Genetically Mediated Nf1 Loss in Mice Promotes Diverse Radiation-Induced Tumors Modeling Second Malignant Neoplasms

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Abstract

Second malignant neoplasms (SMN) are therapy-induced malignancies and a growing problem in cancer survivors, particularly survivors of childhood cancers. The lack of experimental models of SMNs has limited understanding of their pathogenesis. It is currently not possible to predict or prevent this devastating late complication. Individuals with neurofibromatosis I (NF1) are at increased risk of developing therapy-induced cancers for unclear reasons. To model SMNs, we replicated clinical radiotherapy and delivered fractionated abdominal irradiation to Nf1+/- and wild-type mice. Similar to irradiated cancer survivors, irradiated wild-type and Nf1+/- mice developed diverse in-field malignancies. In Nf1+/- mice, fractionated irradiation promoted both classical NF1-associated malignancies and malignancies unassociated with the NF1 syndrome but typical of SMNs. Nf1 heterozygosity potentiated the mutagenic effects of irradiation, as evidenced by the significantly reduced survival after irradiation and tumor development that was often characterized by synchronous primary tumors. Interestingly, diverse radiation-induced tumors arising in wild-type and Nf1+/- mice shared a genetic signature characterized by monoallelic loss of Nf1 and the adjacent Trp53 allele. These findings implicate Nf1 loss as mediating tumorigenesis in a broad range of cell types and organs extending beyond the classical NF1 tumor histologies. Examining clinical SMN samples, we found LOH of Nf1 in SMNs from non-NF1 patients. Nf1 heterozygosity confers broad susceptibility to genotoxin-induced tumorigenesis, and this paradigm serves as an experimental platform for future studies of SMNs. Cancer Res; 72(24); 6425–34. ©2012 AACR.

Introduction

Secondary malignant neoplasms (SMN) are late complications arising after exposure to genotoxins. These cancers can arise from a variety of tissues within an irradiated anatomic compartment (1–3), reflecting the wide range of cell types sharing susceptibility to mutagenesis. It is currently not possible to predict which patients will develop an SMN, nor are there known shared mechanisms linking the pathogenesis of diverse SMN histologies. The incidence of SMNs is growing primarily because the number of pediatric and other at-risk cancer survivors has increased over the last few decades (4–6).

The mechanisms underlying SMN development are very poorly understood, with radiation exposure and young age at the time of treatment being the strongest risk factors (2, 3, 7, 8). Defining the mechanisms responsible for SMN pathogenesis is critical to developing strategies to accurately predict and mitigate this risk in current and future cancer survivors. Mechanistic analyses of both patient and treatment factors are difficult to conduct through retrospective clinical analyses, and thus experimental models of SMNs are needed.

Genetic background contributes to tumor susceptibility in humans and mice, for example, the high frequency of SMNs arising in the setting of Li–Fraumeni syndrome and other genetic disorders of DNA repair (9, 10). Furthermore, experimental models of radiation-induced tumorigenesis do not replicate radiotherapy delivery and often use genetically unstable mouse strains. The resultant tumors do not reconstitute the full spectrum of histologies comprising SMNs, and the latency to tumor development is often extremely abbreviated (within a few months) in contrast to the latency of SMNs in humans, which is over several years and sometimes decades. The inability to broadly model SMN risk in relation to relevant radiotherapy variables of dose, fractionation and targeting has limited our understanding of how clinical treatment, patient variables, and environmental exposures influence SMN risk. As a result, there are no validated approaches to predicting or preventing SMNs in at-risk individuals, such as survivors of childhood cancers.

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Heterozygosity for the NF1 gene causes the Neurofibromatosis I (NF1) syndrome, and individuals with NF1 are at increased risk of developing SMNs after radiotherapy (11). The NF1 gene, and its conserved murine homologue NF1, encode the protein neurofibromin, which is ubiquitously expressed in mammalian cells and necessary for development (12). Neurofibromin possesses a GTPase-activating protein (GAP) domain, a region that negatively regulates Ras signaling (13–16).

