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Exome of Radiation-induced Rat Mammary Carcinoma Shows Copy-number Losses and Mutations in Human-relevant Cancer Genes

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Abstract. *Background/Aim: Our understanding of cancer risk from neutron exposure is limited. We aimed to reveal the characteristics of mammary carcinomas induced by neutrons. Materials and Methods: Mammary carcinomas obtained from female Sprague-Dawley rats irradiated at 7 weeks of age with 0.97 Gy neutrons or 4 Gy γ-rays and from non-irradiated rats were classified into luminal and non-luminal subtypes by immunohistochemistry. Their mutational landscapes were determined by whole-exome sequencing. Results: Neutrons significantly raised the incidence of luminal mammary carcinomas over the non-luminal subtype. Somatic mutations were identified in cancer genes involved in several signalling pathways, including Keap1/Nrf2, Pi3k/Akt and Wnt/β-catenin. Focal copy-number losses involving cancer genes were observed mainly in carcinomas from the irradiated rats. Conclusion: Neutrons increase the incidence of luminal mammary carcinomas, probably through gene mutations similar to those found in human breast cancers, and focal copy-number losses including cancer genes that are characteristics of radiation-induced mammary carcinomas.*

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Epidemiological studies on atomic bomb survivors and patients who have undergone radiotherapy have established that ionizing radiation is a risk factor for breast cancer (1). Neutrons are high linear energy transfer (LET) radiation and produce more complex DNA damage, with stronger carcinogenetic effects as compared with low LET radiation, such as γ-rays (2). Thus, there is concern about the breast cancer risk from neutron exposure caused by nuclear accidents and as a secondary radiation during proton therapy, which is increasingly used in the clinical setting (3-5).

Animal models of carcinogenesis have provided crucial information for estimating the radiation-induced cancer risk in humans when data from human populations are lacking or limited (6). In contrast, comparisons between rodent and human data with respect to spontaneous and chemically induced tumours have shown that particular gene alterations involved in specific tumour types tend to differ across species (6). Therefore, additional data on the carcinogenic mechanisms of radiation exposure are needed to increase the reliability of extrapolation from rodent data the risk in human populations.

The rat is a widely used model to study the risks and mechanisms of breast carcinogenesis because rat mammary carcinomas are similar to human breast cancer with regard to their hormone dependence and pathology (7, 8). We have previously conducted a series of experiments to quantify the incidence of neutron-induced mammary carcinomas in rats (9, 10). Our most recent study revealed that neutrons and γ-rays (at a dose of 0.5 Gy) increase mainly the incidence of luminal mammary carcinoma in rats, probably *via* genetic aberrations associated with human breast cancer (11). In this

previous report, we used array-based comparative genomic hybridization (array-CGH) to identify multiple DNA copy-number aberrations that affect genes for which mutations had been previously reported in human breast cancer. However, none of these aberrations displayed any significant differences among the carcinomas in non-irradiated, neutron-irradiated and γ-ray–irradiated groups. In addition, an important limitation of the array-CGH is the inability to detect gene mutations, and thus further study was warranted using more comprehensive techniques, such as next-generation sequencing.

Breast cancer is a molecularly heterogeneous disease and is classified into several subtypes such as luminal, triple-negative and human epidermal growth factor receptor 2 (HER2)-enriched (12). The subtypes of breast cancer that occur in humans after X-ray exposure have been previously investigated (13-15). However, there has been no consistent evidence that radiation preferentially induces specific subtypes. We thus performed immunohistochemistry and whole-exome sequencing to investigate the subtype(s) and mutational landscape of neutron-induced mammary carcinomas. In this study, to clarify the features of mammary carcinomas induced by neutrons, we further analysed archival mammary carcinomas (16) of rats irradiated with neutrons or γ-rays at the highest doses (0.97 Gy neutron beams or 4 Gy γ-rays) in our previous animal experiments (9).

Materials and Methods

Tumour samples. Mammary carcinoma samples were obtained from our previous animal experiments (9, 17), which were approved by the Institutional Animal Care and Use Committee of the National Institutes for Quantum and Radiological Science and Technology (approval No. 07-1016), and were performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan. Detailed procedures for the animal experiments have been described (9, 17). Briefly, female Sprague-Dawley (Jcl:SD) rats (CLEA Japan Inc., Tokyo, Japan) were whole-body irradiated with $^{9}Be(d, n\gamma)^{10}B$ fast neutrons (0.97 Gy; mean energy, 2 MeV) or ^{137}Cs γ-rays (4 Gy) at 7 weeks of age or were left unirradiated. The biological effects of fast neutrons are known to be high at several MeV (18) and the neutron energy was relevant to that of fission neutrons. Mammary carcinomas were identified by weekly palpation until the age of 90 weeks. All rats were fed a CE-2 diet (CLEA Japan) and were provided sterile water ad libitum. They were maintained under specific pathogen-free conditions in autoclaved cages maintained on a 12-h light/12-h dark cycle. We sacrificed rats and collected their tissues if they showed any signs of deterioration such as rapid body mass loss, severe anaemia or excessive tumour burden. Mammary carcinomas and normal mammary glands from the same animal were stored at –80˚C for molecular analysis and as formalin (10%)-fixed samples for pathological analysis. The characteristics of carcinomas analysed in this study are shown in Table I.

Tumour subtyping. Subtyping of mammary carcinomas was performed as described (11). Briefly, formalin-fixed paraffin-embedded tissues were sectioned (~3 μm thick). The sections, which were placed on silane-coated slides were deparaffinized with xylene, rehydrated through a graded ethanol series and immunostained with antibodies against oestrogen receptor α (ERα) (NCL-L-ER-6F11, clone 6F11; Leica Biosystems, Nussloch, Germany; 1:400), progesterone receptor (PgR) (AM1118PU-S, clone SP42; Acris Antibodies, Herford, Germany; 1:400), HER2 (MS-730-P0, clones e2-4001+3B5; Thermo Fisher Scientific, MA, USA; 1:100) and the proliferation marker Ki-67 (M3064, clone SP6; Spring Bioscience, Pleasanton, CA, USA; 1:200). Methods of immunohistostaining, scanning and evaluation have been described (11). Threshold for positivity of a carcinoma was set at 1% for ERα and PgR; the threshold of Ki-67 positivity was set at the median percentage (15.9%); for HER2, carcinomas were classified as reported (12). The luminal subtype was defined as $HER2^{-}$ and $ER\alpha^{+}$ and/or PgR^{+}, and the non-luminal subtype was any other combination of markers according to the standard classification of human breast cancers (12).

DNA sample preparation. Genomic DNA was extracted from frozen mammary carcinoma or normal mammary gland tissue samples as described (11). Briefly, we prepared 20-μm-thick sections from optimal cutting temperature compound-embedded frozen tissue on a membrane slide (MMI Membrane Slides RNA free, Molecular Machines & Industries, Glattbrugg, Switzerland). These sections were briefly fixed in 2-propanol and stained with hematoxylin and eosin. Cancerous epithelial cells were captured using a microdissection system under the supervision of a pathologist (T.M.), and genomic DNA was isolated from these cells using a QIAmp DNA Micro Kit (Qiagen, Hilden, Germany).

