Development of mammary cancer in γ-irradiated F₁ hybrids of susceptible Sprague-Dawley and resistant Copenhagen rats, with copy-number losses that pinpoint potential tumor suppressors

Mayumi Nishimura¹, Kazuhiro Daino¹, Maki Fukuda²,³, Ikuya Tanaka²,³, Hitomi Moriyama²,³, Kaye Showler²,³, Yukiko Nishimura¹, Masaru Takabatake¹,², Toshiaki Kokubo¹, Atsuko Ishikawa¹, Kazumasa Inoue², Masahiro Fukushima³, Shizuko Kakinuma¹,², Tatsuhiko Imaoka1,²,³*, Yoshiya Shimada¹,²,³

¹ Department of Radiation Effects Research, National Institute of Radiological Sciences, National Institutes for Quantum and Radiological Science and Technology, Chiba, Japan, ² Department of Radiological Sciences, Tokyo Metropolitan University, Tokyo, Japan, ³ Radiobiology for Children’s Health Research Group, Research Center for Radiation Protection, National Institute of Radiological Sciences, Chiba, Japan, 4 Laboratory Animal and Genome Sciences Section, National Institute of Radiological Sciences, National Institutes for Quantum and Radiological Science and Technology, Chiba, Japan

Current address: Radiology Department, St. Luke’s International Hospital, Tokyo, Japan
Current address: Diagnostic Imaging Center, Cancer Institute Hospital of Japanese Foundation for Cancer Research, Tokyo, Japan
Current address: Healthcare Business Headquarters, Konica Minolta Inc., Tokyo, Japan
Current address: Department of Radiology, The Jikei University Hospital, Tokyo, Japan
Current address: Department of Radiobiology, Institute for Environmental Sciences, Aomori, Japan
Current address: Institute for Environmental Sciences, Aomori, Japan

* imaoka.tatsuhiko@qat.go.jp (TI); shimada.yoshiya@ies.or.jp (YS)

Abstract

Copenhagen rats are highly resistant to mammary carcinogenesis, even after treatment with chemical carcinogens and hormones; most studies indicate that this is a dominant genetic trait. To test whether this trait is also dominant after radiation exposure, we characterized the susceptibility of irradiated Copenhagen rats to mammary carcinogenesis, as well as its inheritance, and identified tumor-suppressor genes that, when inactivated or mutated, may contribute to carcinogenesis. To this end, mammary cancer–susceptible Sprague-Dawley rats, resistant Copenhagen rats, and their F₁ hybrids were irradiated with 4 Gy of γ-rays, and tumor development was monitored. Copy-number variations and allelic imbalances of genomic DNA were studied using microarrays and PCR analysis of polymorphic markers. Gene expression was assessed by quantitative PCR in normal tissues and induced mammary cancers of F₁ rats. Irradiated Copenhagen rats exhibited a very low incidence of mammary cancer. Unexpectedly, this resistance trait did not show dominant inheritance in F₁ rats; rather, they exhibited intermediate susceptibility levels (i.e., between those of their parent strains). The susceptibility of irradiated F₁ rats to the development of benign mammary tumors (i.e., fibroadenoma and adenoma) was also intermediate. Copy-number losses were frequently observed in chromosome regions 1q52–54 (24%), 2q12–15 (33%),...
and 3q31–42 (24%), as were focal (38%) and whole (29%) losses of chromosome 5. Some of these chromosomal regions exhibited allelic imbalances. Many cancer-related genes within these regions were downregulated in mammary tumors as compared with normal mammary tissue. Some of the chromosomal losses identified have not been reported previously in chemically induced models, implying a novel mechanism inherent to the irradiated model. Based on these findings, Sprague-Dawley × Copenhagen F1 rats offer a useful model for exploring genes responsible for radiation-induced mammary cancer, which apparently are mainly located in specific regions of chromosomes 1, 2, 3 and 5.

Introduction

Exposure to ionizing radiation is common in the modern world and can induce various types of DNA damage, including double-strand breaks. Some DNA double strand break repair systems are inherently error-prone; therefore, radiation exposure can result in mutations, such as large deletions, translocations, and reversions, and can ultimately disrupt the integrity and/or expression of cancer-related genes [1]. Thus, radiation is a risk factor for cancer development in humans. Epidemiological studies have been conducted on populations exposed to radiation from various sources, including atomic bombs, medical devices, nuclear industry workplaces, contaminated environments, and natural background (see [2] for an example). These studies have clarified that cancer risk increases with radiation dose in a manner compatible with a linear response, without a threshold, at low doses and low dose rates [2]. They also suggest that the response can be modified by individual factors such as age, sex, and lifestyle factors, including cigarette smoking [3]. Genetic variation is another contributor that governs individual susceptibility to cancer. Familial cancer exhibits a high probability of inheritance and constitutes approximately 5% of all cancers [4], and greater numbers of genetic polymorphisms are considered to influence cancer risk in a more subtle manner [5]. Although some genetic factors that influence the risk of developing acute tissue reactions after high doses of radiation have been identified, little is known about genetic factors that interact with radiation-induced cancer [3]. Clarification of such interaction would be of benefit when considering radiation use in clinical settings, as well as for the selection of emergency workers and astronauts who will be exposed to relatively high doses of radiation [3].

The interaction between genetic and environmental factors often affects the risk of cancer development [6]. As extremely large sample sizes are generally required to identify such interactions in human populations [7], good animal models provide a valuable opportunity for their identification. The rat has been widely used for experimental models of breast cancer because its disease mimics the luminal nature and ductal origin of human breast cancer [8]. Moreover, the characteristics of different rat strains offer opportunities to study breast-cancer resistance/susceptibility and their inheritance. The inbred Copenhagen (COP) rat is almost completely resistant to spontaneous, chemically induced, and hormonally induced mammary carcinogenesis [9–12], but the susceptibility of COP rats to mammary carcinogenesis caused by ionizing radiation (a well-known human breast carcinogen [13]) has not been thoroughly investigated.

The resistance of COP rats to chemically induced mammary carcinogenesis exhibits dominant inheritance when animals are bred with a variety of susceptible strains including Sprague-Dawley (SD) and Wistar-Furth (WF) [11]. The SD rat is probably the most widely used model of breast cancer in the long history of radiation biology research because of its
worldwide availability and high susceptibility to radiation-induced mammary carcinogenesis [14–17]. (SD×COP)F₁ rats thus provide a good opportunity to investigate the inheritance of susceptibility to radiation-induced mammary carcinogenesis.

Genetic susceptibility to cancer and environmental factors are often linked to specific types of somatic mutations. For example, non-random genomic changes in chromosome 1 are observed in chemically induced mammary tumors in certain strains, including (WF×COP)F₁ rats, but not in radiation-induced cancers [18, 19]. As a potent tumor-suppressor (or oncogenic) gene(s) is expected to be disrupted (or enhanced) in induced cancers in resistant (or susceptible) strains, non-random changes in tumors strongly suggest the existence of potent tumor-related gene(s). Thus, hybrids between strains with different tumor susceptibility offer ideal experimental tools for identifying potentially causative mutations. A successful example is the F₁ hybrid of susceptible C57BL/6 and resistant C3H mice, which develops radiation-induced thymic lymphoma with genomic changes in chromosomal regions spanning critical tumor-suppressor genes [20, 21].

In the present study, we investigated the susceptibility of irradiated COP rats to mammary cancer and compared the results with those acquired with SD and (SD×COP)F₁ rats to obtain insights into the mode of inheritance. Taking advantage of the mammary carcinomas developed in the (SD×COP)F₁ rats, we then located chromosomal regions exhibiting aberrations with the aim of identifying candidate tumor-suppressor genes associated with carcinogenesis.

