Mol. Cells *27*, 263-268 February 28, 2009
DOI/10.1007/s10059-009-0032-0

Communication

Molecules and Cells

Transcriptomic Analysis of Rat Brain Tissue Following Gamma Knife Surgery: Early and Distinct Bilateral Effects in the Un-Irradiated Striatum

Misato Hirano[1], Junko Shibato[1], Randeep Rakwal[1,2], Nobuo Kouyama[3], Yoko Katayama[3], Motohiro Hayashi[4], and Yoshinori Masuo[1,*]

Gamma knife surgery (GKS) is used for the treatment of various human brain disorders. However, the biological effects of gamma ray irradiation on both the target area, and the surrounding tissues are not well studied. The effects of gamma ray exposure to both targeted and untargeted regions were therefore evaluated by monitoring gene expression changes in the unilateral irradiated (60 Gy) and contralateral un-irradiated striata in the rat. Striata of irradiated and control brains were dissected 16 hours post-irradiation for analysis using a whole genome 44K DNA oligo microarray approach. The results revealed 230 induced and 144 repressed genes in the irradiated striatum and 432 induced and 239 repressed genes in the un-irradiated striatum. Out of these altered genes 39 of the induced and 16 of the reduced genes were common to both irradiated and un-irradiated tissue. Results of semi-quantitative, confirmatory RT-PCR and western blot analyses suggested that γ-irradiation caused cellular damage, including oxidative stress, in the striata of both hemispheres of the brains of treated animals.

INTRODUCTION

Gamma knife surgery (GKS) is a minimally invasive, neurosurgical tool designed as an alternative to open surgery for the treatment of benign and malignant brain, head and neck tumours and vascular malformations (Pan et al., 2008). It is also used to treat certain functional disorders including (but not limited to): trigeminal neuralgia, Parkinson's disease, psychoneurosis and epilepsy (David et al., 2008; Friehs et al., 2007). It utilises highly precise radiation beams to destroy brain tumours and other abnormalities without the need to operate. GKS is known to provide a minimal risk of hemorrhage and infection to targeting and surrounding areas and it is thought that gamma (γ) rays in GKS do not seriously affect the areas of the brain near to/surrounding the target area.

A standard GKS unit is composed of 201 cobalt-60 sources (Co_{60}) with an output of ~30 curies each. These are placed in a spherical array in a heavily shielded unit, with a selected target placed at the centre of the radiation focus. This allows for a curable radiation dose to be delivered in a single, highly focused, treatment. The frequency of GKS use is expanding in both medicine and surgery; however, we know little of the potential biological effects induced by the γ rays used in this procedure.

A simple model in which the effects of irradiation can be evaluated as functional changes in a systematic and controlled manner was recently developed in Japan (Tokumaru et al., 2005). Utilizing this model system in conjunction with two-dimensional gel electrophoresis-based proteomic analysis it was reported that γ rays from the GKS unit resulted in subtle changes in protein patterns in unilateral irradiated striatum brain tissue within 16 h of treatment (Hirano et al., 2007). This finding was intriguing but left open the question of whether γ-ray treatment in the unilateral striatum could also cause distinct molecular changes in the surrounding, un-irradiated tissue? Answering this question was the primary goal of the present study. It should be noted at this point that the aim of this work is not to question the immense usefulness of this widely used surgical technique but merely to study the effects of ionizing γ rays on the brain at the molecular level and thus provide new evidence on the potential risk associated with this type of surgery.

Comprehensive gene expression changes in both unilateral irradiated and contralateral un-irradiated striata were investigated 16 h post-treatment via transcriptomic analysis using rat DNA microarrays. Transcriptomics is a powerful and well-established technique which is widely applied to address biological questions in animals and other organisms. It is routinely employed for unraveling the genome-wide expression profiles

[1]Health Technology Research Center, National Institute of Advanced Industrial Science and Technology (AIST) West, 16-1 Onogawa, Tsukuba 305-8569, Ibaraki, Japan, [2]Research Laboratory for Biotechnology and Biochemistry, Kathmandu, Nepal, [3]Department of Physiology, School of Medicine, Tokyo Women's Medical University, Tokyo 162-8666, Japan, [4]Department of Neurosurgery, School of Medicine, Tokyo Women's Medical University, Tokyo 162-8666, Japan
*Correspondence: y-masuo@aist.go.jp

Received November 5, 2008; revised November 24, 2008; accepted November 25, 2008; published online February 20, 2009

Keywords: brain, gamma irradiation, gene expression, rat model

and for screening genes of interest (Arion et al., 2007; DeRisi, Iyer and Brown, 1997; Zhang et al., 2008). The results presented here reveal distinct gene expression changes in both irradiated and un-irradiated striatum implying that GKS has the potential to affect non target brain regions.