We hypothesized that the clinical susceptibility of NF1 patients to SMN development could be modeled in Nf1-mutant mice to study SMN pathogenesis. In earlier work, we showed that focal, fractionated cranial irradiation of Nf1+/− mice produced both hematologic and in-field solid malignancies (17). To develop a more robust model that (i) tested whether focal fractionated irradiation potentiates tumorigenesis among diverse tissues and (ii) efficiently generated large numbers of tumors in both genetically resistant and susceptible backgrounds for analysis we delivered abdominal irradiation to wild-type and Nf1-mutant mice. We replicated a clinical paradigm using customized techniques to deliver focal, fractionated radiation to the abdomen (Al: 0, 3, 15, or 30 Gy; Fig. 1 and Supplementary Fig. S1). This procedure concentrates radiation exposure to the superficial tissues and generates a dose gradient to deeper internal organs, permitting the safe delivery of radiation doses that would be lethal as whole-body exposures. This application of fractionation mimics clinical radiotherapy practice and was an approach we used in our cranial irradiation model (17).

Both irradiated wild-type and Nf1+/− mice developed diverse in-field malignancies in a dose-dependent manner, with Nf1−/− mice developing greater numbers of malignancies than matched wild-type mice at each radiation dose level. Diverse tumor histologies shared a signature LOH of wild-type Nf1 and Trp53 alleles, rendering tumors from Nf1+/− mice null for Nf1 and consistent with a common pathogenetic mechanism of Nf1-dependent tumorigenesis active in multiple cell types. Interestingly, this signature loss was also present in a subset of radiation-induced tumors from wild-type mice, which lost either parental allele at similar frequencies. This suggests that Nf1 haploinsufficiency can drive radiation-induced tumor formation in the wild-type background as well and may be a genetic mechanism promoting radiation-induced tumors between different genetic backgrounds. We extended our genetic analysis to human radiation-induced breast cancers and identified LOH of NF1 in a subset of these SMNs from unrelated individuals without NF1. These findings suggest that Nf1 loss in our mouse model mirrors Nf1 loss in human SMNs and suggest that targeting the biochemical consequences of Nf1 loss may be a useful strategy against SMN development.

Materials and Methods

Mouse strains, breeding, and treatment

Nf1+/− and wild-type mice were generated as previously described (18). In brief, Nf1+/− mice maintained in the 129/Sv background (19) were crossed with wild-type C57Bl/6 mice. Mice were placed in a cesium-137 source (J.L. Shepherd & Associates) animal irradiator and shielded with an iron collimator that focused the beam width. Five- to 8-week-old mice were given abdominal irradiation at a fractionation of 1 fraction of 3 Gy, 5 fractions of 3 Gy, or 10 fractions of 3 Gy, delivered at a rate of 5 fractions per week, 1 fraction per day. The UCSF IACUC approved all animal procedures.

Pathologic analysis

Animals were followed for a minimum of 18 months postirradiation. Animals with signs of systemic illness were euthanized and visible masses/growth, peripheral blood, and bone marrow were collected immediately. The mice were then perfused with 4% paraformaldehyde, and the following organs were collected: brain, skin in irradiated region, skin outside irradiated region, skull, heart, lungs, spleen, liver, kidneys, and segment of small intestine. Pathologic review was conducted on hematoxylin and eosin–stained sections by A.E. Horvai and S. Kogan. Photographs of histology were taken with an Olympus BX41 microscope, using Olympus UplanFl ×10/0.3 and ×20/0.5 objectives. An attached Olympus DP72 camera and Adobe Photoshop CS2 were used to capture the images. Complete blood counts were conducted on blood samples obtained at the time of sacrifice by cardiac puncture and analyzed immediately on a Hemavet provided by the UCSF Mouse Pathology Core.

Mouse tumor genotyping and mutation analysis

Trp53 LOH was analyzed at the D11Mit29 and D11Mit31 loci by amplifying tumor DNA with the following primers: forward 5′-TTGAGGATGAGGGATAG-3′, reverse 5′-TTTCCCTGATACGCTAG-3′, 5′-TTTCCAGTCAGCGTTGACGT-3′, reverse 5′-AGAATAAGTAACCCACGT-3′. D11Mit29 was amplified with the following primers: forward 5′-TTTCCAGTCAGCGTTGACGT-3′, reverse 5′-AGAATAAGTAACCCACGT-3′. The UCSF Genome Core using Peak
Scanner software from Applied Biosystems conducted PCR fragment analyses. The single-nucleotide polymorphism (SNP) rs13481119 was sequenced using the following primers: forward 5'GCCCCGTACATGCTGAGTGC-3' reverse 5'-GCTTGCTAGGCGCTGAGTGC-3'. SNP products were sequenced using a commercial sequencing service.