Materials and methods
Animal experiments

Animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences (NIRS, approval number 07–1014). Female SD rats (Jcl:SD) were purchased from Clea Japan (Tokyo, Japan). COP rats (COP/Hsd) were obtained from Harlan Sprague Dawley (Madison, WI, USA) and maintained by brother-sister mating. F₁ hybrid rats were created by crossing female SD and male COP rats at NIRS. Rats were maintained under specific pathogen-free conditions and fed a standard CE-2 diet (Clea Japan) and sterile water ad libitum. Rats of the SD, (SD×COP)F₁, and COP strains, born between October 2006 and August 2008, were sequentially irradiated (November 2006–March 2007, May 2007, and July 2007–October 2008, respectively) and observed for overlapping periods of time from November 2006 to September 2010. Experiments were performed as described in detail previously [22]. Briefly, 7-week-old female rats were subjected to whole-body γ-irradiation (³²⁰Cs, 4 Gy, 0.5 Gy/min) and then palpated weekly for the remainder of their lifetime to detect tumors. The dose and dose rate of radiation and the age at irradiation were chosen as they are established to induce maximal mammary carcinogenesis, based on our previous studies [17, 23, 24]. Animals that showed signs of general deterioration (including signs of natural death) were euthanized by exsanguination under isoflurane anesthesia and autopsied; animals found dead were also autopsied. Animals terminated before tumor development were censored. During autopsy, palpable and non-palpable tumors were collected, fixed in 10% formalin, embedded in paraffin, and processed for hematoxylin and eosin staining for histology [25]. The palpation record was used to determine the age at which tumors first developed. Normal mammary tissue was collected from an approximately 1 cm² region proximal to an abdominal nipple to ensure inclusion of mammary epithelium; special care was taken to exclude lymph nodes, skin, and muscle. Remaining portions of mammary carcinomas and normal glands were frozen in liquid nitrogen and stored at –80˚C. Results of the analysis of certain molecular characteristics of carcinomas for the (SD×COP)F₁ cohort were reported
previously [22], as were results pertaining to the development of carcinomas in the SD cohort [17].

**DNA and RNA preparation**

DNA extraction from all frozen tumors that were diagnosed as carcinomas (n = 21) was prioritized; of these 21 tumors, those that were freshly collected and had an available remaining portion (n = 10) were used for RNA extraction. This selection of tumors tended to be larger, but were not biased in terms of location, age at detection, age at collection, or the time interval between detection and collection, relative to the entire set of carcinomas (n = 36) (see S1 Fig). Genomic DNA and total RNA were extracted from the same set of frozen normal mammary glands and mammary carcinoma tissue samples using the Maxwell 16 Instrument and System (Promega, Madison, WI, USA) and used for AI and gene expression analyses. Genomic DNA for microarray analysis was extracted as described previously [26].

**Array-based comparative genomic hybridization**

DNA (1.25 μg) samples from normal ear and mammary carcinoma samples were labeled with cyanine 3- and cyanine 5-dUTP, respectively, and purified using columns (Agilent Technologies, Santa Clara, CA, USA). Labeled DNA was hybridized with microarray probes (Rat CGH, 2x105K; Agilent) at 65˚C with rotation at 20 rpm for 40 h, and then washed with Wash Buffers 1 and 2 (Agilent). The microarray resolution was ~14.5 kb (on average) with 97,973 probes, which were annotated in the rn4 version of assembly. Microarrays were scanned using the Agilent G2565BA microarray scanner. Fluorescence intensity values were obtained from scanned images with Agilent Feature Extraction software (ver. 9.5.1, Agilent) and were analyzed using DNA Analytics software (ver. 4.0.81, Agilent). Rat orthologs of human genes relevant to breast cancer [27] were identified in the rn4 rat genome assembly using the UCSC Genome Browser [28]. Annotations pertaining to the role of genes in cancer were retrieved from the Oncogene Database (http://ongene.bioinfo-minzhao.org/), Tumor Suppressor Gene Database (https://bioinfo.uth.edu/TSGene/), and COSMIC Database (https://cancer.sanger.ac.uk/census). Microarray data are available at the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/; accession number GSE160514).

**Analysis of allelic imbalance (AI)**

AI of a simple sequence length polymorphism genomic DNA locus was assessed by comparing the intensities of two DNA bands amplified with the same PCR primer set. Note that, when intensity of one of the two bands (which represent the two alleles at this locus) is stronger than the other, the imbalance can indicate gain of that allele or loss of the other allele, as the PCR assay used is competitive, rather than quantitative. The primer sequences were obtained from the Rat Genome Database [29]. PCR products were resolved by agarose gel electrophoresis through gels containing ethidium bromide. All AI analyses were performed on genomic DNAs from mammary carcinomas and normal ear skin of the same individual.

**Quantitative PCR**

Complementary DNA was synthesized by reverse transcription (ReverTra Ace, Toyobo, Osaka, Japan). Primer sequences and PCR conditions are shown in Table 1 and were validated to amplify a single product of the correct size for each gene by agarose gel electrophoresis. The PCR amplification program consisted of initial denaturation at 95˚C for 10 s followed by 45 amplification cycles of denaturation at 95˚C for 5 s and annealing/elongation at 60˚C for 20 s.
Table 1. Genes and primers for quantitative PCR.

| Gene symbol | Chromosome band | Location (Mb) | Gene name | Function | Forward primer (5’ → 3’) | Reverse primer (5’ → 3’) |
|-------------|-----------------|---------------|-----------|----------|--------------------------|--------------------------|
| Asah2       | 1q52            | 236.0         | Neutral ceramidase that protects against cytokine-induced apoptosis |
| Fas         | 1q52            | 238.3         | Fas cell surface death receptor | Receptor that conveys death signal |
| Ifit1       | 1q52            | 238.6         | Involved in cellular response to cytokine stimulus |
| Sfrp5       | 1q54            | 248.7         | Involved in several processes including Wnt signaling |
| Sreki       | 2q12            | 34.7          | Member of family of serine/arginine-rich splicing proteins |
| Cenpk       | 2q13            | 35.2          | Subunit of a centromeric complex |
| Ercc8       | 2q14            | 39.4          | Component of nucleotide excision repair |
| Plk2        | 2q14            | 41.8          | Serine/threonine protein kinase with role in normal cell division |
| Gbp1        | 2q14            | 42.8          | GC-rich promoter-specific trans-activating transcription factor |
| Il6st       | 2q14            | 43.8          | Part of cytokine receptor complex |
| Itga1       | 2q14            | 47.2          | Subunit of a cell-surface receptor for collagen and laminin |
| Cat         | 3q32            | 88.7          | Catalase | Involved in hydrogen peroxide catabolic process |
| Meis2       | 3q35            | 101.9         | DNA-binding transcription activator in response to growth factor |
| Bmf         | 3q35            | 105.0         | Induction of apoptosis |
| Rad51       | 3q35            | 105.6         | Involved in homologous recombination and repair of DNA |
| Tp53bp1     | 3q35            | 108.0         | Functions in DNA double-strand break repair pathway choice, promoting 3q36 non-homologous end-joining pathways |
| B2m         | 3q35            | 108.9         | Participates in interleukin-12 signaling pathway |
| Dusp2       | 3q36            | 114.8         | Phosphatase of mitogen-activated protein kinase, involved in negative regulation |
| Mal         | 3q36            | 115.1         | Structural constituent of myelin sheath, implicated in metachromatic leukodystrophy |
| Bcl211      | 3q36            | 115.7         | Interacts with other members of BCL-2 protein family and acts as apoptotic activator |
| Nbl1        | 3q42            | 148.4         | Negative regulation of bone morphogenetic protein signaling pathway |
| Runx3       | 5q36            | 154.0         | DNA-binding transcription factor, implicated in breast cancer |

(Continued)
PCR was performed using the Stratagene Mx3000P real-time PCR system (Agilent) and SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). The expression levels of target genes were normalized to those of Gapdh and expressed relative to the value of an arbitrarily selected normal tissue sample using the $2^{- \Delta \Delta Ct}$ method [30].

Statistics

Tumor incidence was compared using Fisher’s exact test. Tumor number and age of first tumor detection were assessed by the Kruskal-Wallis test followed by pairwise comparison using the Mann-Whitney U test. Tumor-free survival data were analyzed by the log rank test and Cox regression. Gene expression levels in two groups were compared with the Mann-Whitney U test. The significance of correlations was assessed by Spearman’s rank correlation test. P values < 0.05 were considered statistically significant. Analyses were performed on statistical software R [31].

