Jpn. J. Cancer Res. **93**, 994–999, September 2002

Allelic Losses in Mouse Skin Tumors Induced by γ-Irradiation of *p53* Heterozygotes

Tomonori Miyazawa,[1,2] Hiroki Sato,[1] Katsuyoshi Hatakeyama,[2] Tomoyuki Kitagawa[3] and Ryo Kominami[1,4]

[1]Department of Gene Regulation and [2]Department of Regenerative and Transplantation Medicine, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi, Niigata 951-8510 and [3]Cancer Institute, 1-37-1 Kami-Ikebukuro, Tokyo 170-0012

Skin tumors were induced by γ-irradiation in F_1 mice between C3H/He or BALB/c and MSM carrying a *p53*-deficient allele. The incidence was 39.1% (34/87) in *p53*(KO/+) mice of the C3H/MSM genetic background and 14.3% (19/133) in those of the BALB/MSM background. Interestingly, most of the tumors (82%) lost the wild-type *p53* allele and no skin tumor was found in *p53*(+/+) F_1 mice. This suggests a requirement of *p53* loss for the skin cancer development. Genome scan localized a chromosomal locus showing frequent allelic losses near *D12Mit2*, which may harbor a tumor suppressor gene. In addition, 23 loci distributed on 13 chromosomes exhibited allelic losses at frequencies of more than 20%. The genome-wide occurrence of allelic losses suggests that genomic instability of the skin tumors may be implicated in radiation-induced carcinogenesis. The present study is the first to report a mouse model system useful for the analysis of radiation induction of skin cancer in man.

Key words: γ-Ray — Mouse skin tumors — LOH — Tumor suppressor gene — Genomic instability

Ionizing radiation exposure is one of the most well-established risk factors for a number of human solid tumors and hematologic malignancies. However, it is still unknown how the initial radiation-induced damage contributes to the development of cancer occurring many years later. The *p53* tumor suppressor gene is the most frequently mutated gene in a wide variety of human cancers and suppresses tumorigenesis through multiple mechanisms.[1–3] Mice deficient for *p53* (*p53*-KO) develop tumors at a very young age and *p53*(KO/+) heterozygotes are extremely susceptible to tumor development by radiation.[4–6] Although the *p53*-KO mice primarily develop lymphomas, they display strain-dependent differences in the tumor spectrum. In *p53*-KO mice of the C57BL/6 genetic background, the predominant tumor type is thymic lymphoma, whereas testicular teratomas are as common as lymphomas in 129/Sv strain.[7] Hence, these animals would provide a model system to study the mechanism by which radiation contributes to the development of tumors of different types.

One of the radiation-induced processes in carcinogenesis is presumed to be allelic loss or loss of heterozygosity (LOH). A region showing LOH at a high frequency in multiple tumor specimens has been interpreted as harboring a tumor suppressor gene.[1–3] Accordingly, we previously performed a genome-wide scan of LOH in radiation-induced thymic lymphomas of F_1 and N2 backcross mice between BALB/c and MSM strains carrying a *p53*-KO allele. Three loci showing frequent LOH were localized on chromosomes 11, 12 and 16.[8–11]

In addition to lymphomas, we found the development of skin tumors in *p53*(KO/+) mice as well, though the incidence was low in BALB/c×MSM F_1 mice. Skin tumors have never previously been reported in various mouse strains with partial and/or complete deficiency of the *p53* gene.[4–7] In the present experiment we have extended the analysis to *p53*(KO/+) F_1 mice of C3H/He×MSM, and localized the regions showing frequent LOH. Genome-wide occurrence of allelic losses was noted, which may implicate genomic instability in the development of radiation-induced skin tumors. Epidemiological analysis of atomic bomb survivors indicated a clear dose-dependent induction of non-melanoma skin cancers,[12] and the lack of an appropriate model system has hampered the elucidation of the molecular mechanisms involved in radiation induction of skin tumors. The mouse model system of *p53*(KO/+) F_1 mice between C3H/He and MSM is likely to offer a useful system for the analysis of this important cancer in man.

