Brief Report

Identification of mouse mutant cells exhibiting plastic mutant phenotype II; Ionizing radiation-induced mutant phenotype plasticity is not dependent on DNA methylation of the *hypoxanthine phosphoribosyl transferase* gene in mouse FM3A cells

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Summary **As we previously reported, we isolated and examined mouse mutant cells exhibiting phenotypic plasticity. Approximately 10% of 6-thioguanine resistant ($6TG^R$) cells derived from the irradiated cell population exhibited phenotypic plasticity and reverted to wild type HAT resistance (HAT^R). Similar mutant cells were also identified in an un-irradiated wild type cell population, but at a lower frequency. Ionizing irradiation enhanced the frequency of the plastic mutation approximately 24 times in our experiments. Treatment with 5-aza-cytidine did not affect phenotypic plasticity. In this study, we further performed detailed molecular analysis of the promoter region of the *hypoxanthine phosphoribosyl transferase* (*Hprt*) gene. The analysis revealed that most cytidine residues were not methylated, even in $6TG^R$ mutant cells, in which Hprt activity must be down-regulated. These results suggested that DNA methylation was not involved in mutant phenotype plasticity, a new type of genomic instability induced by ionizing radiation. Plasticity in gene regulation may play an important role in radiation carcinogenesis, which is a multiple-stage process.**

Keywords: Phenotype plasticity, genomic instability, ionizing radiation, DNA methylation, hypoxanthine phosphoribosyl transferase (Hprt), mouse FM3A cells

1. Introduction

Accumulating evidence has made it clear that genomic instability plays an important role in mutagenesis (*1-3*) and carcinogenesis (*4-6*) in mammalian cells. Genomic instability is not specific to irradiated cells; normal un-irradiated cells exhibit the same characteristics at lower frequencies. Ionizing radiation (IR) increases the genomic instability of irradiated cells as well as neighboring cells. The latter effect is known as the bystander effect (*7-9*). The bystander effect of IR is controversial, as some researchers claim that observations of the bystander effect may simply be due to experimental error (*10-12*).

Genomic instability induced by IR is characterized by an increased rate of genome alterations such as chromosomal aberrations, micro-nucleation, mutations, microsatellite instability, and cell death (*13*). Increased genomic instability caused by IR is of great concern in the age of advanced medical technologies which use IR, not only chest X-rays and mammography, but also computed tomography (CT) and positron emission tomography (PET), in which higher X-ray doses are often employed. Although IR is currently recognized as a relatively ineffective carcinogen, a significant proportion of cancer incidence has been attributed to

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the recent extensive use of X-ray diagnostics in medical procedures (*14*). Similar speculation has also been argued elsewhere (*15,16*).

Through our investigation of genomic instability induced by X-ray irradiation in mouse cells, we have noted the existence of mutant cell clones that exhibit plasticity in their gene regulation. We previously abandoned such mutants because their plasticity seemed to make them unsuitable for further analyses. However, such unstable mutants were repeatedly isolated in our cell mutation experiments, suggesting that the phenomenon was reproducible. Such unstable mutants sometimes comprised ~ 10% of the mutant population, leading us to hypothesize that these mutants may play an important role in the initiation of radiation carcinogenesis, a process that has not been clearly elucidated.

The genomic instability examined in this study was manifested as reversible drug-resistant phenotypes with drastically elevated mutation frequencies in hypoxanthine phosphoribosyl transferase (Hprt, E.C.2.4.2.8) activity, as we reported previously (*17*). DNA methylation of the promoter region has been reported to suppress Hprt activity (*18-20*); however, the genomic instability identified in this study seemed to involve different mechanisms. Here, we report the molecular characterization of the *Hprt* promoter region in phenotypically plastic mutant clones.

2. Materials and Methods

2.1. *Plastic mutant cells*

Mutant cells exhibiting phenotypic plasticity were isolated from mouse FM3A cells and maintained in ES medium (Nissui, Tokyo, Japan) containing 2% fetal bovine serum (FBS, Nichirei, Tokyo, Japan) using a model 3161 CO_2 incubator (Forma Scientific, Marietta, OH, USA) with 5% CO_2 and 100% humidity, as previously described (*17,21*).