MATERIALS AND METHODS

Gamma knife irradiation (GKS) and dissection of brain tissue

Four adult male Wistar rats were housed in acrylic cages at 24°C and given access to tap water and laboratory chow *ad libitum* (Tokumaru et al., 2005). Gamma knife irradiation was performed as previously described by Tokumaru et al. (2007). The rats were anesthetized with pentobarbital (40 mg/kg intraperitoneally) and placed in a Regis-Valliccioni frame (Neurospace, France). A maximum dose of 60 Gy was administered [for 30 min] to the unilateral striatum with the Leksell gamma knife model C (Elekta Instrument AB) unit using a 4-mm collimator. At 16 h post-irradiation the whole brain of each animal was rapidly [< 30 s] removed and the left and right striata were separated out, on ice, according to the method of Glowinski and Iversen (1996), with minor modifications (Hirano et al., 2007). Each sample was immediately weighed then flash-frozen in liquid nitrogen and stored at -80°C prior to further analysis.

Total RNA extraction and DNA microarray analysis

The irradiated striata, together with the un-irradiated ipsilateral and contralateral striata, were dissected from individual brains and the total RNA of each was extracted. For this, each brain was first ground to a very fine powder using a pre-chilled mortar and pestle and liquid N_2. Total RNA was extracted from ~100 mg subsample of pooled striata powder from each group using the QIAGEN RNeasy Mini Kit (QIAGEN, USA). To verify the quality of this RNA, the yield and purity were determined spectrophotometrically (NanoDrop, USA) and visually confirmed using formaldehyde-agarose gel electrophoresis.

A rat 44K whole genome oligo DNA microarray kit (G4131A, Agilent Technologies, USA) was used for RNA analysis. Total RNA (150 ng) was labeled with either Cy3 or Cy5 dye using an Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Fluorescently labeled targets of control, as well as irradiated or un-irradiated, samples were hybridized to the same microarray slide with 60-mer probes. A flip labeling (dye-swap or reverse labeling with Cy3 and Cy5 dyes) procedure was followed to nullify the dye bias associated with unequal incorporation of the two Cy dyes into cDNA (Hirano et al., 2008). In our experience, the use of a dye-swap approach provides a more stringent selection condition for changed genes than simple replicates. This latter approach is both expensive and, more importantly, overlooks dye bias. The design of the microarray experiment is presented in Supplementary Table 1. Hybridization and wash processes were performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray scanner G2565BA.

For the detection of significantly differentially expressed genes between control and irradiated/un-irradiated samples each slide image was processed by Agilent Feature Extraction software (ver.8.1.1.1). This program measures Cy3 and Cy5 signal intensities of whole probes. Dye-bias tends to be signal intensity dependent, therefore the software selected probes using a set by rank consistency filter for dye-normalization. Said normalization was performed by LOWESS (locally weighted linear regression) which calculates the log ratio of dye-normalized Cy3- and Cy5-signals, as well as the final error of log ratio. The significance (P) value based on the propagate error and universal error models. In this analysis, the threshold of significant differentially expressed genes was < 0.01 (for the confidence that the feature was not differentially expressed). In addition, erroneous data generated due to artifacts were eliminated before data analysis using the software.

