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Instructions for Authors

Aims and Scope

"Genes and Environment" is an official journal of the Japanese Environmental Mutagen Society (JEMS) and published every three months by the society. Papers are published in advance of printing, soon after acceptance, at J-STAGE. The journal publishes six types of manuscripts written in English in the fields of environmental mutagenesis, genomics and epigenetics. Genetic toxicology including risk evaluation for human health, validation studies on testing methods and subjects of guidelines for regulation of chemicals are also within its scope.

A. Types of Manuscript

The journal publishes six types of papers as follows:

1. **Regular articles** report new, significant, innovative and original findings of fundamental and molecular studies. Results and conclusions of genotoxicity testing programs are also acceptable. However, very detailed testing results may only be published electronically (See **C-6**).
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Organization as author

2. Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. J Urol. 2003; 169: 2257–61.

Article republished with corrections

3. Mansharamani M, Chilton BS. The reproductive importance of P-type ATPases. Mol Cell Endocrinol. 2002; 188: 22–5. Corrected and republished from: Mol Cell Endocrinol. 2001; 183: 123–6.

Books and Other Monographs

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4. Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Medical microbiology. 4th ed. St. Louis: Mosby; 2002.

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5. Breedlove GK, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services; 2001.

Chapter in a book

6. Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. p. 93–113.

Scientific or technical report

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7. Yen GG (Oklahoma State University, School

of Electrical and Computer Engineering, Stillwater, OK). Health monitoring on vibration signatures. Final report. Arlington (VA): Air Force Office of Scientific Research (US), Air Force Research Laboratory; 2002 Feb. Report No.: AFRLSRBLTR020123. Contract No.: F496209810049.

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Mutagenic radioadaptation in a human lymphoblastoid cell line

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Abstract

We investigated the mutagenic radioadaptive response of human lymphoblastoid TK6 cells by pretreating them with a low dose (5 cGy) of X-rays followed by a high (2 Gy) dose 6 h later. Pretreatment reduced the 2-Gy-induced mutation frequency (MF) of the *thymidine kinase* (*TK*) gene (18.3×10^{-6}) to 62% of the original level (11.4×10^{-6}). A loss of heterozygosity (LOH) detection analysis applied to the isolated TK^- mutants revealed the mutational events as non-LOH (resulting mostly from a point mutation in the *TK* gene), hemizygous LOH (resulting from a chromosomal deletion), or homozygous LOH (resulting from homologous recombination (HR) between chromosomes). For non-LOH events, pretreatment decreased the frequency to 27% of the original level (from 7.1×10^{-6} to 1.9×10^{-6}). cDNAs prepared from the non-LOH mutants revealed that the decrease was due mainly to the repression of base substitutions. The frequency of hemizygous LOH events, however, was not significantly altered by pretreatment. Mapping analysis of chromosome 17 demonstrated that the distribution and the extent of hemizygous LOH events were also not significantly influenced by pretreatment. For homozygous LOH events, pretreatment reduced the frequency to 61% of the original level (from 5.1×10^{-6} to 3.1×10^{-6}), reflecting an enhancement in HR repair of DNA double-strand breaks. Our findings suggest that the radioadaptive response in TK6 cells follows mainly from mutations at the base-sequence level, not the chromosome level.
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Keywords: Adaptive response; TK6 cells; LOH detection system

1. Introduction

An adaptive cellular response occurs when a mild stress applied before a challenging treatment with a DNA-damaging agent decreases the detrimental effects of the challenge. In radioadaptation, as it is usually defined, exposure to a low dose of ionizing radiation (IR) provides some protection against a high dose. Radioadaptation was first reported by Olivieri et al. [1], who showed that radiation delivered by labeling human lymphocytes with tritiated thymidine causes a decrease in the frequency of chromosomal aberrations induced by subsequent exposure to 15 Gy of IR. That discovery stimulated a series of studies in human lymphocytes and various mammalian cell lines (for review, see refs. [2,3]) and suggested that the adaptive response is an important defense mechanism, especially against low doses of IR. The molecular mechanisms involved, however, remain largely unknown [4–8], and cellular

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responses such as the bystander effect, genetic instability and hyper-radiosensitivity seem tightly related to the adaptive response in a specific low-dose region. One of the hot subjects in recent adaptive response studies is the expression of the genes involved in the mechanism [8–10]. Another is the relationship between the adaptive response and the bystander effect [11–15]. In mammalian cells, for example, bystander mutagenesis may be suppressed by an adaptive response [11].

