuclear atypia (Fig. 3A), and perivascular and perineuronal satellitosis (Supp. Fig 3A). All gliomas examined exhibited prominent mitotic figures and were highly proliferative (Ki67 index >25%) (Fig 3A, Supp. Fig. 3B, C). No histological evidence of less malignant precursor lesions was observed for any of the radiation types, suggesting that IR, regardless of LET, is a potent inducer of primary high-grade gliomas. Next, we examined the expression of cell lineage markers in select mouse brain tumors and normal brain tissue from *NesCre*^{p53+/f}; *Pten*^{+/-} mice by qRT-PCR. We observed decreased expression of the neuronal marker *NeuN* in the brain tumors relative to normal brain, and increased expression of the glial markers *Gfap* and *Olig2*, and the neural stem/progenitor cell marker *Sox2*, a transcription factor pathologically involved in the induction of pluripotency and glioma de-differentiation [44] (Supp. Fig. 3D). Accordingly, upon immunohistochemical staining, the tumors were non-reactive for NeuN, but positive for Gfap, Olig2, and Sox2 (Fig 3B). Thus, both low- and high-LET radiations were capable of inducing poorly-differentiated gliomas that recapitulate the human histo-phenotype.

Neurosphere cultures from radiation-induced gliomas harbor stem-like cells and are highly tumorigenic.

Sox2 expression in gliomas has been linked to malignancy via the maintenance of tumor cell dedifferentiation and plasticity [44]. The high levels of Sox2 expression in the RIGs (Fig 3B, Supp. Fig. 3D) prompted us to examine whether cells from these tumors could be cultured in neural stem cell medium. We found that freshly resected tumors were capable of growth in serum-free medium as tumor neurospheres which maintained high Sox2 expression through several passages in culture (Fig. 4 A, Supp. Fig. 4A). Details of the four glioma lines selected for subsequent studies are provided in Supp. Table 2. In vitro experiments confirmed the ability of these cultures to self-renew and to undergo limited lineage differentiation upon serum supplementation – phenotypes attributed to glioma stem-like cells [45] (Fig. 4 B, C). An in vivo limiting dilution assay was performed to confirm the tumor-initiating capacity of the neurosphere cultures. Tumor development was monitored following injections of decreasing numbers of dissociated tumor neurosphere cells (500,000; 5,000; and 500) or control p53^{+/-};Pten^{+/-} astrocytes (500,000 cells) subcutaneously into nude mice. We found that while the p53^{+/-};Pten^{+/-} astrocytes failed to generate tumors, as few as 500 RIG-derived cells could give rise to subcutaneous tumors in nude mice (Fig. 4 D, Supp. Fig. 4 B; Supp. Table 3). Strikingly, only 500 tumor neurosphere cells were sufficient

to generate tumors upon syngeneic implantation into the brains of immunocompetent FVB/NJ-C57BL6 mixed background mice (Fig. 4 E, Supp. Fig. 4C). Histopathological characteristics of the parental tumors were preserved in the derivative orthotopic tumors; these included parenchymal infiltration (resulting in satellite lesions), nuclear atypia, and the presence of mitotic figures and Ki67-positive cells (Fig. 4 F). Furthermore, the expression of Gfap and Olig2 within the tumor mass indicates that the injected neurosphere cells (negative for glial markers, Fig. 4 C) gave rise to more differentiated progeny *in situ*, recapitulating the cellular lineage hierarchy present in the parental lesions (Fig. 4 G). Based on these results, we conclude that the RIGs harbor cells with cancer stem-cell like properties which can be grown as neurospheres and differentiated into other lineages, and which exhibit tumor-propagating properties.

Loss of heterozygosity of *Trp53* and *Pten* is universal in radiation-induced gliomas.

