Table 2. Spontaneous Spi⁻ Mutation Spectra of *gpt* Delta Mice and Rats

	C57BL/6J mouse		SD rat		F344 rat	
	No.	(%)	No.	(%)	No.	(%)
One bp deletions	95	70.4	5	50.0	30	76.9
GGGG	21	15.6	2	20.0	12	30.8
GGG	4	3.0	0	0.0	2	5.1
GG	6	4.4	0	0.0	2	5.1
G	10	7.4	1	10.0	3	7.7
AAAAAA	18	13.3	0	0.0	2	5.1
AAAAA	29	21.5	2	20.0	8	20.5
AAA	1	0.7	0	0.0	1	2.6
AA	0	0.0	0	0.0	0	0.0
A	6	4.4	0	0.0	0	0.0
> 2 bp deletions	28	20.7	2	20.0	4	10.3
2 bps–1 kb	8	5.9	1	10.0	1	2.6
> 1 kb	20	14.8	1	10.0	3	7.7
Complex	6	4.4	1	10.0	5	12.8
Others[a]	6	4.4	2	20.0	0	0.0
Total No. of Mutations	135	100.0	10	100.0	39	100.0
No. of animal	43		3		10	
Sex	male and female		male		male	
Tissues	brain, colon, liver, bone marrow, epidermis, lung		liver		liver, kidney	
Age	10–20 weeks old		24 weeks old		17–30 weeks old	

a) Deletions coupled with insertions and/or base substitutions.

SPI⁻ ASSAY (SPI⁻ SELECTION) FOR DELETIONS

The Spi⁻ assay is a unique selection that can detect deletions, rather than base substitution mutations. The methodology and the characteristics of the chemical- and radiation-induced Spi⁻ mutations have been described in detail.[1,14] Spi⁻ selection takes advantage of the restricted growth of wild type lambda phage in P2 lysogens.[44] This phenotype is called Spi (sensitive to P2 interference). Only mutant lambda phages that are deficient in the functions of both the *gam* and *red* genes can grow in P2 lysogens and display the Spi⁻ phenotype. Simultaneous inactivation of both the *gam* and *red* genes is usually induced by deletions. Because of the size limitation for *in vitro* packaging reactions (there must be two cos sites separated by 38–51 kb of DNA), the size of deletions detectable by Spi⁻ selection is up to 10 kb. Thus, the mutants are mostly intrachromosomal deletions. However, the tandem array of 80 copies of lambda EG10 DNA in the *gpt* delta mouse provides a potential target of approximately 3.8 megabases.

In previous reports, the spontaneous Spi⁻ MFs of *gpt* delta mice were around $1\text{–}5 \times 10^{-6}$, which is lower than that of other transgenes, such as *lacZ* of Muta mice and *lacI* of Big Blue mice. Because the predominant types of point mutations induced *in vivo* are base substitutions, it could be that the spontaneous Spi⁻ MF, which only detects deletion mutations but not base substitutions, is generally lower than that of *lacZ*, *lacI* or *gpt* MF. Tissue type and sex differences of spontaneous Spi⁻ MFs is not clearly observed,[11] although the number of studies in which multiple tissue types have been compared is very limited. The effect of age has not been well characterized. No significant differences in spontaneous MFs were reported in *p53*, *Atm* or *Parp-1* knockout *gpt* delta mice, although heavy-ion or X-ray irradiation induced more large deletions in knockout mice than in wild type mice.[17,21,26] In the *gpt* delta rat, the spontaneous Spi⁻ MFs we have obtained from 13 rat tissues were between 1.3×10^{-6} and 4.4×10^{-6}, similar to those observed in *gpt* delta mice.[12] No marked strain difference has been observed between SD and F344 rats although additional studies are needed to confirm this finding.

Spontaneous Spi⁻ mutation spectra of *gpt* delta rodents are shown in Table 2. Although the sample sizes of the rats are small, mutational characteristics of spontaneous Spi⁻ mutants are similar between

mice and rats. We have analyzed 135, 10 and 39 Spi$^-$ mutants detected in 43 C57BL/6J mice, 3 SD and 10 F344 rats, respectively. These mutants were rescued from brain, liver, epidermis, bone marrow, colon, lung and kidney (and unpublished data in our laboratory).[16,19,20,28,45] The most frequent mutation is a 1 bp deletion in the repetitive sequences in the *gam* gene. These small deletions are not supposed to induce Spi$^-$ mutations. However, translation of the *gam* and *red* genes is probably linked, and the *gam* gene is first transcribed so that the 1 bp deletions in the *gam* gene may interfere with the start of translation of the downstream *red* gene, thereby functionally inactivating not only *gam* but also *red*.[13] The percentages of the 1 bp deletions are from 50–77%. But these values may be underestimated because we regard identical mutations recovered from the same tissue samples to have resulted from clonal expansion and count them as a single mutation. However, they could also be independent hot spot mutations. If this is the case, they should be counted as multiple independent mutations. Indeed, there are several hot spots of spontaneously occurring Spi$^-$ mutations. Those hot spots are 1-bp deletions of AAAAAA to AAAAA at position 295–300, AAAAA to AAAA at 227–231, GGGG to GGG at 286–289, and CCCC to CCC at 238–241 in the *gam* gene (the number starts from the first ATG of the *gam* gene). We suggest these events are most likely induced by slippage errors of DNA polymerases during DNA replication.

