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Identification of radiation-specific responses from gene expression profile

Woong-Yang Park[1,2], Chang-Il Hwang[2], Chang-Nim Im[2], Min-Ji Kang[2], Jang-Hee Woo[2], Ju-Hoon Kim[2], Yon Su Kim[3], Ju-Han Kim[4], Ho Kim[5], Kyung-A Kim[6], Hyung-Jin Yu[6], Sue-Jae Lee[7], Yun-Sil Lee[7] and Jeong-Sun Seo*[,1,2]

[1]Ilchun Molecular Medicine Institute, Seoul National University, Seoul, Korea; [2]Department of Biochemistry and Molecular Biology, Seoul National University, Seoul, Korea; [3]Department of Internal Medicine, Seoul National University, Seoul, Korea; [4]Department of Preventive Medicine, College of Medicine, Seoul National University, Seoul, Korea; [5]Department of Biostatistics, Graduate School of Public Health, Seoul National University, Seoul, Korea; [6]Macrogen Inc., Seoul, Korea; [7]Laboratory of Radiation Effect, Korea Cancer Center Hospital, Seoul, Korea

The responses to ionizing radiation (IR) in tumors are dependent on cellular context. We investigated radiation-related expression patterns in Jurkat T cells with nonsense mutation in p53 using cDNA microarray. Expression of 2400 genes in γ-irradiated cells was distinct from other stimulations like anti-CD3, phetohemagglutinin (PHA) and concanavalin A (ConA) in unsupervised clustering analysis. Among them, 384 genes were selected for their IR-specific changes to make 'RadChip'. In spite of p53 status, every type of cells showed similar patterns in expression of these genes upon γ-radiation. Moreover, radiation-induced responses were clearly separated from the responses to other genotoxic stress like UV radiation, cisplatin and doxorubicin. We focused on two IR-related genes, phospholipase Cγ2 (PLCG2) and cytosolic epoxide hydrolase (EPHX2), which were increased at 12 h after γ-radiation in RT–PCR. TPCK could suppress the induction of these two genes in either of Jurkat T cells and PBMCs, which might suggest the transcriptional regulation of PLCG2 and EPHX2 by NF-κB upon γ-radiation. From these results, we could identify the IR-specific genes from expression profiling, which can be used as radiation biomarkers to screen radiation exposure as well as probing the mechanism of cellular responses to ionizing radiation.
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Introduction

High energy ionizing radiation (IR) can induce direct and/or indirect damage to DNA, which are followed by a variety of cellular responses like cell-cycle arrest, transformation, and cell death (Lakin and Jackson, 1999; Gong and Almasan, 2000; Backlund *et al*., 2001). IR works via DNA damage and reactive oxygen species (ROS) generation, which can induce the transcription of specific genes through the activation of p53, NF-κB and AP-1 (Liu *et al*., 1996; Khanna and Jackson, 2001; Lu-Hesselmann *et al*., 1997; Nogami *et al*., 1994; Safwat, 2000). Although p53 will play the most important role in cellular response to ionizing radiation, the other pathways through AP-1 and NF-κB will also participate in a p53-independent way (Wahl and Carr, 2001). Because tumor cells are usually devoid of functional p53 (Roth *et al*., 2001), we need to investigate p53-independent responses to understand radiosensitivity of these tumors. Recent reports have claimed that the overall response of tumor cells to ionizing radiation seems to be complex and heterogeneous regardless of their p53 status (Amundson *et al*., 1999; Sally *et al*., 1999).

The identification of new IR-response genes may also provide novel targets for basic research on these topics as well as for future experimental approaches in radiotherapy. Understanding the genetic programs is critical for elucidating the molecular mechanisms of pathologies as diverse as cancer and autoimmunity. However, transcriptional changes are extremely complex because of their dependency on interacting signal transduction pathways, cell type specific factors and the genetic background, which makes it difficult to assess the response associated with a change in a single gene. The application of high-throughput screening like the cDNA microarray may provide us with a clear picture of stress-responsive pathways (Park *et al*., 2001; Amundson *et al*., 2001). The cDNA microarray can permit the analysis of gene expression patterns for large numbers of genes, and enables the dissection of molecular events in the radiation response of tumor cells. In this study, we used the cDNA microarray technique to draw a gene expression map in Jurkat T cells with nonsense mutant p53 after γ-irradiation. We made a profile of p53-independent regulation of gene expression in γ-irradiated cells at different doses or times. The expression pattern of radiation-related genes was similar in every type of cells even with wild type

*Correspondence: J-S Seo, Department of Biochemistry and Molecular Biology, Seoul National University College of Medicine, 28 Yongondong, Chongnogu, Seoul 110-799, Korea;
E-mail: jeongsun@snu.ac.kr
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p53. Among them, we found that the time-dependent elevation of phospholipase Cγ2 and cytoplasmic epoxide hydrolase upon γ-irradiation, which might be used as radiation biomarkers as well as molecular target for radiation research.