Following the report by Olivieri et al., reduced induction of both micronuclei and sister chromatid exchanges was shown in Chinese hamster V79 cells pre-exposed to low doses of γ-rays or ^{3}H β-rays [16]. Subsequent studies reported similar radioadaptive responses, such as reduced mutation frequencies in human lymphocytes [17], mouse SR-1 cells [18] and human–hamster hybrid A_L cells [19], an altered mutation spectrum in human–hamster hybrid A_L cells [19], reduced micronucleus frequencies in human lymphocytes [5] and mouse embryo cells [20], and reduced deletions and rearrangements in human lymphoblast cells [21]. The mechanism underlying those radioadaptations may have been the induction of an efficient chromosome repair system by the priming radiation dose, and in fact, the efficiency of DNA double-strand break (DSB) repair in Chinese hamster V79 cells exposed to γ-rays is enhanced by a priming exposure of 5 cGy of γ-rays [22]. Furthermore, DSBs with either blunt or staggered ends, created by restriction enzymes, induce the adaptive response [3].

The human lymphoblastoid TK6 cell line, isolated by Skopek et al. [23], is heterozygous at the *thymidine kinase* (*TK*) locus. Honma's laboratory developed a loss of heterozygosity (LOH) detection system that can be used for molecular analysis of *TK* mutations as well as for detecting alterations at the chromosome level [24,25]. Using that methodology, we were able to detect IR effects at doses as low as 10 cGy [26–28]. Irradiation of TK6 cells with 10 cGy of X-rays clearly demonstrated radiation-specific types of LOH events or interstitial deletions in chromosome 17 [26]. We also observed more efficient induction of such events after 10 cGy irradiation with an accelerated carbon-ion (135 MeV/u) beam [27], and this was apparent in frozen cells exposed to the same carbon-ion beam [28]. These results strongly suggest that the interstitial deletions were the result of end-joining repair of IR-induced DSBs.

Because the radiation-sensitive LOH analysis system in TK6 cells is effective for detecting the fate of radiation-induced DNA double-strand breaks (DSBs), we use it here to see if the adaptive response could produce measurable changes in IR-induced genetic alterations. The results we obtained were not completely expected, but are interesting.

2. Materials and methods

2.1. Cell culture and adaptive treatment

The methodologies for the detection of *TK*-deficient mutants and the materials and methods used for cell culture and growth have been previously reported [26]. Briefly, TK6 cells were incubated in RPMI1640 medium supplemented with HAT to eliminate pre-existing TK^{-} deficient mutants. The cells were then resuspended in fresh normal medium, and 6 ml cell suspension was dispensed into 6-cm diameter Petri dishes. The cells were pretreated ("primed") with 2.5, 5 or 10 cGy of X-rays (250 kVp) at a rate of 10 cGy/min, and placed in a 5% CO_2 humidified incubator. The cell concentration was adjusted to 8×10^{5} cells/ml at the end of the post-irradiation incubation period of 1.5, 3, 6, 9 or 12 h. The cells were then challenged with 2 Gy X-rays (250 kVp) at 1 Gy/min. Non-primed irradiated cells treated in the same manner as the primed cells served as controls.

2.2. Survival assay and TK mutation assay

To determine the surviving fraction of the challenged cells, we measured the plating efficiency (PE) immediately after irradiation using the limiting dilution method. For mutation expression, we incubated the cells with non-selecting RPMI1640 medium for about 60 h following the X-ray challenge. We measured the PE of incubated cells similarly, determining the *TK* mutation frequency. To select TK^{-} mutant clones, we seeded incubated cells into 96-well plates at 4×10^{4} cells per well in RPMI1640 medium containing 4 μg/ml trifluorothymidine (TFT); we harvested the normally growing clones after 2 weeks and the slow growing clones after 4 weeks.