MATERIALS AND METHODS

Mice, irradiation and tumors MSM is an inbred strain derived from Japanese wild mice, *Mus musculus molossinus*, and the establishment of *p53*(KO/+) MSM mice was described previously.[8] F_1 hybrid mice between BALB/c and *p53*(KO/+) MSM were subjected to γ-ray irradiation,

[4] To whom correspondence should be addressed.
E-mail: rykomina@med.niigata-u.ac.jp

2.5 Gy four times at weekly intervals, starting at the age of 4 weeks. F_1 hybrids between C3H/He and *p53*(KO/+) MSM were irradiated with a single dose of 3 Gy once when mice were 4 weeks old. Skin tumors were diagnosed by inspection and palpation of the mice and were confirmed upon autopsy. Nineteen skin tumors were obtained from F_1 hybrids between BALB/c and *p53*(KO/+) MSM, but four were excluded from the analysis because of severe contamination with normal tissues. Thirty-four skin tumors were obtained from irradiated F_1 mice between C3H/He and *p53*(KO/+) MSM. Tumor specimens were HE-stained and examined under microscope.

Polymerase chain reaction (PCR) analysis Genomic DNA was extracted from skin tumors and normal brain. PCR was carried out by the use of standard protocols and products were separated by electrophoresis on 8% polyacrylamide gel. Microsatellite markers used were as previously reported.[13] *p53* genotyping was carried out by using a set of three primers, as described previously.[8] One primer (F1-53) was located in exon 1 of the *p53* gene, a second primer (R1-53) in a region 5′ to exon 3, and the remaining one (F2-neo) in the *neo* gene inserted.

RESULTS

A total of 276 F_1 mice between BALB/c (C) and *p53*(KO/+) MSM (M) and 164 F_1 mice between C3H/He (H) and *p53*(KO/+) MSM were subjected to γ-irradiation. *p53* genotyping of these CM and HM F_1 mice was performed with PCR using a set of three primers that detected the wild-type *p53* and the inserted *neo* gene.[8] Among the 276 CM F_1 mice, 133 were heterozygous for *p53* and so were 87 of the 164 HM F_1 mice. Table I summarizes the incidences of three different tumors that developed. Almost all of the *p53*(KO/+) mice of the CM F_1 background developed tumors, most of which were thymic lymphoma and only 14.3% of which were skin tumors. On the other hand, those of the HM F_1 background displayed

Table I. Numbers of Tumors Developed in *p53* Wild-type Mice and Mice Carrying a *p53*-deficient Allele after γ-Ray Irradiation

	Lym[a)]	Skin	Hepatoma	Others	No tumor	Total
BALB/c×MSM						
p53 (+/+)	94	0	1	7	41	143
p53 (KO/+)	106	19	1	6	1	133
C3H×MSM						
p53 (+/+)	3	0	14	1	59	77
p53 (KO/+)	11	34	21	6	15	87

a) Lym, thymic lymphoma; Skin, skin tumor; Others, tumors included leukemia, splenic tumors, ovarian cancers.

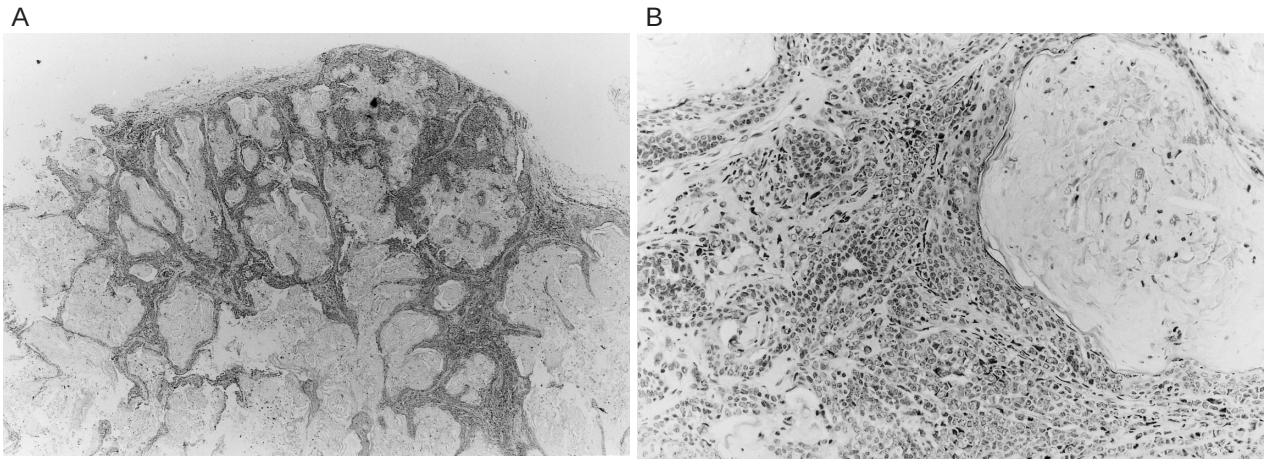

Fig. 1. Histology of skin tumors. Benign demarcated tumor of skin appendage origin showing cyst-like structures containing exfoliated keratinocytes and amorphous material and lined by squamous epithelium, with occasional transition to medullary and double-layered duct patterns. A, 10× magnification; B, 50×.