Results

Irradiated COP rats are less susceptible to mammary cancer than irradiated SD or (SD×COP)F1 rats

To understand the susceptibility of irradiated COP and (SD×COP)F1 rats to mammary carcinogenesis, we irradiated each of SD (n = 20), COP (n = 19), and (SD×COP)F1 (n = 29) rats with 4 Gy of γ-rays and monitored the development of palpable mammary carcinomas and benign tumors. Benign tumors consisted of fibroadenoma (~95%) and adenoma (~5%). All carcinomas and benign tumors were palpable, and no additional tumors in these categories were discovered upon necropsy. Neither the location of tumors (abdomino-inguinal or thoracic) nor the malignant-to-benign ratio differed among strains, although COP rats had more adenomas than SD and (SD×COP)F1 rats as benign tumors (Table 2). Despite the significantly shortened observation period for SD rats (Table 3), the percentage of rats having carcinomas and benign tumors during their lifetime was higher for SD rats than COP rats, and SD rats had a greater number of tumors per rat (Table 3). The age at the first palpation of individual tumors was also lower for SD rats (Table 3). (SD×COP)F1 rats showed susceptibility that was close to that of SD rats (Table 3). Analysis of the time to first palpable mammary carcinoma indicated that COP rats developed carcinoma significantly less frequently than SD and (SD×COP)F1 rats, whereas the difference between SD and (SD×COP)F1 rats was also substantial, albeit with only marginal statistical significance, suggesting intermediate susceptibility of the (SD×COP)F1 rats (Fig 1A and Table 4). Susceptibility of (SD×COP)F1 rats to benign mammary tumors was intermediate between the parental strains (Fig 1B and Table 4). Causes of censoring did not differ among strains (Table 5). Throughout the course of the experiment,

Table 1. (Continued)

| Gene symbol | Chromosome band | Location (Mb) | Gene name | Function | Forward primer (5’ → 3’) | Reverse primer (5’ → 3’) |
|-------------|-----------------|---------------|-----------|----------|------------------------|------------------------|
| Id3         | 5q36            | 154.9         | Inhibitor of DNA binding 3, HLH protein | Involved in positive regulation of apoptosis | GTGATCTCCACAGGACAGAGGA | TGGAGAGAGGGTCCCCAGATT |
| C1qa        | 5q36            | 155.7         | Complement C1q A chain | Participates in coagulation cascade | CGGGTCCTCAAAGGAGAGAGAGG | CCCACATTGCGGGTTT |
| Gapdh       | 4q42            | 161.3         | Glyceraldehyde-3-phosphate dehydrogenase | Participates in gluconeogenesis pathway, used as an internal control | TCAACGGAAGAACCATCAC | TTTGCCCCACCCTTC |

https://doi.org/10.1371/journal.pone.0255968.t001
SD rats were significantly heavier than COP rats, while the weights of (SD×COP)F₁ rats were intermediate (Fig 1C). Thus, irradiated (SD×COP)F₁ rats showed marginally higher mammary-cancer susceptibility, in contrast to the reported dominant inheritance of resistance of COP rats to chemically induced mammary carcinogenesis.

### Mammary carcinomas of (SD×COP) F₁ rats have multiple localized copy-number variations

Previous studies have shown that radiation-induced mammary carcinomas of SD rats harbor multiple copy-number aberrations that do not converge to specific chromosomal regions [26, 32, 33]. In surprising contrast, our present analysis of mammary carcinomas (n = 21) from (SD×COP)F₁ rats revealed multiple copy-number variations in several specific chromosomal regions (Fig 2A). These variations included copy-number losses of chromosome 1q52–54 (observed in 5 carcinomas, 24%), 2q12–15 (7 carcinomas, 33%), and 3q31–42 (5 carcinomas, 24%). Additional large deletions spanning nearly all of chromosome 5 were identified in 6 carcinomas (29%). Focal deletions involving Cdkn2a and Cdkn2b were found at chromosome 5q32 in 2 carcinomas (10%), as had been repeatedly observed in a subset of radiation-induced rat mammary carcinomas [26, 32, 33]. Combined, focal and large deletions affected this chromosomal region in 8 carcinomas (38%). Deletions of 5q36 were found in 7 carcinomas (33%).

Copy-number gains were relatively rare (Fig 2A). We identified many genes relevant to human breast cancer [27] in the chromosomal regions exhibiting copy-number changes

| Strain | Location (carcinoma) | Location (benign) | Tumor type (all) | Tumor type (benign tumors) |
|--------|----------------------|-------------------|-----------------|---------------------------|
|        | Abdomino-inguinal    | Thoracic          | Abdomino-inguinal | Thoracic | Malignant ¹ | Benign ² | Fibroadenoma | Adenoma |
| SD     | 12 (67%)             | 6 (33%)           | 27 (51%)        | 26 (49%) | 18/71 (25%) | 53/71 (75%) | 53/53 (100%) | 0/53 (0%)³³³ |
| COP    | 8 (89%)              | 1 (11%)           | 5 (45%)         | 6 (55%)  | 9/20 (45%)  | 11/20 (55%) | 7/11 (64%)   | 4/11 (36%)   |
| (SD×COP)F₁ | 18 (50%)         | 18 (50%)          | 38 (56%)        | 30 (44%) | 36/104 (35%) | 68/104 (65%) | 65/68 (96%) | 3/68 (4%)⁴⁴⁴ |

¹ Carcinoma, ² fibroadenoma and adenoma. ³³³ P < 0.01, ⁴⁴⁴ P < 0.001 vs. COP.

SD rats were significantly heavier than COP rats, while the weights of (SD×COP)F₁ rats were intermediate (Fig 1C). Thus, irradiated (SD×COP)F₁ rats showed marginally higher mammary-cancer susceptibility, in contrast to the reported dominant inheritance of resistance of COP rats to chemically induced mammary carcinogenesis.

### Mammary carcinomas of (SD×COP) F₁ rats have multiple localized copy-number variations

Previous studies have shown that radiation-induced mammary carcinomas of SD rats harbor multiple copy-number aberrations that do not converge to specific chromosomal regions [26, 32, 33]. In surprising contrast, our present analysis of mammary carcinomas (n = 21) from (SD×COP)F₁ rats revealed multiple copy-number variations in several specific chromosomal regions (Fig 2A). These variations included copy-number losses of chromosome 1q52–54 (observed in 5 carcinomas, 24%), 2q12–15 (7 carcinomas, 33%), and 3q31–42 (5 carcinomas, 24%). Additional large deletions spanning nearly all of chromosome 5 were identified in 6 carcinomas (29%). Focal deletions involving Cdkn2a and Cdkn2b were found at chromosome 5q32 in 2 carcinomas (10%), as had been repeatedly observed in a subset of radiation-induced rat mammary carcinomas [26, 32, 33]. Combined, focal and large deletions affected this chromosomal region in 8 carcinomas (38%). Deletions of 5q36 were found in 7 carcinomas (33%).

Copy-number gains were relatively rare (Fig 2A). We identified many genes relevant to human breast cancer [27] in the chromosomal regions exhibiting copy-number changes
Fig 1. Kaplan-Meier plots of palpable mammary tumor development in Copenhagen (COP), Sprague-Dawley (SD) and hybrid [(SD×COP)F₁] rats. A, carcinoma; B, benign tumors (fibroadenoma and adenoma). Data from SD rats were reported previously [17] and reanalyzed. C, Body weight during the experiment (mean and standard deviation). ***P < 0.001 between strains, two-way analysis of variance.

https://doi.org/10.1371/journal.pone.0255968.g001
Interestingly, the number of genes affected by copy-number loss was positively correlated with age at tumor detection (Fig 2B, $P < 0.01$), which could be explained by correlation with the number of chromosomes with large deletions spanning $>80\%$ of the chromosome (Fig 2C, $P < 0.01$). The frequencies of copy-number losses and gains involving human-relevant tumor suppressors and proto-oncogenes, respectively, were much higher than those reported in a previous study on SD rats [33] (Table 6, two rightmost columns).

Mammary carcinomas of (SD×COP)$F_1$ rats have multiple AIs

A previous study on radiation-induced mammary carcinoma of (WF×COP)$F_1$ rats indicated that AIs occur at very low frequency (4–13\%) in these tumors; the study did not relate these AIs to copy number aberrations [18]. As the SD strain is an outbred population and genetically heterogeneous, we first searched for simple sequence length polymorphism markers that showed heterozygosity in (SD×COP)$F_1$ rats and their parental strains, using genomic DNA obtained from normal ear skin. This search identified 42 markers that showed heterozygosity in tumor-bearing (SD×COP)$F_1$ rats (Fig 3, markers in black letters with asterisks). We found 35 instances of AIs across 20 markers in mammary carcinoma genomes from (SD×COP)$F_1$ rats (Fig 3, COP and SD). The frequently lost regions (Fig 3, light grey) of chromosomes 1, 2, 3 and 5 identified in the microarray analysis coincided with markers D1Rat49, D1Rat67, D1Mgh29 (chromosome 1), D2Rat116, D2Rat17 (chromosome 2), D3Mit7, D3Rat164

### Table 4. Hazard analysis of palpable mammary tumors among strains.

| Strain          | Hazard ratio (vs. SD) | Log rank test (vs. SD) | Hazard ratio (vs. COP) | Log rank test (vs. COP) |
|-----------------|-----------------------|------------------------|------------------------|------------------------|
| Carcinomas      |                       |                        |                        |                        |
| SD              | 1 (referent)          | —                      | 6.2 (2.3, 17)          | $P = 1 \times 10^{-5}$  |
| COP             | 0.16 (0.06, 0.43)     | $P = 1 \times 10^{-5}$ | 1 (referent)           | —                      |
| (SD×COP)$F_1$   | 0.46 (0.21, 1.0)      | $P = 0.1$              | 2.8 (1.2, 6.8)         | $P = 0.02$             |
| Benign tumors   |                       |                        |                        |                        |
| SD              | 1 (referent)          | —                      | 14 (5.5, 36)           | $P = 1 \times 10^{-7}$  |
| COP             | 0.07 (0.03, 0.18)     | $P = 1 \times 10^{-7}$ | 1 (referent)           | —                      |
| (SD×COP)$F_1$   | 0.29 (0.15, 0.57)     | $P = 0.0002$           | 4.1 (1.7, 10)          | $P = 0.0006$           |