In GBM patients, *TP53* and *PTEN* are usually eliminated by deletion or mutation of one allele followed by loss of heterozygosity (LOH) of the wild-type allele [10, 12]. PCR amplification of tumor DNA was carried out to determine the status of the remaining wild-type (WT) allele of each gene in the RIGs from the ^{NesCre}p53^{+/f};Pten^{+/f} mice. As expected, Cre-mediated excision of the conditional (f) *Trp53* allele was observed in the brain (and the RIGs) but not in the pancreas (Fig. 5A). All six tumors tested revealed additional absence of the WT *Trp53* amplification product (Fig. 5A). Loss of the WT *Trp53* allele was confirmed by the absence of p53 protein in glioma-derived primary cultures, and the lack of observable p53 Serine 15 phosphorylation upon irradiation of these cultures [46] (Fig. 5B, Supp. Fig 5A). Functionally, loss of p53 was confirmed by the lack of DNA damage-induced upregulation of transcriptional targets of p53 - p21 and Mdm2 [46] (Fig. 5C, D; Supp. Fig 5B, C). Similar to *Trp53*, Cre-mediated excision of the conditional (f) *Pten* allele was observed in gliomas and brain but not in the pancreas (Fig. 5E). All six tumors tested revealed additional absence of the WT *Pten* amplification product. Loss of *Pten* was further validated by qRT-PCR analysis of five tumors (Fig. 5F). Immunohistochemical analysis of a comprehensive panel of 33 RIGs showed that all tested tumors were non-reactive for Pten protein (Supp. Table 4). Functionally, Pten loss was confirmed by increased phosphorylation of ribosomal protein S6, Erk, and Akt – downstream components of signaling pathways inhibited by the phosphatase activity of Pten [47] (Fig. 5G, H; Supp. Fig 5D). The ubiquitous, concomitant nature of p53 and Pten loss in all tumors tested suggests that these TSGs function independently as barriers to radiation-induced gliomagenesis.

Genomic amplification of *Met* is a frequent event in radiation-induced gliomas.

Human gliomas bear multiple copy number alterations in the PI3K, p53, and RB pathways, and amplification of RTKs is collectively observed in over 70% of cases [1, 2, 10]. Having established loss of both *Trp53* and *Pten* TSGs, we next identified key oncogenic alterations in the mouse RIGs. We analyzed the genomes of 7 radiation-induced tumors by array comparative genomic hybridization (aCGH). To detect significantly reoccurring focal copy number variations (CNV), we used the Genomic Identification of Significant Targets in Cancer (GISTIC2.0) algorithm [42]. Events with q-value <0.05 were considered significant. Approximately 4000 genes fell within the broad genomic regions designated by GISTIC as significantly altered. In the amplified or deleted regions shared by multiple samples, GISTIC

identified 35 genes, falling within the minimum common regions of overlap, as likely cancer driver alterations (Supp. Table 5). A small region on Chr6 containing the RTK *Met* gene ($q\text{-value}=1.15\times 10^{-5}$) was amplified in 43% of tumors tested (Fig. 6A, B). *Met* amplification correlated with robust protein expression and receptor activation, as observed in the corresponding tumor histology sections by staining for total- and phospho-Met (Tyr1234/1235) [48], respectively (Fig. 6C). In contrast, a tumor without *Met* amplification (Supp. Fig. 6A) stained negative for phospho-Met (Supp. Fig. 6B). The immunological analysis for phospho- and total- Met was extended to the panel of 33 RIGs driven by low- or high-LET IR (Supp. Table 4). Met positivity was found in 48.5% of samples, while the receptor was phosphorylated in 36.4% of samples. The prevalence of Met amplification and activation in the radiogenic mouse tumors is in contrast to the low frequency (about 4%) of MET amplification or mutation in sporadic human GBM [10].

