Original Article

Identification of mouse mutant cells exhibiting the plastic mutant phenotype

Masatake Yamauchi[1,*], Kumiko Fukutsu[2], Mari Sakagami[1], Yuji Yamada[2]

[1] *Laboratory of Precancerous Mutation, Experimental Radiobiology for Children's Health Research Group, Research Center for Radiation Protection, National Institute of Radiological Sciences, Inage, Chiba, Japan;*
[2] *Department of Radiation Dosimetry, Research Center for Radiation Emergency Medicine, National Institute of Radiological Sciences, Inage, Chiba, Japan.*

Summary **The initial processes involved in radiation carcinogenesis have not been clearly elucidated. We isolated mouse mutant cells exhibiting plasticity in their mutation phenotypes. These mutant cells were originally isolated from an irradiated cell population as 6-thioguanine resistant ($6TG^R$) mutants that were deficient in hypoxanthine phosphoribosyl transferase (Hprt, E.C.2.4.2.8) activity at the frequency of approximately 6.2×10^{-5}. Approximately 10% of $6TG^R$ cells showed plasticity in their mutant phenotypes and reverted to HAT-resistant (HAT^R), which is Hprt-proficient, wild type phenotype. Eventually we identified the plastic mutants in the un-irradiated wild type cell population as well and found that ionizing irradiation enhanced the frequency of the plastic mutation approximately 24 times. Treatment with 5-aza-cytidine did not affect the plasticity of mutant phenotypes identified in this study, suggesting that DNA methylation was not involved in the plastic changes of the mutant phenotypes. The plastic mutant phenotype identified in our study is a new type of genomic instability induced by ionizing irradiation, and it is likely to be involved in one of the primary changes that occur in the process of radiation carcinogenesis, and may explain one element of carcinogenesis, which is composed of multi-stages.**

Keywords: Plastic mutation, genomic instability, ionizing radiation, mouse FM3A cells, hypoxanthine phosphoribosyl transferase (Hprt)

1. Introduction

The carcinogenic potential of ionizing radiation (IR) was recognized very soon after its discovery in 1895. Frieben reported the first tumor induced by IR in 1902. IR was also the first mutagen shown to increase the mutation rate in an organism (*1*). IR exerts its effects through the deposition of energy in the cells and the subsequent generation of hydroxyl radicals, leading to damage on DNA strands. The comparative importance of the base alterations caused by IR in mutagenesis has been demonstrated in bacteria (*2*), bacteriophages (*3*) and lower eukaryotes (*4*). In mammalian cells, the majority of mutations induced by IR have been shown to be deleterious (*5*). More recently, genomic instability has been shown to play an important role in mutagenesis and carcinogenesis in mammalian cells. However, genomic instability is not a specific phenomenon observed in irradiated cells. Un-irradiated normal cells also exhibit the same characteristics, such as chromosome aberration and microsatellite/minisatellite instability, at lower frequencies. IR increases the genomic instability in the irradiated cells, as well as neighboring cells. Genomic instability induced by IR has been characterized by an increased rate of alteration acquisition in the genome, such as chromosomal aberrations, micro-nucleation, mutations, microsatellite instability, and cell death (*6*). Increased genomic instability caused by IR is of great concern in the age of advanced medical technologies

**Address correspondence to:*
Dr. Masatake Yamauchi, Laboratory of Precancerous Mutation, Experimental Radiobiology for Children's Health Research Group, Research Center for Radiation Protection, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage, Chiba 263-8555, Japan.
e-mail: m_yam@nirs.go.jp

using IR, not only chest X-ray and mammography, but also computed tomography (CT) and positron emission tomography (PET), in which higher doses of X-rays are often employed. Although IR is currently recognized as a relatively ineffective carcinogen, Berrington de Gonzalez and Darby reported that a significant proportion of cancer incidence is attributable to the recent extensive use of these X-ray diagnostic apparatuses in medical procedures (*7*).