Numbers in parentheses denote 95\% confidence interval.

https://doi.org/10.1371/journal.pone.0255968.t004

Table 5. Causes of censoring.

| Analysis       | Cause                | Strain$^\dagger$ |
|----------------|----------------------|------------------|
|                |                      | SD   | COP | (SD×COP)$F_1$ |
| Carcinoma      | Mammary neoplasm     | 4    | 2   | 5           |
|                | Other neoplasms      | 1    | 3   | 3           |
|                | Non-neoplasms        | 0    | 3   | 0           |
|                | Unidentified         | 2    | 4   | 2           |
| Benign tumors  | Mammary neoplasm     | 2    | 1   | 5           |
|                | Other neoplasms      | 1    | 2   | 1           |
|                | Non-neoplasms        | 0    | 1   | 0           |
|                | Unidentified         | 0    | 5   | 2           |

$^\dagger$ There was no significant difference among strains (Fisher’s exact test).

SD, Sprague-Dawley; COP, Copenhagen.

https://doi.org/10.1371/journal.pone.0255968.t005
(chromosome 3), and D5Mit14 (chromosome 5). In AIs of these markers, imbalance was not strongly biased towards either the SD or COP allele, and the trend varied among chromosomal sites (Fig 3, SD and COP). At several sites, copy-number variations identified using microarrays did not accompany AI, indicating the relatively low sensitivity of AI detection, which is understandable, considering the possibility for contamination of tumor samples with non-malignant cells (e.g., stromal cells). The majority of observed AIs (28/35) were accompanied by microarray-identified copy-number loss (Fig 3, SD and COP on light-grey background), suggesting that these AIs reflected a loss of heterozygosity produced by large deletions. In contrast, AIs accompanying microarray-identified copy-number gain were rare (2/35; Fig 3, SD and COP on black background). Some AIs (5/35) were without copy-number variation (Fig 3, SD and COP on white background).
Table 6. Human breast cancer–related genes in chromosomal regions exhibiting meaningful copy-number changes in (SD×COP)1 mammary carcinoma.

| Gene symbol | Role in cancer | Chromosome band | Location (Mb) | Tumors with copy-number change (n = 21) | Moriya et al. [33] (n = 28)
|-------------|----------------|-----------------|---------------|-----------------------------------------|-------------------------------|
|             |                |                 |               | Loss | Gain | Loss | Gain |

Esr1          | POG/TSG        | 1q11            | 35.5–35.8     | 2 (10%) | 0 (0%) | 0 (0%) | 0 (0%) |
Cnot3         | TSG            | 1q12            | 63.9          | 2 (10%) | 0 (0%) | 0 (0%) | 0 (0%) |
Cic           | POG/TSG        | 1q21            | 80.6          | 2 (10%) | 0 (0%) | 0 (0%) | 1 (4%) |
Palb2         | TSG            | 1q36            | 180.9–181.0   | 3 (14%) | 0 (0%) | 0 (0%) | 0 (0%) |
Men1          | TSG            | 1q43            | 209.1         | 2 (10%) | 0 (0%) | 0 (0%) | 0 (0%) |
Pten          | TSG            | 1q52            | 236.8         | 5 (24%) | 0 (0%) | 2 (7%) | 0 (0%) |
Pik3r1        | POG/TSG        | 2q12            | 32.6–32.7     | 7 (33%) | 0 (0%) | 0 (0%) | 0 (0%) |
Map3k1        | POG/TSG        | 2q14            | 43.1          | 7 (33%) | 0 (0%) | 0 (0%) | 0 (0%) |
Fbxw7         | TSG            | 2q34            | 176.7–176.8   | 3 (14%) | 0 (0%) | 0 (0%) | 0 (0%) |
Notch2        | POG/TSG        | 2q34            | 192.8–193.0   | 3 (14%) | 0 (0%) | 0 (0%) | 0 (0%) |
Notch1        | POG/TSG        | 3p13            | 4.6–4.7       | 1 (5%)  | 0 (0%) | 0 (0%) | 1 (4%) |
Bub1b         | TSG            | 3q35            | 105.1         | 5 (24%) | 0 (0%) | 0 (0%) | 0 (0%) |
Foxp1         | POG/TSG        | 4q34            | 133.8–134.0   | 1 (5%)  | 0 (0%) | 0 (0%) | 0 (0%) |
Cdkn1         | POG/TSG        | 4q43            | 171.8         | 1 (5%)  | 0 (0%) | 0 (0%) | 0 (0%) |
Cdkn2a        | TSG            | 5q32            | 108.9         | 8 (38%) | 0 (0%) | 3 (11%) | 0 (0%) |
Cdkn2b        | TSG            | 5q32            | 108.9         | 8 (38%) | 0 (0%) | 3 (11%) | 0 (0%) |
Arid1a        | TSG            | 5q36            | 151.4         | 7 (33%) | 0 (0%) | 0 (0%) | 0 (0%) |
Spen          | TSG            | 5q36            | 160.4         | 7 (33%) | 0 (0%) | 0 (0%) | 0 (0%) |
Msh2          | TSG            | 6q12            | 11.2–11.3     | 2 (10%) | 0 (0%) | 1 (4%) | 0 (0%) |
Dnm3a         | TSG            | 6q14            | 26.8–26.9     | 2 (10%) | 0 (0%) | 0 (0%) | 0 (0%) |
Smarca4       | TSG            | 8q13            | 20.7–20.8     | 2 (10%) | 0 (0%) | 0 (0%) | 0 (0%) |
Atm           | TSG            | 8q24            | 56.9–57.0     | 2 (10%) | 0 (0%) | 0 (0%) | 0 (0%) |
Setsd2        | TSG            | 8q32            | 114.9–115.5   | 2 (10%) | 0 (0%) | 0 (0%) | 0 (0%) |
Mlh1          | TSG            | 8q32            | 115.6–115.7   | 2 (10%) | 0 (0%) | 1 (4%) | 0 (0%) |
Casp8         | TSG            | 9q31            | 57.4          | 2 (10%) | 0 (0%) | 1 (4%) | 0 (0%) |
Crebbp        | POG/TSG        | 10q12           | 11.6–11.7     | 0 (0%)  | 1 (5%) | 0 (0%) | 0 (0%) |
Axin1         | TSG            | 10q12           | 15.4–15.5     | 1 (5%)  | 1 (5%) | 0 (0%) | 0 (0%) |
Ncor1         | TSG            | 10q23           | 48.5–48.6     | 1 (5%)  | 1 (5%) | 0 (0%) | 0 (0%) |
Map2k4        | POG/TSG        | 10q24           | 52.0          | 1 (5%)  | 1 (5%) | 0 (0%) | 0 (0%) |
Tp53          | POG/TSG        | 10q24           | 56.4          | 1 (5%)  | 1 (5%) | 0 (0%) | 0 (0%) |
Nf1           | TSG            | 10q35           | 65.6–65.8     | 1 (5%)  | 1 (5%) | 0 (0%) | 0 (0%) |
Erbb2         | POG            | 10q31           | 87.2          | 0 (0%)  | 1 (5%) | 0 (0%) | 0 (0%) |
Cax1          | POG/TSG        | 12q12           | 21.3–21.5     | 0 (0%)  | 1 (5%) | 0 (0%) | 0 (0%) |
Tbx3          | POG/TSG        | 12q16           | 38.2          | 0 (0%)  | 1 (5%) | 0 (0%) | 0 (0%) |
Nf2           | TSG            | 14q21           | 85.4–85.5     | 1 (5%)  | 0 (0%) | 0 (0%) | 0 (0%) |
Rb1           | TSG            | 15q11           | 53.8–54.0     | 1 (5%)  | 0 (0%) | 1 (4%) | 0 (0%) |
Znf703        | POG            | 16q12.3         | 69.4          | 0 (0%)  | 1 (5%) | 0 (0%) | 0 (0%) |
Fgf1          | POG            | 16q12.4         | 70.9          | 0 (0%)  | 2 (10%)| 0 (0%) | 0 (0%) |
Gata3         | POG/TSG        | 17q12.3         | 80.0          | 3 (14%) | 2 (10%)| 0 (0%) | 0 (0%) |
Apc           | TSG            | 18p12           | 26.7–26.8     | 3 (14%) | 0 (0%) | 0 (0%) | 0 (0%) |
Smad4         | TSG            | 18q12.2         | 70.4–70.5     | 3 (14%) | 0 (0%) | 1 (4%) | 0 (0%) |
Prdm1         | TSG            | 20q13           | 48.4–48.5     | 3 (14%) | 0 (0%) | 0 (0%) | 0 (0%) |
Bcor          | TSG            | Xq12            | 22.7–22.8     | 4 (19%) | 0 (0%) | 1 (4%) | 0 (0%) |
Atrx          | TSG            | Xq22            | 93.9–94.1     | 4 (19%) | 0 (0%) | 1 (4%) | 0 (0%) |