Whole-exome sequencing and data analysis. Whole-exome sequencing was performed on rat mammary carcinoma and matched normal mammary gland tissue samples with sufficient quantity and quality of genomic DNA as described (19). In brief, genomic DNA was fragmented by sonication (Covaris, M&S Instruments Inc., Tokyo, Japan) to 150 bp and was further purified using Agencourt AMPure XP beads. Then, 100 ng of DNA was ligated to specific adapters during library preparation (KAPA Library Preparation Kit, Kapa Biosystems, Woburn, MA, USA). Exon capture probes were designed for targeting available rat genes from the rat reference genome (rn5) RefSeq transcripts, including a total of 15,854 genes, and unannotated transcripts, including 24 known driver genes (*i.e. Pik3ca*, *Kmt2c*, *Arid1b*, *Afdn*, *Fbxw7*, *Ncor1*, *Spen*, *Med23*, *Kdm6a*, *Kmt2d*, *Atr*, *Smad4*, *Atrx*, *Cux1*, *Gnas*, *Phf6*, *Ect2l*, *Pbrm1*, *Tet2*, *Asxl1*, *Braf*, *Bub1b*, *Ercc5* and *Zfp36l1*) in human breast cancers (20). Each library was prepared with sample-specific barcodes and underwent exome enrichment (SeqCap EZ Developer Library, Roche, Tokyo, Japan). Several libraries were pooled and sequenced on NextSeq 500 system (Illumina Inc., Tokyo, Japan).

Data were processed using the pipeline prepared by Amelieff Co. Ltd (Tokyo, Japan) as described previously (19). Briefly, the reads were trimmed by removing low-quality bases and removed if they were shorter than 32 bases or if >80% of any individual read had a quality rating of <20 using the QCleaner tool (ver. 4.1) developed by Amelieff Co. Ltd. The resulting reads were aligned to the rat reference genome (rn6) by the use of the Burrows-Wheeler Alignment tool (ver. 0.7.12). Duplicate reads were removed with SAMtools (ver. 1.2), and base quality recalibration and realignment around insertions/deletions

Table I. *Characteristics of rat mammary carcinomas analysed in this study.*

Sample ID	Group	Subtype	Age at detection (weeks)	Age at autopsy (weeks)
K0679MT2	No irradiation	Luminal	78	93
K0813MT4	No irradiation	Luminal	Discovered at autopsy	90
K1404MT1	No irradiation	Non-luminal	83	92
K1465MT1	No irradiation	Luminal	51	81
K2364MT1	No irradiation	Luminal	74	91
K2403MT1	No irradiation	Luminal	57	92
K2455MT1	No irradiation	Luminal	54	66
K2476MT3	No irradiation	Non-luminal	50	90
K2608MT1	No irradiation	Luminal	64	92
K2861MT1	No irradiation	Luminal	81	90
K2264MT1	Neutrons	Luminal	38	68
K2288MT1	Neutrons	Luminal	30	59
K2315MT1	Neutrons	Luminal	31	61
K2462MT1	Neutrons	Luminal	39	71
K2528MT1	Neutrons	Luminal	16	34
K2578MT1	Neutrons	Luminal	23	37
K2686MT2	Neutrons	Luminal	33	48
K2975MT1	Neutrons	Luminal	52	71
K0886MT2	γ-rays	Luminal	17	24
K0898MT1	γ-rays	Luminal	23	38
K0952MT1	γ-rays	Luminal	24	44
K1009MT2	γ-rays	Luminal	41	52
K1069MT2	γ-rays	Luminal	44	60
K1157MT2	γ-rays	Luminal	52	71
K1163MT3[a]	γ-rays	Undetermined	76	86
K1180MT1	γ-rays	Luminal	35	74
K1254MT8	γ-rays	Non-luminal	43	82
K1328MT4	γ-rays	Luminal	91	98

[a]This sample was obtained from an animal that was found dead and was not subjected to immunohistochemistry.

(InDels) were performed using the Genome Analysis Tool kit (ver. 1.6-13). Somatic single-nucleotide variants (SNVs) or InDels in tumours were called with the VarScan 2 software (ver. 2.4.3). A false-positive filter was then applied to remove sequencing- or alignment-related artifacts. Variants were annotated and the effect on coding sequences predicted using SnpEff software (ver. 4.3). We also required that the variant allele be present in ≥10% of tumour reads and that there were no representations of normal tissue reads. Control-FREEC software (ver. 10.8) was used to identify copy-number changes in tumours as compared with normal tissue from the same animal. Segments that exhibited a copy-number change that was statistically significant relative to normal ploidy were extracted ($p<0.05$, Wilcoxon and Kolmogorov-Smirnov tests). We used the R package Mutational Patterns (ver. 1.4.2) (https://www.bioconductor.org/packages/release/bioc/html/MutationalPatterns.html) to extract and analyse the mutational spectrum of tumours.

Statistical analysis. Statistical analysis was performed using the statistical software R with the aid of the graphical user interface EZR (Jichi Medical University, Saitama, Japan) (21). Comparisons among three groups were performed with the Kruskal-Wallis test. Comparisons between two groups were performed with the Mann-Whitney *U*-test. The correlation coefficient was calculated using Spearman's rank correlation coefficient. Fisher's exact test was used to test the significance of differences in the number of palpable carcinomas or the subtypes of carcinomas. The significance of difference between the means was analysed by F-test. A $p<0.05$ was considered statistically significant.

Results

Neutron exposure increases the risks of luminal and non-luminal rat mammary carcinomas. Here, we investigated mammary carcinomas that developed in two groups of female rats from our previous studies (9, 17), which were irradiated i) with neutrons (0.97 Gy, $n=24$) or ii) with γ-rays (4 Gy, $n=20$), as well as a group of matched non-irradiated rats ($n=285$). The incidence, tumour number, hazard ratio (*i.e*. ratio of the probability of having a new carcinoma per unit time) and the age at which mammary carcinomas were first detected in these groups are summarized in Table II. Neutron or γ-ray exposure significantly increased the incidence and hazard ratio of mammary carcinomas with a significant reduction in the age at which the carcinomas were first detected, as compared with the non-irradiated controls.

To examine the subtype(s) of mammary carcinomas in the non-irradiated and neutron- and γ-ray-irradiated groups, we

Table II. *Characteristics of the cohort of rats included in this study*[a].

Feature	No irradiation	Neutrons	γ-rays
Rats with carcinoma[b]	57/285 (20%)	18/24 (75%)***	13/20 (65%)***
Carcinomas available[c]	45	24	9
Hazard ratio [95%CI]	1.0	26.9 [13.9-52.1]***	10.6 [5.6-20.3]***
Weeks of age at detection[d]	66.3±18.0 (43)	34.0±14.8*** (17)	47.2±22.5** (13)

[a]Data from a previous experiment (9, 17) were used for this analysis. [b]Rats with carcinoma/total number of rats in each group (percentage of rats with carcinoma). [c]Some rats had multiple mammary carcinomas. [d]Mean±SD (number of palpable carcinomas). **$p<0.01$; ***$p<0.001$ *vs.* no irradiation. 95%CI: 95% confidence interval.

then performed immunohistochemical staining for ERα, PgR, HER2 and Ki-67 in all available tumours (Table II). The percentage of positive cells for each antigen did not differ significantly between the groups (Table III). Based on the immunohistochemistry results, we classified the mammary carcinomas as the luminal or non-luminal subtype. The hazard ratio for palpable luminal mammary carcinomas was significantly higher in the neutron- and γ-ray-irradiated groups as compared with the non-irradiated groups (Table IV and Figure 1a). Neutron exposure also increased the hazard ratio of palpable non-luminal carcinomas (Table IV and Figure 1b), although the number of non-luminal carcinomas was relatively small in the neutron-irradiated group. Taken together, these results indicate that neutron exposure increases the risk of luminal and non-luminal mammary carcinomas and that the luminal subtype was mostly involved in the increased incidence of mammary carcinomas in neutron-irradiated rats.

Table III. *Immunohistochemical staining for ERα, PgR and HER2 in rat mammary carcinomas.*

Marker	Positive cells (%)		
	No irradiation	Neutrons	γ-rays
ERα	21.5±15.8[a]	20.7±12.2	9.2±7.2
PgR	14.0±15.8	9.9±11.4	8.1±7.8
HER2	5.7±8.3	2.3±5.4	0.9±2.6
Ki-67	19.2±13.2	20.0±3.8	11.8±10.2

[a]Percentage of positive cells (mean±SD).