Confirmatory RT-PCR

To re-check the microarray data RT-PCR was performed on randomly up- and down-regulated genes using 3′-UTR specific gene primers. Briefly, total RNA samples were first DNase-treated with RNase-free DNase (Stratagene, USA). First-strand cDNA was then synthesized in a 50 μl reaction mixture with a StrataScriptTM RT-PCR Kit (Stratagene) according to the protocol provided by the manufacturer, using 10 μg total RNA isolated from control and irradiated or un-irradiated striata. The 50 μl reaction mixture (in 1X buffer as recommended by the manufacturer of the polymerase) contained 1.0 μl of the first-strand cDNA, 200 mM dNTPs, 10 pmols of each primer set and 0.5 U of taq polymerase (TaKaRa Ex Taq Hot Start Version, TaKaRa Shuzo, Shiga, Japan). Thermal-cycling parameters were as follows: after an initial denaturation at 97°C for 5 m, samples were subjected to a cycling regime of 20 to 35 cycles at 95°C for 45 s, 55°C for 45 s, and 72°C for 1 m. At the end of the final cycle, an additional extension step was carried out for 10 m at 72°C (TaKaRa PCR Thermal Cycle Dice, Model TP600, TaKaRa, Japan). After completion of the PCR the total reaction mixture was mixed with 2.0 μl of 10X loading buffer, vortex mixed, and 10 μl was loaded into wells of a 1.5% agarose (Agarose ME, Iwai Chemicals, Japan) gel. Electrophoresis was then performed for ~30 m at 100 Volts in 1X TAE buffer using a Mupid-ex electrophoresis system (ADVANCE, Japan). The gels were stained (20 μl of 50 mg/ml ethidium bromide in 100 ml 1X TAE buffer) for ~10 min and the stained bands were visualized using an UV-transilluminator (ATTO, Japan).

Total protein extraction, 1-DGE, and Western blot analysis

Extraction of the total protein content was performed using a recently established protocol specifically designed for brain tissue (Hirano et al., 2006). The total protein concentration was determined by a Coomassie Plus™ (PIERCE, USA) protein assay kit, with samples stored at -80°C. Prior to SDS-PAGE, 50 μL of each fractionated protein sample was mixed with 20 μl of SDS sample buffer [62 mM Tris (pH 6.8), 10% (v/v) glycerol, 2.5% (w/v) SDS, and 5% (v/v) 2-mercaptoethanol] and a drop of bromophenol blue. After incubation on a bench (ambient room temperature = 25°C) for 10 m the mixture was centrifuged, and a total of 5 to 80 μg of protein was loaded in each sample well. The anti-superoxide dismutase (SOD) 2 polyclonal (ab13533), anti-catalase polyclonal (ab1877), anti-thioredoxin (Trx) 1 polyclonal (ab16835), anti-Trx 2 polyclonal (ab16836), anti-heat shock protein (HSP) 70 monoclonal (ab6535), and anti-HSP 90 monoclonal (ab13494) antibodies were obtained from Abcam Ltd. (Cambridgeshire, UK). The ECL+plus Western Blotting Detection System protocol for blocking, primary and secondary antibody (using anti-Rabbit IgG and anti-mouse IgG, horseradish peroxidase-linked species-specific whole antibodies; GE healthcare, and anti-Rat IgG-H&L, horseradish peroxidase linked whole antibody; Abcam) incubation set up was as the manufacturer (GE Healthcare, UK) recommended. Immunoassayed proteins were visualized on an X-ray film (X-OMAT AR, Kodak, Japan) using an enhanced chemiluminescence protocol, again performed according to the manufacturer's directions.

