




Interstitial chromosomal deletion of the tuberous sclerosis complex 2 locus is a signature for radiation-associated renal tumors in Eker rats

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Abstract

Ionizing radiation can damage DNA and, therefore, is a risk factor for cancer. Eker rats, which carry a heterozygous germline mutation in the tumor-suppressor gene tuberous sclerosis complex 2 (*Tsc2*), are susceptible to radiation-induced renal carcinogenesis. However, the molecular mechanisms involved in *Tsc2* inactivation are unclear. We subjected Fischer 344 × Eker (Long Evans *Tsc2*^{+/-}) F1 hybrid rats to gamma-irradiation (2 Gy) at gestational day 19 (GD19) or postnatal day 5 (PND5) and investigated the patterns of genomic alterations in the *Tsc2* allele of renal tumors that developed at 1 year after irradiation (N = 24 tumors for GD19, N = 10 for PND5), in comparison with spontaneously developed tumors (N = 8 tumors). Gamma-irradiation significantly increased the multiplicity of renal tumors. The frequency of LOH at the chromosome 10q12 region, including the *Tsc2* locus, was 38%, 29% and 60% in renal carcinomas developed from the nonirradiated, GD19 and PND5 groups, respectively. Array comparative genomic hybridization analysis revealed that the LOH patterns on chromosome 10 in renal carcinomas were classified into chromosomal missegregation, mitotic recombination and chromosomal deletion types. LOH of the interstitial chromosomal deletion type was observed only in radiation-associated carcinomas.

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Sequence analysis for the wild-type *Tsc2* allele in the LOH-negative carcinomas identified deletions (nonirradiated: 26%; GD19: 21%) and base-substitution mutations (GD19: 4%). Reduced expression of *Tsc2* was also observed in the majority of the LOH-negative carcinomas. Our results suggest that interstitial chromosomal deletion is a characteristic mutagenic event caused by ionizing radiation, and it may contribute to the assessment of radiation-induced cancer risk.

KEYWORDS

Eker rat, genomic signature, ionizing radiation, renal carcinoma, *Tsc2*

1 | INTRODUCTION

Ionizing radiation is a mutagenic agent. Epidemiological studies of atomic-bomb survivors and victims of the Chernobyl accident have demonstrated that exposure to ionizing radiation increases the risk of cancer.¹⁻⁴ Recent studies have suggested that computed tomography scans administered during childhood increase the risk of cancer development later in life.⁵⁻⁷ It has also been reported that long-term cancer survivors who have received radiotherapy have a higher risk of secondary cancers compared with the general population.⁸⁻¹¹

Genomic alterations caused by the mutagenic effects of ionizing radiation can lead to cancer.¹²⁻¹⁴ Notably, amplification of several oncogenes, including *C-Myc*, *HER2* and *CLIP2*, has been more frequently observed in breast cancers of atomic bomb survivors and in papillary thyroid cancers of victims of the Chernobyl accident than in control populations.^{15,16} In addition, a high frequency of deletions, balanced inversions and microsatellite alterations has been reported in radiotherapy-induced secondary cancers.¹⁷⁻¹⁹ These radiation-associated alterations in the cancer genome may be useful for estimating the risk of developing radiation-induced cancer.

Identification of signatures of ionizing radiation in human cancers is difficult because cancer occurs as a result of the combined effects of radiation and other physical, chemical and biological factors, such as smoking, diet, ultraviolet radiation, viruses and hormones.²⁰ Animal models are, therefore, important for investigating the contribution of a single factor to carcinogenesis. Characteristic patterns of LOH have been frequently observed in several solid tissues and tumors of mice exposed to ionizing radiation compared with nonirradiated tissues and spontaneous or chemical carcinogen-induced tumors.²¹⁻²⁴ Eker rats spontaneously develop malignant adenocarcinoma via premalignant proliferative lesions of the renal proximal tubular epithelium (eg, phenotypically altered tubules, atypical hyperplasia and adenoma) with 100% incidence by 1 year of age.²⁵ In these rats, a retrotransposon insertion was found in one allele of the gene tuberous sclerosis complex 2 (*Tsc2*), which maps to chromosome 10. Inactivation of the remaining wild-type *Tsc2* allele by spontaneous somatic mutation is responsible for renal carcinogenesis in Eker rats.²⁶⁻²⁸ Rat *Tsc2*, which