Mutational landscape of spontaneous and neutron- and γ-ray-induced rat mammary carcinomas. To profile the somatic mutation spectrum of spontaneous and neutron- or γ-ray-induced mammary carcinomas, whole-exome sequencing was carried out on rat mammary carcinomas from the three groups (non-irradiated, n=10; neutron-irradiated, n=8; γ-ray-irradiated, n=10). As shown in Figure 2a, no significant differences in the number of SNVs) or InDels were observed among the carcinomas from the three groups, *i.e.* non-irradiated (mean=84.7; range=70-96), neutron-irradiated (mean=83.4; range=61-109) and γ-ray-irradiated (mean=85.3; range=54-117). However, mammary carcinomas from the neutron- and γ-ray-irradiated groups showed a large variation in the number of SNVs and InDels among individual cases as compared with spontaneously developed carcinomas. This was statistically significant for the γ-ray-irradiated group and when the neutron- and γ-ray-irradiated groups were combined ($p<0.05$, F-test). The size of insertions and deletions detected in the carcinomas was also similar among the groups (Figure 2b). In contrast, the number of single-nucleotide insertions was significantly lower in the carcinomas from the γ-irradiated group as compared with the other two groups, although the underlying mechanisms for this difference are unknown (Table V). We did not find any other significant differences among the carcinomas in the three groups with regard to the number or spectrum of mutations, the subtype or the age at detection.

In many cancers, the number of somatic mutations increases with age (22, 23). However, no correlation was observed between the number of SNVs in the carcinomas and the age at tumour detection by palpation (data not shown). Cancers do harbour a variable number of somatic mutations that accumulate during their growth as a consequence of diverse cellular process, including the impairment of DNA repair and exposure to endogenous or exogenous DNA-damaging agents (22, 24). To compare the number of somatic mutations per growth period of mammary carcinomas from non-irradiated and neutron- and γ-ray-irradiated groups, we examined the relationship between the numbers of SNVs in the carcinomas and the periods from first palpation of the carcinomas to autopsy. As shown in Figure 3, whereas a significant positive correlation was observed between the total number of somatic mutations (SNVs and InDels) and the growth period of mammary carcinomas in the non-irradiated and neutron-irradiated groups, no correlation was observed in the γ-ray-irradiated group. This result suggests the involvement of some variable factors in the initiation or promotion of breast carcinogenesis induced by γ-ray-

Table IV. *Characteristics of rat mammary carcinoma development by subtype.*

Feature	Subtype[a]	Treatment group		
		No irradiation	Neutrons	γ-rays
Number of palpable carcinomas[b]	L	22 (67%)	21 (91%)†	8 (89%)
	NL	11 (33%)	2 (9%)	1 (11%)
Hazard ratio [95% CI]	L	1.0	36.9 [15.6-87.3]***	14.0 [5.8-33.6]***
	NL	1.0	7.8 [1.6-38.3]*	3.4 [0.4-27.1]
Weeks of age at detection[c]	L	66.8±17.6	41.6±12.6***	40.9±23.5**
	NL	74.3±18.9	48.7±18.0	43.0

[a]L: Luminal; NL: non-luminal. [b]Some samples were obtained from an animal that was found dead and were not subjected to immunohistochemistry. [c]Mean±SD. †p=0.05, *p<0.05, **p<0.01, ***p<0.001 *vs*. no irradiation. 95%CI: 95% confidence interval.

exposure. In contrast, when the carcinomas of all groups were collectively analysed, a significant positive correlation was observed between the total number of somatic mutations (SNVs and InDels) and the duration of tumour growth (Figure 4a). In addition, a significant positive correlation was observed between the number of C:G>T:A transitions at CpG sites, which is known as an age-associated mutation (22), and the duration of tumour growth (Figure 4b). Although the underlying mechanisms are unknown, we also observed a significant positive correlation between the number of T:A>C:G transitions and the duration of tumour growth (Figure 4c).

To compare the mutational spectrum of mammary carcinomas from non-irradiated and neutron- and γ-ray-irradiated groups, we then analysed the frequency of seven single base-pair substitutions in the carcinomas based on the observation of a comparable number of SNVs between the carcinomas from three groups. As shown in Figures 5a and 6 and Table V, the mutation spectrum of single base-pair substitutions was dominated by C:G>T:A transitions, and this was common among spontaneous and neutron- and γ-ray-induced carcinomas. In contrast, although no statistically significant difference was found, an increase in C:G>A:T transversions, which are induced by reactive oxygen species (ROS) (25), was observed in some mammary carcinomas in the irradiated groups (sample IDs K2264MT1, K2462MT1, K2578MT1 and K0886MT2) as compared with those in the non-irradiated group. To further characterize the mutational spectrum of spontaneous and neutron- and γ-ray-induced rat mammary carcinomas, we then performed a mutational signature analysis (22). As shown in Figure 5b, the mutational patterns of the carcinomas showed similarities to the mutational signatures observed in human breast cancers (namely signatures 1, 5 and 30) as listed in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (26). This analysis further revealed that the mutational patterns in a subset of the carcinomas (grouped into cluster A) were similar to the signatures

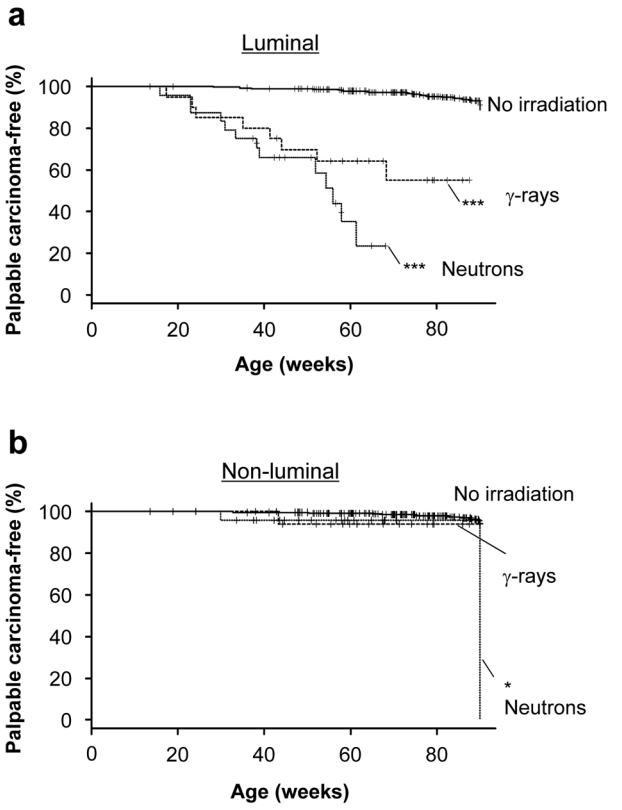

Figure 1. *Incidence of mammary carcinoma among the three treatment groups of rats. (a, b) Kaplan-Meier plots showing the onset of luminal (a) and non-luminal (b) palpable mammary carcinomas following irradiation with neutrons or γ-rays and the spontaneous development of carcinomas in the non-irradiated control. *p<0.05, ***p<0.001 vs. no irradiation.*

associated with mismatch repair deficiency (signatures 6, 15 and 20). The remaining carcinomas were then grouped into two clusters: i) cluster B, which showed a similarity to the signatures for tobacco exposure (signatures 4 and 29) and BRCA