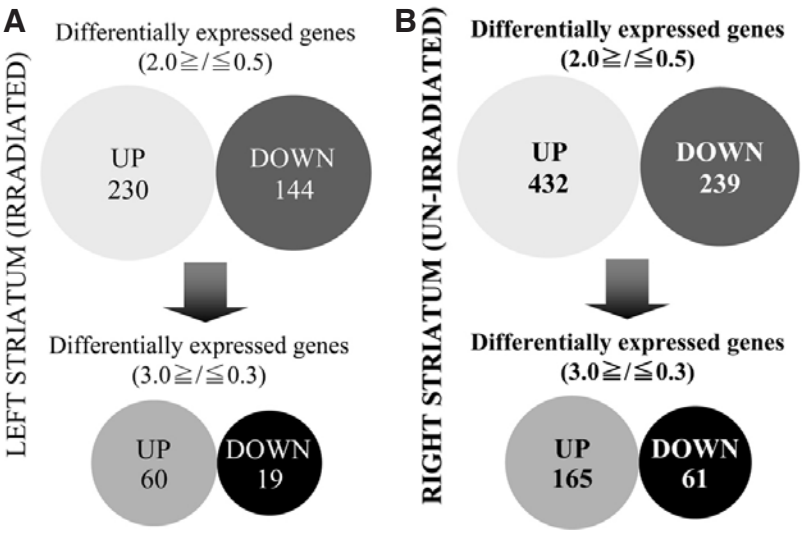

Fig. 1. Differentially expressed genes in the irradiated (A) and un-irradiated striata (B). Light grey/black and dark grey/black circles denote up- and down-regulated genes respectively. The numbers in the circles signify differentially expressed genes compared to their respective controls. Numbers in parentheses indicate fold changes from the microarray analyses.

RESULTS AND DISCUSSION

DNA microarray analysis of irradiated and un-irradiated striata

To investigate the alterations of gene profiles in irradiated and un-irradiated striata as a result of GKS, the transcriptional level of approximately 44,000 rat genes was examined using the DNA microarray chip as described in the materials and methods section. For the microarray experiment, total RNA was pooled from two rat striata in each group; followed by cDNA synthesis, labeling, and hybridization (Supplementary Table 1). The resulting data was sorted using GeneSpring (Version 7) software for normalization. Fold changes ($\geq$ 2.0-fold and $\leq$ 0.5-fold) were calculated from normalized log ratios.

Overview of global gene expression after γ-irradiation

The results showed that 230 genes were up-regulated and 144 genes were down-regulated in the irradiated striatum. Interestingly, 432/239 genes were found to be up/down-regulated in the un-irradiated striatum respectively (Figs. 1A and 1B). Using the NCBI or RGD databases, these genes were functionally categorized according to their biological function/process (Supplementary Figs. 1A and 1B - left panel, and supplementary Tables 2-5). Microarray analysis revealed that more genes were altered in un-irradiated striatum than the irradiated striatum but that 39 of the up-regulated and 16 of the down-regulated genes were common in each tissue (Fig. 4, and supplementary Tables 2-5). Moreover, 6 up-regulated and 4 down-regulated genes, including NM_001061616 (oxidation resistance 1), NM_022689 (synaptosomal-associated protein 23), NM_207586 (chitinase, acidic), NM_001009685 (developmentally regulated GTP binding protein 1), AC119382, AA858927, NM_013105 (cytochrome P450, family 3, subfamily a, polypeptide 23/polypeptide 1), NM_001004086 (paraoxonase 3), NM_001106882 (unc-5 homolog C (*C. elegans*)-like), and NM_001108175 (TBC1 domain family, member 2B) showed opposite expression profiles in the striata of both brain hemispheres. While a large number of genes were found to be induced or repressed, for clarity, this list was narrowed down to genes with $\geq$ 3-fold changes compared to controls. This left 60 induced and 19 repressed genes present in the irradiated striatum, and 165 induced and 61 repressed genes in the un-irradiated striatum compared to controls. These genes were again categorized according to their biological process/function (Supplementary Figs. 1A and 1B - right panel).

Functional category analysis of the genes expression changes in the irradiated striatum revealed that those involved in systemic interactions with the environment, cell cycle/DNA processing, and protein fate were highly induced, whereas genes involved in signal transduction, binding function, and development were highly suppressed (Fig. 4). Genes categorized into the systemic interactions with the environment group were the most highly-induced and contained six chemokine genes. Interestingly, five of these genes were also induced in the un-irradiated striatum; these were NM_139089 [chemokine (C-X-C motif) ligand 10 (CXCL10)], NM_031530 [chemokine (C-C motif) ligand 2) (CCL2)], BC088260 [similar to Small inducible cytokine B13 precursor (CXCL13)], XM_342824 [chemokine (C-C motif) ligand 19 (CCL19)], and NM_013025 [chemokine (C-C motif) ligand 3 (CCL3)]. Previous reports showed that CCL2, also known as macrophage chemoattractant protein 1 (MCP-1), is induced after radiation treatment and protects neurons and astrocytes from apoptosis (Eugenin et al., 2003; Inoue et al., 2004).