Clinical FFPE tissue DNA isolation and amplification

Formalin-fixed, paraffin-embedded (FFPE) sections of 8 radiation-induced breast cancers were obtained from City of Hope Medical Center (Duarte, CA). Hematoxylin and eosin (H&E) slides of FFPE sections were reviewed by a pathologist (A.E. Horvai), who delineated normal and normal areas for dissection. Tumor and normal tissues were then dissected off (unstained sections), and gDNA was isolated and subjected to whole-genome amplification according to manufacturer’s instructions using the REPLI-g FFPE Kit (Qiagen). DNA was purified using sodium acetate and quantified using Picogreen (Qiagen).

TaqMan SNP genotyping

Analysis of clinical samples was conducted with approval from the UCSF Committee on Human Research. Clinical DNA samples were genotyped at 4 selected SNPs in the human NF1 gene (NCBI ref SNPS rs2952994, rs2953014, rs9902893, and rs2107359 using C1547650_10, C2557613_10, C2533273_10, and C16832374_10, all from Applied Biosystems, respectively). Primers flanking selected SNPs were designed using Integrated DNA Technologies primer design program. Assay C2557613_10 was used to genotype using the Taq amplification method in a 7900 HT Fast Real-Time PCR system (Applied Biosystems). Five microliter PCR reactions were carried out using 2.42 μL DNA in H2O (1 ng total) or H2O for negative controls, 0.08 μL probe (20 μmol), and 2.5 μL Master Mix (20 μmol; Applied Biosystems). Reactions underwent heat activation at 95°C for 10 minutes, then cycled 40 times with a denaturation step of 92°C for 15 seconds, and an annealing and elongation step of 60°C for 90 seconds. All samples were run in triplicates of 6. Allelic discrimination plots generated by SDS3.0, an Applied Biosystems program used to make genotyping calls.

Trp53 sequencing

Whole-exomic sequencing was conducted to assess for the presence of mutations in the Trp53 gene. Two-kb fragments of the Trp53 gene spanning exons 2–4, 5–7, and 8–10 were PCR-amplified from all primary tumor and select matching tail DNA samples. Similarly, 1-kb fragments were PCR-amplified from exons 1 and 11. PCR amplification was conducted with Qiagen Taq DNA polymerase. PCR cleanup was done with ExoSAP-IT (Affymetrix). The PCR fragments were then sequenced (Quintara Biosciences) with sequencing primers (listed in Supplementary Table S2). The Catalogue of Somatic Mutations in Cancer Database (COSMIC, www.sanger.ac.uk/genetics/CGP/cosmic, Wellcome Trust Sanger Institute, UK) was used to search for references to human tumors also possessing the identified mutations.

Statistical analysis

Survival curves are calculated from Kaplan–Meier product limit estimators to determine the association of confidence interval on mortality. Log-rank tests are used to test for differences in survival curves between groups. All analyses were conducted using Prism v.4 (GraphPad).

Results

High-dose AI reduces survival in Nf1-mutant mice but not wild-type mice

In irradiated cancer survivors, the risk of SMNs is well recognized, although the precise SMN histology can be difficult to predict. Furthermore, cancer survivors may develop multiple subsequent malignancies and diverse SMNs over time (1). Multiorgan SMN development is therefore not well-modeled using tissue-restricted conditional mutations. To replicate whole organismal tumor susceptibility, we sought to model the germline Nf1 loss responsible for the NF1 syndrome.

To establish a homogeneous genetic background, we intercrossed wild-type (WT) C57BL6 and heterozygous Nf1-mutant (Nf1+/-) Sv129 mice to generate F1 Nf1+/- and control cohorts. Between 5 and 8 weeks of age, mice were assigned to 1 of 4 AI treatment regimens: 0 Gy, 3 Gy (single dose), 15 Gy (5 daily doses of 3 Gy), or 30 Gy (10 daily doses of 3 Gy; Fig. 1A and Supplementary Fig. S1). To accurately reflect current clinical practice, the mice were irradiated 5 times per week, 1 fraction per day. They were then observed until they developed signs of illness necessitating euthanasia or until 18 months postirradiation.