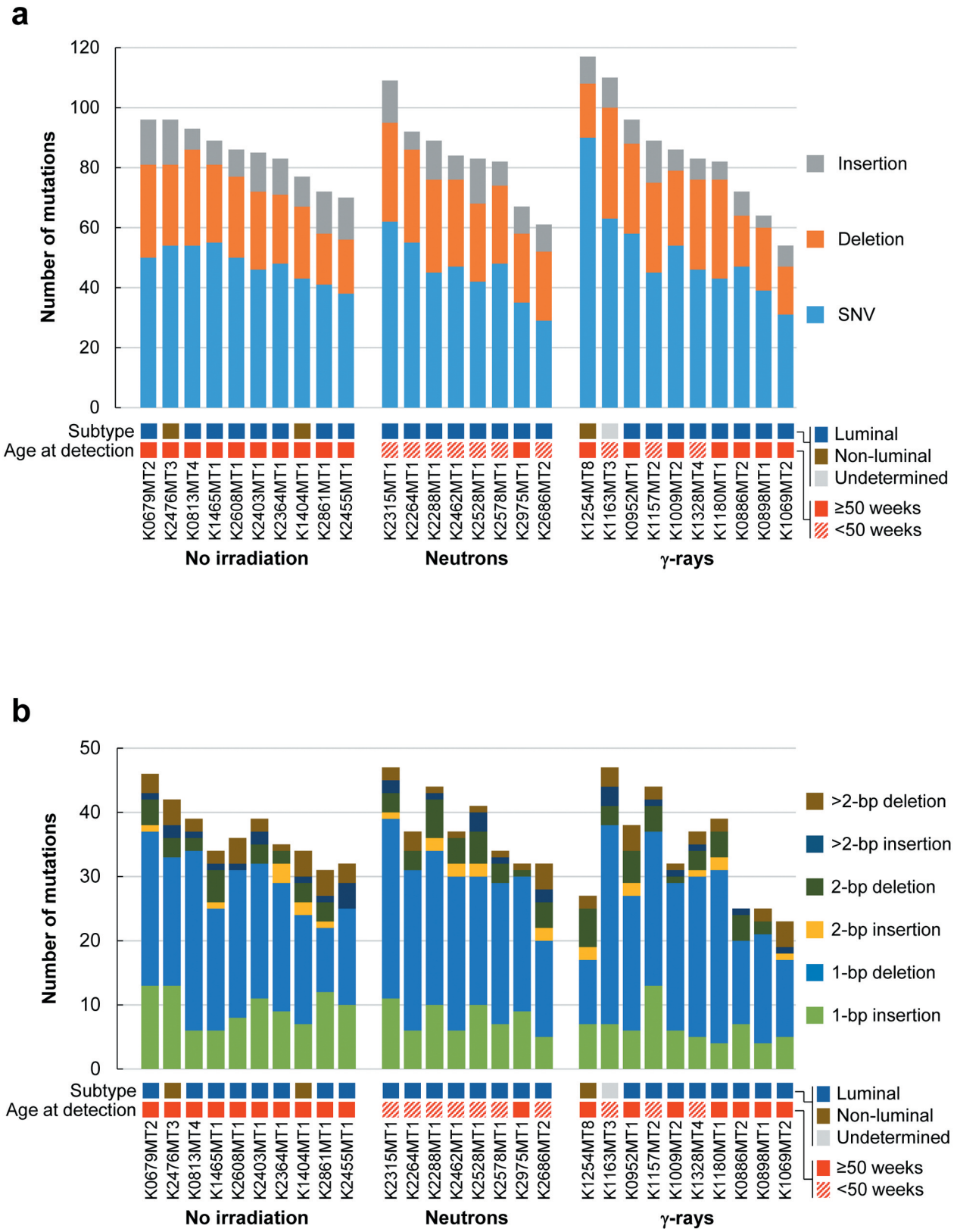

Figure 2. *The number of somatic mutations identified in rat mammary carcinomas. (a) Mutations are separated into single nucleotide variants (SNVs), insertions and deletions. (b) The number of insertions and deletions (InDels) classified by their length.*

Table V. *The pattern and number of somatic mutations detected in rat mammary carcinomas.*

Mutation pattern	No irradiation	Neutrons	γ-rays	*p*-Value	Adjusted *p*-value
SNVs					
Total number of SNVs	47.9±.5[a]	45.4±9.8	51.6±15.5	0.77	NA
C:G>T:A (at non-CpG sites)	11.2±3.0	9.8±4.1	12.0±5.9	0.50	NA
C:G>T:A (at CpG sites)	6.2±2.3	7.1±2.0	5.6±3.3	0.36	NA
C:G>A:T	7.9±3.4	10.1±5.1	10.5±4.6	0.45	NA
C:G>G:C	4.2±2.1	2.4±1.4	4.8±2.7	0.10	NA
T:A>C:G	8.5±1.5	6.3±1.9	8.2±2.7	0.06	NA
T:A>A:T	5.9±2.3	5.3±1.3	6.5±2.6	0.41	NA
T:A>G:C	4.0±1.6	4.5±2.3	4.0±1.7	0.60	NA
InDels					
Total number of insertions	11.7±2.8	10.3±3.1	8.0±2.5[†]	0.04	0.03
Total number of deletions	25.1±4.6	27.8±3.6	25.7±7.0	0.66	NA
1-bp insertions	9.5±2.6	8.0±2.1	6.4±2.5[†]	0.04	0.03
1-bp deletions	19.7±4.7	22.4±3.6	20.3±6.6	0.47	NA
2-bp insertions	0.8±1.0	1.1±0.9	0.8±0.9	0.70	NA
2-bp deletions	2.5±1.5	3.6±1.4	3.2±1.7	0.31	NA
>2-bp insertions	1.4±1.0	1.1±1.1	0.8±0.9	0.35	NA
>2-bp deletions	2.9±1.0	1.8±1.1	2.2±1.2	0.10	NA

[a]All data are shown as the mean±SD. Data were analyzed with Kruskal-Wallis test followed by *post-hoc* Steel test. [†]$p=0.05$ *vs.* no irradiation. NA: Not applicable.

deficiency [signatures 3 and 8; the aetiology of signature 8 is unknown, but its presence has been observed in breast cancer linked to BRCA deficiency (27)], and ii) cluster C, which showed weak similarity to multiple signatures associated with clusters A and B. There was, however, no obvious difference in the mutational signatures among the spontaneous and neutron- and γ-ray-induced rat mammary carcinomas. In addition, no significant difference was observed among the carcinomas in the three groups with regard to the spectrum of mutations, the subtype or the age at detection. These results indicate that spontaneous and neutron- and γ-ray-induced rat mammary carcinomas all have a similar mutation spectrum.

Genes commonly targeted for mutation in spontaneous and neutron- and γ-ray-induced rat mammary carcinomas. To identify gene mutations potentially involved in the development of rat mammary carcinomas, nonsynonymous mutations, including missense, nonsense and splice site mutations, were extracted from the whole-exome sequencing data. As shown in Figure 7, we identified mutations in cancer genes listed in the COSMIC Cancer Gene Census (CGC) database (28) that are involved in DNA repair (*e.g. Rad50, Brca2, Fance*), cell cycle (*e.g. Cdkn2a, Lzts1, Lats1*), transcription (*e.g. Prdm5, Camta1, Tp53, Cux1, Mef2b*), chromatin remodelling (*e.g. Smarca2, Smarca4*), cytoskeleton (*e.g. Flna, Fat4*), metabolism (*e.g. Mtap*), membrane transport (*e.g. Abcg2*) and signal transduction in MAPK (*e.g. Epha2, Dusp6*), Keap1/Nrf2 (e.g. *Nfe2l2, Cul3*), Pi3k/Akt (*e.g. Pik3r1*) or Wnt/β-catenin (*e.g. Cxxc4*) pathways. Notably, focal copy-number losses in cancer genes were dominantly found in the carcinomas from the irradiated groups. In addition, we identified recurrent copy-number gains in 16p16 (*Rps24* and *Spin1*) and 20p12 (*Srsf3*) and losses in 2q34 (*Sycp1*), 5q32 (*Mtap, Cdkn2a* and *Cdkn2b*), and 15p12 (*Xkr6*) regions in the carcinomas (Figure 8). Moreover, copy-number losses in 5q32 (*Mtap, Cdkn2a* and *Cdkn2b*) and 15p12 (*Xkr6*) regions were identified only in the carcinomas from irradiated groups. These results suggest that mammary carcinomas in this rat model are induced probably through the induction of gene mutations similar to those found in human breast cancers and that mammary carcinomas induced by neutrons and γ-rays show focal copy-number losses in cancer genes as a radiation signature.