2.2. *DNA methylation analysis*

Genomic DNA was extracted from the cells using proteinase K-sodium dodecyl sulfate (SDS) treatment and purified by phenol-chloroform extraction, as described previously (*22*). Purified genomic DNA was amplified with oligonucleotide primers F71 5'-CAAA TGTATGTGTGCAATCC-3' and R81 5'-GTGTTCC CTGGCCGCCAAAC-3'. For nucleotide sequencing analysis of the PCR product, oligonucleotide primers F71-2 5'-TGTTGTATAAGATTGAACCCAG-3', F71-3 5'-ACCAAAAAAAAAAAAAAAAAGAT-3', R81-2 5'-GGCAAAAAGCGGTCTGAGG-3', and R81-3 5'-A TGGTTTAAAAAAAAAAAAAGG-3' were used.

For methylation analysis, genomic DNA was denatured and treated with sodium bisulfite, as described previously (*23*). The treated DNA was used as a template for PCR amplification using a set of oligonucleotide primers Meth-1064F 5'-ATGAGGAGG GAGAAAAATG-3' and Meth-R4-RV 5'-AAAACTCT ACTAAAATCCCCTTAAC-3'. Nucleotide sequences of the PCR products were determined by direct sequencing using the same oligonucleotide primers (Meth-1064F and Meth-R4-RV) or by TA cloning (Takara Bio Inc., Shiga, Japan) using universal primers M4 and RV.

3. Results and Discussion

3.1. *Identification of $6TG^R/HAT^R$ mutants exhibiting phenotypic plasticity*

As previously described, $6TG^R/HAT^R$ mutant cells exhibiting phenotypic plasticity were originally identified in a cell population irradiated with 5 Gy X-rays (*17*). Mutant cells exhibiting the same phenotypic plasticity were eventually isolated from un-irradiated cells. As summarized in Figure 1, X-ray exposure induced 192 $6TG^R$ mutants from 3.1×10^6 cells at a mutation frequency of 6.2×10^{-5}, 187 spontaneous $6TG^R$ mutants were obtained from the un-irradiated cell population at a frequency of 1.2×10^{-5}. Five Gy X-ray exposure enhanced the frequency of *Hprt*-deficient mutations 5-fold. Loss-of-heterozygosity at the *Hprt* locus in $6TG^R$ cells was examined by PCR using the UniSTS 178186 primers, as previously described (*17*). The *Hprt* locus was not detected in 94 clones of the 187 spontaneous mutants and in 138 clones of the 192 irradiated mutants, as shown in Figure 1. Cells that did not yield PCR products were regarded as having a deletion mutation in the *Hprt* allele and were not employed in further experiments. Using the $6TG^R$ mutant cells without LOH, namely, 93 spontaneous mutants and 54 irradiated mutants, we isolated revertants by culturing $6TG^R$ cells in HAT medium, as previously described (*17*).

As summarized in Figure 1, we isolated 4 revertant clones from 93 spontaneous $6TG^R$ mutants that did not show LOH, and 19 from 54 irradiated mutants. The reversion frequency was 4.3% among spontaneous mutants and 35.2% among irradiated mutants. As a result, the irradiated $6TG^R$ mutants contained approximately 10-fold 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. The plastic mutants changed their phenotype at a frequency of approximately 10^{-2}. The remaining $6TG^R$ mutants exhibited a stable phenotype and did not grow in HAT medium.