(Continued)
Reduced expression of certain potential cancer-related genes in chromosomal regions with copy-number losses

The observation of frequent copy-number losses in specific chromosomal regions implies that important tumor-suppressor genes may be located within these regions. Among genes known to be related to human breast cancer [27], we previously reported the reduced expression of Pten (on 1q52, 0.53-fold), Pik3r1 (on 2q12, 0.61-fold), and Map3k1 (on 2q14, 0.75-fold) in mammary carcinomas of (SD × COP)F₁ rats [22]. Here, we investigated the expression of 24 different potentially cancer-related genes found in the chromosomal regions showing copy-number losses. Genes were selected based on functions reported in the NCBI Gene Database (https://www.ncbi.nlm.nih.gov/gene/). Quantitative PCR analysis revealed 11 putative tumor-suppressor genes that had reduced expression in radiation-induced carcinomas (Fig 4). These included Asah2, Fas, Sfrp5 (selected from chromosome 1q52–54), Il6st, Itga1 (chromosome 2q12–15), Meis2, B2m, Mal, Nbl1 (chromosome 3q31–42), and Id3 and C1qa (chromosome 5) (Fig 4). Genes for which expression did not change included Ifit1 (chromosome 1q52), Srek1, Cenpk, Ercc8, Gpibp1 (chromosome 2q12–14), Cat, Bmf, Rad51, Tp53bp1, Dusp2, Bcl2l1 (chromosome 3q31–42), and Runx3 (chromosome 5). Plk2 (chromosome 2q14) was significantly upregulated. The downregulation of these 11 genes supports their potential relevance to radiation-induced mammary carcinogenesis.

Discussion

Epidemiological studies have established that radiation exposure is a risk factor for cancer development in humans, and determining the genetic factors that interact with radiation in this context is vital to understanding individuals’ responses to radiation [3]; however, screening of genetic polymorphisms related to environmental cancer risk in humans generally requires a massive sample size [7]. In this regard, animal models are useful for studying the role of gene-environment interactions in cancer susceptibility. Analyses of F₁ hybrids of cancer-susceptible and -resistant strains are also advantageous for identifying driver genes in cancer using experimental animal models, including those of radiation-induced carcinogenesis. We examined the mammary-cancer susceptibility of irradiated (SD × COP)F₁ hybrids of susceptible SD and resistant COP rats and found that they had intermediate susceptibility levels; thus, they are useful for exploring cancer-causing gene mutations. Our approach of combining analyses of copy-number variations and AIs in mammary cancer successfully identified marginally frequent (14–38%) copy-number losses in chromosome regions 1q52–54, 2q12–15,
and 3q31–42 as well as chromosome 5, with many genes in these regions showing reduced expression. This frequency is higher than the AI frequency reported in radiation-induced mammary cancer of (WF×COP)F\textsubscript{1} rats (4–13\%) \cite{18}, and higher than the frequency of copy number changes in breast cancer–relevant genes in SD rats (4–11\%) \cite{33}. Thus, (SD×COP)F\textsubscript{1} rats offer a new option in the search for causative genes of radiation-induced mammary cancer.

The present study is the first report of mammary carcinogenesis in irradiated COP and (SD×COP)F\textsubscript{1} rats. COP rats are completely resistant to chemically induced mammary cancer, whereas radiation-induced mammary cancer induces tumors in both the irradiated and non-irradiated rats. The present study demonstrates that the radiation-induced mammary cancer is more frequent and more aggressive in (SD×COP)F\textsubscript{1} rats than in COP rats. This suggests that radiation-induced mammary cancer is more sensitive to genetic changes than chemically induced mammary cancer.

### Table 1

| Marker | Chromosome band | Location (Mb) | Tumors with AI |
|--------|-----------------|---------------|----------------|
| D1Rat4* | 1p11 | 29.7 | COP |
| D1Rat4* | 1q32 | 157.5 | COP |
| D1Rat7* | 1q11 | 188.6 | SD |
| D1Mga3* | 1q5 | 234.6 \(1\) | SD |
| D2Rat11* | 2q12 | 33.8 | COP |
| D2Rat11* | 2q14 | 48.2 | SD |
| D2Rat6* | 2q34 | 106.5 | SD |
| D2Rat6* | 2q43 | 232.6 | SD |
| D3Mga19* | 3q11 | 15 | COP |
| D3Mga19* | 3q22 | 88.6 | COP |
| D3Rat16* | 3q55 | 108.1 | COP |
| D4Rat27* | 4q22 | 56.6 | SD |
| D4Rat114* | 4q34 | 128.8 | SD |
| D5Rat11* | 5p26 | 138.5 | SD |
| D5Rat11* | 5p36 | 165.1 | SD |
| D6Rat20* | 6p16 | 48.2 | SD |
| D6Rat45* | 6p33 | 108.5 | SD |
| D6Rat17* | 6q31 | 108.8 | SD |
| D7Rat19* | 7q13 | 29.1 | SD |
| D8Rat16* | 8p13 | 29.4 | SD |
| D8Rat32* | 8p24 | 76.0 | SD |
| D9Rat11* | 9q12 | 10.4 | SD |
| D9Rat11* | 9p22 | 37.5 | SD |
| D9Rat12* | 9p38 | 111.5 | SD |
| D10Rat21* | 10q12 | 16.8 | SD |
| D10Rat12* | 10q34 | 51.5 | SD |
| D10Rat27* | 10q35 | 52.9 | SD |
| D13Mga11* | 13q31 | 60.2 | SD |
| D11Rat34* | 11q21 | 63.8 | SD |
| D11Rat38* | 11q33 | 79 | SD |
| D12Rat4* | 12q11 | 13.6 | SD |
| D12Rat5* | 12q12 | 40.4 | SD |
| D12Rat5* | 12q13 | 43.5 | SD |
| D13Rat32* | 13q32 | 66.5 | SD |
| D14Rat8* | 14q22 | 7.7 | SD |
| D14Rat55 | 14q22 | 101.5 | SD |
| D15Rat5 | 15q13 | 11.1 | SD |
| D15Rat5 | 15q32 | 100 | SD |
| D16Mga1* | 16q14 | 116.2 | SD |
| D16Rat4* | 16q31 | 75.8 | SD |
| D17Rat2* | 17q14 | 35.7 | SD |
| D17Rat2* | 17q21 | 36.1 | SD |
| D18Rat7* | 18q12 | 84.6 | SD |
| D19Rat7* | 19q12 | 12.9 | SD |
| D2Rat2 | 2p11 | 6 | SD |
| D3Rat5* | 3p11 | 18.4 | SD |
| D3Rat6* | 3q12 | 45.9 | SD |

Fig3. Allelic imbalance (AI) sites observed in (SD×COP)F\textsubscript{1} mammary carcinomas. Results of the AI analysis along with information from microarray-based copy-number analysis (Fig 2), indicated in grey scale. Columns indicate individual tumors. Note that copy-number variations irrelevant to the indicated markers are not shown. *Markers with (black with asterisk) or without (grey) heterozygous individuals. \textsuperscript{a}No data present in the rn4 rat genome assembly; values in parentheses are from the Celera assembly. \textsuperscript{b}H, markers exhibiting homozygosity, where allelic analyses were impossible.

https://doi.org/10.1371/journal.pone.0255968.g003
Fig 4. Expression of 24 genes located in chromosomes 1q52–54, 2q12–15, 3q31–42, and 5 showing copy-number losses in mammary carcinomas of irradiated (SD×COP)F1 rats. Relative mRNA expression levels of the indicated genes in carcinomas and matched normal mammary glands are shown. N, normal tissues (n = 7–8); T, tumors (n = 10). Data are presented as box plots with median values indicated by horizontal bars within the boxes. Boxes represent values between the 25th and 75th percentiles, whiskers extend to the 5th and 95th percentiles, and circles represent outliers. *P < 0.05; **P < 0.01, ***P < 0.001 (Mann-Whitney U test). Relative expression was normalized to a specific sample.

https://doi.org/10.1371/journal.pone.0255968.g004
carcinogenesis [11]. In the present study, mammary carcinoma developed in irradiated COP rats after 60 weeks post-irradiation. As chemically induced tumor development usually occurs earlier (typically within 30 weeks post-induction [11]), the observation period tends to be shorter for chemically induced carcinogenesis experiments than for radiation-induced carcinogenesis experiments (e.g., 300 days in Isaacs 1988 [11] vs. >100 weeks in the present study). Thus, the lack of mammary carcinomas following chemical induction may be due to the shortened observation period.