MET has been linked to the induction and maintenance of cancer stem cells in glioma and breast cancer [32, 49–52]. To determine the effect of Met expression in the context of p53 and Pten loss, p53^{−/−};Pten^{−/−} primary murine astrocytes were transduced with lentiviral vectors expressing V5-tagged human MET or β -Gal as control. The presence of V5-tagged proteins was confirmed in cell lysates by western blotting (Fig. 6D). The functionality of MET in the neurospheres was determined by the ability of the MET ligand – hepatocyte growth factor (HGF), to induce MET phosphorylation upon supplementation to the growth medium (Supp. Fig. 6C). Exogenous expression of MET enabled neurosphere formation and growth under serum-deprived conditions (Fig. 6E). Unlike β -Gal transfected astrocytes, V5-MET-expressing cells plated as single cells in serum-free neurosphere medium successfully formed neurospheres at a high frequency in a sphere-formation assay (Fig. 6F). Taken together, our results suggest that RIGs might be driven by oncogenic changes (like Met amplification) that differ from those in sporadic GBM, and that enhanced RTK signaling in the p53- and Pten-deficient mouse RIGs likely contributes to the preponderance of GBM stem-like cells in these tumors.

Discussion

In this study, we examined the roles of TP53 and PTEN as barriers to radiation-induced gliomagenesis by using mouse models with brain-targeted incremental allelic deletions of these TSGs. Although single-allele loss of either gene predisposed the mice to RIG development, combined loss of one copy of each TSG significantly increased RIG frequency. These results indicate that p53 and PTEN both play a critical role in preventing the development of RIG. The importance of both genes as barriers to IR-induced transformation is underscored by the universal LOH of p53 and Pten in the gliomas that developed in the irradiated ^{NesCre}p53^{+/f};Pten^{+/f} mice. The elevated susceptibility of the ^{NesCre}p53^{+/f};Pten^{+/f} mice to RIG, combined with a low frequency of spontaneous GBM development, makes them a highly suitable model for mechanistic analysis of radiation-induced tumorigenesis. Using this model, we examined the effects of radiation quality on transformation by interrogating a range of charged particles (H, C, Si and Fe) in comparison with radiotherapy-grade X-rays. We observed that both electromagnetic and particle radiation are capable of inducing high-grade gliomas. At the low end of the LET spectrum, we observed equal frequencies of glioma induction following irradiation with therapy-grade

X-rays and H ions. The frequency of glioma induction strongly correlated with LET for values up to 79 keV/ μ m ($r^2=0.988$), with the peak tumor incidence seen with Si. The maximal in vivo effects observed with silicon are in agreement with recent studies in the lung, where silicon ions were similarly shown to be more tumorigenic than X-rays or Fe ions [53]. The increase in tumor frequencies with heavier ions correlates with the higher complexity of DNA damage which becomes increasingly refractory to repair with increasing LET, as seen in this study and others [25, 26], which may increase the likelihood of oncogenic events such as the observed Met amplification. The decrease in tumor frequency observed with the most energetic ion (Fe; LET of 180 keV/ μ m) may reflect a shift in the balance between transformation and cell killing, as Fe ions are more cytotoxic than Si as seen in this study and others [26, 53]. Regardless, it is clear that particle radiation (especially ions heavier than H) could be a concern from the standpoints of primary cancer development from GCR exposure and secondary cancer development after particle therapy. Our conclusions are consistent with findings in genetically-engineered mouse models of lung, intestinal, and hepatocellular cancer [53–57], further attesting to the carcinogenic properties of particle radiation. Importantly, pre-clinical animal models, like the ^{NesCre}p53+/f;Pten+/f mouse described here, can complement ongoing clinical trials [58] and risk-estimation models [59] for the assessment of the efficacy and safety profile of charged particles relative to X-rays or gamma rays with regard to brain tumor development. The advantages of this particular mouse model are manifold: it harbors deletions of TSGs relevant to human GBM, does not exhibit spontaneous gliomas, is susceptible to IR-induced glioma development with a high frequency and short latency, and exhibits brain tumors that histopathologically resemble human Grade IV gliomas. Such a mouse model could be useful both for understanding mechanisms underlying radiation carcinogenesis as well for testing preventative therapeutic approaches.