5-Aza-cytidine is a chemical compound that is incorporated into DNA molecules through cell

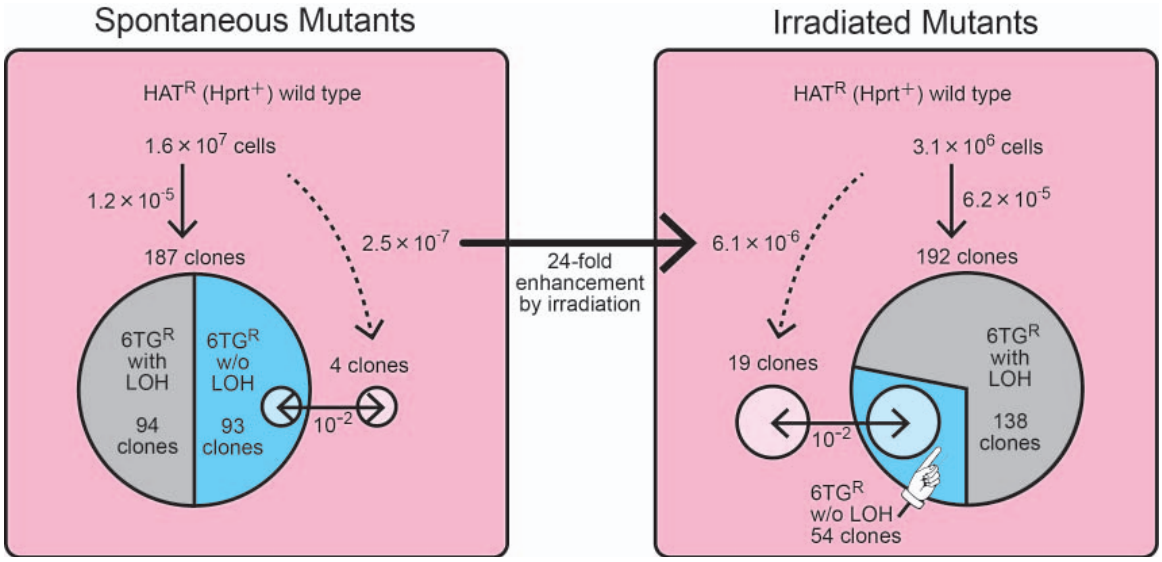

Figure 1. Isolation of the plastic mutants. The box on the left shows the un-irradiated cell population including the HAT^R wild-type phenotype. The box on the right shows the irradiated cell population. Total number of cells used for the drug selection experiments, number of $6TG^R$ clones and plastic mutants, and their frequencies are provided. Within the box, $6TG^R$ clones isolated from each wild-type population are enclosed in a circle. The grey shaded portion represents the $6TG^R$ clones with LOH, and the blue portion represents the $6TG^R$ clones without LOH. Small circles represent the plastic mutants identified in the $6TG^R$ clones; pale-red, HAT^R and pale-blue, $6TG^R$. The bi-directional arrows show the phenotypic plasticity, and the frequency at which the plastic mutants change their phenotypes is approximately 10^{-2}.

metabolism. The presence of 5-aza-cytidine in DNA strands inhibits methylation at the 5th position of the pyrimidine ring of cytidine molecules. To examine involvement of DNA methylation in phenotype plasticity, the effect of 5-aza-cytidine was investigated. As previously reported, inclusion of 5-aza-cytidine did not affect plasticity of the mutant phenotypes (*17*). Plastic mutants isolated in our experiments changed from HAT^R wild-type to a $6TG^R$ mutant phenotype, from a $6TG^R$ mutant to HAT^R wild-type in the presence of 5-aza-cytidine at an average frequency of approximately 10^{-2}.

3.2. *DNA methylation analysis of the Hprt promoter region*

In addition to the incorporation of 5-aza-cytidine in cell culture experiments, we further examined the methylation status of the *Hprt* promoter region utilizing bisulfite-induced modification of genomic DNA, whereby cytosine was converted to uracil, but 5-methyl-cytosine remained non-reactive. Positions and directions of the oligonucleotide primers are shown in Figure 2.

Prior to bisulfite treatment, the nucleotide sequence of the *Hprt* promoter region was amplified from genomic DNA with primers 71 and 81, and confirmed by sequencing with primers 71-2, 71-3, 81-2, and 81-3. The original nucleotide sequence of the *Hprt* promoter region is provided as clone number 0 in Figure 3.