a higher incidence of skin tumors (39.1%). Histological examination of the radiation-induced tumors provided an impression of trichofolliculoma, benign demarcated tumor of skin appendage origin (Fig. 1). This type of tumor has not been observed previously in *p53*(KO/+) mice.[4–7] Interestingly, no skin tumor developed in *p53*(+/+) mice of either background. *p53* genotyping of the skin tumors showed loss of the wild-type allele at a frequency of as high as 81.6% (40/49), suggesting *p53* inactivation in almost all of the skin tumors. As for hepatomas, the incidence was much higher in the HM F_1 mice (21.3%) than in the CM mice (0.72%). However, the incidences in *p53*(+/+) and *p53*(KO/+) HM F_1 mice did not much differ.

A primary screen was made on 25 HM F_1 skin tumors for LOH at 52 microsatellite marker loci which were distributed throughout the autosomes. Fig. 2 shows an example of such analyses with the *D12Mit2* probe and Table II

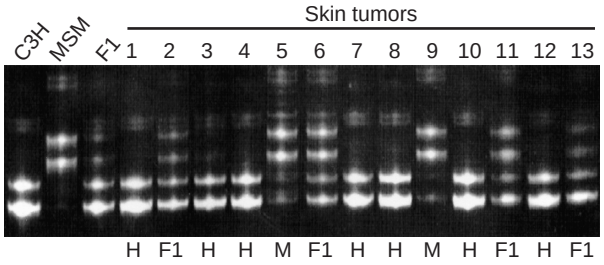

Fig. 2. Allelic loss analysis of the *D12Mit2* locus. PCR products were subjected to polyacrylamide gel electrophoresis. The first three lanes on panels represent control DNA of C3H/He, MSM and F_1 mice. Other lanes display tumor DNA. The numbering of skin tumors is arbitrary. Presence or absence of allelic loss in each tumor is indicated at the bottom: H, loss of M allele; M, loss of H allele; and F_1, no allelic loss.

Table II. Frequencies of LOH in Marker Loci Distributed throughout the Mouse Genome

Locus	CM[a)]	%[b)]	Locus	CM[a)]	%[b)]
D1Mit4	8.7	4	*D11Mit62*	2.2	65.3
D1Mit9	84.0	12	*D11Mit12*	75.4	32
D1Mit17	110.4	32	*D12Mit2*	16.4	51.0
D2Mit3	7.7	12	*D12Mit6*	40.4	51.0
D2Mit15	50.3	12	*D12Mit279*	50.3	51.0
D2Mit25	85.2	28.6	*D13Mit14*	3.3	16
D3Mit3	16.4	12	*D13Mit9*	26.2	20
D3Mit17	50.3	36	*D13Mit150*	50.3	24
D4Mit149	0.0	28	*D14Mit2*	6.6	4
D4Mit12	54.6	4	*D14Mit237*	40.4	26.5
D4Mit344	79.8	4	*D14Mit7*	52.5	34.7
D5Mit3	14.2	0	*D14Mit8*	59	29.4
D5Mit5	26.2	0	*D15Mit11*	5.5	0
D5Mit101	74.3	0	*D15Mit16*	63.4	12
D6Mit1	3.3	20	*D16Mit122*	6.6	16
D6Mit12	49.2	32	*D16Mit4*	25.1	12
D7Mit74	1.1	36	*D16Mit7*	45.9	24
D7Mit17	37.2	20	*D17Mit11*	10.9	0
D8Mit1	4.4	28	*D17Mit119*	33.9	0
D8Mit13	68.9	20	*D17Mit123*	50.3	4
D9Mit1	4.4	0	*D18Mit19*	0.0	8
D9Mit20	60.1	12	*D18Mit7*	35.0	0
D10Mit15	25.1	16	*D19Mit32*	0.0	0
D10Mit7	40.4	26.5	*D19Mit14*	15.3	8
D10Mit70	57.9	44.9	*D19Mit123*	38.3	16
D10Mit14	69.9	36.7	*D19Mit19*	26.2	12

a) Distance from the centromere in centi-Morgan.
b) Integers indicate % of 25 tumors examined and the others % of 49 tumors.