Genes in the protein fate category (one of highly-induced gene categories in both striata) included many related to the ubiquitin cycle and protein modification. The ubiquitin cycle regulates many cellular signalling networks; for example the DNA repair pathway. Protein modification is also important in ubiquitin binding to substrates. Ubiquitin-conjugating enzyme E2Z (Ube2Z), induced in the un-irradiated striatum, is one of the ubiquitin conjugating (E2) enzymes and catalyzes the covalent attachment of ubiquitin to other proteins. It has been previously suggested that the ubiquitination state of substrate proteins regulates apoptosis (O'Donnell et al., 2007). Although the present study does not offer proof of this, it is possible to speculate that up-regulation of the Ube2Z mRNA level may be involved in the regulation of pro-survival signals in the un-irradiated striatum.

RT-PCR and western analysis

To validate the microarray data, semi-quantitative RT-PCR was performed with several, randomly selected genes in both irradiated and un-irradiated striata (Fig. 2). The mRNA level changes corresponding to the up- and down-regulation of the genes in the whole-genome profiling experiment validated the microarray

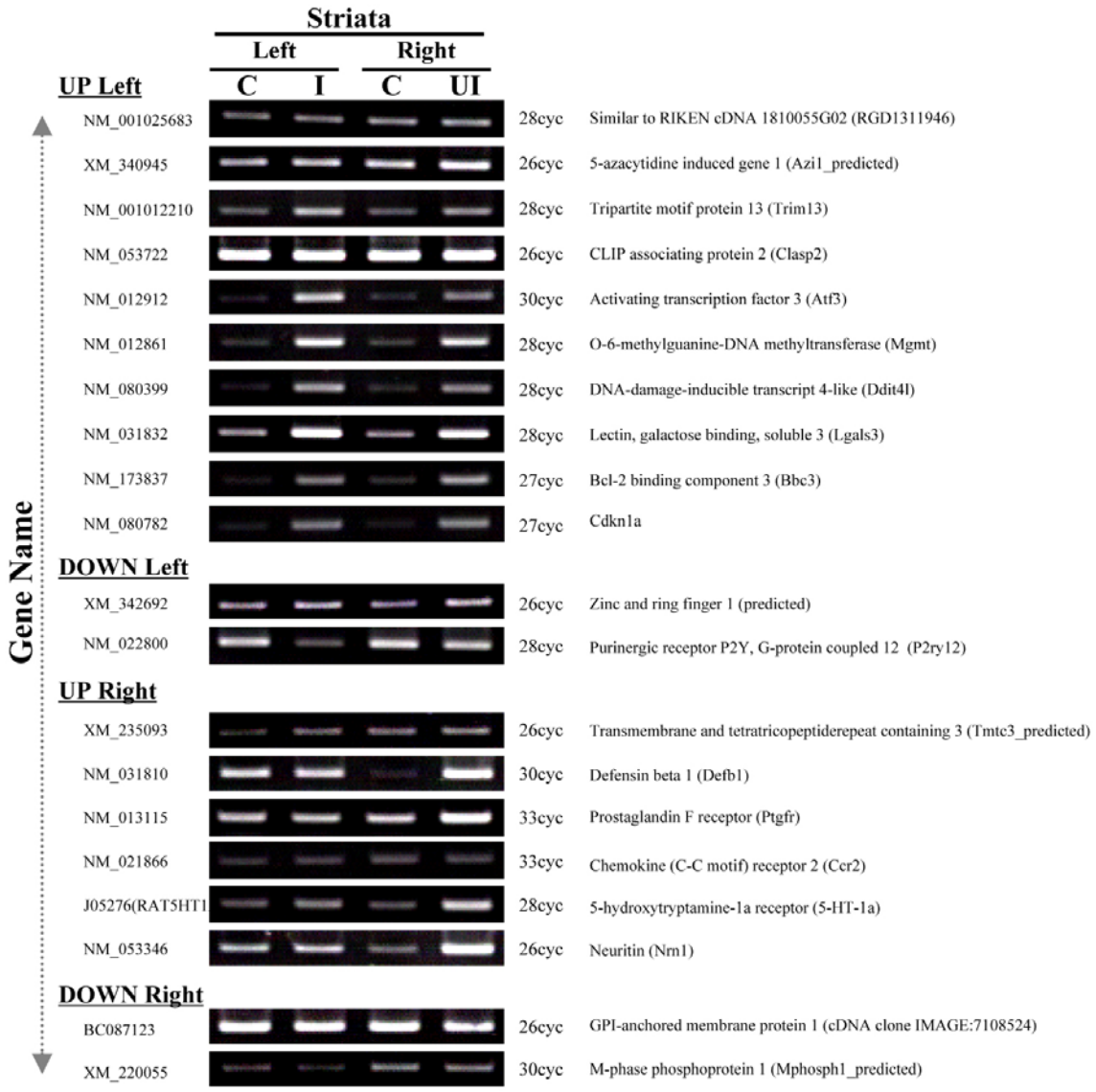

Fig. 2. RT-PCR analysis of differentially expressed genes. Accession numbers are given on the left hand-side of the gel images and are subdivided into up-regulated and down-regulated genes. The gene names and PCR cycles are marked on the right hand-side. C, control; I, irradiated; UI, un-irradiated.

data. Furthermore, the RT-PCR analysis also revealed the alterations of seven genes (XM_340945, NM_001012210, NM_031832, NM_080782, XM_235093, J05276, and NM_053346) in either of the striata, these gene expression changes were not detected in the microarray experiment indicating the value of a two pronged approach.