Discussion

Consistent with our previous report (11), this study demonstrated an increased incidence of hormone receptor-positive luminal mammary carcinoma in rats after neutron- and γ-ray exposure as compared with non-irradiated controls. However, whole-exome sequencing of these mammary carcinomas revealed no significant difference in the number of somatic mutations among rat mammary carcinomas from non-irradiated and neutron- and γ-ray-irradiated groups. As shown in Tables II and IV, we also found that the period until the first palpation of mammary carcinomas in the irradiated groups was significantly shorter than that of the non-irradiated group, indicating an earlier induction of tumours in the irradiated groups. However, no increase in the number of somatic mutations was observed in the mammary carcinomas from the irradiated groups. These observations suggest that

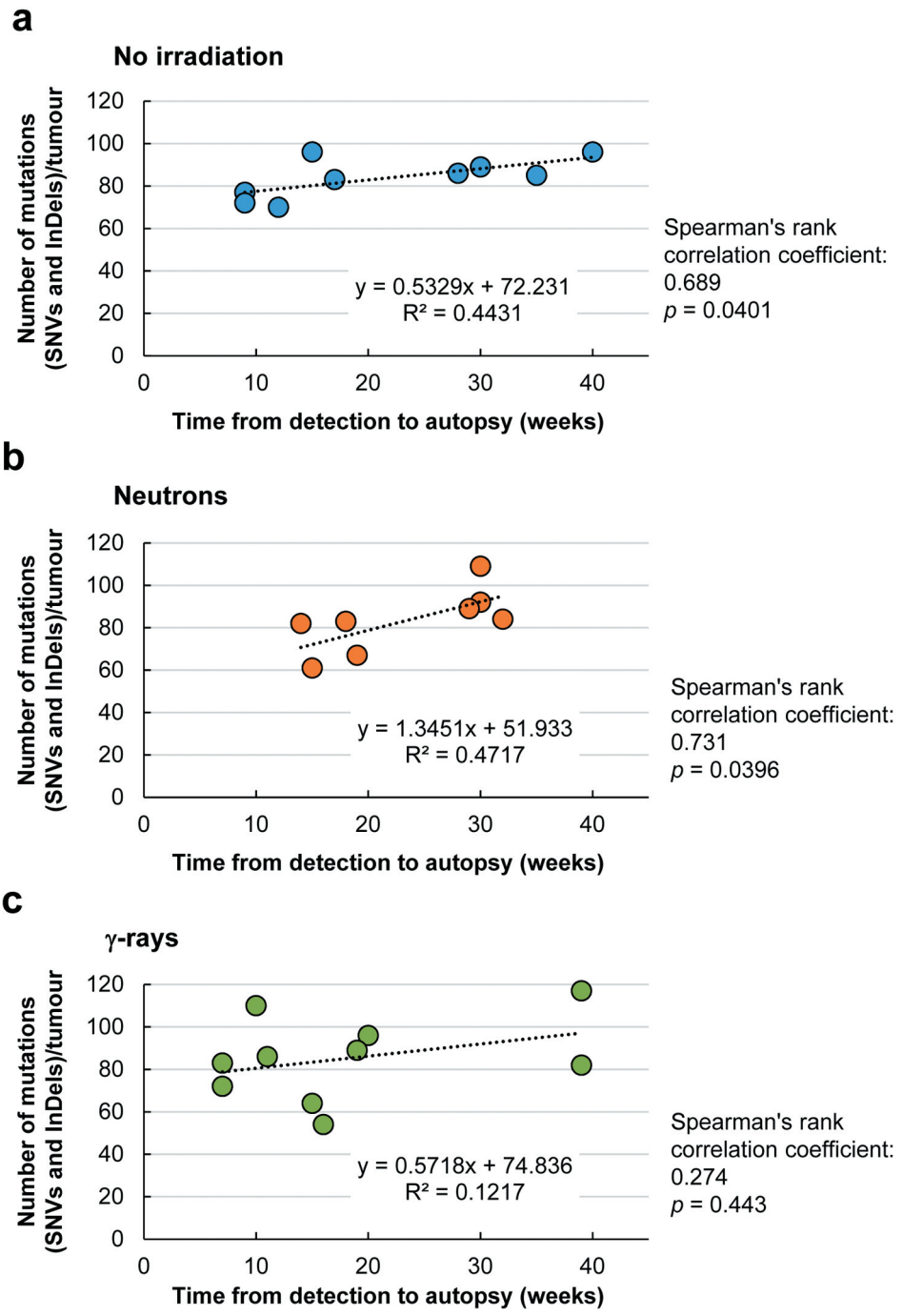

Figure 3. *Correlation between the total number of somatic mutations (SNVs and InDels) and the growth period of rat mammary carcinomas. (a) Carcinomas (n=9) from non-irradiated rats. (b) Carcinomas (n=8) from neutron-irradiated rats. (c) Carcinomas (n=10) from γ-ray–irradiated rats.*

radiation exposure increased the rate of somatic mutations for malignant transformation. Although ionizing radiation is known to induce genomic deletions (29), we noted relatively few deletion mutations in the mammary carcinomas from the irradiated groups. In addition, no difference in the spectrum of somatic mutations was observed among the carcinomas from the non-irradiated and neutron- and γ-ray-irradiated groups. Concerning this, it has been reported that genomic instability induced by ionizing radiation produces mutations across a spectrum similar to that occurring naturally (30).