2.3. Determination of optimum irradiation conditions for mutagenic adaptation

To determine the optimum conditions for evoking the mutagenic radioadaptive-response, we tested the MF induced by 2 Gy at 0, 1.5, 3, 6, 9 and 12 h after a priming dose of 10 cGy, selected the optimum interval time, and then tested the MF induced by 2 Gy at that interval time after priming doses of 0, 2.5, 5 and 10 cGy.

2.4. LOH analysis of TK^{-} mutants

Fig. 1 illustrates how we classified TK^{-} mutants. We first determined *TK* LOH by PCR analysis of exons 4 and 7 [29]. If the PCR products of both were similar to those of the parental *TK* heterozygous cells, we classified the mutant

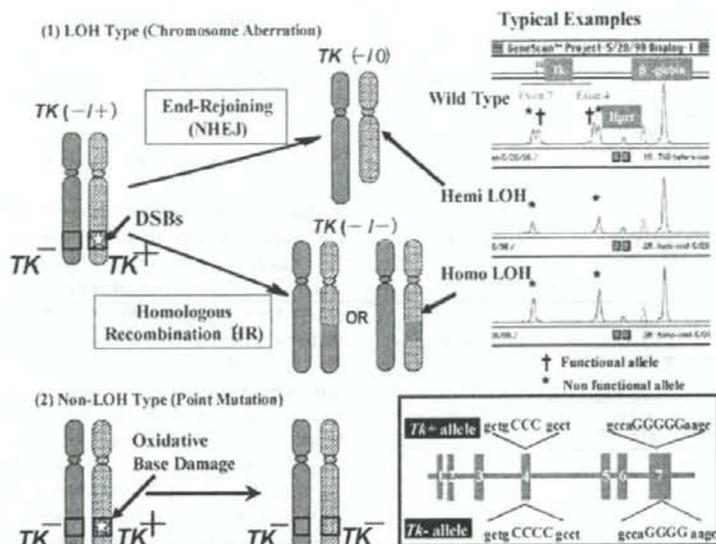

Fig. 1. LOH classifications of TK^- mutants. The first step in the genetic analysis of selected TK^- mutants was to judge whether there was a loss of *TK* heterozygosity (LOH). This was accomplished by PCR amplification of exons 4 and 7 regions of the *TK* locus. This step also distinguished between hemizygous LOH (loss of the functional *TK* allele) and homozygous LOH (replacement of the functional *TK* allele by a mutated TK^- allele (see ref. [29]).

as a "non-LOH" mutant. We used the same technique to distinguish between hemizygous LOH (in which the functional *TK* allele is lost) and homozygous LOH (in which the functional *TK* allele is replaced by TK^-). To determine the extent and size of the deleted or substituted portions of the chromosome involved, we analyzed 11 microsatellite regions (D17S588, D17S1784, D17S785, D17S789, D17S802, D17S807, D17S928, D17S932, D17S1299, D17S1566 and THRA) on chromosome 17 using multiple PCR reactions as described previously [29]. The fine structure of the recovered TK^- LOH mutations was determined by chromosome mapping analysis.

2.5. Base sequencing of non-LOH mutants

For a precise analysis of non-LOH mutants, we extracted RNA using Isogen (Nippon Gene, Japan), and obtained cDNA using a First-Strand cDNA Synthesis Kit, Amersham, USA). Following PCR amplification, the purified 807-bp fragments were sequenced by Takara Bio (Japan). The primers 5′-AGAGTACTCGGGTTCGTGAA-3′ and 5′-GCAGCATGCAGGGCAGCGTG-3′ (forward and reverse, respectively) were used for cDNA synthesis, PCR amplification and base sequencing [30]. To prevent the overestimation of mutational events, we counted identical mutations originating from a single irradiated dish as a single event.

