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Analysis of differentially expressed genes on human X chromosome harboring large deletion induced by X-rays

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ABSTRACT

We examined here normal human cells with large deletions encompassing the hypoxanthine-phosphoribosyltransferase 1 (*HPRT1*) gene on X chromosome. Expression levels of genes on X chromosome were analyzed by microarray and RT-qPCR method, and differentially expressed genes (DEGs) were extracted. We found that DEGs were not limited to the genes flanking deleted regions but spread over the entire X chromosome. Interestingly, the gene regulation patterns were similar to a large extent among independent clones that have similar-sized large deletions involving the *HPRT1* gene. Thus, it is indicated that an impact of large deletion on possible epigenetic transcriptional regulation is not limited to the regions proximal to the deletion region.

Keywords: radiation; mutation; deletion; gene expression; microarray; polymerase chain reaction (PCR)

INTRODUCTION

It is well established that DNA double-strand breaks (DSBs) cause gross chromosome rearrangements, particularly, large deletions, which potentially induce genomic instability over several generations, involving radiation-induced carcinogenesis [1–3]. However, the mechanism, by which large deletion triggers various delayed phenotypes, has not been fully understood yet.

Recent advances in chromosome conformation capture technologies have made it possible to illustrate 3D nuclear organization. It now turns out to be clear that interphase chromosomes fold into two compartments [4]. Below the compartment scale, chromatin interactions were enriched within 100 kb-1 Mb domains, which are called topologically associating domains (TADs). TADs are demarcated by CCCTC-binding factor (CTCF) binding and regulated transcriptional activity, suggesting that large deletions, eradicating CTCF-binding sites, result in perturbation of gene expression, as reported previously [5, 6]. Furthermore, accumulating evidence has now demonstrated that 3D nuclear environment is significantly disturbed by DSB induction and its repair [7–9]. Thus, it can be hypothesized that DSB-induced alteration in nuclear architecture leads to unscheduled regulation of gene expression, which has never been examined.

Previously, we isolated 6-thioguanine (6-TG) resistant clones, harboring mutations in the *HPRT1* locus, using SV40-immortalized normal human fibroblasts, GM638, exposed to X-rays [2, 10]. Dose-dependent induction of clones with deletion of the *HPRT1* locus was quite obvious, so that we determined deletion size encompassing the *HPRT1* locus using real-time quantitative polymerase chain reaction (RT-qPCR) analysis with 31 sequence-tagged site (STS) markers and 15 originally designed primers. Large deletions over 2 Mb were commonly identified in multiple clones, along with a patchwork appearance of deletions in a genome close to the largest deletion involving the *HPRT1* locus [10]. As these clones are ideal objects to examine large deletion-induced perturbation of transcriptional control, they were subjected to DNA microarray analysis. Consequently, differentially expressed genes (DEGs) were identified not only in the vicinity of the deletion region but also throughout the entire X chromosome. We confirmed that a part of the up- and down-regulated genes were common among deletion clones with different deletion size.

Our results demonstrate that large deletions are indeed have a transcriptional impact on the entire chromosomal regions, not just on genes flanking deletions, providing a whole new insight into the biological effect of genome alterations caused by ionizing radiation.

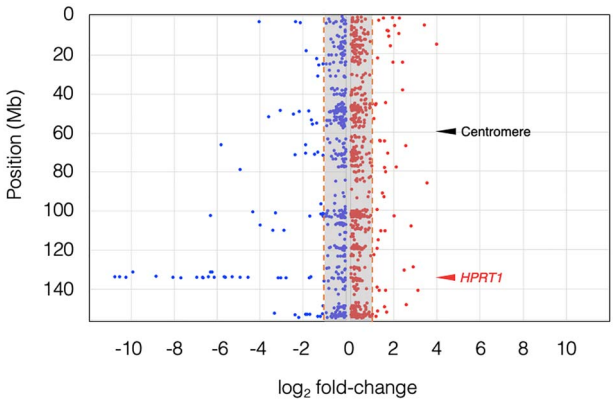

Fig. 1. Expression of genes determined by microarray analysis. Data represented as $\log_2$FC of the X chromosome in 3X-40 cells given as relative to GM638 cells. The horizontal axes represent the genomic position according to the GRCh37/hg19. The blue circles indicate down-regulated genes, while the red circles indicate up-regulated genes. The central shaded area within two orange dotted lines includes genes that had no significant differences ($< \log_2\text{FC} = 1$).