Other than 1 bp deletions, we observed about 20% of the Spi$^-$ mutations are larger deletions more than 2 bps in sizes. In mice, based on the sequence characteristics of the junctions and the neighboring regions, the Spi$^-$ deletions more than 1 kb in size could be classified as either those having junctions exhibiting short homology (1–12 bps) (13/20 = 65%) or those having flush junctions (7/20 = 35%). Deletions with short or no homologous sequences at their junctions have been observed in a number of mutant genes implicated in human diseases, including cancer. About 40% of large deletions in human disorders are characterized by the presence of very short sequence homologies at the breakpoints.[46] We suggest that non-homologous end-joining (NHEJ) repair plays an important role in the generation of intrachromosomal deletions such as Spi$^-$ mutants.[14] This pathway involves the DNA end-binding heterodimer Ku70/Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), X-ray repair cross complementing (XRCC)4, and DNA ligase IV.[47,48] Although some of these proteins play an essential role in the maintenance of genome stability and suppression of tumorigenesis, NHEJ also has the potential to induce deletion mutations. If two incompatible ends are generated by DNA double-strand breaks (DSBs), they first have to be converted to ligatable ends by enzymatic processing, which often causes deletions.[49] The middle size Spi$^-$ mutants (2 bps to 1 kb) might be caused by DNA replication error or by NHEJ. We have also detected complex type deletions containing genome rearrangement. This type of the Spi$^-$ mutation is sometime difficult to analyze by DNA sequencing. Other deletions coupled with short insertions at junctions or base substitutions are also observed in spontaneous Spi$^-$ mutations. We did not identify hot spots in the Spi$^-$ large deletions either in untreated or mutagen-treated mice. This may indicate that DSBs are randomly induced in the neutral transgene region. Further work is required to understand the mechanism by which Spi$^-$ deletions are generated.

CONCLUSION REMARK

The human genome is continuously exposed to various exogenous and endogenous DNA damaging agents including reactive oxygen species. To survive and protect the genome against DNA damage, cells employ many repair mechanisms such as mismatch repair, base excision repair, nucleotide excision repair, translesion DNA synthesis, and homologous and non-homologous recombination repair mechanisms. However, some repair mechanisms appear to be involved in error-prone DNA replication process or the induction of genome rearrangements such as deletions. To analyze the various types of *in vivo* mutations, *gpt* delta transgenic rodents were established to detect deletions as well as point mutations. Here we reported the characteristics of the spontaneous *gpt* (point mutations) and Spi$^-$ (deletions) mutations obtained from *gpt* delta mice and rats. The results suggested that the assays permit the efficient and quantitative detection of mutations in various tissues of mice and rats, and analysis of mutations at the molecular level. The spontaneous mutations observed in the tissues of mice and rats included both base substitutions and deletions. The predominant types of mutations are G:C to A:T transitions at 5′-CpG-3′ sites, G:C to T:A transversions, 1 bp deletions at repetitive se-

quences, and larger deletions of more than 1 kb. It suggests that deamination of methylated cytosines at CpG sites, oxidative damage to DNA, such as 8-oxoguanine lesions, slippage errors in DNA replication, and error-prone DSB repair may contribute to spontaneous mutations in the rodents. Further studies are necessary to investigate whether oxidative damage in DNA and dNTPs pool induces base substitutions and deletions in mammals and to determine the molecular characteristics of such mutations. Accumulative mutations with age might be related to endogenous oxidative stresses. Genome rearrangements associated with oxidative stress are also important in the field of mutagenesis and carcinogenesis. Because oxidation of DNA is often caused indirectly by malnutrition, the relationship between nutrition and genome rearrangements mediated via oxidative stresses could be an important and interesting topic. To investigate the mechanisms of carcinogenesis in target organs, *gpt* delta rat could be useful because most of carcinogenesis studies are undertaken in rats rather than mice. The mouse model is also useful to investigate specific gene function by crossing with gene knockout mice.

Transgenic mutation assays have the ability to evaluate mutagenesis *in vivo* in a broad range of tissues using neutral reporter genes integrated into genome. Recently, Bielas and Loeb reported a method to directly detect random point mutations in genomic and mitochondrial DNA from mouse and human cells.[50–52] This technique, called random mutation capture, is based on gene capture by hybridization with oligonucleotide probes, followed by cleavage by a restriction enzyme and quantification of the non-cleavable mutants by real-time quantitative PCR. Such a direct detection concept could be extended to quantify mutation in any cell types, at different sites in the genome, in coding and non-coding regions. In another approach, Jiang *et al.* systematically examined the mutational spectrum of the entire human genome and categorized regions using 1.8 million human single nucleotide polymorphisms (SNPs).[53] Although the mutational analysis using SNPs is limited to single base substitutions, extensive and comprehensive sequencing analysis may lead to new perspectives on *in vivo* mutagenesis.

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Differential effects of low- and high-dose X-rays on *N*-ethyl-*N*-nitrosourea-induced mutagenesis in thymocytes of B6C3F1 *gpt*-delta mice

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Abstract

Carcinogenesis in humans is thought to result from exposure to numerous environmental factors. Little is known, however, about how these different factors work in combination to cause cancer. Because thymic lymphoma is a good model of research for combined exposure, we examined the occurrence of mutations in thymic DNA following exposure of B6C3F1 *gpt*-delta mice to both ionizing radiation and *N*-ethyl-*N*-nitrosourea (ENU). Mice were exposed weekly to whole body X-irradiation (0.2 or 1.0 Gy), ENU (200 ppm) in the drinking water, or X-irradiation followed by ENU treatment. Thereafter, genomic DNA was prepared from the thymus and the number and types of mutations in the reporter transgene *gpt* was determined. ENU exposure alone increased mutant frequency by 10-fold compared to untreated controls and over 80% of mutants had expanded clonally. X-irradiation alone, at either low or high dose, unexpectedly, reduced mutant frequency. Combined exposure to 0.2 Gy X-rays with ENU dramatically decreased mutant frequency, specifically G:C to A:T and A:T to T:A mutations, compared to ENU treatment alone. In contrast, 1.0 Gy X-rays enhanced mutant frequency by about 30-fold and appeared to accelerate clonal expansion of mutated cells. In conclusion, repeated irradiation with 0.2 Gy X-rays not only reduced background mutation levels, but also suppressed ENU-induced mutations and clonal expansion. In contrast, 1.0 Gy irradiation in combination with ENU accelerated clonal expansion of mutated cells. These results indicate that the mode of the combined mutagenic effect is dose dependent.
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Keywords: Combined genotoxic effect; *gpt*-delta mouse; *N*-Ethyl-*N*-nitrosourea; Radiation; Clonal expansion