Table 1a
TK mutation frequency (MF) at various time intervals between priming and challenging X-ray exposures (priming dose, 10 cGy; challenging dose, 2 Gy)

Time interval (h)	0	1.5	3	6	9	12
TK MF ($\times10^{-6}$)	19.8	18.1	14.4	13.5	17.8	19.7

3. Results

3.1. Optimum conditions for mutagenic adaptation

For inducing an adaptive response to X-ray irradiation, the optimum interval between a 10-cGy priming dose and a 2-Gy challenging dose was 6 h (Table 1a), and the optimum priming dose 6 h prior to a 2-Gy challenging dose was 5 cGy (Table 1b). We therefore decided to characterize the induced *TK* mutants by repeating

Table 1b
TK mutation frequency at various priming X-ray doses (challenging dose, 2 Gy; interval between 2 exposures, 6 h)

Priming X-ray dose (cGy)	0	2.5	5	10
TK MF ($\times10^{-6}$)	13.3	15.8	4.5	6.3

Table 2
Surviving fractions of primed and non-primed TK6 cells following challenge exposure to 2 Gy X-rays

Experiment	Surviving fraction	
	Non-primed cells	Primed cells (5 cGy)
I	0.043	0.047
II	0.047	0.070
III	0.049	0.040
Mean ± S.D.	0.046 ± 0.0031*	0.052 ± 0.016*

* $P = 0.58$; t-test.

Table 3
TK mutation frequency in primed and non-primed TK6 cells following challenge exposure to 2 Gy X-rays

Experiment	*TK* mutation frequencies ($\times 10^{-6}$)	
	Non-primed cells	Primed cells (5 cGy)
I	13.3	4.5
II	13.3	10.5
III-a	20.4	15.1
III-b	21.0	15.6
Mean ± S.D.	18.3 ± 4.3*	11.4 ± 5.1*

Experiments III-a and III-b were carried out concurrently with survival assay III, but they were independent mutation assays.
* $P = 0.020$; t-test.

our mutation experiments under those conditions (5 cGy followed 6 h later with 2 Gy).

3.2. Survival assay and TK mutation assay

Table 2 shows the surviving fraction, expressed as PE (2 Gy X-ray irradiated cells)/PE (unirradiated cells) of primed and unprimed cells immediately after the 2-Gy challenge exposure. Irradiation with the priming dose of 5 cGy did not influence the PE of unchallenged cells (data not shown). The effect of priming on survival after 2 Gy X-ray irradiation was 1.1 (0.052/0.046; $P = 0.58$, t-test). Thus, priming did not significantly affect survival after the challenge exposure.

On the other hand, priming did affect the *TK* MF induced by the challenge. Data from 4 independent experiments showed that priming reduced the MF to 62% of the unprimed MF ($P = 0.020$, t-test) (Table 3).

3.3. LOH analysis of TK⁻ mutants

Table 4 shows the distributions of LOH classes among the isolated TK^- mutants as determined by PCR analysis. We isolated non- and "small" LOH mutants (see Sections 3.4 & 3.5) as normal growth mutants in the first selection, except for a few cases. We isolated the remaining LOH mutants as slow growth mutants in the second selection. We estimated the pre-exposure effect from the proportion of each mutational event as follows: (i) 7.1×10^{-6} to 1.9×10^{-6} reduction in corresponding MF of non-LOH events, (ii) 6.4×10^{-6} to 6.1×10^{-6} change in corresponding MF of hemizygous LOH events and (iii) 5.1×10^{-6} to 3.1×10^{-6} reduction in corresponding MF of homozygous LOH events. Thus, the MF of a non-LOH event in primed cells was reduced to 27% of the non-primed MF. The induction of hemizygous events, on the other hand, was barely influenced by priming. As far as homozygous events go, their corresponding MF was reduced to 61% of the original level, which was similar to level of reduction in total MF (62%).

3.4. Analysis of LOH tracts on chromosome 17

Fig. 2 shows the deleted or replaced regions of chromosome 17 in each LOH mutant. Mutants reflected both type 1 and type 2 LOH events. Type 1 defines a terminal event; that is, the deleted or exchanged chromosome segment extends to the telomere marker (D17S928). Type 2 defines an interstitial deletion; the altered segment does not reach the telomere marker.

In the present study, most hemizygous LOH mutations, which are considered to be the result of DSB non-homologous end-joining (NHEJ) repair, reflected

Table 4
Distribution of mutational classes among the isolated *TK* mutants

Mutational class	Number of identified mutants (Exp. I, II, III-a, III-b) [MF $\times 10^{-6}$]	
	Non-primed cells	Primed cells (5 cGy)
Non-LOH	18 (5, 4, 6, 3) [7.1]	8 (1, 3, 2, 2) [1.9]
LOH		
Hemizygous	15 (3, 3, 7, 2) [6.0]	27 (8, 7[a], 5, 7) [6.4]
Homozygous	13 (3, 4, 3, 3) [5.1]	13 (2, 3, 5, 3) [3.1]
Total	46 (11, 11, 16, 8) [18.3]	48 (11, 13, 12, 12) [11.4]

[a] One of the seven mutants was a mixed hemizygous/homozygous type.