A previous proteomics based study had suggested the possibility that γ-ray exposure causes oxidative stress in both irradiated and un-irradiated striata (Hirano et al., 2007). Therefore mRNA and protein expression levels of oxidative stress-related proteins were also investigated using RT-PCR (Fig. 3A) and western blot analysis (Fig. 3B). RT-PCR revealed the alteration of peroxiredoxin (Prx) family mRNA expression in both treatments. The Prx proteins are involved in the protection of cells against oxidative stress and in apoptosis/anti-apoptosis (Cullingfordet al., 2006). In this experiment, *Prx1*, *4*, and *5* mRNA levels were increased in the un-irradiated striatum, whereas the *Prx3* mRNA level was reduced in the irradiated striatum. The *Prx2* and *6* mRNA expressions were not significantly changed in either tissue.

Using western blot analysis it was also found that the level of SOD 2 protein, which can provide radiation protection (Epperly et al., 2002), was also increased in both the irradiated and un-irradiated striata. However, SOD2 was more highly induced in the un-irradiated striatum. In addition, the Trx1 protein was present at high levels in un-irradiated striatum. A previous report has demonstrated that Trx1 was increased and is a radiosensitivity modulator in the non-malignant human bronchial epithelial cell line (Demizu et al., 2008). Conversely the HSP90 protein was slightly reduced in un-irradiated striatum.

Interestingly, the results outlined above reveal more alteration in the un-irradiated compared to irradiated striatum. These results again suggest that γ-irradiation caused cellular damage, including oxidative stress, in both striata.

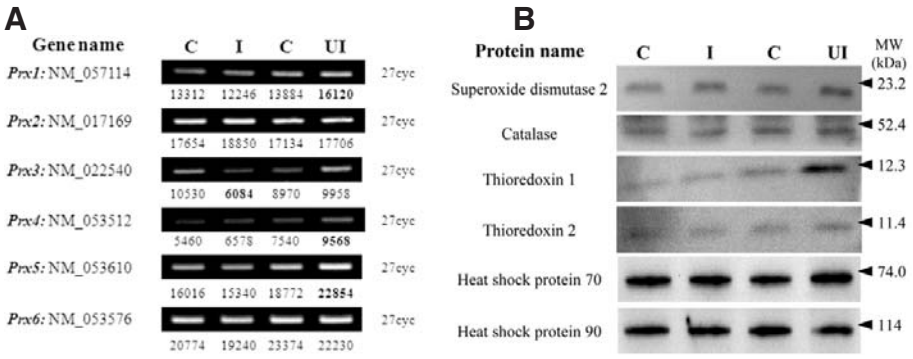

Fig. 3. RT-PCR (A) and western blot (B) analyses for oxidative stress-related genes/proteins. Gene names and accession numbers are on the left hand-side of gel images. Protein names are given on the left hand-side of the images and molecular mass of proteins on the right. Numbers in bold indicate differentially expressed genes compared to controls. Prx; peroxiredoxin. C, control; I, irradiated; UI, un-irradiated.