The survival of wild-type mice was not significantly reduced after any AI dose, and median survival was not reached by any experimental group (Fig. 1B). In contrast, radiation exposure reduced the overall survival of Nf1+/- mice in a dose-dependent manner (log-rank test, P < 0.0001; Fig. 1B) at higher dose levels (15 and 30 Gy). The median survivals of Nf1+/- mice receiving 0, 3, 15, and 30 Gy were 583, 652, 469, and 410 days, respectively.

AI drives multiorgan tumorigenesis in WT and Nf1+/- mice

Necropsy revealed multiorgan abnormalities after irradiation. Hematologic malignancies such as myeloproliferative neoplasms and leukemias can arise as secondary malignancies after radiation exposure (20). To screen for this, we assessed spleen and liver weight and conducted a complete blood count (CBC) at the time of euthanasia and found significantly increased mean terminal splenic weight in unirradiated Nf1+/- mice as compared with WT mice (Supplementary Fig. S2A). However, splenic weight was not significantly affected by radiation dose in either the WT or Nf1+/- cohorts. Similarly, terminal hemoglobin levels did not significantly differ between any genotype/radiation dose combination, except for Nf1+/-/3 Gy and Nf1+/-/15 Gy (Supplementary Fig. S2B). Examination of H&E-stained spleen, liver and bone marrow sections from animals showing splenomegaly and/or peripheral anemia or leukocytosis revealed changes consistent with varied hematologic
diseases such as histiocytic sarcoma, myeloproliferative neoplasms, and lymphoma.

Radiation-induced SMNs in humans consist of varied malignant histologies, predominantly solid tumors, reflecting a breadth of cell types and tissues that are susceptible to genotoxin-induced mutagenesis (6). Capturing this diversity is a critical requirement for preclinical platforms in which to test SMN-modifying approaches for multiple at-risk tissues. Comprehensive necropsy of experimental animals revealed solid tumor development as the cause of death in most animals (Fig. 2; Supplementary Table S1). Similar to irradiated patients, irradiated mice developed diverse solid tumors originating from organs in the irradiated field (in-field tumors). Tumors were more common in Nf1<sup>+/−</sup> mice than in WT mice, and for Nf1<sup>+/−</sup> mice, tumor incidence increased with increasing radiation dose (\( P < 0.0001 \) by \( \chi^2 \) analysis), whereas for WT mice, a relationship between tumor incidence and radiation dose failed to reach significance (\( P = 0.0511 \) by \( \chi^2 \) analysis). Synchronous tumorigenesis occurred in both WT and Nf1<sup>+/−</sup> mice, with synchronous malignancies in the Nf1<sup>+/−</sup> background increasing with increasing radiation doses (\( P = 0.0264 \) by \( \chi^2 \) analysis). WT, wild-type; HET, Nf1-mutant.

**Figure 2.** Tumor incidence in experimental groups. Percentages of mice developing tumors (A), multiple (synchronous) primary tumors (B), and metastases (C) are shown for each experimental group. Tumors were more common in Nf1<sup>+/−</sup> mice than in WT mice, and for Nf1<sup>+/−</sup> mice, tumor incidence increased with increasing radiation dose (\( P < 0.0001 \) by \( \chi^2 \) analysis), whereas for WT mice, a relationship between tumor incidence and radiation dose failed to reach significance (\( P = 0.0511 \) by \( \chi^2 \) analysis). Synchronous tumorigenesis occurred in both WT and Nf1<sup>+/−</sup> mice, with synchronous malignancies in the Nf1<sup>+/−</sup> background increasing with increasing radiation doses (\( P = 0.0264 \) by \( \chi^2 \) analysis). WT, wild-type; HET, Nf1-mutant.