Through our investigation on the genomic instability induced by X-ray irradiation in mouse cells, we identified mutant cell clones that exhibited plasticity in their gene regulation. This genomic instability was manifested as reversible drug resistant phenotypes with increased mutation frequencies concerning hypoxanthine phosphoribosyl transferase (Hprt, E.C.2.4.2.8) activity. It has been shown that the resistance to the cytotoxic drug 6-thioguanine (6TG) could be achieved by the loss of Hprt activity (*8,9*). Involvement of DNA methylation at the promoter region has been reported in the event of suppressing Hprt activity (*10,11*); however, the genomic instability identified in this study seemed to involve different mechanisms. Here, we report the isolation and preliminary molecular characterization of the mutant cells exhibiting the plastic mutant phenotype.

2. Materials and Methods

2.1. *Cell culture*

Mouse FM3A cells were maintained in ES medium (Nissui, Tokyo, Japan) containing 2% fetal bovine serum (FBS) (Nichirei, Tokyo, Japan), as described previously (*12*).

2.2. *X-ray exposure*

Cells were exposed to 5 Gy of 250 keV X-ray using the Shimadzu Pantak model HF-250 (Shimadzu, Kyoto, Japan) at a dose of 0.5 Gy/min. After X-ray exposure, cells were allowed to recover for 24-48 h prior to further treatment.

2.3. *Isolation of 6TG-resistant ($6TG^R$) mutants*

Prior to the selection experiment using 6TG, cells were cultured in HAT medium (*13*) containing 10^{-4} M hypoxanthine, 10^{-6} M aminopterin (also known as methotrexate, MTX), and 10^{-5} M thymidine for 48 h, and then in HT medium containing 10^{-4} M hypoxanthine and 10^{-5} M thymidine for 24 h. Cells were plated onto ES plates containing 5% FBS, 0.5% agarose, and 10^{-5} M 6TG; 0.05 mM of 5-aza-cytidine was also included in the selection plates in the experiments examining DNA methylation (*14*). The number of the cells employed in the drug selection experiment was estimated by the number of colonies formed on the ES plates without 6TG using the appropriate dilution of the cell suspension. Colonies formed on the selection plates containing 6TG were independently isolated and cultured for further analyses. All chemicals were obtained from Wako Chemical (Osaka, Japan), unless otherwise specified.

2.4. *Isolation of HAT-resistant (HAT^R) revertants*

Prior to HAT selection, $6TG^R$ clones were transferred to normal growth medium without 6TG for 24 h. HAT^R clones were selected using ES liquid medium containing 2% FBS and the HAT contents described above or ES plates containing 5% FBS, 0.5% agarose, and the HAT contents; 0.05 mM of 5-aza-cytidine was included in the selection plates in the experiments examining DNA methylation. The number of cells employed in the HAT selection was estimated using the ES plates without HAT.

2.5. *Loss-of-heterozygosity (LOH) analysis*

Genomic DNA was extracted from the cells by proteinase K-SDS treatment and purified by the phenol-chloroform extraction method, as described previously (*15*). LOH at the *Hprt* locus coding the *hypoxanthine phosphoribosyl transferase (Hprt)* gene was examined by polymerase chain reaction (PCR) with the oligonucleotide primers UniSTS178186 *Hprt* F 5'-GAA ATG TCA GTT GCT GCG TC-3' and UniSTS178186 *Hprt* R 5'-GCC AAC ACT GCT GAA ACA TG-3' (*16*). The PCR mixture was prepared as recommended by the manufacturer (Takara, Shiga, Japan). The reaction was started with 5 min at 94°C, which was followed by 40 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C using the GeneAmp PCR System 9700 (Applied Biosystems Inc., Carlsbad, CA, USA). PCR products were analyzed by 3% agarose gel electrophoresis.