shares 92% sequence similarity with human *TSC2*,²⁹ encodes tuberin, which contains a GTPase-activating protein (GAP) domain.³⁰ *Tsc2* can associate with GTP-bound Rheb (a small GTPase and Ras homolog), thereby promoting GTP hydrolysis, which results in inhibition of mTOR activation.³¹ In cells deficient in *TSC2*, mTOR activation enhances the phosphorylation of the downstream substrates p70 S6 kinase and 4E-BP1, which are involved in translation, thereby promoting cell proliferation and carcinogenesis.^{32,33} A study using Eker rats demonstrated that exposure to ionizing radiation increases the incidence of renal tumors with a linear dose-response relationship.³⁴

We previously found that Eker rats are most susceptible to the development of renal tumors when irradiated with 2 Gy of gamma-rays during the perinatal period.^{35,36} The multiplicity of renal tumors developed at the age of 27 weeks was significantly increased in irradiated rats compared with nonirradiated rats. However, we could not perform molecular analyses owing to the small size of tumors. Thus, in this study, we extended the observation period from 27 to 52 weeks to obtain sufficiently large tumors to allow investigation of the patterns of genomic aberrations at the *Tsc2* allele in spontaneous and radiation-associated renal tumors of Eker rats and to identify radiation-associated mutational events.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

A total of 59 F1 hybrid rats produced by mating Fischer 344 female rats with Long Evans male rats carrying the heterozygous Eker mutation (*Tsc2*^{+/−}) were used in this study. Among them, 13 F1 hybrid rats were used as the control group, while the remaining 26 and 20 F1 hybrid rats were used as gamma-irradiated at gestational day 19 (GD19) and at postnatal day 5 (PND5) groups, respectively. All experimental protocols in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences (NIRS; approval no. 07-1075) and were performed in strict accordance with the NIRS Guide for Care and Use of Laboratory Animals.

2.2 | Irradiation and sample preparation

Pregnant rats were either not irradiated (controls) or subjected to whole-body gamma-irradiation (2 Gy) at GD19, or the pups were not irradiated or irradiated at PND5. The irradiation was conducted using a Gammacell 40 irradiator containing Cs-137 source (Atomic Energy of Canada) with a dose rate of 0.7 Gy/min. When the offspring reached 52 weeks of age, both kidneys were removed and fixed in 10% neutral buffered formalin at room temperature for 24 hours and then cut into five transverse sections per kidney. These sections were embedded in paraffin and further sectioned to a thickness of 4 μ m for histopathological examination. Renal tumors (>2 mm diameter) removed during dissection were frozen in liquid nitrogen. Genomic DNA and total RNA were isolated from frozen renal tumors using an AllPrep DNA/RNA/Protein Mini Kit (Qiagen).

2.3 | Multiplicity of renal proliferative lesions

H&E-stained kidney sections from nonirradiated rats and from rats irradiated at GD19 or PND5 were used to count the multiplicity of renal proliferative lesions. The renal lesions were classified into three histological types: (a) phenotypically altered tubule; (b) atypical hyperplasia; or (c) adenoma or adenocarcinoma.

2.4 | LOH analysis

LOH analysis for the *Tsc2* locus and the 10 microsatellite loci spanning rat chromosome 10 were performed as described³⁵ using genomic DNA isolated from 8 tumor samples of nonirradiated rats and 24 and 10 tumor samples of rats irradiated at GD19 and PND5, respectively. PCR for the 10 microsatellite loci was carried out in 25 μ L reaction volume containing 50 ng genomic DNA, 0.2 mM dNTP, 1.4 mM MgCl₂, 0.2 μ M of each primer and 0.625 U rTaq polymerase. The primer sequences and PCR conditions are listed in Table S1. The PCR products were resolved on an acrylamide gel using capillary electrophoresis (HAD-GT12, eGene, Qiagen).