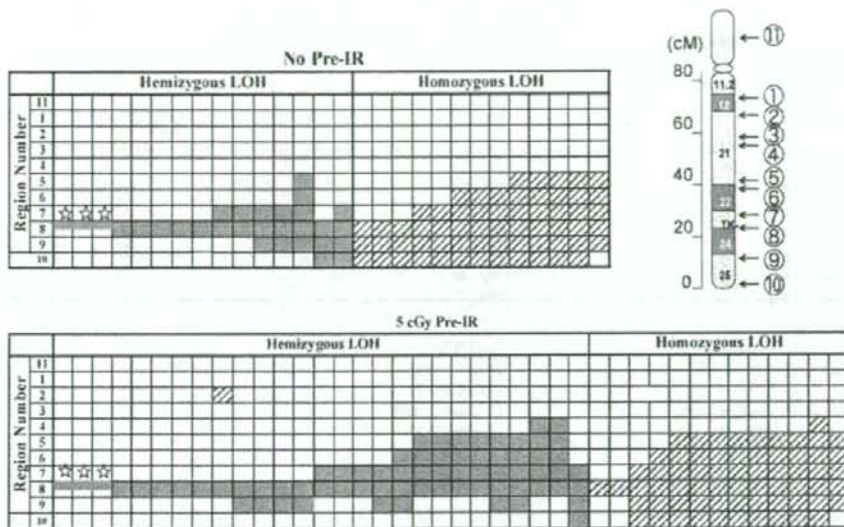

Fig. 2. Chromosome mapping of the LOH mutants. We analyzed the LOH mutants selected after a 2 Gy of challenging X-ray irradiation to determine the extent of the deleted or exchanged portions of the chromosome. The upper panel shows the profiles of 28 LOH mutants selected from non-primed cells, and the lower panel shows the profiles of 40 LOH mutants selected from cells primed with 5 cGy of X-rays. Each column represents a single LOH mutant. The rows represent regions of chromosome 17 diagrammed in the upper right insert. Shaded squares represent deleted regions and hatched squares represent exchanged regions (see text). The region numbers refer to the 11 microsatellite regions: (1) D17S588; (2) D17S1784; (3) D17S785; (4) D17S789; (5) D17S802; (6) D17S807; (7) D17S928; (8) D17S932; (9) D17S1299; (10) D17S1566; (11) THRA (see ref. [29]). The star symbol represents a "small" type 2 hemizygous event in which the deletion is restricted to *TK* locus.

type 2 events in both the non-primed (13 of 15 mutants) and primed (26 of 27 mutants) groups. Small type 2 deletions – those restricted to the *TK* locus (Fig. 2) – were infrequent in both groups (3 of 15 mutants in non-primed cells and 3 of 27 in primed cells). Similarly, the proportion of large deletion mutants (expanding to the region beyond region 8, Fig. 2) was also similar in the primed (18 of 27 mutants) and non-primed (7 of 15 mutants) groups. Homozygous LOH events, on the other hand, which are considered to be the result of homologous recombination (HR) repair of DSBs, were primarily identified as type 1 events in both primed (10 of 13) and non-primed (12 of 13) groups. Interestingly, small homozygous LOH events (where only a single region was replaced, Fig. 2) were recovered from the primed cells (2 of 13 (3 Of 14)), but not from the non-primed cells (0 of 13).

3.5. Analysis of non-LOH-mutants

We detected many types of alterations in the non-LOH mutant cDNAs (Table 5). The proportion of single base-substitutions among all the mutations identified as this class was 1/8 (13%) in the primed cells, and this value was clearly lower than 7/18 (87%) in the unprimed cells. G and C bases were targeted in base substitution mutations, except for a single case of an A to T transversion (Table 5). Most (4/5) of the double-base changes consisted of a single base deletion (causing a frameshift) and a base substitution, except for a single case of a GC to TA double transversion in a radioadapted mutant. It is difficult to estimate the effect of priming on the induction of the double-base change events from the limited number of cells involved. Similar difficulties were also found in the other mutational events in this class such as triple-base changes, multiple-base changes and exon skipping. In addition, the proportion of abnormal transcription events (both functional and non-functional *TK* alleles are equally transcribed) was also similar in the radioadapted (1/8, 13%) and the non-adapted (3/18, 17%) group, although its origin was not identified.