3. Results

3.1. *Isolation of $6TG^R$ mutants*

Prior to the drug selection experiments using 6TG, mouse FM3A cells were cultured in HAT medium for 48 h to eliminate naturally ocurring Hprt-deficient cells. Since Hprt activity is not an absolute requirement for survival, cells that lack Hprt activity will grow in normal growth medium and affect the results of experiments examining the mutation frequency.

Cells were allowed to recover from the toxic effects of MTX in HT medium for 24 h. MTX is a drug that inhibits dihydrofolate reductase activity, leading to the deprivation of *de novo* biosynthesis of both purine and pyrimidine nucleotides. If the cells grown in HAT medium were transferred immediately to the medium

containing 6TG without recovery of the *de novo* syntheses of nucleotides, not only Hprt-proficient wild-type cells, but also Hprt-deficient mutant cells would not survive.

Cells were then exposed to 5 Gy X-ray to induce the Hprt-deficient mutations. Hprt-deficient mutants were selected as the $6TG^R$ phenotype. 6TG is a toxic nucleotide analogue that is incorporated into the cell metabolism through Hprt enzymatic activity, leading to cell death. The mutant cells deficient in Hprt activity do not incorporate the toxic analogue into the nucleotide metabolism and thus will survive and grow to form colonies in the presence of 6TG.

In our experiments, X-ray exposure induced 192 $6TG^R$ mutants from 3.1×10^6 cells at a mutation frequency of 6.2×10^{-5}, as shown in Table 1. Additionally, 187 spontaneous $6TG^R$ mutants were obtained from the un-irradiated cell population at a frequency of 1.2×10^{-5}. Thus, 5 Gy X-ray exposure enhanced the frequency of Hprt-deficient mutations about 5 times.

3.2. *LOH analysis at the Hprt locus*

The genomic structure at the *Hprt* locus in $6TG^R$ cells was examined by PCR using a set of UniSTS primers. The *Hprt* locus was not detected in 94 clones of the 187 spontaneous mutants and in 138 clones of the 192 irradiated mutants. DNA sequencing analysis of the genomic *Hprt* gene was not carried out. The cells that did not provide PCR products were regarded as having deleterious mutations in the *Hprt* allele and were not employed in further experiments.

3.3. *Isolation of HAT-resistant reversion mutants*

Using the $6TG^R$ mutant cells without LOH, namely, 93 spontaneous mutants and 54 irradiated mutants, we attempted to isolate revertants by culturing $6TG^R$ cells in HAT medium. MTX inhibits the biosynthetic pathways of both types of nucleotides, purine and pyrimidine, and the cells growing in HAT medium must incorporate hypoxanthine as a substrate for purine nucleotides through the salvage pathway catalyzed by Hprt activity and thymidine for pyrimidine nucleotides through thymidine kinase activity. The cells deficient in Hprt activity do not incorporate hypoxanthine for purine biosynthesis and will not survive in HAT medium. Thus, revertant cells that reactivated Hprt activity will survive in HAT medium.

As summarized in Table 1, we isolated 4 reversion mutant clones from 93 spontaneous $6TG^R$ mutants without the deletion and 19 from 54 irradiated mutants. The frequency of reversible mutants was 4.3% among spontaneous mutants and 35.2% among irradiated mutants. As a result, the irradiated $6TG^R$ mutants contained approximately 10 times more reversible mutant cell clones than the spontaneous mutants. The frequency of the plastic mutant was approximately 2.5×10^{-7} in the normal cell population and approximately 6.1×10^{-6} in the irradiated population, indicating that IR induced approximately 24 times more phenotypic plasticity in mouse FM3A cells. Primary screening of reversion mutants was carried out in ES liquid culture medium containing 2% FBS and the HAT contents. For calculation of the reversion frequencies, the cells were plated on ES agarose plates containing 5% FBS and the HAT contents, and the number of colonies formed was counted. The plastic mutants changed their phenotype at a frequency of approximately 10^{-2} on average, as summarized in Figure 1. The remaining $6TG^R$ mutants exhibited the stable 6TG-resistant phenotype and did not grow in HAT medium.