The longer observation period in the present study also enabled evaluation of susceptibility to the development of benign mammary tumors (fibroadenoma and adenoma), which showed the same tendency as susceptibility to mammary carcinoma. This similarity might be understood given that each of these lesions is of epithelial origin and that the resistance exhibited by COP rats has been attributed to epithelial cells [34].

The inheritance of COP rat resistance to chemically induced mammary carcinogenesis has been reported to be dominant under a short observation period [11]. This is in stark contrast to our finding that the susceptibility of (SD×COP)F1 hybrids was intermediate, i.e., between that of its parent strains, which is logical, as this trait is known to be polygenic [19]. The inheritance of benign mammary tumors has not yet been reported because most of the previous experiments used chemical carcinogens, which mainly induce carcinoma. The present finding indicates that inheritance of benign tumor resistance in (SD×COP)F1 hybrids is similar to that of carcinoma, suggesting a common mechanism of resistance to carcinoma and benign tumors in COP rats.

The present study identifies chromosome regions 1q52–54, 2q12–15, and 3q31–42 and the entire chromosome 5 as sites of potentially relevant cancer-related genes. We previously reported aberrations of genes related to the phosphoinositide 3-kinase (PI3K) pathway, including downregulation of Pten (1q52) and mutations of Pik3r1 (2q12), in mammary cancers from the same cohort of irradiated (SD×COP)F1 rats [22], but the frequency of these mutations was low (i.e., 1 of 14 for each mutation). Copy number loss of Pten observed in (SD×COP)F1 rats (24%) has also been reported in SD rats [33]. Chromosome 1q52–54 coincides with the location of the mammary-cancer susceptibility quantitative trait locus (QTL) Ms17. The SD allele of this QTL is associated with an increased number of chemically induced mammary carcinomas compared with the COP allele [35]. By contrast, in our data, the polymorphic marker D1Mgh29, which is located on chromosome 1q52–54, shows a higher rate of loss of the potential susceptibility SD allele (2 of 3), suggesting a different role for this allele in the present model. Analysis of the human counterpart of this region (10q23) revealed frequent losses in sporadic breast cancers [36]. On the other hand, rat chromosome 2q14 (which harbors Gphp1, Map3k1 and Il6st) coincides with Mamtr3 (also known as Ms1b or Ms10), another mammary-cancer susceptibility QTL for which the COP allele confers resistance to chemically induced mammary carcinogenesis [37]. Map3k1 (43.1 Mb of chromosome 2) and Mier3 (43.0 Mb) have been identified as candidate susceptibility genes within this region [38]. Our data indicate that loss of the markers D2Rat116 and D2Rat17, which flank Map3k1 and Mier3, are biased to the potentially resistant COP allele. Analysis in humans indicated that MAP3K1 is the gene in the corresponding region (human chromosome 5q11.2) with the greatest influence on risk of breast-cancer development [39]. No QTL has been reported on chromosome 3q31–42, suggesting the existence of important unidentified determinants of susceptibility. A further search for candidate causative genes in this region is thus warranted, and downregulated genes identified in the current study, namely Meis2, B2m, Mal, and Nbl1, are candidates for further study. Comparison with previous measurements of gene expression changes in radiation-induced SD rat mammary carcinoma [24] (S2 Fig) supports downregulation of Fas, Sfrp5 (chromosome 1q52–54), Itga1 (chromosome 2q12–15), Meis2, B2m, Mal (chromosome 3q31–
42), Id3, Clqα, and Nbl1 (chromosome 5), reinforcing the role of these genes as potential tumor suppressors. A previous study on irradiated (WF×COP)F₁ rats revealed somewhat less frequent AI of markers located on 1q32–56 (1–3 of 24 tumors, 4–12%) and 2q24–34 (1–2 of 25 tumors, 4–8%) [18], implying that the mechanisms underlying mammary carcinogenesis differ between SD and WF rats.

Our present analysis indicated a positive correlation between large deletions and age at tumor detection, implying another benefit of the long observation period. This observation suggests that some tumors develop via accumulation of large chromosomal deletions which requires time, whereas those that develop early may use other mechanisms, such as point mutations, inversions, and translocations.

Beside the above-mentioned susceptibility loci, several factors should be considered that may have caused the observed strain difference. Burden of mammary tumor virus has been related to mammary tumor susceptibility in some mouse strains [40], and a counterpart tumor virus has been reported in rats [41], but has not been confirmed extensively; however, we cannot completely rule out the possibility that the strain difference observed in the current study is due to virus burden. Genetic contamination has been reported as another factor influencing disease susceptibility in specific rat strains [42, 43]. The current SD and COP strains were maintained under strict management; nevertheless, as genetic tests were not conducted, the possibility of contamination cannot be excluded. It is also possible that systemic factors affect tumor development. Long-term hormone administration to COP rats has been reported to promote development of mammary tumors that would otherwise undergo spontaneous regression [44]. Fat tissue is a source of estrogen, especially after senescence of ovarian function; thus, obesity and being overweight promote breast cancer development [45]. The difference in body weight among rat strains reported herein, which is concordant with that associated with mammary cancer susceptibility, is therefore a plausible factor that could explain the observed susceptibility.

In humans, sporadic breast cancers generally exhibit multiple DNA copy number aberrations, consistent with the present animal model. By contrast, there is little evidence of genetic aberrations in radiation-induced breast cancer in humans. In the present study, the regions showing copy number aberrations in tumors from (SD×COP)F₁ rats did not necessarily correspond to those previously reported in SD rats [33]. This strain difference suggests that copy number aberrations in radiation-induced breast cancer are subject to the influence of genetic background. In fact, in a study of human breast cancers that developed as second primary cancers after radiotherapy (n = 3 tumors), no common deletions or inversions that could be causative were reported; rather, multiple deletions and inversions of non-coding regions were reported [46]. Larger investigations are thus warranted to clarify the common genetic changes detected in radiation-induced human cancers.

The present study has the following limitations. First, results should be compared between irradiated and non-irradiated groups to determine extent to which the present findings can be attributed to radiation exposure or genetic characteristics. Second, our results may have been affected by variation in exposure conditions, such as radiation dose, dose rate, fractionation regimen, radiation type, age at time of exposure, hormonal conditions at time of exposure, and whether the whole or partial body was irradiated. This point remains open for future study. Third, it is unclear whether the mammary carcinoma subtype is strain-dependent. Our previous studies on mammary carcinomas of (SD×COP)F₁ (n = 24 tumors) and SD (n = 85 tumors) rats indicated that they are mainly (70%–90%) of the luminal subtype [22, 32, 33], consistent with reports from other laboratories [47, 48]. Further studies are required to clarify if this applies to COP rats, as the number of tumors obtained in our study was small (n = 9) due to the high resistance of the strain.
Taken together, the present study clearly indicates that irradiated (SD×COP)F₁ hybrid rats are intermediately susceptible to mammary carcinogenesis, and hence are a useful model for exploring potentially causative gene mutations in mammary cancer. Chromosome regions 1q52–54, 2q12–15, and 3q31–42 and chromosome 5 are expected to harbor driver mutations relevant to mammary carcinogenesis. This study also suggests that mammary cancer in (SD×COP)F₁ rats involves many genetic aberrations that are relevant to human breast cancer and thus offers a good model for basic research.

**Supporting information**

**S1 Dataset. Unprocessed data.** Sheet 1: Animal and tumor development data used for Fig 1 and Tables 3–5. Sheet 2: Tumor location and tumor type data in Table 2 and age at first tumor detection data used for Table 3. Sheet 3: Quantitative PCR data used for Fig 4. Sheet 4: Copy-number aberration data used for Table 6. Sheet 5: Previous expression microarray data mentioned in Discussion.

(XLSX)

**S1 Fig. Effect of selection of tumors.** Distribution of tumor weight (A), age at tumor detection (B), age at autopsy (C), and tumor age (i.e., interval between tumor detection and autopsy) (D). Circles, individual tumors; horizontal and vertical bars, mean and SD. *P* values, Welch’s *t* test.