1. Introduction

Humans are continuously and simultaneously exposed to numerous environmental mutagens. It is important, therefore, to determine the dose–effect relationship of combined exposure when assessing human health risks. Mutagenic DNA damage arises from interaction of DNA with a myriad of endogenous and exogenous agents [1]. Ionizing radiation induces DNA strand breaks and base modifications, directly or indirectly via reactive oxygen species. Such DNA damages result in base substitutions, insertions and deletions. Thus, the recent increase in the utilization of, and hence chance for exposure to, medical ionizing radiation has raised an aspect of social concern. Alkylating agents also modify DNA, thereby inducing base substitutions that lead to point mutations [2,3]. These agents are found in plants, food, cigarette smoke, fuel combustion products, and commonly used industrial solvents. In addition, ionizing radiation and some alkylating agents are used for cancer chemotherapy. *N*-Ethyl-*N*-nitrosourea (ENU) is a potent alkylating mutagen and carcinogen that induces G:C to A:T transitions,

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A:T to G:C transitions and A:T to T:A transversions *in vivo* [4–8]. Since ionizing radiation and alkylating agents are widely distributed and utilized, understanding their toxicity and the cellular response mechanism(s) is important. Occasionally, we are exposed to these agents simultaneously in the environment or for therapeutic reasons. However, currently available data are not sufficient to delineate the mode and the mechanism of combined action of these agents.

The combined effect of radiation and alkylating agents has been examined in animal tumor models, including thymic lymphomas, brain tumors, mammary tumors and colon tumors [9–12]. Thymic lymphoma (TL) is an excellent model for human T-cell acute lymphoblastic leukemia and has been used for the study of radiation carcinogenesis. Concomitant exposure to butylnitrosourea and X-rays for 12 weeks accelerates the development of thymic lymphoma in BDF1 mice, in a manner dependent on the dose of radiation [9,13]. Irradiation 2–5 weeks before methylnitrosourea exposure accelerates thymic lymphoma development [13]. Irradiation for 5 days, but not 30 days, before administrating ENU treatment enhances lymphomagenesis [9].

DNA mutations play a central role in carcinogenesis. The frequency and type of mutations that result from combined treatment may shed light on the molecular mechanism(s) underlying the carcinogenic effects of combined exposure to alkylating agents and radiation. In order to delineate such mechanisms, we have examined the occurrence of mutations in thymic cells of B6C3F1 (*gpt*+/−) mice after combined exposure to X-rays and ENU. Repeated exposure to high-dose X-rays (1.0 Gy) followed by ENU increased the frequency of mutants and facilitated clonal expansion of mutated cells. However, unexpectedly, when low-dose X-rays (0.2 Gy) preceded ENU, mutant frequency was reduced primarily due to a decrease in G:C to A:T and A:T to T:A mutations. This is the first report, to our knowledge, that provides a new evidence on the mode and mechanism of combined exposure, which clearly differs between low and high dose of radiation.

2. Materials and methods

2.1. Mice

Male C3H/He mice were purchased from Charles River Laboratories (Kanagawa, Japan). Female *gpt*-delta C57BL/6J mice carrying approximately 80 copies of λEG10 DNA in haploid genome were obtained from Japan SLC (Shizuoka, Japan) [14]. Mice were housed five to a cage in a room maintained at 23 ± 2 °C and 50 ± 10% humidity, with a 12 h dark–light cycle. All mice were fed standard laboratory diet MB-1 (Funabashi Farm Co., Ltd., Chiba, Japan) and water *ad libitum*.

2.2. Chemicals

ENU (CAS No. 759-73-9) was purchased from Nakarai Tesque (Kyoto, Japan). Reagents for M9 buffer preparation were purchased from Nakarai Tesque and Wako Pure Chemical Industries (Osaka, Japan). Dimethyl sulfoxide and 6-thioguanine (6-TG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chloramphenicol (Cm) was purchased from Wako Pure Chemical Industries. Bacto yeast extract, Bacto tryptone and Bacto agar were obtained from Difco (Franklin Lakes, NJ, USA).

2.3. Irradiation

X-ray irradiation was performed using a Pantak HF-320 machine (PANTAK Ltd., East Haven, CT, USA) at 200 kV, 20 mA, and a dose rate of 0.7 Gy/min.

2.4. Combined X-ray exposure and ENU treatment

Mice (4–5 weeks old) were exposed weekly to 0.2 or 1.0 Gy X-rays for 4 consecutive weeks, because this X-ray exposure method is the most leukemogenic [15]. ENU was dissolved in reverse osmosis water (Elix UV10, Millipore, Bedford, MA) at pH 6.0 to prepare 200 ppm (1.17 mM) solution. To avoid degradation, ENU solution was administered in drinking water in brown bottle immediately after preparation. Freshly prepared ENU solution was exchanged on every Monday, Wednesday, Friday and Saturday during treatment. The amount of ENU solution up-taken was approximately 3 ml per mouse per day. For combined exposures, X-ray irradiation at 0.2 or 1.0 Gy for 4 consecutive weeks was followed by 4 weeks of ENU treatment (Fig. 1). Four weeks after the end of ENU treatment, mice were sacrificed and thymuses were collected and frozen immediately in liquid nitrogen, then stored at −80 °C until use. These animal experiments were conducted in compliance with guidelines for animal experiments of the National Institute of Radiological Sciences for the care and use of laboratory animals.

2.5. Genomic DNA extraction and in vitro packaging

The thymuses (20 mg) were homogenized in a Dounce homogenizer (Kontes, Vineland, NJ, USA) and genomic DNA was isolated using the RecoverEase DNA isolation kit (Stratagene, La Jolla, CA, USA). Transpack Packaging Extract (Stratagene, La Jolla, CA, USA) was used to rescue λEG10 phages from genomic DNA (10 μl) isolated from the thymus.

2.6. gpt mutation assay

The *gpt* mutagenesis assay was performed on five mice per group according to methods previously described [16]. Briefly, λEG10 phage was incubated with *E. coli* YG6020 at 37 °C for 20 min. After incubation, *E. coli* was incubated at 37 °C with vigorous agitation for 30 min. *E. coli* was mixed with 0.6% molten soft agar with or without 6-TG and the entire contents poured onto the M9 + Cm + 6-TG or M9 + Cm plates and incubated at 37 °C for 3 or 2 days. After incubation, colonies were counted and on M9 + Cm + 6-TG plates were subject to colony PCR for *gpt* gene. At least three independent experiments were performed per mouse.