**Figure 3.** Solid tumor and hematologic disease-free survival after AI. Kaplan–Meier survival analysis of wild-type (WT) mice shows no significant dose response for death due to either solid tumor or hematologic disease (log-rank test, \( P = 0.13 \) for solid tumor-free survival and \( P = 0.68 \) for hematologic disease-free survival). In contrast, Nf1<sup>−/−</sup> mutant mice show a dose response for both solid tumor and hematologic disease-related death (log-rank test, \( P < 0.0001 \) for solid tumor-free survival and \( P = 0.0001 \) for hematologic disease-free survival).
Multiorgan examination revealed that radiation-induced tumors displayed important features of aggressive malignancies, including local invasiveness and distant metastases (Fig. 2C). Appropriate site-specific SMN presentations were observed in irradiated *Nf1*+/− mice, for example, a locally invasive chest wall sarcoma causing a pathologic fracture of an adjacent rib (Fig. 4). Metastatic disease in the lungs and liver, common clinical manifestations of advanced and aggressive cancers, were the sites of metastatic disease in this mouse model (Fig. 2C). The most common metastatic histology was pheochromocytoma, followed by sarcoma and carcinoma. Individual patients can develop multiple independent SMNs in different organs within an irradiated region (1), and this aspect of SMN risk has not been modeled to date. The irradiation of the large anatomic compartment enclosed by the peritoneum concurrently mutagenizes multiple diverse tissues (e.g., hematopoietic, gastrointestinal, endocrine, and connective). Furthermore, irradiating *Nf1*+/− mice capitalizes on the large volume mutagenesis of AI and allows tests of multiorgan *Nf1*+/− dependent susceptibility to radiation-induced tumors. Synchronous tumorigenesis occurred in both WT and *Nf1*+/− mice, with synchronous malignancies in the *Nf1*+/− background increasing with increasing radiation doses (*P* = 0.0264 by χ² analysis). Consistent with the enhanced and broad susceptibility conferred by *Nf1* heterozygosity, synchronous malignancies were more common in the irradiated *Nf1*+/− background (Fig. 2B). Synchronously occurring malignancies in both genotypic backgrounds included pheochromocytomas, sarcomas, and carcinomas.

**Concurrent loss of *Nf1* and *Trp53* is a signature genetic lesion in radiation-induced mouse tumors**

The genetic mechanisms driving SMN development are poorly understood. While genetic background clearly modulates tumorigenesis risk, a common signature of genetic alterations has not been defined for SMNs. The human *NF1* and *TP53* tumor suppressor genes are located 21.8 Mb apart on chromosome 17. Similarly, the homologous murine genes *Nf1* and *Trp53* are both on chromosome 11, and the intervening sequences are conserved in both species. Previous mouse models have shown that engineered *Nf1* and *Trp53* loss results in tumor development (24, 25), but spontaneous cooperativity between these 2 tumor suppressor genes is less well established. We conducted microsatellite analysis of 4 markers spanning these 2 genes (D11Mit31, D11Mit4, D11Mit219, D11Mit38) and also sequenced the SNP rs13481119, located in exon 41 of *Nf1*, to determine whether LOH at these loci occurred and which parental allele was lost (Fig. 5). Microsatellite and SNP sequencing analysis revealed that LOH was a common event in tumors isolated from *Nf1*-mutant mice (Fig. 5), and the frequency of LOH at rs13481119 was similar across the 3 major classes of solid malignancies (pheochromocytoma, carcinoma, and sarcoma).