(TIF)

**S2 Fig. Expression of genes in SD rat mammary carcinomas.** Genes in Fig 4 in mammary carcinomas from SD rats from a previous microarray analysis [24]. Expression levels are standardized against the 75th percentiles of all genes on individual microarrays and are expressed as log₂ values. Probe IDs are shown above the gene symbols. Data are presented as the mean and SD. * *P* < 0.05, Mann-Whitney U test.

(TIF)

**Acknowledgments**

We thank Harumi Osada and Masami Ootawara for animal breeding and specimen preparation as well as the staff at the Laboratory Animal and Genome Sciences Section for animal facility management. A portion of this work was conducted in association with the QST-NIRS project of the Japan StoreHouse of Animal Radiobiology Experiments (J-SHARE) [49].

**Author Contributions**

**Conceptualization:** Kazumasa Inoue, Masahiro Fukushi, Shizuko Kakinuma, Yoshiya Shimada.

**Formal analysis:** Kazuhiro Daino, Atsuko Ishikawa, Tatsuhiko Imaoka.

**Funding acquisition:** Kazuhiro Daino, Shizuko Kakinuma, Tatsuhiko Imaoka.

**Investigation:** Mayumi Nishimura, Kazuhiro Daino, Maki Fukuda, Ikuya Tanaka, Hitomi Moriyama, Kaye Showler, Yukiko Nishimura, Masaru Takabatake, Toshiaki Kokubo, Tatsuhiko Imaoka.

**Project administration:** Shizuko Kakinuma, Yoshiya Shimada.

**Software:** Atsuko Ishikawa.

**Supervision:** Kazumasa Inoue, Masahiro Fukushi, Yoshiya Shimada.
Writing – original draft: Mayumi Nishimura, Kazuhiro Daino, Tatsuhiko Imaoka, Yoshiya Shimada.

Writing – review & editing: Maki Fukuda, Ikuya Tanaka, Hitomi Moriyama, Kaye Showler, Yukiko Nishimura, Masaru Takabatake, Toshiaki Kokubo, Atsuko Ishikawa, Kazumasa Inoue, Masahiro Fukushima, Shizuko Kakinuma.

References

1. Chauhan V, Stricklin D, Cool D. The Integration of the Adverse Outcome Pathway Framework to Radiation Risk Assessment. Int J Radiat Biol. 2020:1–21. Epub 2020/05/14. https://doi.org/10.1080/09553002.2020.1761570 PMID: 32397918.

2. National Council on Radiation Protection and Measurements. Implications of recent epidemiologic studies for the linear no-threshold model and radiation protection. Bethesda, Maryland: National Council on Radiation Protection and Measurements; 2018. ix, 199 pages p.

3. Applegate KE, Ruhm W, Wojcik A, Bourquin M, Brenner A, Hamasaki K, et al. Individual response of humans to ionising radiation: governing factors and importance for radiological protection. Radiat Environ Biophys. 2020; 59(2):185–209. Epub 2020/03/09. https://doi.org/10.1007/s00411-020-00837-y PMID: 32146555.

4. Pomerantz MM, Freedman ML. The genetics of cancer risk. Cancer J. 2011; 17(6):416–22. Epub 2011/12/14. https://doi.org/10.1097/PPO.0b013e31823e5387 PMID: 22157285.

5. Stewart BW, Wild C, International Agency for Research on Cancer, World Health Organization. World cancer report 2014. Lyon, France, Geneva, Switzerland: International Agency for Research on Cancer, WHO Press; 2014. xiv, 630 pages p.

6. Carbone M, Amelio I, Affar EB, Brugarolas J, Cannon-Albright LA, Cantley LC, et al. Consensus report of the 8 and 9th Weinman Symposia on Gene x Environment Interaction in carcinogenesis: novel opportunities for precision medicine. Cell Death Differ. 2018; 25(11):1885–904. Epub 2018/10/17. https://doi.org/10.1038/s41418-018-0213-5 PMID: 30323273.

7. Simonds NI, Ghazarian AA, Pimentel CB, Schully SD, Ellison GL, Gillanders EM, et al. Review of the Gene-Environment Interaction Literature in Cancer: What Do We Know? Genet Epidemiol. 2016; 40(5):356–65. Epub 2016/04/12. https://doi.org/10.1002/gepi.21967 PMID: 27061572.

8. Gould MN. Rodent models for the study of etiology, prevention and treatment of breast cancer. Semin Cancer Biol. 1995; 6(3):147–52. https://doi.org/10.1006/scbi.1995.0023 PMID: 7495982.

9. Dunning WF, Curtis MR. The respective roles of longevity and genetic specificity in the occurrence of spontaneous tumors in the hybrids between two inbred lines of rats. Cancer Res. 1946; 6:81–8. Epub 1946/02/01. PMID: 21010873.

10. Isaacs JT. Genetic control of resistance to chemically induced mammary adenocarcinogenesis in the rat. Cancer Res. 1986; 46(8):3958–63. Epub 1986/08/01. PMID: 3089584.

11. Isaacs JT. Inheritance of a genetic factor from the Copenhagen rat and the suppression of chemically induced mammary adenocarcinogenesis. Cancer Res. 1988; 48(2):2204–13. Epub 1988/04/15. PMID: 3127047.

12. Gould MN, Zhang R. Genetic regulation of mammary carcinogenesis in the rat by susceptibility and suppressor genes. Environ Health Perspect. 1991; 93:161–7. Epub 1991/06/01. https://doi.org/10.1289/ehp.9193161 PMID: 1773877.

13. Brenner AV, Preston DL, Sakata R, Sugiyama H, de Gonzalez AB, French B, et al. Incidence of Breast Cancer in the Life Span Study of Atomic Bomb Survivors: 1958–2009. Radiat Res. 2018. https://doi.org/10.1667/RR15015.1 PMID: 30044713.

14. Cronkite EP, Shellabarger CJ, Bond VP, Lippincott. Studies on radiation-induced mammary gland neoplasia in the rat. I. The role of the ovary in the neoplastic response of the breast tissue to total- or partial-body x-irradiation. Radiat Res. 1980; 10:62–93. Epub 1980/01/01. PMID: 13612937.

15. Welsch CW, Goodrich-Smith M, Brown CK, Migliore N, Clifton KH. Effect of an estrogen antagonist (tamoxifen) on the initiation and progression of gamma-irradiation-induced mammary tumors in female Sprague-Dawley rats. Eur J Cancer Clin Oncol. 1981; 17(12):1255–6. Epub 1981/12/01. https://doi.org/10.1016/0959-8049(81)90004-8 PMID: 7200024.

16. Broersse JJ, Hennen LA, Klipwijk WM, Solleveld HA. Mammary carcinogenesis in different rat strains after irradiation and hormone administration. Int J Radiat Biol Relat Stud Phys Chem Med. 1987; 51(6):1091–100. Epub 1987/06/01. https://doi.org/10.1080/09653008714551381 PMID: 3496299.
PLOS ONE | https://doi.org/10.1371/journal.pone.0255968 August 13, 2021 19 / 20

18. Haag JD, Hsu LC, Newton MA, Gould MN. Allelic imbalance in mammary carcinomas induced by either 7,12-dimethylbenz[a]anthracene or ionizing radiation in rats carrying genes conferring differential susceptibilities to mammary carcinogenesis. Mol Carcinog. 1996; 17(3):134–43. PMID: 8944073.

19. Gould MN, Lubet RA, Kelloff GJ, Haag JD. Inherited susceptibility and acquired allelic imbalance in rat mammary carcinogenesis. J Cell Biochem Suppl. 1996; 25:37–40. Epub 1996/01/01. PMID: 9027596.

20. Shimada Y, Nishimura K, Ikaros, Okamoto M, Shirosi T, Clifton KH, et al. Radiation-associated loss of heterozygosity at the Znf11a1 (ikaros) locus on chromosome 11 in murine thymic lymphomas. Radiat Res. 2000; 154(3):293–300. PMID: 10956435.

21. Okamoto M, Mori N, Myashita N, Moriwaki K, Imai S, Haga S, et al. Radiation-induced lymphomas in MSM, (BALB/cHeA x MSM) F1 and (BALB/cHeA x STS/A) F1 hybrid mice. Exp Anim. 1995; 44(1):43–8. Epub 1995/01/01. https://doi.org/10.1538/expanim.44.43 PMID: 7705478.

22. Showler K, Nishimura M, Daino K, Imaoka T, Moroika T, et al. Analysis of genes involved in the P13K/Akt pathway in radiation- and MNU-induced rat mammary carcinomas. J Radiat Res. 2017; 58(2):183–94. Epub 2017/04/14. https://doi.org/10.1093/jrr/jrw097 PMID: 27738081.