2.5 | Array comparative genomic hybridization analysis

Array comparative genomic hybridization (CGH) analysis was performed using the Agilent Rat Genome CGH Microarray Kit (Agilent Technologies). Fluorescent labeling of DNA, microarray hybridization and post-hybridization washing were conducted as per the manufacturer's protocol. The array images were scanned using an Agilent Microarray Scanner (G2565CA), and signal intensities were measured with Feature Extraction software ver. 10.5.1. The obtained data were imported into Agilent Genomic Workbench 6.5 and analyzed using the Aberration Detection Method 2 algorithm.

2.6 | Sequence analysis

Reverse transcription was performed to generate cDNA using 5 μ g of total RNA, ReverTra Ace (Toyobo) and random primers. Then, the cDNA was amplified using four primer pairs covering the coding region of *Tsc2*. PCR was carried out in 25 μ L reaction volume containing 0.2 μ M of each primer, 0.2 mM dNTP, 1.5 mM MgCl₂, 0.625 U AmpliTaq Gold polymerase (Perkin Elmer Japan) and 1 μ L cDNA. The primer sequences and PCR conditions are listed in Table S1. PCR products were resolved by 2% agarose gel electrophoresis and then sequenced directly using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). To identify the mutations, the cDNA sequences were compared with the rat *Tsc2* gene sequence registered in the GenBank database (accession: NM_012680.3).

2.7 | Quantitative RT-PCR

Quantitative RT-PCR was performed on an Mx3000P real-time PCR system (Agilent Technologies) using SYBR Premix Ex Taq (Takara Bio Inc). Analysis of the cDNA sequence revealed a splice-site variant lacking exon 32 that was predominantly expressed in almost all tumors without LOH or mutation in *Tsc2* (18 of 19 cases). We therefore examined the expression of this variant as a *Tsc2* wild-allele-derived transcript. The expression of the housekeeping gene *Actin* was used to normalize the data. The primer sequences and PCR conditions are listed in Table S1. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method.²⁴ A mean value of *Tsc2* expression derived from the kidneys of 6 Eker rats with *Tsc2*^{+/+} (52 weeks of age) was used as a reference value.

2.8 | Immunohistochemistry

Fixed sections were deparaffinized through a graded ethanol series, rehydrated, and washed with TBS containing 0.1% (w/v) Tween 20. Sections were then incubated overnight at 4°C with the following primary antibodies at the indicated dilution: (a) phospho-Akt1 (Ser473) (D9E) rabbit mAb (1:25) (Cell Signaling Technology); (b) phospho-mTOR (Ser2448) (49F9) rabbit mAb (1:50) (Cell Signaling Technology); (c) phospho-S6 ribosomal protein (Ser235/236) (91B2) rabbit mAb (1:75) (Cell Signaling Technology); and (d) phospho-4E-BP1 (Thr37/46) (236B4) rabbit mAb (1:400) (Cell Signaling Technology). Bound antibodies were visualized with a Vectastain Elite ABC Kit (Vector Laboratories) using a biotinylated secondary antibody against rabbit IgG and diaminobenzidine as a chromogen. All sections were then counterstained with hematoxylin.

2.9 | Statistical analysis

Statistical analysis was performed with the Wilcoxon rank sum test to assess the significance of differences in the multiplicity of renal

lesions between groups. The differences were considered significant at values of $P < 0.05$.

3 | RESULTS

3.1 | Multiplicity of renal proliferative lesions in Eker rats exposed to ionizing radiation

Representative histopathological images of renal lesions from irradiated *Tsc2*^{+/-} F1 rats are shown in Figure 1. Lesion histology did not differ between the nonirradiated and irradiated groups. The multiplicities of renal lesions for each group are shown in Table 1. Compared with the nonirradiated group, the multiplicity of phenotypically altered tubules was significantly greater in males irradiated at PND5 ($P < 0.05$). The multiplicity of atypical hyperplasias was significantly greater in both males and females irradiated at GD19 and PND5 ($P < 0.05$). The multiplicity of renal tumors (adenoma and adenocarcinoma) was significantly greater in males and females irradiated at GD19 and PND5 ($P < 0.05$). These results indicated that ionizing radiation has a carcinogenic effect in the *Tsc2*^{+/-} rats and increases the total number of renal tumors at 52 weeks of age by hastening the advance of renal carcinogenesis.