MATERIALS AND METHODS

GM638-derived clones with large deletions at the *HPRT1* locus were selected previously [2,10]. Three 6-TG resistant clones, 3X-40, 3X-41 and 3X-45, were used in this study, and their possible deletion sizes were 2.94 Mb, 4.33 Mb and 3.02 Mb, respectively (Supplementary Fig. 1).

Two-color microarray was used to compare gene expressions between GM638 and 3X-40 cells. For target preparation, total RNAs were extracted from cells using QIAGEN RNeasy mini kit (Hilden, German). Fluorescently labelled cRNA were generated and amplified using Low Input Quick Amp Labeling Kit (Agilent, California, USA). After hybridization using Gene Expression Hybridization Kit (Agilent, California, USA) and washing by Gene Expression Wash Pack (Agilent, California, USA), the microarrays were scanned with the Agilent DNA Microarray Scanner. Agilent's Feature Extraction software (ver.12.1.11) (Agilent, California, USA) was used to quantify the scan results. Raw data were normalized using GeneSpring software (ver.14.9.1) (Agilent, California, USA) processed by the 75th percentile shift method.

Total RNA was extracted from each clone using a RNeasy Plus Mini kit (QIAGEN, Hilden, Germany), and then cDNA was synthesized using a QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany). RT-qPCR analysis was performed as described previously using the Eco Real-Time PCR system (Illumina, California, USA) [10].

RESULTS AND DISCUSSION

Microarray analysis was performed using 3X-40 and GM638. The $\log_2$ fold change ($\log_2$FC) values were obtained in comparison with the parental cells (Fig. 1). It was obvious that the *HPRT1* gene and genes flanking to the *HPRT1* gene showed significantly negative $\log_2$FC compared to the other genes, which was due to large deletion around the *HPRT1* locus as previously reported [10]. Besides, there were several genes showing differential expression, some of which were located away from the deletion. These included genes on the short arm of X chromosome, not just those on the long arm, where the *HPRT1* gene is located, indicating that deletion could have an impact on transcriptional regulation of genes throughout the entire X chromosome.

Since genes with the value of $\log_2$FC lower than −6 showed limited distribution around the *HPRT1* gene, $\log_2$FC of the genes flanking the deletion in 3X-40 were analyzed in more detail (Fig. 2). Negative $\log_2$FC values were observed in many of the genes flanking the *HPRT1* gene, however, two genes, the *STK26* and the *RAP2C*, also showed significantly negative $\log_2$FC (Supplementary Table 1). Thus, it was confirmed that transcription of genes adjacent to the deletion region could be affected. We assumed that large deletion causes ripples over the entire X chromosome, one possible mechanism was that deletion altered long-range epigenetic regulation as indicated previously [11]. However, another possibility could be down-regulation of the gene expression caused by DNA deletion by itself, which could be defined by whole X chromosome sequencing.

Number of up- and down-regulated genes on each cytoband of X chromosome of 3X-40 was obtained from the microarray assay, and summarized in Supplementary Table 2. Percentage of DEGs in each cytoband was calculated (Fig. 3), showing that regulated genes were not only limited to the cytobands containing the *HPRT1* locus but also spread over the whole X chromosome. For example, cytobands p22.32, q11.2 and q27.2 showed approximately 50% of genes were up-regulated. Therefore, we decided to validate the result in other clones with large deletion (3X-41 and 3X-45) by selecting 12 genes considering the results of the microarray analysis and their $\log_2$FC (Supplementary Table 3). A gene was selected from each cytoband of interest and its expression was analyzed by RT-qPCR (Fig. 4). The expression pattern of each gene was largely comparable to the results obtained from microarray assay. Most of the examined genes showed similar trends of up- or down-regulations as typically seen for the *MIR221*, *HMGN5*, *PHEX*, *MIR503HG*, or *GARBA3* genes (Supplementary Table 4), however, some of the genes demonstrated a slightly different regulation pattern among three clones, the reasons for which are currently unknown.

Our observation implied that a common mechanism could be at work in three independent clones with large deletion, strongly suggesting that similar change in 3D chromatin structure was caused by those large deletions. While altered gene expression might be cause by 6-TG administration by itself, the possibility could be eliminated, since DEG patterns were unchanged, when 3X-41 and 3X-45 were maintained and analyzed without 6-TG (data not shown). DSBs have been shown to perturb TAD formation and structure, and the TAD compartment boundary disruption was frequently observed in human cancer cells [8, 9, 12], which resulted in rewiring of gene-enhancer interactions regulated by CTCF-associated TAD domain formation [13]. Consequently, it is likely that large deletions cause unscheduled gene expression, which could work as a key towards carcinogenesis. In fact, it has been demonstrated that deletions alter expression of neighboring genes through disruption of higher-order genome architecture in congenital disorders, malignancies and development [14–16]. To confirm recent sophisticated technologies, such as the Hi-C method, might be needed for further studies [4].