The RIGs that develop in the ^{NesCre}p53+/f;Pten+/f mouse model exhibit the hallmarks of human grade IV glioma, including diffuse parenchymal infiltration, pseudopalisading necrosis, and profound nuclear atypia [38]. Interestingly, these tumors also have a prominent cancer stem cell phenotype, as exemplified by high levels of Sox2 expression, and the ability of primary ex-vivo cultures to give rise to differentiated CNS cells and to propagate the tumor hierarchy of the parental lesions when injected orthotopically. This stem cell phenotype could be driven by genetic alterations acquired in the process of radiation-induced tumorigenesis. We find that the RTK Met was frequently amplified in RIGs in the ^{NesCre}p53+/f;Pten+/f model as well as in a mouse RIG model driven by *Cdkn2a/b* loss described previously [32]. It has been reported that MET amplification in human tumors may be a consequence of DNA breakages occurring within chromosomal fragile sites [60]. Specifically, the *MET* gene locus falls within the FRA7G common fragile site which is implicated in esophageal and prostate cancer [61, 62]. Such amplification of Met in the RIGs may have consequences beyond aberrant cancer cell proliferation. MET was recently shown to be a functional marker of a GBM cancer stem cell (CSC) subtype [52]. MET signaling activates the MAPK, PI3K-Akt, NF- κ B, and STAT pathways thereby driving a comprehensive rewiring of gene expression and cell identity [48]. Recent studies indicate that MET maintains a GBM CSC phenotype via the induction of reprogramming transcription factors, including Sox2, c-Myc, Klf4, Oct4, and Nanog [49, 50, 52]. Given that

p53 functions as a barrier to reprogramming [63], increased expression of Met in the p53-deficient RIGs likely serves as a potent inducer of the CSC phenotype. Such cooperativity between p53 loss and Met in the induction of “stemness” was recently described in a Wnt/ β -Catenin-driven mouse model of basal-like breast cancer [51], while cooperation between p53 deficiency and RTK signaling through Ras activation has similarly been reported to drive astrocyte de-differentiation [64]. In accord with these studies, we find that that ectopic MET over-expression in p53^{-/-};Pten^{-/-} astrocytes causes a phenotypic shift towards a more stem cell-like state as evidenced by growth and continued propagation as neurospheres in neural stem cell media. The preponderance of Met amplification in RIGs in the NesCre⁺p53^{+/-};Pten^{+/-} (this study) and the Cdkn2a/b^{-/-} [32] mouse models is in agreement with a recent study assessing the genetic landscape of RIGs in human patients where MET amplification was prominently featured [65]. It is important to point out that PTEN mutations were not observed in the human RIGs though biallelic TP53 inactivation was common. Thus, while commonalities (such as RTK amplification and TP53 inactivation) exist between mouse and human RIGs, the precise genetic signatures that define RIGs in mouse differ from those in humans. Regardless, our mouse results, along with this human study [65], suggest that RIGs exhibit distinct genetic alterations and molecular signatures such as MET amplification that could be targeted for treating secondary or recurrent GBM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

This study uncovers mechanisms by which ionizing radiation, especially particle radiation, promote GBM development or recurrence.

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Figure 1. Trp53 and Pten are barriers to radiation-induced gliomagenesis.
A. Table shows genotypes of mice mock-irradiated or intracranially irradiated with 0.5 Gy of Fe ions (600 MeV/n) along with the corresponding glioma frequencies. Kaplan–Meier curves show Fe-irradiated mice surviving without intracranial lesions for the indicated genetic backgrounds. Numbers in parentheses indicate fraction of mice that developed gliomas for each cohort. In the ^{NesCre}p53+/f;Pten+/f cohort, significantly more glioma-associated deaths were observed following irradiation compared to mock treatment (p=0.0005). **B.** H&E-stained sections of mock-irradiated (glioma-free) and Fe-irradiated (tumor-bearing) ^{NesCre}p53+/f;Pten+/f brains; “f” denotes conditional (floxed) alleles targeted for Nestin-Cre-mediated excision.