3.2 | Frequency and pattern of LOH on chromosome 10

As shown in Figure 2A, the frequency of LOH at the *Tsc2* locus in spontaneous tumors and tumors developed after irradiation at GD19 and PND5 was 38% (3 of 8), 29% (7 of 24) and 60% (6 of 10),

respectively. Three distinct LOH patterns were detected: (a) LOH spanning from locus D10MIT14 to locus D10RAT7 (covering the entire chromosome 10) (pattern a); (b) LOH spanning from the *Tsc2* locus to the distal locus (D10RAT7) (pattern b); and (c) LOH ranging from the *Tsc2* locus to the center of the chromosome (D10RAT32) (pattern c). Interestingly, pattern c was observed only in irradiated groups, whereas patterns a and b were observed in all experimental groups. To evaluate the pattern of genomic copy-number aberrations in the tumors, 1 tumor sample of each LOH type was chosen from each group (nonirradiated: 2, irradiated at GD19: 3, PND5: 3) for array CGH analysis (Figure 2B). Tumor samples with continuous LOH patterns a and b had normal copy numbers throughout chromosome 10, indicating that the LOH was caused by chromosomal missegregation or mitotic recombination. In contrast, genomic copy-number losses spanning from chromosome 10q12 to q24 were detected in the tumor samples exhibiting pattern c from groups GD19 and PND5, indicating that this LOH was caused by interstitial deletion. Notably, this interstitial chromosomal deletion was not found when we examined LOH in an additional 11 spontaneous renal tumors from fixed kidney sections (data not shown). These results suggested that there is a quality difference in the LOH patterns between spontaneous and radiation-associated tumors and that the LOH with interstitial chromosomal deletion is a signature for radiation-associated tumors.

3.3 | *Tsc2* sequence analysis

Sequence analysis of *Tsc2* in LOH-negative tumor samples (5, 17 and 4 samples from nonirradiated control, GD19 and PND5 groups, respectively) was performed to identify the mutations occurring in the wild-type *Tsc2* allele. As shown in Table 2 and Figure 3, two and

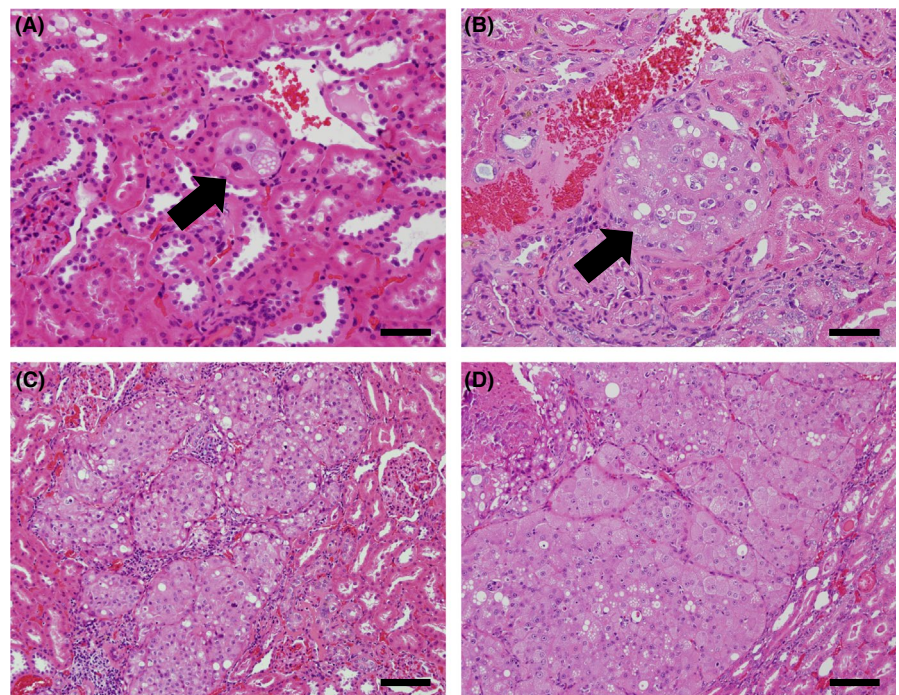


FIGURE 1 Representative histopathological identification of lesions in the kidneys of gamma-irradiated *Tsc2*^{+/-} F1 rats. H&E-stained sections revealed phenotypically altered tubules (A), atypical hyperplasia (B), adenoma (C) and adenocarcinoma (D). A phenotypically altered tubule and atypical hyperplasia are indicated by arrows. Scale bars, in (A) and (B), 50 μ m; (C) and (D), 100 μ m