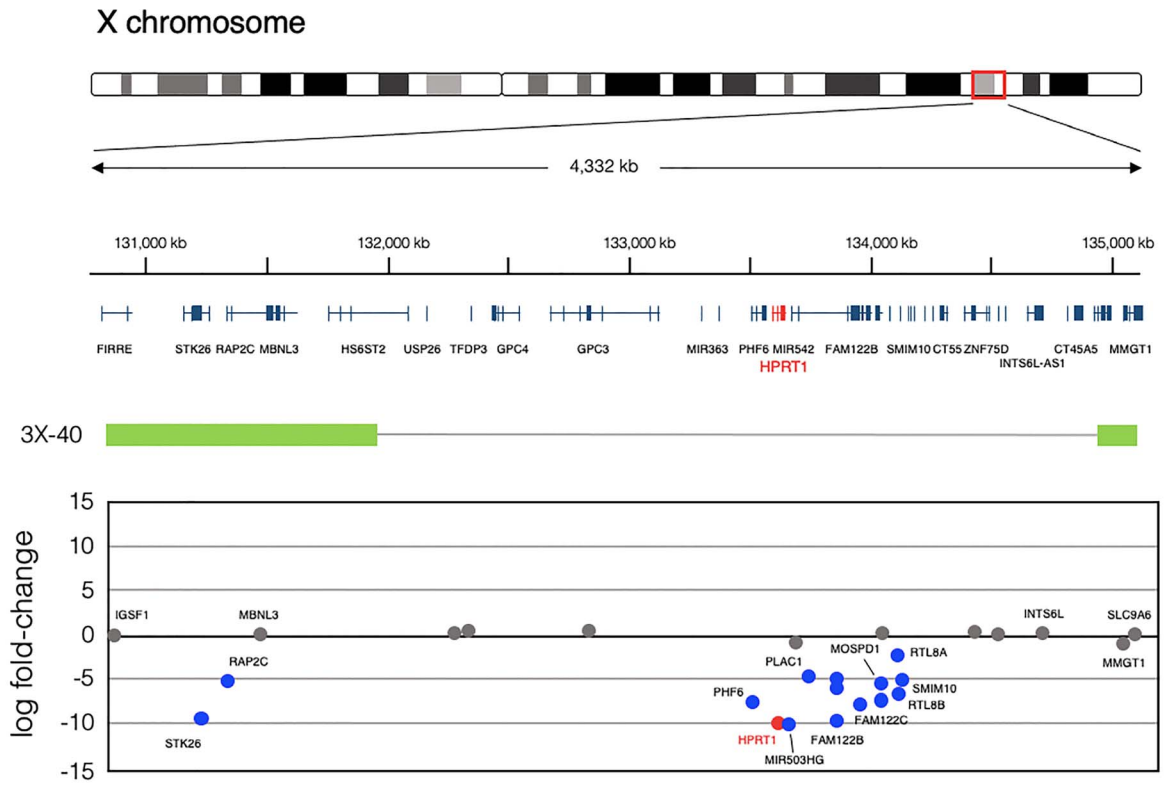

Fig. 2. Expression of genes flanking to deletion determined by microarray analysis. The horizontal axes indicate data represented as $\log_2$FC of the X chromosome in 3X-40 cells given as relative to GM638 cells. Genes between 131 Mb and 135 Mb positions of X chromosome are presented. A gray line between green bars indicates deletion. The blue circles indicate down-regulated genes, while the gray circles indicate genes showing no significant differences ($< \log_2\text{FC} = 1$).