Mutant frequency was calculated by dividing the number of colonies growing on M9 + Cm + 6-TG plates by the number of colonies growing on M9 + Cm plates.

Recurrent mutations derived from the same tissue of a single animal could be the result of clonal expansion that occurred early after mutagen treatment. When multiple identical mutations were recovered from an individual mouse, the

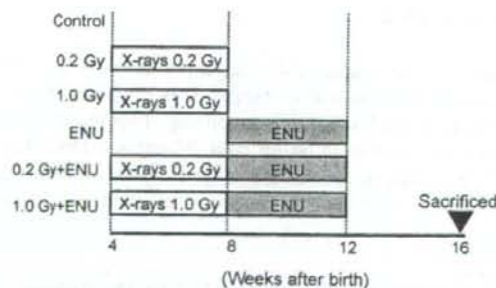

Fig. 1. Experimental design for *gpt* mutation analysis of thymic DNA from mice treated with X-ray irradiation, ENU or a combination of the two. Mice were exposed to X-rays weekly. ENU was administered at a concentration of 200 ppm in drinking water.

data were corrected for any clonal expressions by counting just the mutation as defined by independent mutation [17,18]. *Mutation frequency* was calculated as the ratio of calculated total independent mutants to the total number of colonies growing on M9 + Cm plates. Mutational common hot spots were defined as sites where the same mutation was observed in three or more mice from the same group. Clonality was calculated as the ratio of clonal (total − independent) mutations to the total mutations [19,20].

2.7. PCR and DNA sequencing analysis of gpt

A 739-bp DNA fragment containing *gpt* was amplified by PCR using two primers (primer 1 (forward): 5′-TACCACTTTATCCCGCGTCAGG-3′, primer 2 (reverse): 5′-ACAGGGTTTCGCTCAGGTTTGC-3′). The reaction mixture contained 5 pmol of each primer and 200 μM of each dNTP. PCR amplification was carried out using Taq DNA polymerase (Takara Bio, Shiga, Japan) with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The reaction was started by incubation at 96 °C for 180 s, followed by 29 cycles of 30 s at 94 °C, 30 s at 58 °C, 60 s at 72 °C and an additional 180 s incubation at 72 °C after the final cycle.

PCR products were purified using Exo-SAP It (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). DNA sequencing of *gpt* was performed using Big Dye Terminator v3.1 (Applied Biosystems) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with sequencing primer (5′-ATCTCTATAATCTCGCGCAACC-3′) according to the manufacturer's instructions. Oligonucleotide primers were obtained from Hokkaido System Science (Sapporo, Japan).

2.8. Statistical analysis

Mutant frequency is presented as mean ± S.D. Statistical significance was evaluated with the Student's *t*-test and Fisher's exact test using Graphpad Prism software (Graphpad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Thymus weight after treatment with ENU and X-rays

First, we measured body and thymus weight after combined exposure to X-rays and ENU (Table 1). The thymus weight of mice exposed to 0.2 or 1.0 Gy X-rays, ENU alone, or 0.2 Gy X-rays followed by ENU did not differ from that of non-irradiated controls. Thymus from mice exposed to 1.0 Gy X-rays followed by ENU weighed slightly greater than control thymus (68 ± 22 mg vs. 43 ± 9 mg; $P < 0.05$), suggestive of an onset of leukemogenesis. The body weight of all treated mice, except the 0.2 Gy followed by ENU mice, did not differ significantly from control.

3.2. Thymus gpt mutant frequency analysis

The *gpt* reporter transgene was used to analyze the occurrence of mutations in the thymus. The frequency of *gpt* mutants in each control and treated thymus is shown in Table 1 and Fig. 2. It is evident that ENU increased mutant frequency by 10-fold relative to untreated controls. Surprisingly, the mutant frequency in mice exposed to 0.2 or 1.0 Gy X-rays alone was significantly reduced compared to the control ($P < 0.05$). The mutant frequency in mice exposed to 0.2 Gy X-rays in combination with ENU was also, unexpectedly, reduced compared to ENU treatment alone, almost to the level of the untreated controls. In contrast, exposure to 1.0 Gy X-rays in combination with ENU increased the mutant frequency by 3-fold compared to ENU treatment alone. In mouse #30 of ENU group, mutant frequency was not different from that in control group (0.91×10^{-5} vs. 1.20×10^{-5}), but the weight of thymus was significantly increased compared with control (Table 1). These results suggested that the thymocyte with no *gpt* mutation expanded in this mouse.

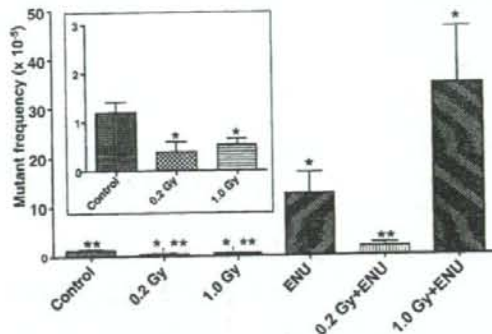

Fig. 2. Mutant frequency analysis of gpt^- recovered from thymus DNA from control, irradiated (0.2 or 1.0 Gy), ENU-treated, and irradiated/ENU-treated mice. The inset shows an expanded scale for mutant frequency for the first three conditions. $^*P < 0.05$, significantly different from control. $^{**}P < 0.05$, significantly different from ENU. Bars represent mean ± S.D.

3.3. Characteristics of the gpt mutants

The class- and site-distribution of mutations are shown in Tables 2 and 3, respectively.

In control group, G:C to A:T mutation was predominantly detected, especially at both non-CpG and CpG sites (0.24×10^{-5} and 0.30×10^{-5}, respectively). In 0.2 and 1.0 Gy groups, however, G:C to A:T mutation at CpG was reduced by 6-fold compared to control, respectively (0.2 Gy, 0.05×10^{-5}; 1.0 Gy, 0.05×10^{-5}). In ENU group, G:C to A:T mutation was generated at non-CpG sites (8.26×10^{-5}) especially at positions 86 and 409. These sites might be hot spot for mutation by ENU, because four out of five mice commonly had these mutations. In addition, mutants with G:C to T:A and A:T to T:A in ENU group were also increased at the rate of 1.47×10^{-5} and 2.36×10^{-5}, respectively.