LOH of *Nf1* was accompanied by LOH of at least one of the microsatellites in 87% of all tumors, and, in fact, LOH commonly involved all 4 tested microsatellites and intragenic SNP from the same parental strand, consistent with a loss of a chromosomal segment spanning *Nf1* and *Trp53*. Comparison to parental control DNA revealed that LOH in tumors arising in *Nf1*+/− mice commonly involved the C57Bl/6-derived allele at all positions (Fig. 5A–C). This led to loss of the wild-type *Nf1* allele and adjacent *Trp53* allele (Fig. 5D). LOH of the wild-type *Nf1* allele and the adjacent *Trp53* allele occurred at similar frequencies for the major tumor classes (carcinomas, sarcomas, and pheochromocytomas), indicating that *Nf1* nullizygosity is a shared genetic mechanism of tumorigenesis in different tissue lineages. Because our mouse model uses a genetic background in which mice possess 2 wild-type copies of *Trp53*, loss of a chromosomal segment spanning *Nf1* and *Trp53* would render tumors heterozygous for *Trp53*. To determine whether tumors in this model are driven by nullizygosity for *Trp53* in addition to nullizygosity for *Nf1*, we assessed the remaining *Trp53* allele by exon sequencing. Of 123 primary and metastatic tumors, we isolated from this mouse model that *Trp53* exon sequencing was obtained on 98 (79%). Interestingly, most tumors retained a copy of wild-type *Trp53* allele, and we identified only 5 mutations in the remaining *Trp53* allele (Fig. 6). These included point mutations and small deletions localizing to the DNA-binding domain, a known hotspot for *Trp53* mutations, and occurring in conjunction with LOH of *NF1* (Fig. 6A). Examination of these mutations in the Catalogue of Somatic Mutations in Cancer (COSMIC, Wellcome Trust Sanger Institute) revealed that each of these mutations occur in diverse human cancers (Fig. 6A).

![Figure 4. Radiation-induced tumors in mice replicate clinical SMN presentations. A, ×100 magnification. B, ×200 magnification images of H&E-stained radiation-induced chest wall sarcoma arising in an *Nf1*-mutant mouse. The soft tissue mass surrounds and invades an adjacent rib (R) with tumor cells present in the marrow. C, local invasiveness of the sarcoma evidenced by invasion of the adjacent skeletal muscle by tumor cells.](image-url)
In contrast to tumors arising in \( Nf1 \)-mutant mice, most tumors from WT mice showed intact heterozygosity at the tested markers (Fig. 5D). However, surprisingly in 5 WT tumors, we identified segmental loss of \( Nf1 \) and \( Trp53 \), similar to tumors arising in \( Nf1^{-/-} \) mice. This suggests that concurrent loss of these genes is also an important mechanism for radiation-induced tumorigenesis in a wild-type background. Among these wild-type tumors, segmental LOH involved either parental allele at similar frequency (Fig. 5D), consistent with allelic loss that is unbiased by an inactivated allele on either parental strand. This pattern stands in contrast to segmental LOH in tumors derived from the \( Nf1^{-/-}/C0 \) mice, in which the mutant \( Nf1 \) allele is maintained on the Sv129-derived allele and preferential loss of the C57Bl/6-derived allele occurred.

From 11 mice identified to have developed metastatic disease, we isolated sufficient quantities of metastatic tumor DNA from 7 (6 primary tumors, each with associated metastasis, and one pair of metastases developing from an unknown primary), permitting comparisons of genetic loss between primary and metastatic disease. Assessing for LOH at \( Nf1 \) and \( Trp53 \) in metastatic disease revealed partial concordance with the primary, as 3 of 7 metastases shared \( Nf1 \) LOH status with their associated primary tumor, whereas 3 metastases did not. In one animal bearing both lung and liver metastases, both metastases showed loss of the C57Bl/6-derived wild-type \( Nf1 \) allele, suggesting that \( Nf1 \) nullizygosity preceded metastases development. Only one tumor showed LOH at 2 tested loci but involving opposite strands; an osteosarcoma metastasis arising in an irradiated WT animal showed loss of the C57Bl/6-derived allele at the D1Mit31 locus and loss of the Sv129-derived allele at rs13481119, located in exon 41 of \( Nf1 \). As a consequence, this metastasis lost \( Trp53 \) and \( Nf1 \) on opposite parental strands, whereas interestingly its matched primary osteosarcoma maintained intact heterozygosity at these loci.

Concurrent distinct primary malignancies (synchronous primaries) are occasionally diagnosed in patients, with synchronous primaries being more common in individuals with tumor predisposition syndromes. Synchronous primary tumors were more common in \( Nf1^{-/-} \) mice and, for both genotypes, high radiation doses (Fig. 2). Synchronously developing tumors are unique reagents for analysis as these tumors arise in the same individual and therefore share the same organismal history genetically and environmentally. We analyzed the synchronously arising tumors in our model to determine whether tumors from a given individual, initiated by the same mutagenic exposure, share genetic alterations at \( Nf1 \) and \( Trp53 \).