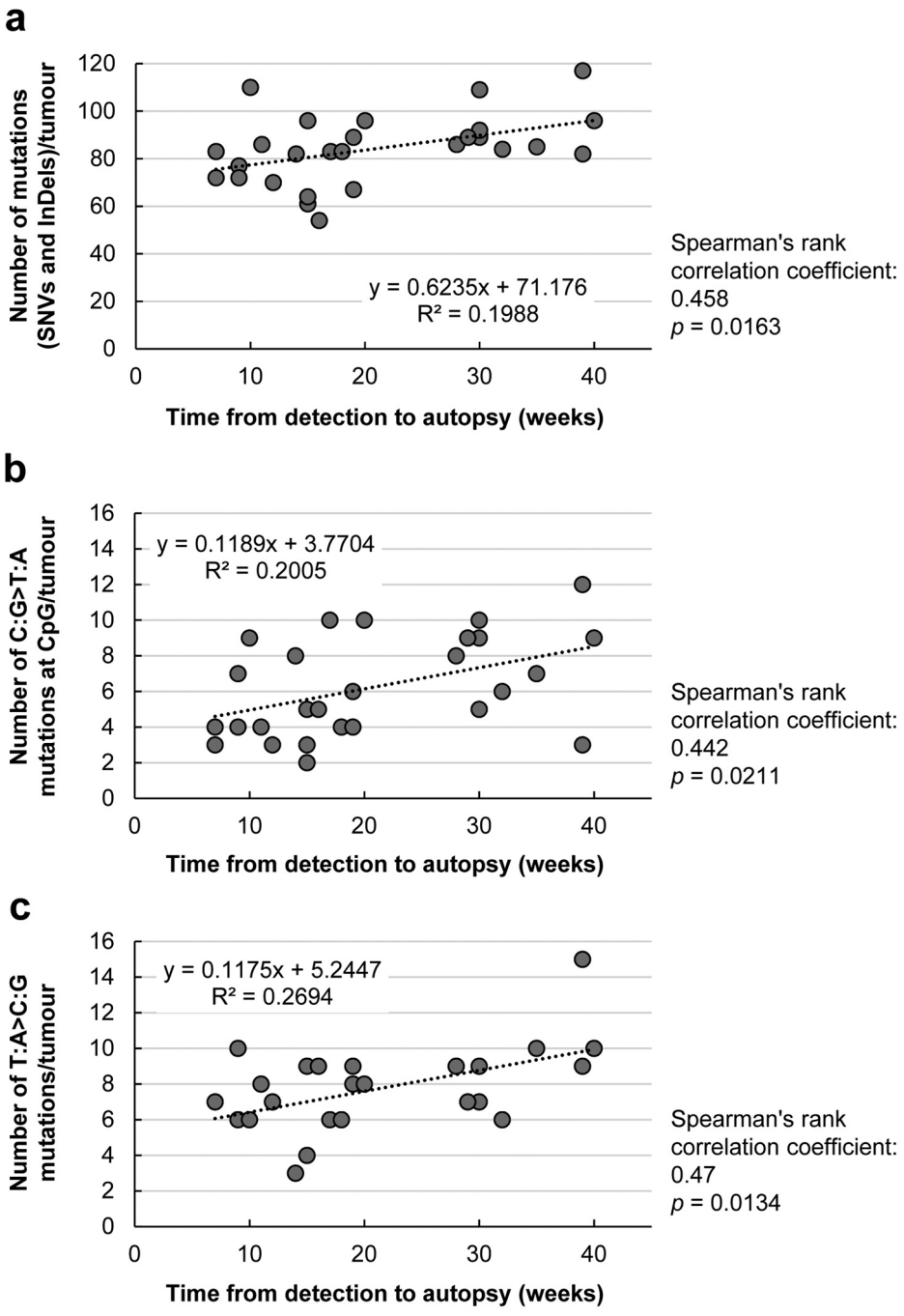

Figure 4. *Correlation between the number of somatic mutations and the growth period of rat mammary carcinomas. (a) Correlation between the total number of somatic mutations (SNVs and InDels) and the duration of tumour growth for all palpable carcinomas (n=27). (b) Correlation between the number of C:G > T:A transitions at CpG sites and the duration of tumour growth for all palpable carcinomas (n=27). (c) Correlation between the number of T:A > C:G transitions and the duration of tumour growth for all palpable carcinomas (n=27).*

Thus, our findings suggest that radiation exposure accelerates the accumulation of somatic mutations required for tumour formation through the induction of a small number of mutations, such as deletions, directly induced by the radiation exposure and a large number of mutations induced by radiation-induced genomic instability.

Similar to spontaneous mammary carcinomas, the mutation spectrum of SNVs in mammary carcinomas from

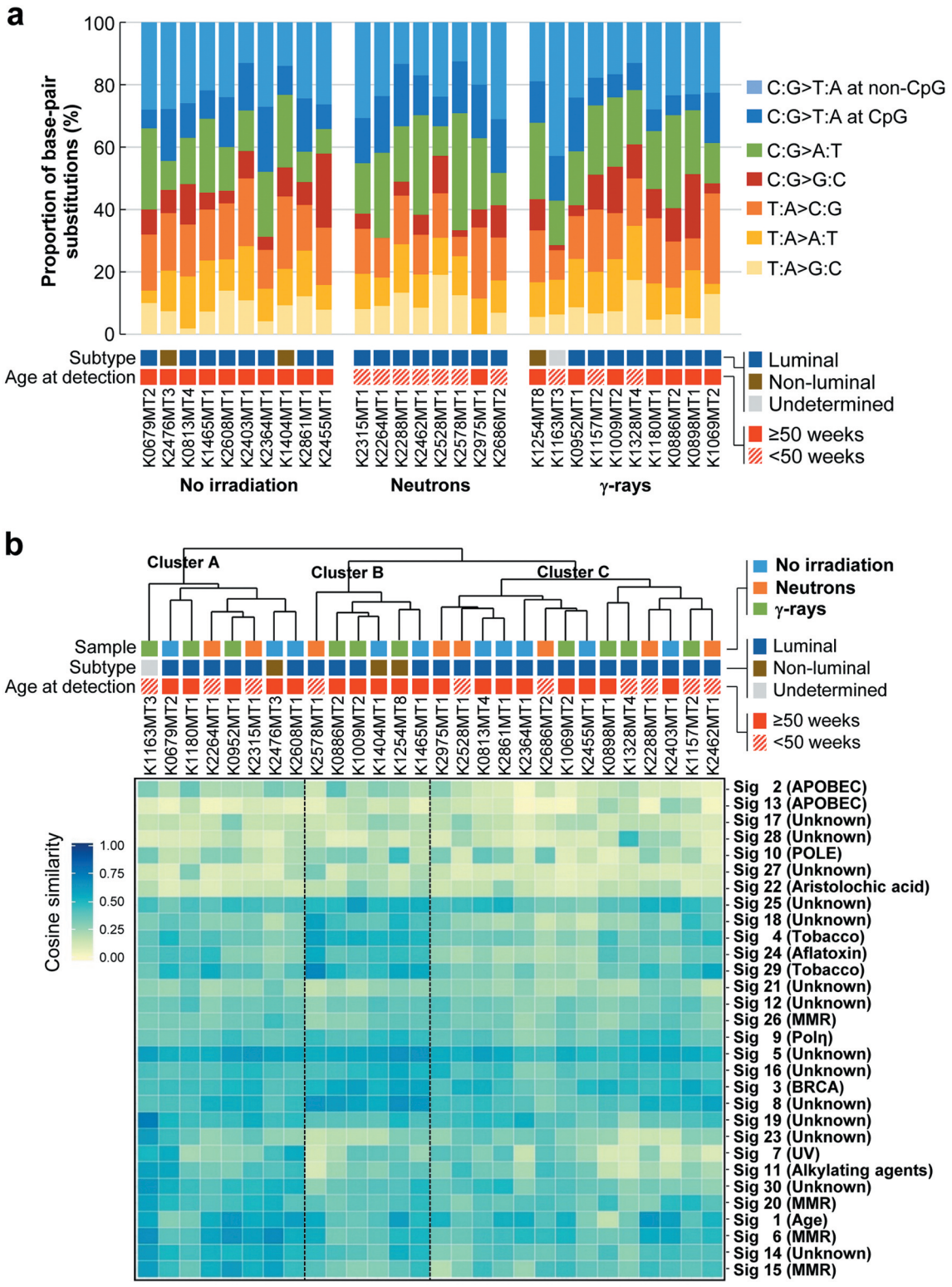

Figure 5. *Mutational signatures in rat mammary carcinomas. (a) The proportions of seven possible base-pair substitutions among mutations for each carcinoma. (b) Similarity between mutational profiles of rat mammary carcinomas and COSMIC signatures. A heatmap showing the cosine similarity between the mutational profile of each rat mammary carcinoma and COSMIC signatures (Sig). Samples were classified by unsupervised hierarchical clustering.*