In 0.2 Gy followed by ENU group, however, the mutant frequency of G:C to A:T and A:T to T:A was significantly reduced compared to the ENU-treated group ($P < 0.001$). Particularly, mutants with G:C to A:T transitions decreased by 37-fold (ENU, 8.26×10^{-5}; 0.2 Gy + ENU, 0.24×10^{-5}).

In three mice of 1.0 Gy followed by ENU group, more than 1000 mutants were detected per thymus (#26, 27 and 29); 201, 168 and 135 mutants were analyzed for each mouse, respectively (Table 3). The class- and site-distribution of mutations differed between mice and jackpot mutation was shown. In mouse #26, 143 of 201 mutations were G:C to A:T transitions at position 87. Almost all of the mutations (164 of 168) detected in mouse #27 were A:T to C:G transversions at position 106 (Table 3). Ninety-eight of 135 mutations in mouse #29 were A:T to G:C transition

Table 1
Mutant and mutation frequency in thymus DNA from control, irradiated, ENU-treated, and combined treated mice

Treatment	Mouse ID	Body weight (g)	Average ± S.D. (mg)	Thymus weight (mg)	Average ± S.D. (mg)	Number of Cm colonies ($\times 10^5$)	Number of mutants	Mutant frequency ($\times 10^{-5}$)	Average ± S.D. ($\times 10^{-5}$)	Number of mutants sequenced	Number of independent mutants	Independent mutants (%)	Mutation frequency ($\times 10^{-5}$)	Average ± S.D. ($\times 10^{-5}$)
Control	1	25.7		45		18.3	32	1.75		28	22	79	1.38	
	2	25.8		39		12.7	16	1.26		14	13	93	1.17	
	3	24.6	26.5 ± 1.7	57	43 ± 9	16.2	21	1.30	1.2 ± 0.48	15	10	67	0.86	1.00 ± 0.43
	4	28.6		38		13.4	17	1.27		9	9	100	1.27	
	5	28.1		36		35.8	15	0.42		14	10	71	0.30	
	6	27.0		48		5.3	6	1.14		4	4	100	1.14	
	7	28.4		49		31.5	1	0.03		0	0	N.D.	N.D.	
0.2 Gy	8	24.6	26.3 ± 1.6	39	46 ± 6	28.3	4	0.14	0.39 ± 0.46*	1	1	100	0.14	0.59 ± 0.51[a]
	9	27.1		53		6.9	1	0.14		0	0	N.D.	N.D.	
	10	24.7		56		6.0	3	0.50		3	3	100	0.50	
	11	25.2		45		18.3	15	0.82		12	10	83	0.68	
	12	25.6		48		23.4	8	0.34		8	7	88	0.30	
1.0 Gy	13	24.6	25.0 ± 0.5	45	46 ± 1	47.8	12	0.25	0.53 ± 0.29*	9	9	100	0.25	0.49 ± 0.27
	14	24.5		45		15.0	13	0.87		10	10	100	0.87	
	15	25.5		47		8.5	3	0.35		2	2	100	0.35	
	16	24.5		35		17.7	110	6.21		110	15	14	0.85	
	17	26.7		43		2.3	66	28.21		58	12	21	5.84	
ENU	18	24.6	25.1 ± 1.0	36	41 ± 5	20.6	281	13.62	12.56 ± 9.67*	274	25	9	1.24	2.01 ± 2.15
	19	25.2		45		53.4	616	11.54		110	9	8	0.94	
	20	24.4		45		13.3	43	3.23		38	14	37	1.19	
	21	24.0		39		8.0	6	0.75		6	6	100	0.75	
	22	24.9		41		28.8	31	1.08		31	19	61	0.66	
0.2 Gy + ENU	23	24.6	24.4 ± 0.4*	35	40 ± 4	7.4	11	1.48	1.74 ± 1.76	10	9	90	1.34	1.12 ± 0.79
	24	24.8		47		5.0	24	4.82		22	11	50	2.41	
	25	24.0		38		31.3	18	0.57		17	13	76	0.44	
	26	24.4		64		18.5	1028	55.72		201	7	3	1.94	
	27	25.4		43		20.9	1001	47.94		168	5	3	1.43	
1.0 Gy + ENU	28	24.6	24.8 ± 0.9	100	68 ± 22*	5.7	75	13.09	34.74 ± 25.90*	59	8	14	1.77	1.61 ± 0.50
	29	25.9		54		27.5	1539	56.07		135	5	4	2.08	
	30	23.8		77		15.4	14	0.91		11	10	91	0.83	

N.D.: not determined.
[a] $n = 3$.
* $P < 0.05$, statistically significant difference vs. control.

Table 2

Classification and mutant frequency (M.F.) and mutation frequency (m.f.) of *gpt* mutations recovered from thymus DNA from control, irradiated, ENU-treated, and combined treated mice