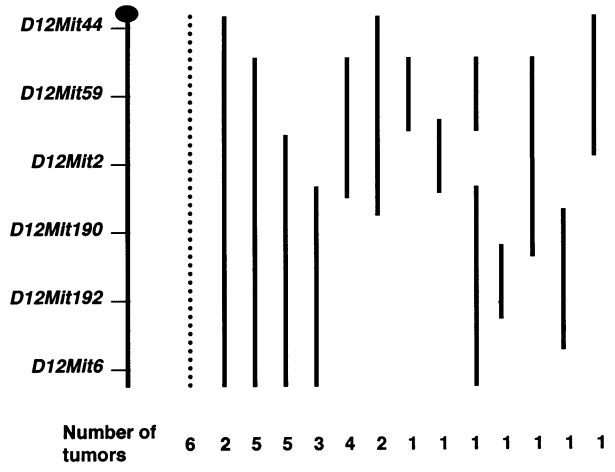

Fig. 3. Allelic loss mapping of chromosome 12 in skin tumors induced by γ-irradiation. Vertical lines represent regions showing allelic loss and a dotted line indicates no allelic loss. The number of tumors having each type of allelic loss is shown under each vertical line.

summarizes the results. There were eight loci exhibiting LOH at frequencies higher than 30%, and the LOH frequencies for *D11Mit62* and *D12Mit2* loci were even higher than 50%. To further localize these regions, marker loci in the vicinities were typed for all 49 F_1 tumors. Fig. 3 shows the extent of allelic losses in the 34 HM F_1 tumors on chromosome 12. Loss of a whole chromosome was found in two tumors, whereas LOH in part of the chromosome was detected in 26 tumors. Therefore, the region between *D12Mit2* and *D12Mit190* underwent allelic loss at the highest frequency in these tumors. In addition, the loss of *D12Mit2* was biased toward the MSM allele (P=0.018); the loss of the MSM allele accounted for 17 cases of the 20 losses and that of the C3H allele for three. As for the 15 CM F_1 tumors examined, only five showed allelic loss at *D12Mit2*, with BALB/c allele-loss in three and MSM allele-loss in two.

Allelic loss at *D11Mit62* had a preference for the C3H and BALB/c chromosome carrying the wild-type *p53* allele. Loss of the C3H and BALB/c alleles was detected in 26 tumors and 6 such losses were noted for the MSM allele. Besides, 24 of the 26 tumors underwent loss of the wild-type *p53* allele. The result suggested that the preference noted at *D11Mit62* was caused by loss of the wild-type *p53* allele on the same chromosome 11.

DISCUSSION

The present study describes the development of skin tumors by γ-ray irradiation of F_1 mice between C3H/He or BALB/c and MSM carrying a *p53*-deficient allele. Histological examination suggests that the tumors are of epithelial cell origin (Fig. 1). Most of the tumors had lost the wild-type *p53* allele and no tumor was induced in $p53^{+/+}$ mice of the same genetic background (Table I). These results strongly suggest a requirement of *p53* loss for development of the tumors under the conditions employed in the present study. Interestingly, the incidence of skin tumors was higher in F_1 mice of the HM genetic background than in those of the CM background (Table I). The difference may reflect a modifier(s) that contributes to the tumor development in combinations with *p53* deficiency. A modifier of testicular teratomas was previously reported for *p53*-deficient mice of the C57BL/6 and 129/Sv strains.[7]

Genome-wide analyses revealed that the *D12Mit2* locus underwent allelic loss at a frequency of more than 50% and the loss appeared to have a preference for the MSM allele in HM F_1 mice. Therefore, this locus may harbor a tumor suppressor gene for skin tumor development and the MSM allele could be a functional gene while the C3H allele mutated. Database search did not provide any candidate gene within this chromosomal region. The *D11Mit62* locus exhibited a high LOH frequency. However, most of the allelic losses were accompanied by the loss of the wild-type *p53* allele on the same chromosome 11. Therefore, a tumor suppressor gene is less likely to reside in the region. In addition, there were many other loci exhibiting LOH at high frequencies, six showing more than 30% and five more than 20% (Table II). This may suggest the existence of tumor suppressor genes in these regions. Alternatively, such genome-wide allelic losses may be secondary results of genomic instability associated with the development of skin tumors, although a high frequency of changes does not always reflect a high rate of genomic alterations.[14]