From 25 total mice bearing synchronous primary malignancies, we analyzed the previously named microsatellites and SNP in 47 primaries derived from 20 mice. Malignancies in 13 of these 20 mice showed discordance between tumors with regard to LOH at the \( Nf1 \) locus at rs13481119. Interestingly, segmental loss of \( Nf1 \) and \( Trp53 \) was not always shared even among synchronous tumors that were of the same histology, for example, among 7 \( Nf1^{-/-} \) mice that developed bilateral pheochromocytomas, 3 pairs showed genetic discordance characterized by LOH of C57Bl/6-derived \( Nf1 \) and \( Trp53 \)-associated markers in one tumor and intact heterozygosity in the other.

**Somatic loss of \( NF1 \) occurs in human radiation–induced breast cancers**

The genetic basis of human SMNs is poorly understood, most likely due to the scarcity of high-quality SMN tissue.
samples. Our mouse model implicates *Nf1* loss as an important driver of SMN development, but the incidence and significance of *Nf1* loss in human SMNs are not known. We assessed *NF1* status in human radiation-induced breast cancers, which are well-recognized SMNs developing in women who receive chest irradiation as children (7, 26).

Radiation-induced breast cancers and matched normal tissue from patients (8 total patients, none known to have the NF1 syndrome) were analyzed using TaqMan-based SNP genotyping at multiple loci across the *NF1* gene. Allelic discrimination plots show 2 radiation-induced breast cancers (Fig. 7, 2 pairs of matched control normal tissue and tumor, designated E and H shown, Supplementary Fig. S3) whose normal tissues cluster with heterozygous controls (green, A/T genotype) but whose tumors show LOH at SNP rs9902893 in *NF1* (clustering with either T/T or A/A genotype controls). To our knowledge, this is the first evidence of somatic *NF1* gene alterations in radiation-induced SMNs. Further analyses of larger numbers of SMN samples are necessary to more accurately estimate the incidence of *NF1* gene alterations. However, these data suggest concordance between clinical SMNs and our mouse model and justify further comparative studies.

**Discussion**

The pathogenesis of SMNs is not well understood, but clinical analyses indicate that the process is influenced by both cancer therapies and features of patients with cancer themselves. In addition to radiotherapy, chemotherapeutic agents such as alkylating agents and topoisomerase II inhibitors such as epipodophyllotoxin have been associated with SMN development (23, 27). In particular, leukemias as therapy-induced malignancies have been associated with chemotherapies (28, 29). Genetic tumor predisposition syndromes are associated with SMN risk (7), and individuals with strong family histories of cancer...
also have enhanced risk. SMNs are substantially more likely to arise in patients treated as young children (6). Post-treatment endocrinopathies and obesity are common in survivors of pediatric cancers and may also influence risk of SMNs known to be hormone-sensitive such as breast cancer (30).

The AI model replicates the central elements of clinical radiotherapy, namely, anatomic targeting and dose fractionation, in resistant (WT) and susceptible (\(N\text{F1}^{++/-}\)) genetic backgrounds. The resulting radiation-induced diseases reproduce the wide range of SMN histologies developing in irradiated patients. In contrast to an earlier mouse model, we developed that used fractionated focal cranial irradiation (17), the AI model revealed a radiation dose-dependent reduction in survival in the \(N\text{F1}^{+/+}\)-mutant background only. Overall, large numbers of radiation-induced tumors were generated in the AI model, likely explained by the relatively large volume of tissue irradiated with AI. Furthermore, the increased tumor incidence associated with higher doses was driven by the development of heterogeneous tumor types, revealing the general susceptibility of the \(N\text{F1}^{\text{wild-type}}\)-mutant background to mutagen-induced tumorigenesis.

Segmental chromosomal loss identified in radiation-induced tumors from our model appears consistent with a mutagenesis mechanism driven by double-strand breaks, as are classically induced by ionizing radiation. Germline point mutations and microdeletions are well-described genetic mechanisms for \(N\text{F1}^{\text{loss}}\) in individuals with the \(N\text{F1}\) syndrome (31, 32) rather than large chromosomal losses and thus the mechanism of somatic loss of \(N\text{F1}\) in tumors from wild-type and \(N\text{F1}^{\text{mutant}}\)-mutant mice may be unique to radiation-induced mutagenesis.