	Control				0.2 Gy				1.0 Gy				ENU				0.2 Gy + ENU				1.0 Gy + ENU			
	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{-5}$)	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{5}$)[a]	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{-5}$)	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{-5}$)	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{-5}$)	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{-5}$)
G:C to A:T (at non-CpG)	16	0.24	15	0.23	5	0.24	5	0.37	8	0.10	7	0.09	388	8.26	20	0.54	12	0.24	11	0.21	149	9.02	6	0.28
G:C to A:T (at CpG)[b]	20	0.30	11	0.17	1	0.05	1	0.07	4	0.05	3	0.04	1	0.02	1	0.03	7	0.14	5	0.10	5	0.30	4	0.18
G:C to T:A	16	0.24	15	0.23	0	<0.05	0	<0.07	12	0.16	12	0.15	69	1.47	15	0.40	15	0.30	10	0.19	20	1.21	4	0.18
G:C to C:G	3	0.05	3	0.05	0	<0.05	0	<0.07	1	0.01	1	0.01	2	0.04	2	0.05	0	<0.02	0	<0.02	1	0.06	1	0.05
Total G:C[c]	55	0.83	44	0.69	6	0.29	6	0.44	25	0.32	23	0.30	460	9.79	38	1.02	34	0.69	26	0.50	175	10.59	15	0.69
A:T to G:C	11	0.17	7	0.11	0	<0.05	0	<0.07	7	0.09	6	0.08	4	0.09	4	0.11	17	0.34	8	0.15	103	6.23	6	0.28
A:T to T:A	0	<0.02	0	<0.02	1	0.05	1	0.07	1	0.01	1	0.01	111	2.36	24	0.64	31	0.63	20	0.39	129	7.81	10	0.46
A:T to C:G	1	0.02	1	0.02	1	0.05	1	0.07	1	0.01	1	0.01	13	0.28	7	0.19	2	0.04	2	0.04	164	9.93	1	0.05
Total A:T[c]	12	0.18	8	0.13	2	0.10	2	0.15	9	0.12	8	0.10	128	2.72	35	0.94	50	1.01	30	0.58	396	23.97	17	0.78
1 bp deletion	6	0.09	6	0.09	0	<0.05	0	<0.07	6	0.08	6	0.08	2	0.04	2	0.05	1	0.02	1	0.02	2	0.12	2	0.09
1 bp insertion	2	0.03	2	0.03	0	<0.05	0	<0.07	1	0.01	1	0.01	0	<0.02	0	<0.02	1	0.02	1	0.02	1	0.06	1	0.05
>2 bp frameshifts	5	0.08	4	0.06	0	<0.05	0	<0.07	0	<0.01	0	<0.01	0	<0.02	0	<0.02	0	<0.02	0	<0.02	0	<0.06	0	<0.05
Total frameshifts	13	0.20	12	0.19	0	<0.05	0	<0.07	7	0.09	7	0.09	2	0.04	2	0.05	2	0.04	2	0.04	3	0.18	3	0.14
Total	80	1.20	64	1.00	8	0.39	8	0.59	41	0.53	38	0.49	590	12.56	75	2.01	86	1.74	58	1.12	574	34.74	35	1.61

No.: number of mutation(s). M.F.: specific mutant frequency was calculated by multiplying the mutant frequency by the ratio of the number of mutations in each class among the total number of *gpt* mutants. m.f.: specific mutation frequency was calculated by multiplying the mutation frequency by the ratio of independent mutation in each class to the total number of independent *gpt* mutant.

[a] $n = 3$.

[b] Number of occurrences of the G:C to A:T mutation at the 5′-CpG-3′ site.

[c] Total number of mutations occurring at G:C or A:T base pairs.

at position 419. These results indicate that the increased frequency of mutation in this group was caused by clonal expansion of cells possessing a unique mutation. None of the mutation sites were identical to those observed as hot spots in mice treated with ENU alone.

3.4. Mutation frequency

Unlike mutant frequency, difference in overall mutation frequency after ENU was calculated to be small among groups. However, A:T to T:A mutation frequency in ENU group, either combined or not combined with 1.0 Gy X-rays, was increased compared with control or X-ray irradiation groups (Table 2), while mutation at G:C sites remained unchanged statistically. The total mutation frequency by ENU group reduced from 2.01×10^{-5} to 1.12×10^{-5} when combined with 0.2 Gy, although statistically not significant. In contrast, repeated 1.0 Gy did not alter the overall frequency of ENU-induced mutation (1.61×10^{-5}).

3.5. Clonality of gpt mutations

The mutant frequency was significantly larger than mutation frequency in the groups treated with ENU alone, and with 1.0 Gy combined with ENU (Table 1). This means that mutation occurrence in ENU and 1.0 Gy combined with ENU groups were mainly caused by clonal expansion. We established the approximate level of clonality for each group. Clonality was less than 20% in the control group, which was the same as that observed in *lacI* transgenic mice [19]. Clonalities in 0.2 Gy X-ray and 1.0 Gy X-ray groups were also similar to that in the control group. It increased dramatically to over 80% in the ENU-treated group (Fig. 3). When 0.2 Gy X-ray exposure was combined with ENU, clonality was drastically reduced compared with ENU alone (24.6% vs. 82.2%, $P < 0.001$). In contrast, 1.0 Gy X-rays, except mouse #30, facilitated clonal expansion of mutated cells, as shown by the high percentage of subpopulations with specific mutations and the shift from an oligoclonal to monoclonal population (Table 3).

4. Discussion

In this study we investigated the combined effect of ENU and X-rays on the occurrence of mutations in the reporter transgene *gpt* in mouse thymus DNA. Repeated irradiation with 0.2 Gy X-rays not only reduced background mutation levels, but also suppressed ENU-induced mutations and clonal expansion. In contrast, 1.0 Gy irradiation in combination with ENU accelerated clonal expansion of mutated cells.

Reduction of the background mutation frequency by repeated dosing with 0.2 Gy X-rays or 1.0 Gy X-rays was unexpected. A decrease in G:C site mutations, including G:C to A:T at the CpG site, primarily accounted for this effect. The *gpt* in *gpt*-delta transgenic mice may be methylated at the CpG site [21]. Mammalian cell, in general, is heavily methylated at C-5 of cytosine residues at CpG dinucleotides. When 5-methylcytosine was deaminated, it converts to thymine, which results in G:C

Table 3
Mutational spectrum of *gpt* gene mutations recovered from thymus DNA from non-, X-ray-, ENU- and combined treated mice