	N	Phenotypically altered tubule Multiplicity (number of lesions in five kidney sections/animal)	Atypical hyperplasia	Adenoma and adenocarcinoma
Male				
Control (0 Gy)	8	30.1 ± 10.0 ^a	4.1 ± 3.4	2.9 ± 1.5 ^a
GD19 (2 Gy)	8	33.4 ± 15.0 ^a	25.8 ± 12.7*,a	7.6 ± 1.6*,a
PND5 (2 Gy)	9	73.6 ± 20.6*,†	54.0 ± 12.6*,†,a	6.2 ± 2.8*,a
Female				
Control (0 Gy)	5	50.8 ± 18.6	3.4 ± 2.3	0.4 ± 0.5
GD19 (2 Gy)	18	48.1 ± 21.5	9.7 ± 5.0*	3.3 ± 1.7*
PND5 (2 Gy)	11	66.0 ± 14.8 [†]	18.8 ± 5.0*,†	2.2 ± 1.3*
Male and female				
Control (0 Gy)	13	38.1 ± 16.8	3.8 ± 2.9	1.9 ± 1.7
GD19 (2 Gy)	26	43.5 ± 21.4	14.6 ± 10.9*	4.6 ± 2.6*
PND5 (2 Gy)	20	69.4 ± 17.6*,†	34.7 ± 20.1*,†	4.0 ± 2.9*

Note: Data represent the mean ± SD.

GD, gestational day; PND, postnatal day.

* $P < 0.05$, Control vs gestational day (GD19) or postnatal day 5 (PND5); † $P < 0.05$, GD19 vs PND5;

^a $P < 0.05$, male vs female.

TABLE 1 Multiplicity of renal proliferative lesions in *Tsc2*^{+/-} F1 rats with or without exposure to gamma radiation

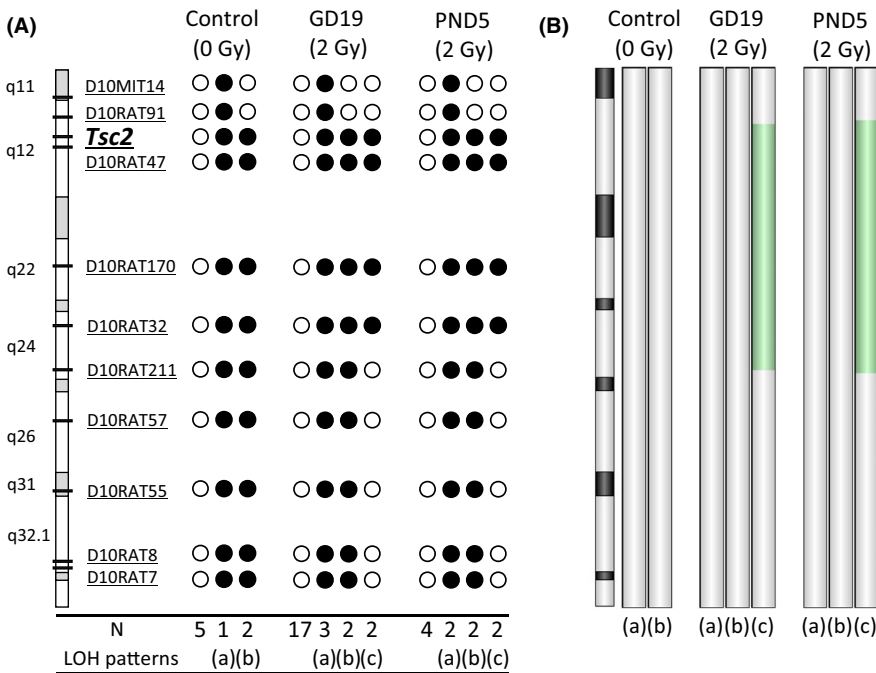


FIGURE 2 LOH patterns reveal different mutational events in spontaneous and radiation-associated renal tumors from *Tsc2*^{+/-} F1 rats. A, Polymorphic markers and their positions are shown in the chromosome schematic. Closed circles (●) represent LOH. N, number of cases that have the same LOH pattern. B, Changes in DNA copy number revealed by array comparative genomic hybridization (CGH) analysis. Deleted regions detected by array CGH analysis are shown in green