The genomic DNA samples were prepared from both $6TG^R$ and HAT^R clones, and treated with sodium bisulfite. The promoter region of *Hprt* was then amplified by PCR with primers Meth-1064F and Meth-R4-RV. Meth-1064F was designed to bind at a position where bisulfite treatment did not affect the nucleotide sequence. On the other hand, Meth-R4-RV was designed by converting all cytosine residues to thymines at the 3'-downstream end of exon 1 (*24*), as shown in Figure 3.

PCR products were sequenced directly to provide strand-specific average sequences, and compared between $6TG^R$ and HAT^R cells, as well as between spontaneous and X-ray induced mutants. Surprisingly, all cytidine nucleotides in the *Hprt* promoter region were un-methylated in both active and inactive genes. In other words, there were no methyl-cytosine residues even in the $6TG^R$ clones, in which *Hprt* expression was expected to be suppressed.

PCR products were cloned into TA-plasmid vectors and sequenced to provide methylation maps of single DNA molecules. Sequence analysis of the TA-clones revealed the absence of consensus methylated cytosines between 10 clones as shown in Figure 3, which represents typical results obtained from DNA methylation analysis of a $6TG^R$ clone. Sample 0 represents the nucleotide sequence of the un-treated *Hprt* promoter region confirmed in this study. Of the 443 nucleotides in the region, the number of cytidine nucleotides was 125. The results of DNA sequencing obtained from 10 TA-clones of the PCR products amplified from the irradiated $6TG^R$ clone with Meth-1064F and Meth-R4-RV are presented as sample numbers 1-10.