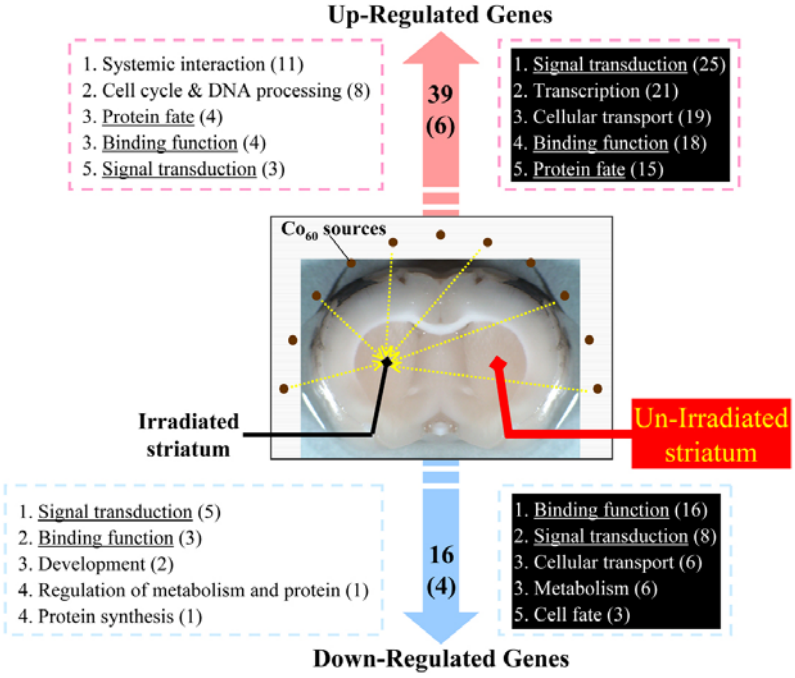

Fig. 4. Functional categories of the differentially expressed up-/down-regulated genes. The picture in the center illustrates the point where the unilateral striatum was irradiated by the GKS unit. The main photo is a coronal view of rat brain including bilateral striata. Brown dots and yellow arrows show Co_{60} and the γ ray source. The pink boxes indicate the categories of up-regulated genes in the irradiated (left) and un-irradiated (right) striata. The blue boxes indicate down-regulated genes in irradiated (left) and un-irradiated (right) striata. Underlined categories were common between irradiated and un-irradiated striata. Numbers on the left hand side of category labels indicate the order while numbers in parentheses show the number of up-/down-regulated genes belonging to each category.

CONCLUSIONS

This study provided an approach for further experimentation on the effects of GKS in un-targeted brain regions using an animal model; with potential use for understanding the risks in human patients. The work is the first such investigation to identify differentially expressed genes after γ-irradiation during GKS in both the unilateral irradiated and contralateral un-irradiated striata. Microarray analysis revealed a surprisingly large number of induced and suppressed genes in the un-irradiated striatum. This result pointed to the bilateral effects previously discussed by various authors (Hirano et al., 2007; Masuo et al., 1990a; 1990b; Rohatgi et al., 2003). Furthermore, the RT-PCR and western blot analyses showed that some genes and proteins involved in the oxidative stress response were also highly altered in the un-irradiated striatum. These results indicate that unilateral irradiation during GKS seemed to cause the spread of radiation induced metabolic changes to the un-irradiated striatum. The difference of expression changes between the irradiated and un-irradiated striata may be a result of the body priming the un-irradiated striatum against potential future damage.

Data availability

All microarray data from this work is available in the NCBI Gene Expression Omnibus (GEO, (www.ncbi.nlm.nih.gov/geo) database under the series entry GSE13220.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

We thank Drs. Kouji Satoh and Shoshi Kikuchi (Plant Genome Research Unit, National Institute of Agrobiological Sciences, Tsukuba, Japan) for expert help with the Agilent scanner and data analysis. We also appreciate a critical reading of the manuscript by Dr. Oliver A. H. Jones at the University of Cambridge, UK.

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