six mutations were identified in the tumors of the nonirradiated and GD19 groups, respectively. Among them, seven mutations were deletions and the eighth was a base substitution. One sample (TK718) had two deletions in the *Tsc2* gene. Five of the eight mutations were predicted to produce aberrant truncated proteins owing to a frameshift. These results suggested that there is no qualitative difference in the patterns of the *Tsc2* mutations between spontaneous and radiation-associated tumors and that deletion is the major type of mutation that causes the inactivation of *Tsc2* function in tumors.

3.4 | Expression of *Tsc2*

To examine the downregulation of *Tsc2* in the LOH-negative or *Tsc2* mutation-negative tumors, expression of a remaining *Tsc2* wild-allele-derived splicing variant was analyzed in LOH-negative or *Tsc2* mutation-negative renal tumor samples (3, 12 and 4 samples from nonirradiated control, GD19 and PND5 groups, respectively) as well as normal kidney tissues (Figure 4). The expression of *Tsc2* was less than half of the average level of normal kidney tissues in 67% (2 of 3), 58% (7 of 12) and 100%

TABLE 2 Mutations in *Tsc2* in spontaneous and radiation-associated renal tumors

Number	Group	Sample ID	DNA	Exon	Protein alteration	Size of deletion (bp)
1	Control (0 Gy)	TK711	c.1120_1257del	12	p.Asn374_Pro419del	138
2		TK727	c.4387delC	34	p.Arg1463Glu, fs17	1
3	GD19 (2 Gy)	TK756	c.444_445delCA	5	p.Asp148Glu, fs44	2
4		TK718	c.1802_2541del	17	p.Leu604Thr, fs962	740
5		TK718	c.4735_4846del	37	p.Gly1579Ser, fs11	112
6		TK709	c.3351delG	29	p.Gln1118Ser, fs73	1
7		TK719	c.4943T > A	38	p.Val1648Asp	n.a.
8		TK708	c.4996_5074del	39	p.Gly1666_Asn1722del	79

del, deletion; GD19, gestational day 19; fs, frameshift; n.a., not applicable.

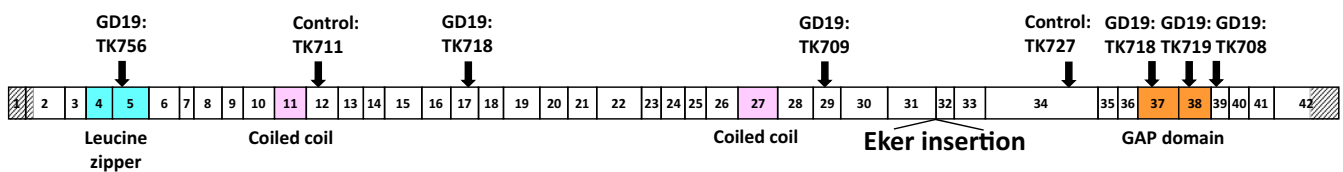


FIGURE 3 Distribution of *Tsc2* mutations in spontaneous and radiation-associated rat renal tumors. A schematic depicts the structure of rat *Tsc2*, including 42 numbered exons and the locations of the functional domains (colored). Hatched boxes indicate the 5' and 3' UTR. Arrows indicate the positions of the identified mutations

(4 of 4) of the tumors of the nonirradiated, GD19 and PND5 groups, respectively. These results suggested that *Tsc2* expression is downregulated in the majority of tumors without LOH or mutation in *Tsc2*, suggesting that inactivation of *Tsc2* may involve an epigenetic mechanism.

3.5 | Activation of the mTOR pathway

To examine the activation of the mTOR pathway in the LOH-negative renal tumors, immunohistochemical staining of several regulators in the mTOR pathway was assessed. Representative images of phospho-Akt1 (Ser473), phospho-mTOR (Ser2448), phospho-4E-BP1 (Thr37/46), and phospho-S6 ribosomal protein (Ser235/236) staining in renal adenocarcinomas are shown in Figure S1. As expected, positive staining for these regulators was observed in all tumors, indicating that the mTOR pathway was activated in the LOH-negative renal tumors.