4. Discussion

The radioadaptation conditions used in this study (5 cGy of priming X-rays followed in 6 h by 2 Gy of challenging X-rays) were similar to those used in other studies [4,6,11,12,14,16]. The *TK* mutation frequency

Table 5
Nature of the isolated non-LOH mutants

Type of mutation		Number of identified mutants	
Specific changes	[Position: exon]	Non-primed exposure	Primed (5 cGy) pre-X-ray
Single base substitutions		7	1
G → A (Gly → Glu)	[56:1]	3	0
C → T (Gln → Stop)	[64:1]	1	0
C → A (Ser → Stop)	[89:2]	1	0
A → T (Ser → Cys)	[97:2]	1	0
G → C (Leu → Phe)	[108:2]	0	1
G → A (Glu → Lys)	[430:6]	1	0
Double base changes		3	2
G → A (Gly → Glu)/Del. C	[56:1/676:7]	1	0
G → C (Leu → Phe)/Del. A	[108:2/686:7]	0	1
Add. C/Del. G	[232:4/641:7]	1	0
GC → TA (Leu Asp → Leu Asn)	[372 and 373:5]	0	1
G → A (Glu → Lys)/Del. G	[430:6/447:6]	1	0
Triple base changes		0	1
Del. G/C → T (Ile → Ile)/C → T (Leu → Leu)	[92:1/288:4/561:7]	0	1
Multiple base changes		1	1
CC → AT (Thr Gln → Thr Stop)/G → A (Gln → Gln)/G → A (Gln → Gln)/Add. C/Del. G	[51 and 52:1/66:1/667:7/232:4/641:7]	0	1
Base changes at 20 sites		1	0
Exon skipping, abnormal splicing and deletion		3	2
Del. of a part of exon 1 (48 bases)	[161–209: 1]	1	1
Abnormal splicing of intron (between exons 1 and 2)		1	0
Skipping of exon 3 (Del. 111 bases)	[99–203: 3]	1	0
Skipping of exon 5 (Del. 90 bases)	[304–393: 5]	0	1
Abnormal transcription		3	1
Both functional and non-functional alleles are equally transcribed			
Unidentified		1	0
Total		18	8

we observed after the challenge X-rays (18.3×10^{-6}) was reduced by the 5-cGy priming exposure to about 62% of the non-primed level (11.4×10^{-6}). Taking into consideration the *TK* spontaneous mutation frequency observed in our recent study (3.0×10^{-6}) [32], the increase in MF induced by 2 Gy of X-rays was reduced from 6.1-fold to 3.8-fold.

We originally planned this study to determine whether radioadaptation would alter the characteristics of X-ray-induced LOH events. X-ray-induced interstitial deletions are likely to be the result of NHEJ repair of DSBs, and this type of mutation was the one we recovered most frequently after 2 Gy X-ray irradiation in our previous study [24]. We also found that carbon-ion beam irradiation induced interstitial deletions more efficiently than the same dose of X-rays [26,27], which we interpreted as the result of a higher occurrence of inaccurately repaired DSBs. In the present study, however, we found that the frequency of hemizygous LOH mutations, as well as their size and the distribution of deleted regions on chromosome 17, was similar for radioadapted and non-adapted cells. Those results are not consistent with reports suggesting that enhanced repair of DSBs reduces chromosomal alterations [21,22]. An entire genome assay might lead to results similar to the ones in those reports, but our observations were restricted to the *TK* locus on chromosome 17.

On the other hand, we observed a decrease in the induction of homozygous LOH events in the primed cells, which suggests that priming enhanced the HR repair of DSBs. We recently constructed a model system to follow the fate of a single DSB introduced by the restriction enzyme I-sceI at a specific site in the *TK* gene in TK6 cells [31]. In preliminary exper-