Radiation is known to induce skin cancer in man.[12] However, no animal model system for γ-ray-induced skin carcinogenesis has been developed. Previous models develop skin cancers after repeated local β-irradiation of the backs of mice and rats.[15, 16] Heterozygous mice with a *p53*-deficient allele exhibit enhanced susceptibility to various cancers after irradiation[6, 17] and the present study has revealed that *p53*(KO/+) F_1 mice between C3H/He and MSM strains were sensitive to radiation induction of skin tumors. The MSM strain is an inbred line derived from Japanese wild mice, *Mus musculus molossinus*, and exhibits resistance to radiogenic cancers.[8, 18] This model system offers an opportunity to analyze the molecular mechanisms of radiation carcinogenesis.

Two hypotheses have been proposed regarding the mechanism of radiation carcinogenesis.[19–22] Radiation may lead directly to oncogenic mutations as a result of misrepair and misreplication of DNA damage, and all progeny of the mutant cell would carry the same mutation

and generate the malignant clone. It is possible therefore that allelic losses found in the skin tumors, including loss of the wild-type *p53* allele, were initiated directly by radiation-induced damage. An alternative is the indirect mechanism, in which radiation initially induces genomic instability that is transmitted through subsequent cell divisions. Radiation is known to induce delayed mutation and such a process increases the probability of later occurrence of transforming mutations. In this scenario, the allelic losses are the secondary result of some as yet unidentified genomic alteration induced by ionizing radiation. The data obtained in this study cannot determine which hypothesis is more probable. On the other hand, genomic instability is a well-established feature of cells with mutations in the *p53* gene. *p53* is an archetypal regulator of cell-cycle checkpoints and apoptosis, and responds to DNA damage induced by genotoxic agents such as γ-rays.[2, 3, 6, 23, 24] Hence, the frequency of allelic losses or genomic instability in tumors may well be affected by the loss of *p53*.

ACKNOWLEDGMENTS

We thank O. Niwa for critical reading of this manuscript and Y. Saito for technical assistance. This work was supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan.

(Received May 28, 2002/Revised July 4, 2002/Accepted July 8, 2002)