3.4. *Effect of X-ray exposure and 5-aza-cytidine treatment on plastic mutation*

We examined the effect of X-ray exposure on the induction of reversion mutation from the plastic mutants isolated in this study. A total of 93 clones of the spontaneous $6TG^R$ mutants without the deletion and 54 clones from the irradiated mutants were exposed to 5 Gy X-ray and cultured in liquid ES medium containing 2% FBS and the HAT contents. As a result, the HAT^R revertants appeared from the same $6TG^R$ clones, but no enhancements by X-ray irradiation were observed in the number of HAT^R clones or in the frequency of HAT^R clones obtained. The inclusion of 5-aza-cytidine also did not affect the plasticity of the mutant phenotypes. The plastic mutants isolated in our experiments changed their phenotypes from HAT^R wild-type to $6TG^R$ mutant phenotype, as well as from $6TG^R$ mutant to HAT^R wild-

Table 1. Summary of the cell culture experiments isolating the plastic mutants from the un-irradiated or irradiated cell population

	0 Gy	5 Gy X-ray
No. of cells selected	1.6×10^7	3.1×10^6
No. of 6TG-resistant clones	187	192
Frequency of 6TG-resistant mutation	1.2×10^{-5}	6.2×10^{-5}
No. of clones with/without LOH in *Hprt* allele	94/93	138/54
No. of HAT-resistant clones	4	19
Frequency of plastic mutants	2.5×10^{-7}	6.1×10^{-6}