A.

Radiation type	X-rays	Hydrogen (H)	Carbon (C)	Silicon (Si)	Iron (Fe)
Atomic number	-	1	6	14	26
Energy	250 kV	150 MeV/n	293 MeV/n	238 MeV/n	600 MeV/n
LET (keV/ μ m)	2	0.5	13	79	174
Glioma frequency (%)	16.2	12.5	22.5	50	27.5

B.

Figure 2. Glioma frequencies vary as a function of radiation quality.

A. Table shows atomic numbers, energies, and LETs of ions used to irradiate $NesCre^{p53+/f}; Pten^{+/f}$ mice along with the corresponding glioma frequencies. Kaplan–Meier curves show irradiated $NesCre^{p53+/f}; Pten^{+/f}$ mice surviving without intracranial lesions. Mice were irradiated with 0.5 Gy of X-rays or the indicated ions (n=40 animals per radiation type). Data for Fe ions from Figure 1B is included for comparison. **B.** Glioma frequencies for mouse cohorts irradiated with X-rays or the indicated ions. Glioma frequency was found to be significantly different among the 5 groups ($p=0.0013$, 2 by 5 χ^2 test). Pair-wise

comparison showed that Si was significantly more tumorigenic than other ions. * designates a p-value 0.05; ** designates a p-value 0.005; *** designates a p-value 0.0005.

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Figure 3. Radiation-induced gliomas exhibit key features of human grade IV disease.

A. H&E-stained sections of representative $NesCre^{p53+/f};Pten+/f$ brains irradiated with X-rays or with the indicated ions. Please note massive unilateral tumors infiltrating contralaterally through the corpus callosum. Examples of GBM-characteristic pseudopalisading necrosis, nuclear atypia, and mitotic figures are shown. **B.** Immunohistochemical staining of normal and tumor-bearing brains for neural (NeuN), glial (Gfap and Olig2), and stem cell (Sox2) markers. High magnification images show representative areas within the normal cerebral cortex or tumor mass (black boxes). Scale bar = 50 μ m.

Figure 4. Neurospheres derived from radiation-induced gliomas resemble cancer stem-like cells.

A. Neurosphere formation by tumor-derived cells cultured in serum-free, neural stem cell medium (bright field images, top panel). Expression of the stem/progenitor marker Sox2 was assessed by immunofluorescence staining (green, bottom panel). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). **B.** Cells were plated in neural stem cell medium at single-cell density in 96-well plates to assay for sphere formation. The percentages of cells forming neurospheres are plotted for three lines derived from radiation-induced tumors and p53+/-;Pten+/- astrocytes as control. ** designates a p-value 0.005; *** designates a p-value 0.0005. Error bars represent SD from three independent experiments. **C.** Tumor-derived neurospheres were grown in neural stem cell medium or neural stem cell medium supplemented with 10% fetal bovine serum. Expression of lineage markers Gfap (green) and Tuj1 (red) was assessed by immunofluorescence staining. Nuclei were stained with DAPI. Each image is representative of at least 3 independent experiments. **D.** Tumor growth rates following subcutaneous injections of tumor-derived cells (T 2634) or control p53+/-;Pten+/- astrocytes into the flanks of immunocompromised mice. Plot labels indicate the number of cells injected and the tumor take rate in parentheses; error bars represent SEM. **E.** H&E-stained section of a representative brain of immunocompetent FVB/NJ-C57BL6 mouse

intracranially injected with syngeneic T 2634 cells (500 cells per injection; n=5 animals/group). **F.** Histopathological features of orthotopic gliomas generated from T 2634 cells. Examples of tumor infiltration beyond the site of injection (satellite lesion below the main tumor mass), nuclear atypia, and mitotic figures are shown. Ki67 staining was used as a marker of proliferation. **G.** Cell lineage markers expressed in the orthotopic tumors generated by T 2634 cells as determined by immunohistochemical staining. Scale bars = 50 μm .