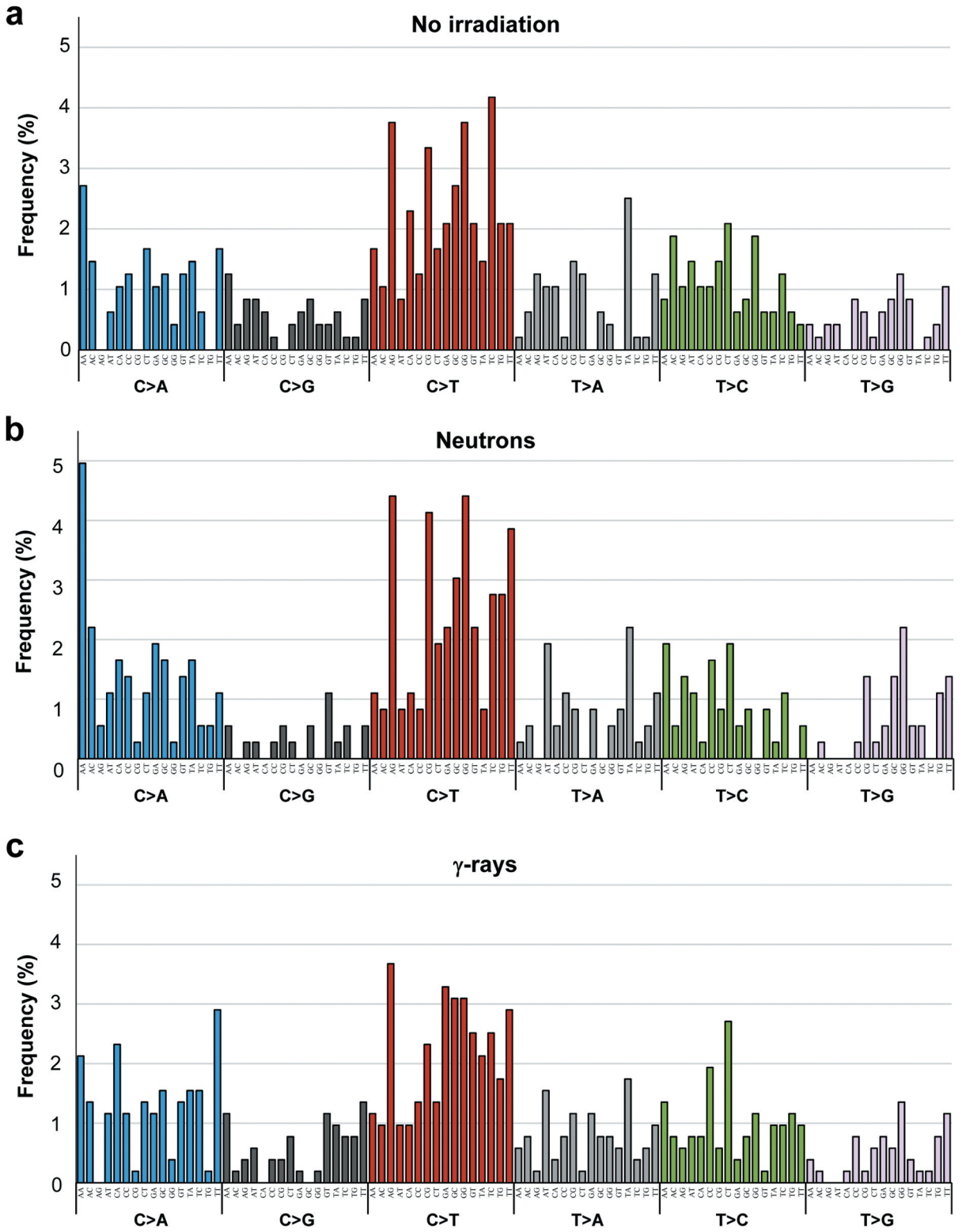

Figure 6. *Mutational signatures identified in mammary carcinomas. (a-c) Data are shown for carcinomas from non-irradiated (a), neutron-irradiated (b) and γ-ray–irradiated (c) groups. Each signature is represented by the relative contribution (y axis) of 96 classes of mutations defined by base substitution and tri-nucleotide sequence context of the mutated base (x axis). The signatures were extracted from the SNV data pooled from 9 carcinomas from non-irradiated (a), 8 carcinomas from neutron-irradiated (b) and 10 carcinomas from γ-ray–irradiated (c) rats.*

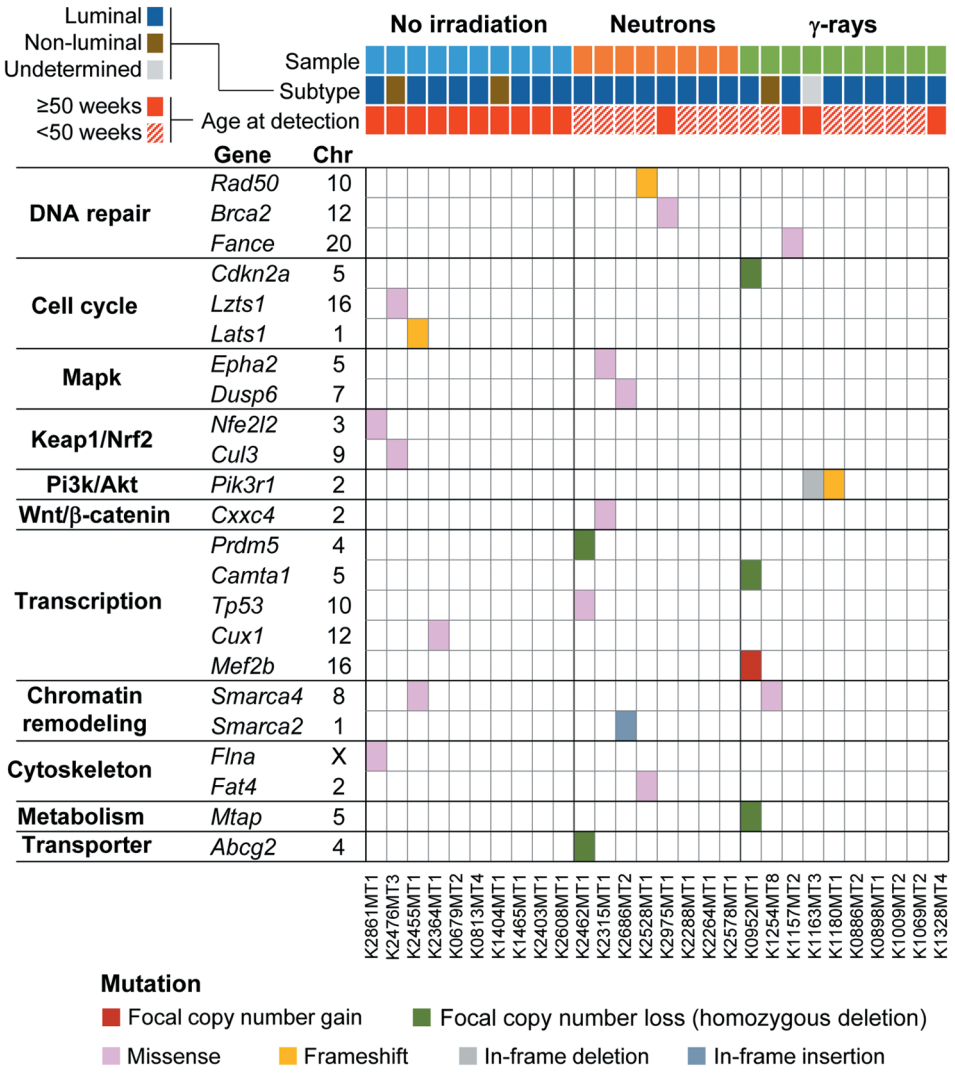

Figure 7. *Cancer gene mutations identified in rat mammary carcinomas. Nonsynonymous somatic mutations and focal copy-number changes either oncogenes or tumour-suppressor genes listed in the COSMIC CGC are shown. Gene aberrations predicted to lead to the activation of at least a single allele of oncogenes or to inactivation of both alleles of tumour-suppressor genes were extracted.*

the neutron- and γ-ray-irradiated groups was dominated by C:G>T:A transitions (Figures 5a and 6). This observation is consistent with several reports that have shown genome-wide mutational patterns in rodent and human neoplasms associated with radiation exposure (31-33). Notably, although we did not found the mutation spectrum that distinguishs spontaneous and radiation-associated mammary carcinomas, Davidson *et al.* have reported mutational signatures associated with ionizing radiation in mouse and human tumours induced by fractionated high-dose irradiation (a total of 30-60 Gy at 2-3 Gy per fraction) (32). Thus, this discrepancy may reflect the difference in the total dose of irradiation. Ionizing radiation is also known to generate ROS, which can induce premutagenic modifications of DNA bases, leading to the formation of 8-oxoguanine (25, 34). However, no statistically significant difference in the frequency of C:G>A:T transversion, which can be generated by misrepair of 8-oxoguanine lesion, was observed among the carcinomas from the non-irradiated and irradiated groups. Therefore, the production of ROS by ionizing radiation may have little contribution to the induction of gene mutations, although an increase in C:G>A:T transversions was observed in some mammary carcinomas in the irradiated groups as compared with those in the non-irradiated group.