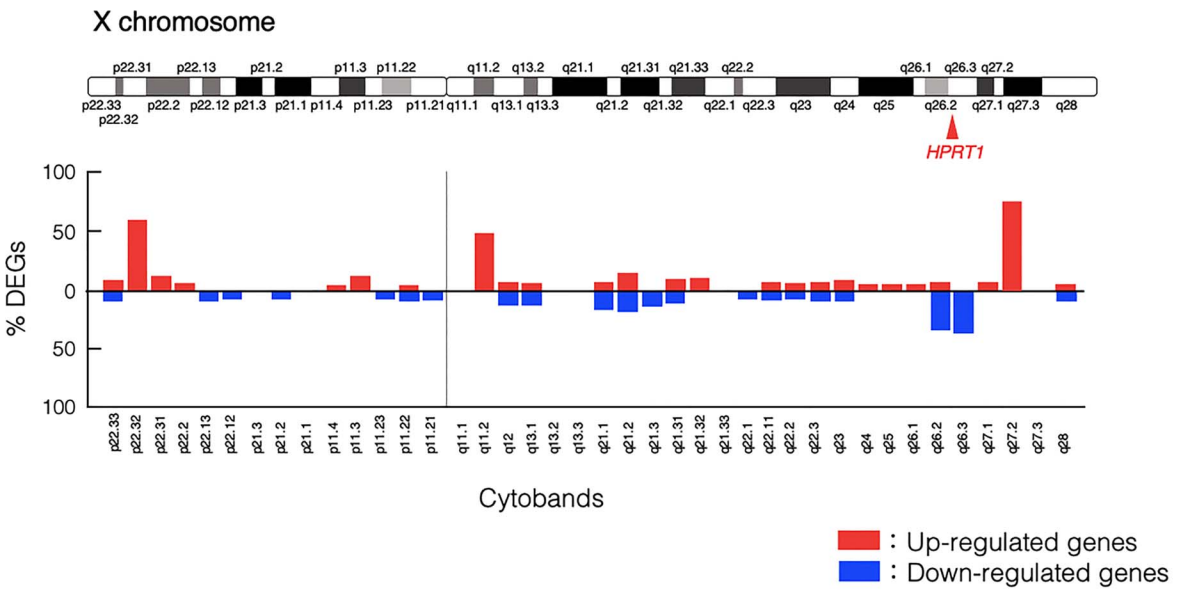

Fig. 3. Percentage of DEGs of the X chromosome in 3X-40 cells. The horizontal axis represents the X-chromosome cytobands, while the vertical axis represents the percentage of DEGs. The red and blue bars indicate percentages of up- and down- regulated genes, respectively.

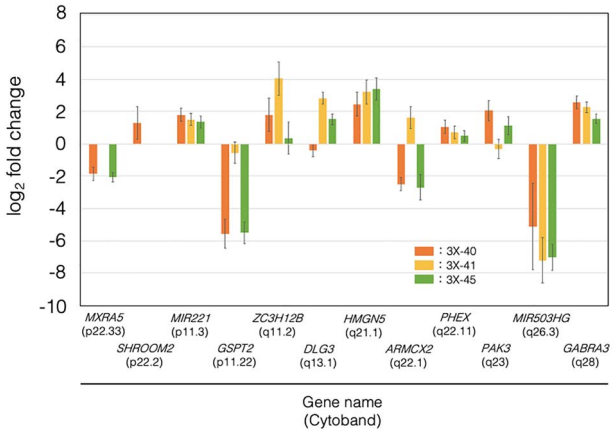

Fig. 4. Results of RT-qPCR analysis for the 12 selected genes. Twelve genes were selected from each cytoband by considering $\log_2$FC obtained from the microarray analysis. All data were normalized to the reference gene, ACTB (β-actin) and given as relative to control, GM638 cells. Data for each gene were calculated using the $\Delta\Delta$Ct method. The orange, yellow and green boxes indicate genes expressed in 3X-40, 3X-41 and 3X-45, respectively. No applicable (N/A) data were indicated as blanks. Error bars represent the standard deviation ($n = 3$).

In summary, we examined expression levels of genes on X chromosome by microarray and RT-qPCR method and extracted several DEGs. Interestingly, DEGs were not limited to the genes flanking deleted regions but spread over the entire X chromosome. As the gene regulation patterns were similar among three independent clones that have similar-sized large deletions, it is indicated that an impact of large deletion on possible epigenetic transcriptional regulation is not limited to the regions proximal to the deletion region, suggesting that large deletion could alter the 3D higher-order structure of chromosome. Our results could provide a new insight into the biological effect of genome alterations caused by ionizing radiation.

SUPPLEMENTARY DATA

Supplementary data is available at *RADRES Journal* online.

DATA AVAILABILITY

The data underlying this article will be shared on reasonable request to the corresponding author.

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CONFLICT OF INTEREST

All the authors declare no conflicts of interest.

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