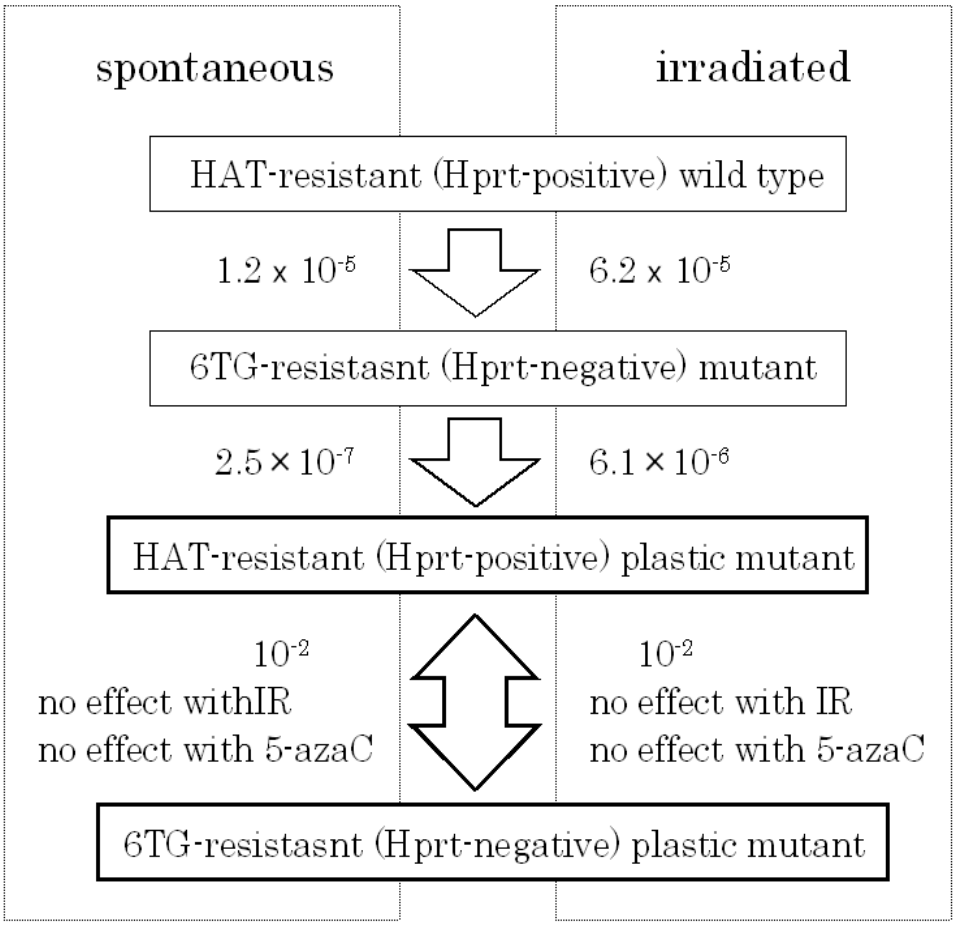

Figure 1. Characteristics of the plastic mutation. The frequencies leading to isolation of plastic mutants are given. Note the direction of the arrows, one directional or bi-directional. Hprt-positive means Hprt-proficient and Hprt-negative means Hprt-deficient.

type in the presence of 5-aza-cytidine at a frequency of approximately 10^{-2} on average, as shown in Figure 1.

4. Discussion

In this manuscript, we described the identification of a new class of genomic instability in cultured mouse FM3A cells, which shows plasticity in its mutant phenotype. The forward mutation from HAT^R wild-type to $6TG^R$ mutant phenotype occurred at a rate of 10^{-5} and the mutation frequency was increased approximately 5 times by irradiation with 5 Gy X-ray. These results are consistent with previous observations (*17-19*).

We then isolated the mutant clones that reverted from $6TG^R$ mutant phenotype to HAT^R wild-type phenotype. We first identified such plastic mutants only among the $6TG^R$ clones isolated from the irradiated cell populations; however, we eventually also succeeded in isolating the $6TG^R$ mutants from un-irradiated cell populations, showing the plasticity of their $6TG^R$ mutant phenotype.

The frequency of the plastic mutant in the spontaneous cell population was calculated to be 2.5×10^{-7}, whereas it was 6.1×10^{-6} in the irradiated cell population, as presented in Table 1 and Figure 1. Thus, the frequency of $6TG^R$ clones showing plasticity of the mutant phenotype was increased by X-ray irradiation approximately 24 times. In other words, IR induced the plasticity of gene regulation in mouse FM3A cells. Approximately 35% of the $6TG^R$ mutants without LOH derived from the irradiated cell population exhibited the plasticity in their mutant phenotype.

The reversion frequency from $6TG^R$ to HAT^R of each clone was approximately 10^{-2}. This frequency is almost equivalent to the one observed in the germline mutation of hyper-variable minisatellite repeats, such as Ms6-hm (*15*). However, the molecular mechanism involved in the hyper-variable repeat instability appears to differ from the plastic mutation observed here. Repeat instability through cell division has been explained by replication slippage. Additionally, instability of hyper-variable repeats has been observed in cultured cell lines at much lower frequencies, ranging from 10^{-5} to 10^{-8} (*20*).

The plastic mutation phenotype identified here appeared to be stable. Once the genomic instability was acquired by the cell lines, it was transmitted stably to the daughter cells for at least three months through more than 100 cell divisions (data not shown). Interestingly, additional radiation exposure of the

plastic mutant cells derived from both spontaneous and irradiated cell populations did not affect the frequency of phenotypic changes in either direction.

We speculated that these phenotypic changes could be attributable to the change in transcriptional level of the *Hprt* gene through DNA methylation. DNA methylation is one of the most common mechanisms involved in the regulation of gene transcription, especially in the gene suppression often observed in the inactivation of the X chromosome (*10*,*11*). As observed in this study, 5-aza-cytidine treatment did not affect the frequency of plastic mutations in either direction, implying that DNA methylation was not involved in the plasticity of the mutant phenotypes we examined. Histone modification may be another mechanism involved in the transcriptional regulation of these genes (*21*-*23*). However, histone modifications have been reported to be frequently associated with DNA methylation (*24*,*25*). A detailed examination of the methylation status at the promoter region of the *Hprt* gene may provide useful information for the understanding of the molecular mechanisms involved in the plasticity of the mutant phenotype identified in our study. An examination of the DNase I sensitivity of the *Hprt* gene promoter region (*26*,*27*) may also provide useful information for understanding the underlying molecular mechanisms.