Animal studies have long been one of the principal sources of data for estimating the cancer risk of radiation

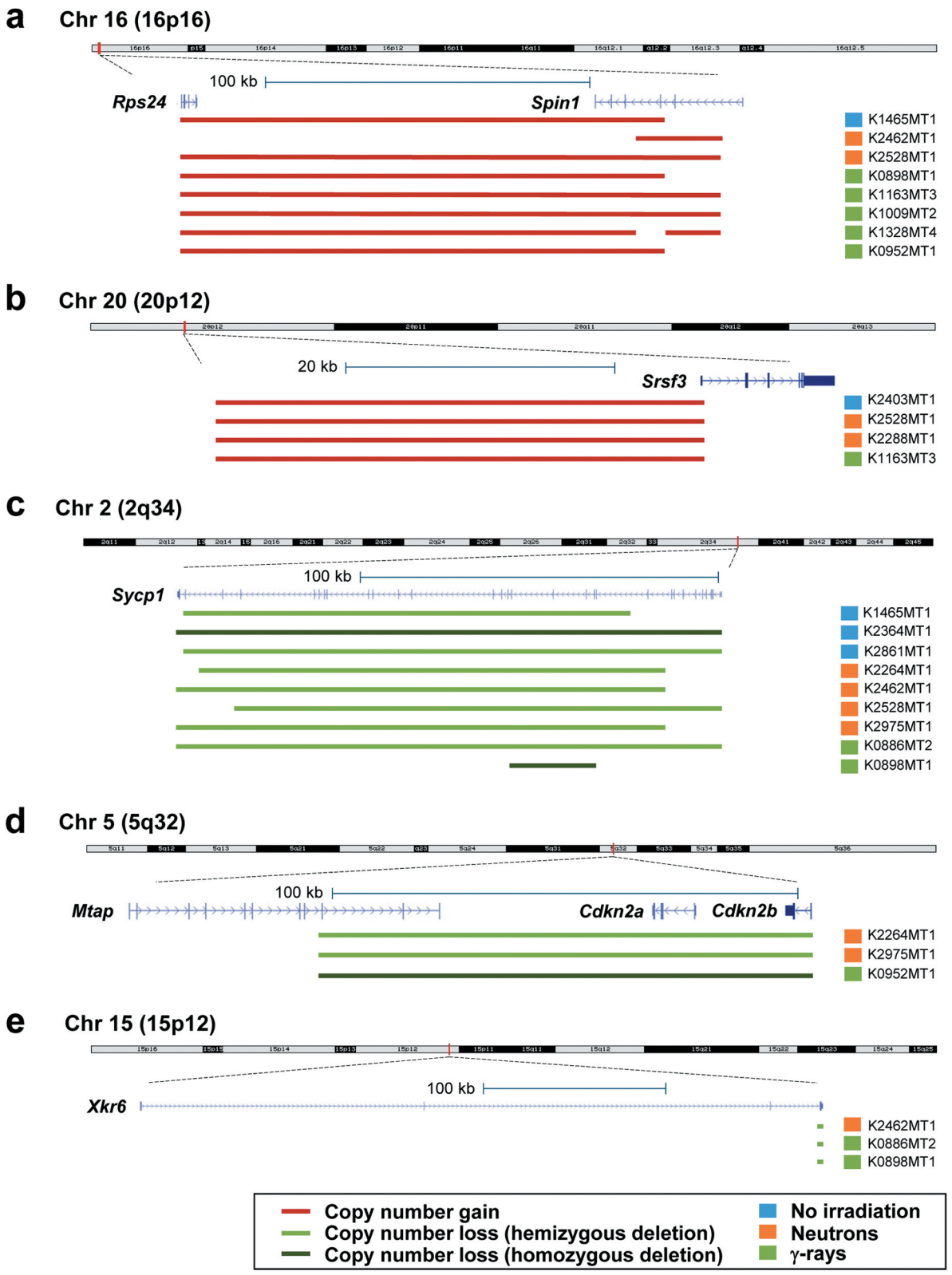

Figure 8. *Recurrent copy-number alterations in rat mammary carcinomas. (a–e) Copy-number gains in 16p16 (a) and 20p12 (b) and losses in 2q34 (c), 5q32 (d), and 15p12 (e) regions are shown.*

exposure in humans due to a lack of or limited data for human populations (6). Additional data on the carcinogenic mechanisms related to radiation exposure are, however, needed to improve the level of confidence in extrapolating the results from animal studies to humans, because it has been shown that the particular gene alterations involved in a specific tumour type tend to differ across species based on comparisons of the rodent and human data of spontaneous and chemically induced tumours, with only limited data being available on radiation-induced tumours (6). Our whole-exome sequencing analysis revealed that mammary carcinomas that developed in those rats showed mutational patterns and gene mutations that were similar to those observed in human breast cancer (Figures 5b and 7), suggesting that this is a suitable model for human breast cancer. Notably, nonsynonymous somatic mutations were identified in the rat mammary carcinomas in cancer genes, including *Cdkn2a*, *Tp53*, and *Smarca 2/4*, that are frequently mutated in various human cancers (20, 35). Mutations were also identified in genes involved in several pivotal signalling pathways, including Keap1/Nrf2, Pi3k/Akt and Wnt/β-catenin, in human breast cancers (20, 36, 37). These similarities strengthen our confidence in the extrapolation from data on rat mammary carcinoma to human breast cancer.

In the present study, focal copy-number losses were observed in rat mammary carcinomas predominately in the irradiated groups (Figures 7 and 8c-e). In addition, as shown in Figure 8d, copy-number loss of chromosome 5 including the *Cdkn2a* locus was observed in mammary carcinomas only in the irradiated groups. With respect to this, our previous array-CGH analysis of rat mammary carcinomas also showed copy-number losses in this chromosomal region in the carcinomas in the irradiated groups (11, 38). Interestingly, consistent with these findings, loss of heterozygosity at chromosomal region 9q21 (*CDKN2A* locus) has been found to be significantly more common in human breast cancers after radiation therapy for Hodgkin's lymphoma than sporadic breast cancers (39). These observations suggest that focal copy-number loss in cancer genes is a signature for radiation-induced breast cancer.

Finally, the present data from this rat mammary cancer model provide fundamental new insights related to the extrapolation from the results obtained with this model to a better understanding of the risk and biology of human breast cancer induced by neutron and γ-ray exposure. Our findings may also be valuable for understanding the aetiology of cancers induced by radiation exposure.

Conflicts of Interest

The Authors declare no competing interests in relation to this study.

Authors' Contributions

HM, KD, TI and YS conceived and designed the experiments. HM, KD, TI, MN, YN and MT performed the primary experiments, including animal experiments. HM and TM evaluated pathology. HM, MT and TI performed tumour subtyping. HM and AI performed the bioinformatics and statistical analysis. HM and KD analysed the results and wrote the manuscript. KD, TI, KI, MF, YS and SK supervised the study and reviewed the manuscript. All Authors read and approved the final manuscript.

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