REFERENCES

1) Greenblatt, M. S., Bennent, W. P., Hollstein, M. and Harris, C. C. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.*, **54**, 4855–4878 (1994).
2) Giaccia, A. J. and Kastan, M. B. The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev.*, **12**, 2973–2983 (1998).
3) Sionv, R. V. and Haupt, Y. The cellular response to p53: the decision between life and death. *Oncogene*, **18**, 6145–6157 (1999).
4) Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Butel, J. S. and Bradley, A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, **356**, 215–221 (1992).
5) Harvey, M., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., Bradley, A. and Donehower, L. A. Spontaneous and carcinogen-induced tumorigenesis in *p53*-deficient mice. *Nat. Genet.*, **5**, 225–229 (1993).
6) Kemp, C. J., Wheldon, T. and Balmain, A. p53-deficient mice are extremely susceptible to radiation-induced tumorigenesis. *Nat. Genet.*, **8**, 66–69 (1994).
7) Donehower, L. A., Harvey, M., Vogel, H., McArthur, M. J., Montgomery, C. A., Park, S. H., Thompson, T., Ford, R. J. and Bradley, A. Effects of genetic background on tumorigenesis in p53-deficient mice. *Mol. Carcinog.*, **14**, 16–22 (1995).
8) Matsumoto, Y., Kosugi, S., Shinbo, T., Chou, D., Ohashi, M., Wakabayashi, Y., Sakai, K., Okumoto, M., Mori, N., Aizawa, S., Niwa, O. and Kominami, R. Allelic loss analysis of gamma-ray-induced mouse thymic lymphomas: two candidate tumor suppressor gene loci on chromosomes 12 and 16. *Oncogene*, **16**, 2747–2754 (1998).
9) Shinbo, T., Matsuki, A., Matsumoto, Y., Kosugi, S., Takahashi, Y., Niwa, O. and Kominami, R. Allelic loss mapping and physical delineation of a region harboring a putative thymic lymphoma suppressor gene on chromosome 12. *Oncogene*, **18**, 4131–4136 (1999).
10) Okano, H., Saito, Y., Miyazawa, T., Shinbo, T., Chou, D., Kosugi, S., Takahashi, Y., Odani, S., Niwa, O. and Kominami, R. Homozygous deletions and point mutations of the Ikaros gene in γ-ray-induced mouse thymic lymphomas. *Oncogene*, **18**, 6677–6683 (1999).
11) Matsuki, A., Kosugi-Okano, H., Ochiai, Y., Kosugi, S., Miyazawa, T., Wakabayashi, Y., Hatakeyama, K., Niwa, O. and Kominami, R. Allelic loss mapping and physical delineation of a region harboring a thymic lymphoma suppressor gene on mouse chromosome 16. *Biochem. Biophys. Res. Commun.*, **282**, 16–20 (2001).
12) Little, M. P. and Charles, M. W. The risk of non-melanoma skin cancer incidence in the Japanese atomic bomb survivors. *Int. J. Radiat. Biol.*, **71**, 589–602 (1997).
13) Dietrich, W. F., Miller, J., Steen, R., Merchant, M. A., Damron-Boles, D., Husain, Z., Dredge, R., Daly, M. J., Ingalls, K. A., O'Connor, T. J., Evans, C. A., DeAngelis, M. M., Levinson, D. M., Kruglyak, L., Goodman, N., Copeland, N. G., Jenkins, N. A., Hawkins, T. L., Tein, L., Page, D. C. and Lander, E. S. A comprehensive genetic map of the mouse genome. *Nature*, **380**, 149–152 (1996).
14) Lengauer, C., Kinzler, K. W. and Vogelstein, B. Genetic instabilities in human cancers. *Nature*, **396**, 643–649 (1998).
15) Ootsuyama, A. and Tanooka, H. One hundred percent induction in mouse skin after repeated beta irradiation in a limited dose range. *Radiat. Res.*, **115**, 488–494 (1988).
16) Coggle, J. E. and Williams, J. P. Experimental studies of radiation carcinogenesis in the skin: a review. *Int. J. Radiat. Biol.*, **57**, 797–808 (1990).
17) Backlund, M. G., Trasti, S. L., Backlund, D. C., Cressman, V. L., Godfrey, V. and Koller, B. H. Impact of ionizing radiation and genetic background on mammary tumorigenesis in p53-deficient mice. *Cancer Res.*, **61**, 6577–6582 (2001).
18) Saito, Y., Ochiai, Y., Kodama, Y., Tamura, Y., Togashi, T., Kosugi-Okano, H., Miyazawa, T., Wakabayashi, Y., Hatakeyama, K., Wakana, S., Niwa, O. and Kominami, R. Genetic loci controlling susceptibility to γ-ray-induced thy-

mic lymphoma. *Oncogene*, **20**, 5243–5247 (2001).
19) International Commission on Radiological Protection. "1990 Recommendations of the International Commission on Radiological Protection," ICRP Publication 60 (1991). Pergamon Press, Oxford, UK.
20) Little, J. B. Failla Memorial Lecture. Changing views of cellular radiosensitivity. *Radiat. Res.*, **140**, 299–311 (1994).
21) Kronenberg, A. Radiation-induced genomic instability. *Int. J. Radiat. Biol.*, **66**, 603–609 (1994).
22) Sadamoto, S., Suzuki, S., Kamiya, K., Kominami, R., Dohi, K. and Niwa, O. Radiation induction of germline mutation at a hypervariable mouse minisatellite locus. *Int. J. Radiat. Biol.*, **65**, 549–557 (1994).
23) Lee, J. M., Abrahamson, J. L., Kandel, R., Donehower, L. A. and Bernstein, A. Susceptibility to radiation-carcinogenesis and accumulation of chromosomal breakage in p53 deficient mice. *Oncogene*, **9**, 3731–3736 (1994).
24) Boyle, J. M., Spreadborough, A. R., Greaves, M. J., Birch, J. M., Varley, J. M. and Scott, D. Delayed chromosome changes in gamma-irradiated normal and Li-Fraumeni fibroblasts. *Radiat. Res.*, **157**, 158–165 (2002).