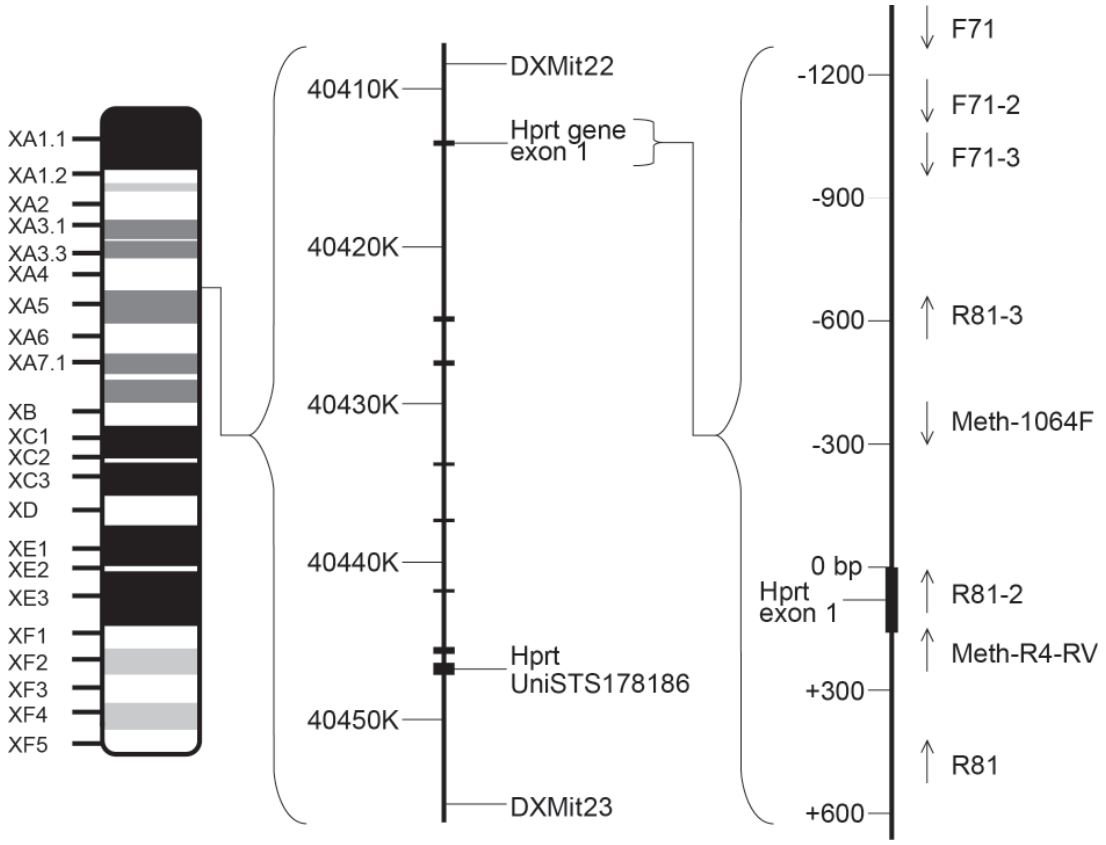

Figure 2. Genomic organization of the mouse *Hprt* allele and oligonucleotide primer design. Chromosomal location of the mouse *Hprt* gene is shown on the left. The center figure describes the genomic structure of the gene and the locations of the UniSTS primers and *Hprt* exon 1. The right figure describes the location of oligonucleotide primers used for the determination of nucleotide sequences of the normal and bisulfite-treated *Hprt* promoter regions.

Almost all of the cytidine residues were converted to thymines, suggesting that these cytidine residues were not methylated in the $6TG^R$ clone. Of 125 cytidine nucleotides in the region, all were converted to thymines in 7 TA-clones, 1 cytidine residue remained un-converted in 1 clone (#8), 2 cytidines remained in 1 clone (#1), and 5 remained in 1 clone (#9). There seemed to be no consensus in the position of methylated cytidine nucleotides. These results showed that DNA methylation was not involved in silencing of *Hprt* transcription.

In this report, we described the identification and DNA methylation analysis of a new class of genomic instability in cultured mouse FM3A cells, which exhibit mutant phenotypic plasticity. The frequency of plastic $6TG^R$ clones was enhanced approximately 24-fold by X-ray irradiation, as shown in Figure 1. In other words, IR increased the plasticity in *Hprt* gene regulation approximately 24-fold in mouse FM3A cells. Mutation frequency was drastically increased to approximately 10^{-2} in plastic mutants.

Interestingly, additional radiation exposure of the plastic mutants derived from both spontaneous and irradiated cells did not affect the frequency of phenotypic changes in either direction, as reported previously (*17*). The plastic mutation phenotype identified here appeared to be stable. Once the genomic instability was acquired by the cells, it was transmitted stably to the daughter cells for at least three months, implying that the genomic instability induced by IR and manifested as phenotypic plasticity can be transmitted stably to daughter cells and may contribute to carcinogenesis.

We speculated that these phenotypic changes could be attributable to the change in *Hprt* expression mediated by DNA methylation. DNA methylation is one of the most common mechanisms in the regulation of transcription, especially in gene suppression often observed in X chromosome inactivation (*18-20*). As observed in our previous 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. In this study, the speculation was confirmed by detailed DNA methylation analysis of the promoter region of *Hprt* in both $6TG^R$ and HAT^R clones derived from the same parental cells. Cytidine nucleotides in the promoter region of *Hprt* genes were totally un-methylated even in $6TG^R$ clones, in which transcription of the *Hprt* gene must be down regulated. We therefore concluded that DNA methylation was not involved in plastic mutations and that Hprt activity was regulated by some different mechanism(s).

Figure 3. DNA methylation analysis of the *Hprt* promoter region in a 6TG^R clone. As mentioned in the text, sample 0 represents the nucleotide sequence of the untreated *Hprt* promoter region. Samples 1-10 represent the nucleotide sequences of TA-clones derived from a 6TG^R clone that showed plasticity in its mutant phenotype. Positions where unconverted cytidines were identified are marked with asterisks. Positions of oligonucleotide primers for sequencing of the converted DNA are shown. Exon 1 of *Hprt* is shown in the open black box. The green box represents the first ATG codon of *Hprt*. The number of cytidine nucleotides found in each sequence is also given.

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