23. Takabatake M, Daino K, Imaoka T, Blyth BJ, Kokubo T, Nishimura Y, et al. Differential effect of parity on rat mammary carcinogenesis after pre- or post-pubertal exposure to radiation. Sci Rep. 2018; 8(1):14325. Epub 2018/09/27. https://doi.org/10.1038/s41598-018-32406-1 PMID: 30254198.

24. Imaoka T, Nishimura M, Izuik D, Nishimura Y, Ohmachi Y, Shimada Y. Pre- and postpubertal irradiation induces mammary cancers with distinct expression of hormone receptors, ErbB ligands, and developmental genes in rats. Mol Carcinog. 2011; 50(7):539–52. Epub 2011/03/05. https://doi.org/10.1002/mc.20746 PMID: 21374731.

25. Mohr U. International Classification of Rodent Tumours. Part I: The Rat. 5. Integumentary Systems. Lyon: Oxford University Press; 1993. 22–37 p.

26. Izuik D, Imaoka T, Takabatake T, Nishimura K, Nishimura Y, et al. DNA copy number aberrations and disruption of the p16ink4a/Rb pathway in radiation-induced and spontaneous rat mammary carcinomas. Radiat Res. 2010; 174(2):206–15. Epub 2010/08/05. https://doi.org/10.1667/RR2006.1 PMID: 20818787.

27. Nık-Zaınal S, Davies H, Staal J, Ramakrishna M, Gledzik D, Zou X, et al. Landscape of somatic mutations in 560 breast cancer whole-genome sequences. Nature. 2016; 534(7605):47–54. Epub 2016/05/03. https://doi.org/10.1038/nature17676 PMID: 27135926.

28. Karolich D, Baertsch R, Diekhans M, Furey TS, Hinrichs A, Lu YT, et al. The UCSC Genome Browser Database. Nucleic Acids Res. 2003; 31(1):51–4. Epub 2003/01/10. https://doi.org/10.1093/nar/gkg129 PMID: 12519945.

29. Smith JR, Hayman GT, Wang SJ, Launderdkind SJF, Hoffman MJ, Kaldunski ML, et al. The Year of the Rat: The Rat Genome Database at 20: a multi-species knowledgebase and analysis platform. Nucleic Acids Res. 2020; 48(1):D731–D42. Epub 2019/11/13. https://doi.org/10.1093/nar/gkz1041 PMID: 31713623.

30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001; 25(4):402–8. Epub 2002/02/16. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609.

31. R Core Team. R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2013.

32. Moriyama H, Daino K, Imaoka T, Nishimura M, Nishimura Y, Takabatake M, et al. Neutron-induced Rat Mammary Carcinomas Are Mainly of Luminal Subtype and Have Multiple Copy Number Aberrations. Anticancer Res. 2019; 39(3):1135–42. Epub 2019/03/08. https://doi.org/10.21873/anticancerres.13222 PMID: 30842142.

33. Moriyama H, Daino K, Ishikawa A, Imaoka T, Nishimura M, Nishimura Y, et al. Exome of Radiation-induced Rat Mammary Carcinoma Shows Copy-number Losses and Mutations in Human-relevant Cancer Genes. Anticancer Res. 2021; 41(1):55–70. Epub 2021/01/10. https://doi.org/10.21873/anticancerres.14751 PMID: 33419799.

34. Isaacs JT. A mammary cancer suppressor gene and its site of action in the rat. Cancer Res. 1991; 51(6):1591–5. Epub 1991/03/15. PMID: 1900214.

35. Quan X, Laes JF, Stieber D, Rivi ere M, Russo J, Wedekind D, et al. Genetic identification of distinct loci controlling mammary tumor multiplicity, latency, and aggressiveness in the rat. Mamm Genome. 2006; 17(4):310–21. Epub 2006/04/06. https://doi.org/10.1007/s00335-005-0125-9 PMID: 16596452.

36. Felletor HE, Coulon V, McVeigh JL, Boag AH, Dorion-Bonnet F, Duboue B, et al. Analysis of the 10q23 chromosomal region and the PTEN gene in human sporadic breast carcinoma. Br J Cancer. 1999; 79(5–6):718–23. Epub 1999/03/10. https://doi.org/10.1038/sj.bjc.6690115 PMID: 10070859.
37. Haag JD, Shepel LA, Kolman BD, Monson DM, Benton ME, Watts KT, et al. Congenic rats reveal three independent Copenhagen alleles within the Mcs1 quantitative trait locus that confer resistance to mammary cancer. Cancer Res. 2003; 63(18):5808–12. Epub 2003/10/03. PMID: 14522903.

38. denDekker AD, Xu X, Vaughn MD, Puckett AH, Gardner LL, Lambring CJ, et al. Rat Mcs1b is concordant to the genome-wide association-identified breast cancer risk locus at human 5q11.2 and MIER3 is a candidate cancer susceptibility gene. Cancer Res. 2012; 72(22):6002–12. Epub 2012/09/21. https://doi.org/10.1158/0008-5472.CAN-12-0748 PMID: 22993404.

39. Glubb DM, Maranian MJ, Michailidou K, Pooley KA, Meyer KB, Kar S, et al. Fine-scale mapping of the 5q11.2 breast cancer locus reveals at least three independent risk variants regulating MAP3K1. Am J Hum Genet. 2015; 96(1):5–20. Epub 2014/12/23. https://doi.org/10.1016/j.ajhg.2014.11.009 PMID: 25529635.

40. Callahan R, Smith GH. MMTV-induced mammary tumorogenesis: gene discovery, progression to malignancy and cellular pathways. Oncogene. 2000; 19(8):992–1001. Epub 2000/03/14. https://doi.org/10.1038/sj.onc.1203276 PMID: 10713682.

41. Bogden AE, Cobb WR, Ahmed M, Alex S, Mason MM. Oncogenicity of the R-35 rat mammary tumor virus. J Natl Cancer Inst. 1974; 53(4):1073–7. Epub 1974/10/01. https://doi.org/10.1093/jnci/53.4.1073 PMID: 4372364.

42. St Lezin EM, Pravenec M, Wong A, Wang JM, Merriouns T, Newton S, et al. Genetic contamination of Dahl SS/Jr rats. Impact on studies of salt-sensitive hypertension. Hypertension. 1994; 23(6 Pt 1):786–90. Epub 1994/06/01. https://doi.org/10.1161/01.hyp.23.6.786 PMID: 8260578.

43. Jones RE, Weinberg A, Bourdette D. Evidence for genetic contamination of inbred buffalo rats (RT-1b) obtained from a commercial vendor. J Neuroimmunol. 1994; 52(2):215–6. Epub 1994/07/01. https://doi.org/10.1016/0165-5728(94)90115-5 PMID: 8034760.

44. Rajkumar L, Arumugam A, Elsayed A, Schecter S, Kotkowski E, Castillo R, et al. Long-term hormonal promotion overcomes genetic resistance to mammary cancer. Steroids. 2011; 76(1–2):31–7. Epub 2010/08/25. https://doi.org/10.1016/j.steroids.2010.08.004 PMID: 20732338.

45. Mohanty SS, Mohanty PK. Obesity as potential breast cancer risk factor for postmenopausal women. Genes Dis. 2021; 8(2):117–23. Epub 2019/10/10. https://doi.org/10.1016/j.gendis.2019.09.006 PMID: 33997158.

46. Behjati S, Gundem G, Wedge DC, Roberts ND, Tarpey PS, Cooke SL, et al. Mutational signatures of ionizing radiation in second malignancies. Nat Commun. 2016; 7:12605. Epub 2016/09/13. https://doi.org/10.1038/ncomms12605 PMID: 27615322.

47. Kinoshita Y, Yoshizawa K, Hamazaki K, Emoto Y, Yuri T, Yuki M, et al. Dietary effects of mead acid on N-methyl-N-nitrosourea-induced mammary cancers in female Sprague-Dawley rats. Biomed Rep. 2016; 4(1):33–9. Epub 2016/02/13. https://doi.org/10.3892/br.2015.530 PMID: 26870330.

48. Russo J. Significance of rat mammary tumors for human risk assessment. Toxicol Pathol. 2015; 43(2):145–70. Epub 2015/02/26. https://doi.org/10.1177/0192623314532036 PMID: 25714400.

49. Moroka T, Blyth BJ, Imaoka T, Nishimura M, Takeshita H, Shimomura T, et al. Establishing the Japan-Store house of animal radiobiology experiments (J-SHARE), a large-scale necropsy and histopathology archive providing international access to important radiobiology data. Int J Radiat Biol. 2019; 95(10):1372–7. Epub 2019/05/31. https://doi.org/10.1080/09553002.2019.1625456 PMID: 31145030.