In this report, we demonstrated the induction of plasticity in the regulation of Hprt activity by IR. Examination of this new type of genomic instability in other cell lines, as well as primary culture cells, may provide useful information on genomic plasticity. The plastic mutant phenotype identified in our study appeared to be a new type of genomic instability induced by IR. Involvement of this plastic mutation in the initial processes of radiation carcinogenesis, which is composed of multiple stages, would be of great concern in the age of advanced medical technologies using IR, assuming that the linear-non-threshold (LNT) model (*28*-*30*) could also be applicable for the induction of plastic mutation by IR. The LNT model basically says that there is no safe dose of radiation, and it is the current basis of radiation protection rules imposed by the United Nations Scientific Committee on the Effect of Atomic Radiation (UNSCEAR). We employed a relatively acute dosage of X-ray irradiation in the primary induction of the plastic mutation phenotype. The effect of irradiation dose, fractionated irradiation, and the type of radiation in the induction of the plastic mutation phenotype should be examined in future experiments.

Acknowledgements

The authors thank Mrs. Reiko Ishikawa and Mrs. Takako Hatada for their excellent technical assistance. This study was supported by the grant-in-aid for scientific research provided by the Japan Society for the Promotion of Science (JSPS).

References

1. Muller HJ. Artificial transmutation of the gene. Science. 1927; 66:84-87.
2. Glickman BW, Rietveld K, Aaron CS. Gamma-ray induced mutational spectrum in the lacI gene of Escherichia coli: Comparison of induced and spontaneous spectra at the molecular level. Mutat Res. 1980; 69:1-12.
3. Conkling MA, Grunau JA, Drake JW. Gamma-ray mutagenesis in bacteriophage T4. Genetics. 1976; 82:565-575.
4. Malling HV, De Serres FJ. Genetic alterations at the molecular level in X-ray induced ad-3B mutants of Neurospora crassa. Radiat Res. 1973; 53:77-87.
5. Stankowski LF Jr, Hsie AW. Quantitative and molecular analyses of radiation-induced mutation in AS52 cells. Radiat Res. 1986; 105:37-48.
6. Kovalchuk O, Baulch JE. Epigenetic changes and nontargeted radiation effects – is there a link? Environ Mol Mutagen. 2008; 49:16-25.
7. Berrington de González A, Darby S. Risk of cancer from diagnostic X-rays: Estimates for the UK and 14 other countries. Lancet. 2004; 363:345-351.
8. Tachibana A, Ohbayashi T, Takebe H, Tatsumi K. Molecular changes in UV-induced and gamma-ray-induced mutations in human lymphoblastoid cells. Mutat Res. 1990; 230:159-166.
9. Porter MB, Fournier RE. Isolation and characterization of HPRT-deficient human hepatoma cells. Somat Cell Mol Genet. 1996; 22:341-348.
10. Litt MD, Hornstra IK, Yang TP. *In vivo* footprinting and high-resolution methylation analysis of the mouse hypoxanthine phosphoribosyltransferase gene 5' region on the active and inactive X chromosomes. Mol Cell Biol. 1996; 16:6190-6199.
11. Chen C, Yang MC, Yang TP. Evidence that silencing of the *HPRT* promoter by DNA methylation is mediated by critical CpG sites. J Biol Chem. 2001; 276:320-328.
12. Yamauchi M, Ayusawa D, Shimizu K, Seno T, Matsuhashi M. Two types of mouse FM3A cell mutants deficient in 5-aminoimidazole-4-carboxamide ribonucleotide transformylase and their transformants isolated by human chromosome-mediated gene transfer. Somat Cell Mol Genet. 1989; 15:39-48.
13. Bakay B, Nyhan WL, Croce CM, Koprowski H. Reversion in expression of hypoxanthine-guanine phosphoribosyl transferase following cell hybridization. J Cell Sci. 1975; 17:567-578.
14. Hockey AJ, Adra CN, McBurney MW. Reactivation of hprt on the inactive X chromosome with DNA demethylating agents. Somat Cell Mol Genet. 1989; 15:421-434.
15. Yamauchi M, Nishimura M, Tsuji S, Terada M, Sasanuma M, Shimada Y. Effect of SCID mutation on the occurrence of mouse Pc-1 (Ms6-hm) germline mutations. Mutat Res. 2002; 503:43-49.
16. *http://www.ncbi.nlm.nih.gov/genome/sts/sts.cgi?uid=178186* (accessed May 11, 2010).
17. Kunugita N, Mei N, Goncharova T, Norimura T. Measurement of mutant frequency in T-cell receptor (TCR) gene by flow cytometry after X-irradiation