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Figure 5. Concomitant loss of wild-type *p53* and *Pten* alleles in radiation-induced gliomas.

A. Genomic DNA isolated from *NesCre*^{p53^{+/f};Pten^{+/f} mouse pancreas, brain, and brain tumor tissues was assayed by PCR for genotyping *p53*. “WT” and “f” designate the wild-type and conditional (floxed) alleles respectively. **B.** Phosphorylation and stabilization of p53 in *p53^{+/f};Pten^{+/f}* and *p53^{-/-};Pten^{-/-}* astrocytes, and tumor-derived lines (T2089 and T2262) following irradiation with 10 Gy of γ -rays as assessed by western blotting. Actin was used as a loading control. **C, D.** Relative expression of p21 (C) and Mdm2 (D) mRNA following exposure of *p53^{+/f};Pten^{+/f}* and *p53^{-/-};Pten^{-/-}* astrocytes and tumor-derived lines (T 2089 and T 2262) to 10 Gy of γ -rays as determined by qRT-PCR. All measurements are relative to 18s mRNA levels. ** designates p-values ≤ 0.005 ; error bars represent SD from three independent experiments. **E.** The tissue samples shown in (A) were used to assess the status of the WT and floxed (f) *Pten* alleles by a PCR-based assay. **F.** Levels of Pten}

mRNA in radiogenic gliomas, as determined by qRT-PCR, in five tumors relative to Pten expression in ^{NesCre}p53+/f;Pten+/f normal brain. All measurements were normalized to 18S mRNA levels. Error bars represent SD between three technical replicates. **G.** Immunoreactivity of tumor and normal brain tissues from ^{NesCre}p53+/f;Pten+/f animals for Pten, phospho-S6, and phospho-Erk as determined by IHC staining. Scale bar = 50 μ m. **H.** Protein lysates from p53+/-Pten+/- and p53-/-;Pten-/- astrocytes and tumor-derived lines were assessed for Pten and total and phosphorylated Akt by western blotting. Actin was used as a loading control.

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Figure 6. Genomic amplification of *Met* is the primary oncogenic alteration in radiation-induced gliomas.

A. Circos plot representing the genome of a radiation-induced tumor (T 1679) with *Met* amplification. Copy number (CN) gain is shown in green; red color represents CN loss. Genomic regions with CN change of 1 or more are designated by black borders. Magnified view of the boxed region containing chromosome 6 is also shown. **B.** Genomic region of chromosome 6 containing the *Met* locus (x-axis). Three samples with local CN gain (T 1035, T 2634, T 1679) are shown together with one sample with no amplification (T 2089). Arrows demarcate the borders of the amplified regions. Log2 ratios of CN are plotted on the y-axis. **C.** Immunohistochemical staining for Met and phospho-Met of a radiation-induced glioma with focal amplification of chr6 (T 1679). IHC staining of normal brain is shown for comparison. Scale bar = 50 μ m. **D.** Western blotting analysis of p53^{-/-};Pten^{-/-} astrocytes ectopically expressing V5-tagged MET or β -Gal as control. Actin was used as a loading

control. **E.** β -Gal or MET-expressing p53^{-/-};Pten^{-/-} astrocytes grown in serum-supplemented or serum-free neural stem cell media. Scale bar = 400 μ m. **F.** β -Gal or MET-expressing p53^{-/-};Pten^{-/-} astrocytes were plated in neural stem cell medium at single-cell density in 96-well plates to assay for sphere formation. The percentages of cells forming neurospheres are plotted. *** designates a p-value ≤ 0.0005 . Error bars represent SD between three technical replicates.