Base positions[a]	CpG site	Control		0.2 Gy		1.0 Gy		ENU		0.2 Gy + ENU		1.0 Gy + ENU	
		No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)[b]	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)
G:C to A:T													
3		2	2 (1, 4)										
7	CpG					1	1 (14)						
37		1	1 (5)										
58	CpG											1	1 (28)
64	CpG	12	4 (1,2,3, 5)	1	1 (6)	2	1 (11)			3	2 (21,25)		
86								194	4 (16,17,18,19)				
87								1	1 (18)			143	1 (26)
92				2	2 (6,8)					1	1 (22)	1	1 (26)
107		2	2 (1, 2)										
109	CpG	2	1 (5)										
110	CpG	1	1 (3)							3	2 (21,22)	2	1 (26)
113				1	1 (10)			2	2 (19,20)				
115	CpG	3	3 (2,3,4)			1	1 (12)	1	1 (17)	1	1 (22)	1	1 (27)
116								19	2 (16,18)				
128		1	1 (1)					83	2 (19,20)				
176						1	1 (11)	2	2 (17,19)				
189								1	1 (19)				
262						1	1 (12)						
274		2	2 (1,2)	1	1 (10)			1	1 (19)	1	1 (21)	2	1 (30)
280	CpG	1	1 (2)										
281		1	1 (1)			1	1 (15)					1	1 (30)
287								1	1 (16)				
301	CpG											1	1 (28)
391										1	1 (22)		
401				1	1 (6)	1	1 (13)			1	1 (22)		
402										2	2 (21,23)	1	1 (26)
406		3	2 (1,2)							1	1 (22)		
409								84	4 (16,17,18,19)			1	1 (26)
413	CpG	1	1 (2)										
417		1	1 (2)			1	1 (13)						
418		3	3 (1,2,5)			3	2 (11,12)			5	4 (22,23,24,25)		
Total at non-CpG		16	15	5	5	8	7	388*	20	12	11	149*	6
Total at CpG		20	11	1	1	4	3	1*	1	7	5	5*	4
G:C to T:A													
3												1	1 (30)
7	CpG					1	1 (11)	32	2 (16,18)				
15								1	1 (18)				
19	CpG									1	1 (24)		
26						1	1 (12)						
37						1	1 (13)						

79										1	1(27)
109	CpG	1	1 (4)								
110	CpG	1	1 (4)								
113						1	1 (16)				
115	CpG	1	1 (1)	1	1 (13)			1	1 (24)		
116		1	1 (3)	1	1 (15)	1	1 (16)				
143	CpG	1	1 (3)					1	1 (22)		
176				1	1 (14)						
185		1	1 (2)								
189								1	1 (23)		
190	CpG							1	1 (22)		
208	CpG					1	1 (18)	9	4 (22,23,24,25)	1	1(28)
232		1	1 (1)								
244	CpG	1	1 (5)	1	1 (13)	8	2 (18,19)				
265						3	2 (16,18)				
304		2	2 (1,2)								
401		1	1 (5)			19	3 (16,17,18)				
402		3	2 (1,5)	3	3 (11,13,14)						
406		1	1 (1)	1	1 (14)	1	1 (17)				
409		1	1 (1)			2	1 (17)	1	1 (24)	17	1(29)
418				1	1 (13)						
Total		16	15	12	12	69*	15	15	10	20*	4
G:C to C:G											
7	CpG					1	1 (17)			1	1(30)
115	CpG	1	1 (1)								
125	CpG					1	1 (18)				
289		1	1 (1)								
402				1	1 (15)						
436	CpG	1	1 (4)								
Total		3	3	1	1	2	2			1	1
A:T to G:C											
2		2	2 (4,5)							1	1(30)
8								1	1 (25)	1	1(29)
25		4	3 (1,2,4)								
56				1	1 (14)						
119								9	2 (22,25)		
149								1	1 (24)		
175				1	1 (11)						
181						1	1 (17)				
187						1	1 (17)			1	1(30)
188								1	1 (22)		
191								1	1 (21)	1	1(30)
269		2	1 (5)	1	1 (14)			1	1 (25)		
275										1	1(27)
277								3	1 (22)		
343				1	1 (14)						
419		3	1 (1)	3	2 (11,14)	1	1 (17)			98	1(29)
457						1	1 (18)				
Total		11	7	7	6	4	4	17	8	103*	6
A:T to T:A											
2						2	2 (18,19)	1	1 (25)		
12				1	1 (13)						
35								1	1 (25)	18	1(29)
52						5	2 (16,18)			1	1(27)
56						1	1 (18)				
81								1	1 (22)		
88						14	1 (19)	8	2 (23,24)	43	2(26, 28)
95								1	1 (25)		
134						16	4 (16,17,18,19)				

Table 3 (*Continued*)

Base positions[a]	CpG site	Control		0.2Gy		1.0Gy		ENU		0.2 Gy + ENU		1.0 Gy + ENU	
		No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)[b]	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)
164				1	1(6)			3	3 (16,18,19)	2	2 (24,25)		
173								1	1 (18)				
177										1	1 (23)		
214										1	1 (24)		
223												1	1(30)
254												1	1(28)
257								2	1 (16)			1	1(28)
260										1	1 (25)		
263								8	2 (18,19)	7	3 (22,23,24)	63	2(26, 28)
312								1	1 (19)	2	1 (22)		
329								1	1 (18)			1	1(30)
345								56	4 (16,17,18,19)				
375										1	1 (22)		
400										2	2 (23,24)		
407										1	1 (23)		
420								1	1 (19)	1	1 (22)		
Total				1	1	1	1	111*	24*	31*	20*	129*	10*
A:T to C:G													
1								8	4 (16,17,18,19)				
9								1	1 (19)				
65				1	1(10)								
106								2	1 (19)			164	1(27)
218		1 (1)	1										
312										1	1 (25)		
331										1	1 (25)		
345						1	1 (14)	2	1 (18)				
Total		1	1	1	1	1	1	13	7	2	2	164*	1

No. of mutants: total number of mutations detected, include clonal mutations. Total number was shown the total number of mutations. No. of independent mutations: total number was shown the total number of independent mutations. The data were corrected for any clonal expansions by counting only one mutation when multiple identical mutations were recovered form an individual mouse. ID of mice with a mutation was shown in parentheses on the right-hand side of the total number of independent mutations.

[a] Position in the *gpt* coding sequence when A of start codon is labeled as 1.

[b] $n = 3$.

* $P < 0.05$ vs. control.