on EL-4 mice lymphoma cells. J Toxicol Sci. 2007; 32:377-386.
18. Furuno-Fukushi I, Aoki K, Matsudaira H. Mutation induction by different dose rates of gamma rays in near-diploid mouse cells in plateau- and log-phase culture. Radiat Res. 1993; 136:97-102.
19. Knaap AG, Simons JW. A mutational assay system for L5178Y mouse lymphoma cells, using hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT) -deficiency as marker. The occurrence of a long expression time for mutations induced by X-rays and EMS. Mutat Res. 1975; 30:97-110.
20. Boyer JC, Yamada NA, Roques CN, Hatch SB, Riess K, Farber RA. Sequence dependent instability of mononucleotide microsatellites in cultured mismatch repair proficient and deficient mammalian cells. Hum Mol Genet. 2002; 11:707-713.
21. Keohane AM, O'Neill LP, Belyaev ND, Lavender JS, Turner BM. X-Inactivation and histone H4 acetylation in embryonic stem cells. Dev Biol. 1996; 180:618-630.
22. Csankovszki G, Nagy A, Jaenisch R. Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. J Cell Biol. 2001; 153:773-784.
23. Pradhan S, Chin HG, Estève PO, Jacobsen SE. SET7/9 mediated methylation of non-histone proteins in mammalian cells. Epigenetics. 2009; 4:383-387.
24. Ikegami K, Ohgane J, Tanaka S, Yagi S, Shiota K. Interplay between DNA methylation, histone modification and chromatin remodeling in stem cells and during development. Int J Dev Biol. 2009; 53:203-214.
25. Cheng X, Blumenthal RM. Coordinated chromatin control: Structural and functional linkage of DNA and histone methylation. Biochemistry. 2010; 49:2999-3008.
26. Chen C, Yang TP. Nucleosomes are translationally positioned on the active allele and rotationally positioned on the inactive allele of the *HPRT* promoter. Mol Cell Biol. 2001; 21:7682-7695.
27. Yang TP, Caskey CT. Nuclease sensitivity of the mouse *HPRT* gene promoter region: Differential sensitivity on the active and inactive X chromosomes. Mol Cell Biol. 1987; 7:2994-2998.
28. Preston RJ. Update on linear non-threshold dose-response model and implications for diagnostic radiology procedures. Health Phys. 2008; 95:541-546.
29. Mothersill C, Seymour C. Implications for environmental health of multiple stressors. J Radiol Prot. 2009; 29: A21-A28.
30. Little MP. Do non-targeted effects increase or decrease low dose risk in relation to the linear-non-threshold (LNT) model? Mutat Res. 2010; 687:17-27.

(Received May 26, 2010; Revised June 22, 2010; Accepted June 27, 2010)