4 | DISCUSSION

The present study aimed to investigate genomic aberrations at the *Tsc2* allele in radiation-associated renal tumors of *Tsc2*^{+/-} rats. We showed that: (a) the multiplicity of renal tumors that had developed in irradiated rats by 52 weeks of age significantly increased compared with that of nonirradiated rats; (b) LOH at the *Tsc2* locus and mutations of *Tsc2* were identified in the renal tumors; (c) interstitial chromosomal deletion on chromosome 10, including the *Tsc2* locus,

was found only in the tumors of irradiated rats; and (d) *Tsc2* expression in the majority of tumors was lower than that of normal kidney tissues.

The multiplicity of atypical hyperplasia and renal tumors was significantly increased in the irradiated groups compared with the nonirradiated control group (Table 1). Consistent with our previous study,^{35,36} this result indicates that the actively growing kidney during the perinatal period is susceptible to radiation-induced renal carcinogenesis. However, in our previous observations of nonirradiated rats at 27 weeks of age, female rats were more predisposed to renal preneoplastic lesions than were males, whereas no difference in the multiplicity of neoplastic lesions was observed between male and female rats.^{35,36} Consistent with this finding, estrogen has been shown to promote the development of preneoplastic lesions in kidneys of female Eker rats and to inhibit the progression of human renal carcinoma cells through activation of estrogen receptor β .^{37,38} In the present study, by extending the observation period from 27 to 52 weeks, we found that the multiplicity of neoplastic lesions was more prevalent in male rats than in female rats at 52 weeks of age. In humans, a similar gender difference is observed in kidney cancer, where the cancer incidence is twofold higher in men than in women.³⁹ In this regard, it has been speculated that androgen promotes tumorigenesis and progression in kidneys, whereas estrogen inhibits tumorigenesis/progression.³⁹

LOH is a major mechanism for inactivation of tumor-suppressor genes in cancer. In this study, LOH at the *Tsc2* locus was detected in both spontaneous (38%) and radiation-associated tumor samples (29-60%) (Figure 2A.). The frequency of LOH did not differ significantly between the tumors. Consistent with our results, other

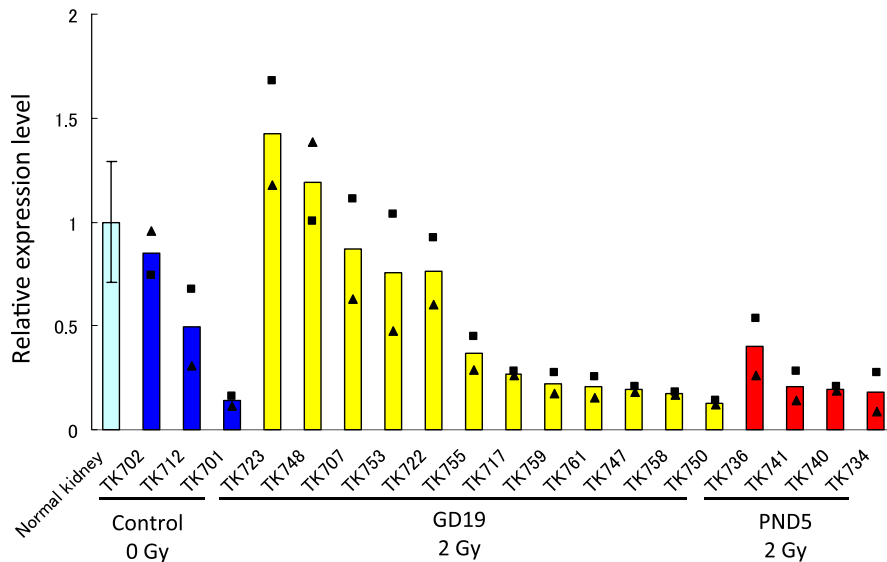


FIGURE 4 Quantitative RT-PCR assessment of *Tsc2* expression in normal rat kidney tissues, spontaneous renal tumors and radiation-associated renal tumors. The data for normal kidney tissues (the mean \pm SD of 6 samples) and for renal tumors (the average of two independent experiments, ■ and ▲) are shown. The data for tumors were normalized to the average expression value for normal kidney tissues

studies using Eker rats have reported that the frequencies of LOH at the *Tsc2* locus are 44%–60% in spontaneous^{40,41} and 29% in radiation-induced renal tumors.⁴²