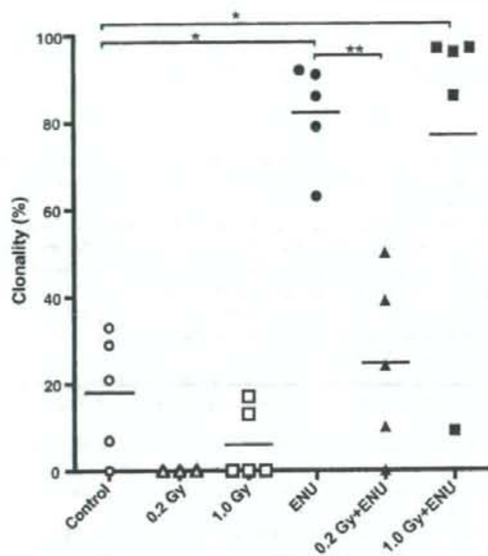

Fig. 3. Mutation clonality was enhanced by ENU as well as by 1.0 Gy X-rays in combination with ENU, but was suppressed by repeated irradiation with 0.2 Gy X-rays before ENU treatment. $^{*}P<0.05$, significantly different from control. $^{**}P<0.05$, significantly different from ENU. Bars represent means.

to A:T mutation after DNA replication if G:T mismatch is not repaired [22]. Repeated irradiation with 0.5 Gy X-rays leads to a decrease in DNA methylation via loss of histone H4-Lys20 trimethylation in thymus tissue [23], which could account for the reduced G:C to A:T mutation occurrence at CpG sites. Low-dose radiation (within 0.2 Gy) reduces thymic DNA fragmentation and apoptosis relative to non-irradiated background levels [24], suggesting that activation of DNA repair mechanisms may also contribute to the reduced frequency of mutations.

We show here that repeated 0.2 Gy irradiation also dramatically reduces ENU-induced mutations and the number of hot spot sites, primarily by decreasing the number of G:C to A:T transitions and G:C to T:A and A:T to T:A transversions. ENU produces ethylated base damage such as O^6-ethylguanine, O^4-ethylthymine and O^2-ethylthymine, which induces G:C to A:T transition, A:T to G:C transition and A:T to T:A transversion, respectively [4–8]. Base damage can be removed by multiple DNA repair systems. O^6-Methylguanine-DNA methyltransferase (*Mgmt*) recognizes and directly binds O^6-ethylguanine and removes the ethyl residue, thereby preventing G:C to A:T transition [25–28]. It is shown that transient expression of *Mgmt* is stimulated by X-irradiation both *in vitro* and *in vivo* within 3–48 h [29–31]. Importantly, the inductive effect of X-irradiation on O^6-alkylguanine-DNA alkyltransferase activity corresponds with a reduced incidence of rat CNS tumors after X-irradiation combination with ENU [10]. We have also examined if the expression of *Mgmt* mRNA increased after repeated X-irradiation of 0.2 Gy followed by ENU treatment. It turned out that the induction of *Mgmt* was negligible in irradiated thymus: fold increase was just 1.1 after the last irradiation. Subsequent ENU treatment increased *Mgmt* expression as a function of time. The 2-fold induction of *Mgmt* in thymus with 0.2 Gy followed by ENU, however, was smaller than 3.9-fold induction in thymus with ENU alone. Therefore, decrease in mutation frequency by 0.2 Gy could not be explained by expression level of *Mgmt*. Mechanism(s) other than *Mgmt* induction might be involved. Nucleotide excision repair can also repair alkylating base damage [32–34]. Chinese hamster ovary cells lacking *XPD/ERCC2* are highly susceptible to ENU-induced *Hprt* mutations [32]. DNA microarray studies indicate that *XPC* is up-regulated in human blood lymphocytes after 0.2 Gy X-ray exposure [35]. Taken together, it is required to determine activation or inactivation of other DNA repair pathways that reduce or enhance the overall rate of ENU-induced mutations.

We noticed that ENU mutations were predominantly induced at A:T site. It is reported that O^6-ethylguanine was repaired fast by *Mgmt* and nucleotide excision repair, while O^4-ethylthymine and O^2-ethylthymine were persistent lesions and the repair of these lesions occurred only at a very slow rate [25,32,36]. *In vivo* mutagenicity assays in mouse T lymphocytes have shown that predominant mutations induced by ENU are A:T to T:A [37].

Cells with hot spot point mutations expanded oligoclonally after ENU treatment. Clonal thymic lymphomas selected from oligoclonal preleukemic cells have been reported in virus-induced or radiation-induced T-cell lymphomagenesis [38,39]. Interestingly, the treatment with 0.2 Gy X-rays followed by ENU reduced oligoclonality, whereas 1.0 Gy X-rays accelerated ENU-induced clonal expansion. It is reported that high-dose whole body irradiation at 1.0 Gy induced p53 dependent transcription of *Noxa*, *Killer/DR5*, *Fas*, *Pidd* and *Perp* genes in thymus, whereas irradiation at 0.2 Gy did not induce these gene expressions [40]. Low-dose irradiation likely again activates multiple repair pathways and cell cycle control. In contrast, high-dose radiation may select specific clones with a growth advantage.

Point mutations of K-*ras*, *p53* and *Ikaros* occur frequently in ENU-induced T-cell lymphomas [41,42]. These mutations may enhance responsiveness of cells to growth factor induction of proliferation or resistance to apoptosis, thereby improving survival and increasing clonal expansion. When myeloma cells are cultured on normal bone marrow stromal cells, or in the presence of IL-6, activation of the K-*ras* oncogene provides a growth advantage over cells lacking activated K-*ras* [43]. The progression from low grade to high-grade brain tumors is associated with clonal expansion of cells that have acquired a *p53* mutation that endows the cells with a selective growth advantage [44]. T-cells with reduced or dominant-negative Ikaros activity, which may result from either a lack of or a point mutation in the zinc finger responsible for DNA binding, exhibit a greater proliferative response to IL-2 [45,46]. Irradiation of thymic epithelial cells enhances IL-7 production, and thymocytes at preleukemic stage proliferate more vigorously in response to IL-7 [47,48]. Taken together, these results suggest that high-dose radiation provides a thymic microenvironment ripe for the occurrence of prelymphoma cells, which harbor growth-advantageous mutations following ENU treatment.

In conclusion, low-dose X-rays (0.2 Gy) reduce not only the frequency of spontaneously occurring but also ENU-induced

mutations, suggestive of an adaptive response. Low-dose X-rays also reduce the clonal expansion of cells following ENU treatment, whereas 1.0 Gy X-rays accelerate cell expansion. Thus, low- and high-dose radiations play two different roles in lymphomagenesis when combined with ENU exposure.

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