Wild-type \(N\text{F1}\) loss occurred jointly with \(T\text{rp53}\) in tumors from both wild-type and \(N\text{F1}^{\text{mutant}}\)-mutant backgrounds, suggesting that concurrent loss of these genes can serve as a central event in radiation-induced tumorigenesis regardless of genetic background. In wild-type tumors, \(L\text{OH}\) involving \(N\text{F1}\) may prime affected cells for tumorigenesis similar to \(N\text{F1}^{++/-}\) mice. \(L\text{OH}\) of \(T\text{rp53}\) was also accompanied by somatic mutations of the remaining allele in a minority of tumors analyzed by \(T\text{rp53}\) exon sequencing. The several mutations identified included substitutions and deletions in conserved regions in several tumors that serve as examples of bi-allelic inactivation of \(T\text{rp53}\). \(p\text{53}\) is mutated in 50% of human cancers, with mutations in regulators in \(p\text{53}\) occurring in many tumors lacking \(T\text{rp53}\) mutations (33). It is possible that in this model, dysregulation of \(p\text{53}\) function may occur through alternative mutations. Comprehensive genomic analyses of our mouse tumors lacking bi-allelic \(T\text{rp53}\) loss might reveal these alternative mechanisms of \(T\text{rp53}\) inactivation and are planned for future studies.

An important strength of the AI model is the robust cancer phenotype that includes metastatic disease as well as multiple synchronous tumors. These disease features lend the AI model to comparative studies between (i) primary and metastatic disease and (ii) primary tumors arising from different organs. In the case of synchronously arising primary tumors, the genetic background and tumor-initiating mutagen are identical, and thus comparisons may identify shared as well as organ-specific mechanisms of pathogenesis. Comparisons of matched primary and metastatic disease may identify common and required mechanisms of tumor growth, which similarly holds translational potential for advanced cancers.

Comparative oncogenomics uses experimental mouse and human cancer genetics to gain insights into conserved and robust mechanisms of disease. The genetic and histopathologic analysis of human SMNs has been limited by the relative scarcity of well-archived samples, and thus these tumors are poorly described than their non–therapy-associated counterparts. Harnessing robust animal models to precious human SMN samples may be a particularly productive approach toward gaining insights into SMN pathophysiology. To our knowledge, comparative genetic analyses between a mouse model and clinical SMNs have not been conducted. The presence of \(L\text{OH}\) of \(N\text{F1}\) in radiation-induced human breast cancers suggests a role for \(N\text{F1}\) in SMN pathogenesis. Somatic \(N\text{F1}\) loss in irradiated tissues may promote SMN development. Further analyses of both mouse and human SMN samples are needed to determine the degree of concordance between these systems and to identify potential pathways that may be modified for chemoprevention.

Taken together, our data in wild-type and \(N\text{F1}^{++/-}\) mice suggest that genomic injury after radiation exposure resulting in monoallelic somatic loss of \(N\text{F1}\) and \(T\text{rp53}\) is a common genetic mechanism that promotes radiation-induced tumors. Loss or inactivation of the remaining \(N\text{F1}\) allele may be a critical secondary event that accelerates tumorigenesis in multiple tissue types, as evidenced by the marked susceptibility of \(N\text{F1}^{++/-}\) mice, which already bear the first of presumably 2 required losses. However, as we detected \(L\text{OH}\) of \(N\text{F1}\) in human samples, another possibility is that the upregulation of Ras signaling apparent with monoallelic \(N\text{F1}\) loss is a primed signaling backdrop that increases the efficiency with which additional alterations, for example, in growth factor–mediated signaling, promote cell proliferation and transformation. Genomic and proteomic analyses of these tumors may be particularly productive for identifying these cooperating pathways.

Genome-wide association studies have identified potential predictors of cancer susceptibility in cancer survivors (34, 35). Experimental validation is needed to justify and optimize testing chemoprevention strategies for patients. As a result, our model fills a vital role in studies of SMN susceptibility and the preclinical validation of chemopreventive strategies against SMNs.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: J.L. Nakamura

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