Interestingly, the interstitial LOH pattern has also been reported in some radiation-induced tumors, including mice models of medulloblastoma in heterozygous *Ptch1*^{23,24} and of intestinal tumors in heterozygous *Apc*.^{43,44} A high frequency of the interstitial LOH pattern at the tumor-suppressor *Ikaros* locus has also been reported in radiation-induced thymic lymphomas in mice, compared with spontaneously developed or alkylating agent-induced lymphomas.²¹ Furthermore, using mouse kidney cell lines, it has been reported that the interstitial LOH pattern is induced as a delayed effect of gamma, proton and iron-ion radiation exposure.⁴⁵ In those studies, however, it was not clear whether the LOH was caused by chromosomal missegregation, mitotic recombination or deletion. Therefore, in the present study we examined the copy number of the *Tsc2* locus by array CGH analysis and found that LOH of the interstitial deletion type was observed only in tumors of irradiated groups (Figure 2B). Because the interstitial deletion is likely the result of the misrejoining of two DNA double-strand breaks induced by ionizing radiation within the same chromosome,⁴⁶ these data indicate that interstitial chromosomal deletion is a radiation-specific mutational event.

Kobayashi et al reported *Tsc2* mutations in 33% (7 of 21) of spontaneous and 33% (3 of 9) of ethylnitrosourea-induced renal tumors without LOH in Eker rats.²⁹ They also reported that deletion, duplication or base-substitution mutations in *Tsc2* were observed in spontaneous tumors, whereas only base-substitution mutations were observed in ethylnitrosourea-induced tumors. In our study, sequence analysis of *Tsc2* in LOH-negative tumor samples identified two deletions in spontaneous tumors and five deletions and one base substitution in radiation-associated tumors (Table 2). These mutations were predicted to produce truncated proteins, except for 1 sample, which produced an aberrant protein. It should be noted that, in the present study, small deletions were found in the tumors in both nonirradiated and irradiated groups. In addition, no difference

in the size of the deletions was observed between the groups. Given that small deletions are also frequently observed as spontaneous mutations in other models,^{47–50} our data suggest that small deletions are induced by ionizing radiation but not a specific mutational event for radiation exposure.

Approximately 50% of the tumor samples we assessed had no *Tsc2* mutations. This result was unexpected because tumorigenesis in this model is associated with either the loss or the inactivation of a wild-type allele according to Knudson's two-hit theory.⁵¹ However, we found that *Tsc2* expression was downregulated in the majority of tumors (Figure 4). Activation of the *Tsc2*-mTOR pathway in these tumors was also confirmed by immunohistochemistry (Figure S1). These results indicate the involvement of epigenetic alterations in the inactivation of the *Tsc2* wild-type allele. In fact, promoter DNA hypermethylation and silencing of *TSC2* have been reported in human oral squamous cell cancer and acute leukemia.^{52,53}

Concerning the difference in the dependency of age-at-irradiation, high LOH frequency was observed in the PND5 group (60%, 6 of 10) compared with that in the GD19 group (29%, 7 of 24) (Figure 2). In contrast, the *Tsc2* mutation frequency in the GD19 group (29%, 5 of 17) was higher than that in the PND 5 group (0%, 0 of 4) (Table 2). Although, in both cases, no statistically significant difference was observed between the groups, radiation exposure to actively growing kidney cells at perinatal stage (GD19) and subsequent perturbation of normal kidney development³⁵ may be involved in the increase in the *Tsc2* gene mutations and other mechanisms, such as the promoter DNA hypermethylation, resulting in the decrease of LOH frequency, in the GD19 group.

In conclusion, our study identified the genomic mutational events for the inactivation of the tumor-suppressor gene *Tsc2* in renal carcinogenesis of Eker rats exposed to ionizing radiation. We also identified interstitial chromosomal deletions in the tumors as a mutagenic event induced by radiation exposure. Identification of radiation-associated signatures will inform the assessment of cancer risk caused by ionizing radiation.

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DISCLOSURE

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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