Results

Responses to γ-irradiation in p53-mutant Jurkat T cells

Jurkat T cells have a nonsense mutation in p53 (Iwamoto *et al*., 1996), but it can show many characteristics of T cells upon various stimulations including ionizing radiation (Moretta *et al*., 1987; Abraham, 2000). We investigated IR-related cellular responses in Jurkat T cells. As shown in Figure 1a, ionizing radiation could slow down the cell growth rate in dose-dependent manner in this cell line. This gross retardation in cell growth after γ-irradiation might be due to cell cycle arrest and/or cell death induced by DNA damage (Figure 1b,c). Jurkat T cells were stuck in the G2/M phase even at 2 Gy IR, which might be mediated by Cdc25C and PCNA (Kawabe *et al*., 2002). In addition, IR-exposed cells showed increased apoptotic fractions upon γ-irradiation (Vigorito *et al*., 1999). These results suggest that in spite of nonfunctional p53, Jurkat T cells can respond to γ-irradiation by growth arrest or apoptosis, which might be similar to any other cells with wild type p53.

Gene expression pattern of responses to γ-irradiation

Jurkat T cells could respond to γ-radiation by arresting cell cycle as well as apoptotic cell death. To understand the cellular responses in this cell without functional p53, we tried to make a time-sequence profile of gene expression in Jurkat T cells after γ-radiation using a DNA microarray containing 2400 human cDNAs. At 4, 12 and 24 h, we collected total RNA from γ-irradiated or control Jurkat T cells and labeled with Cy5-dUTP and Cy3-dUTP, respectively. After hybridization and scanning the cDNA microarray, the data were converted to relative ratio of fold activation changes in γ-irradiated cells against unirradiated control. All expression databases can be browsed at our homepage, http://medicine.snu.ac.kr/parklab. Each value means relative increase or decrease in γ-irradiated cells against unirradiated control. We also used a series of samples that had been irradiated at different doses (4, 8 and 16 Gy). From those time-series or dose-series datasets, we could select the radiation-related responses, which showed time-dependent as well as dose-dependent changes in gene expression. In addition, we adapted the second criteria for selecting radiation-related responses by comparing this profiles to other types of T-cell stimulations like anti-CD3, PHA and ConA treatment.

We normalized the data according to the LOWESS method (Quackenbush, 2001) and analysed 11×2400 datasets using GeneCluster (http://rana.stanford.edu/clustering/) (Eisen *et al*., 1998) or MDS (http://www.netlib.org/mds/) (Dysvik and Jonassen, 2001) for unsupervised data clustering. As shown in Figure 2, 11 sets of experiments were divided into two big clusters by either algorithm. Although most of the genes behaved in a similar manner, the responses after γ-irradiation could be distinguished from other types of T-cell stimulations. We conclude that the cellular responses to ionizing radiation were distinct from the other three nonspecific T cell stimulation responses. From these clustering data, we could make a list of genes that seemed to be changed specifically upon γ-irradiation.

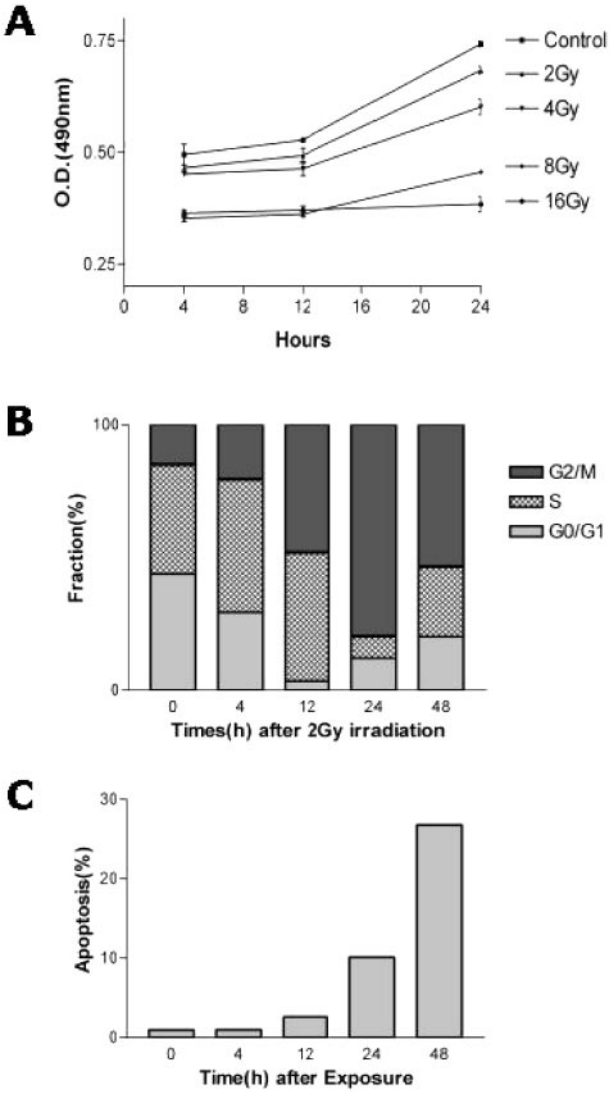

Figure 1 The effect of γ-irradiation on Jurkat T cells. The survival of the cells after treatment with 2–16 Gy of γ-radiation was analysed by MTS assay (**a**). The values represent means of triplicate measurements. After the exposure to 2 Gy γ-irradiation, cell cycle distribution (**b**) and apoptosis (**c**) were analysed by FACS

Expression profile of radiation-related genes

After the raw data had been scaled and normalized, we selected changes specific to IR and listed these in

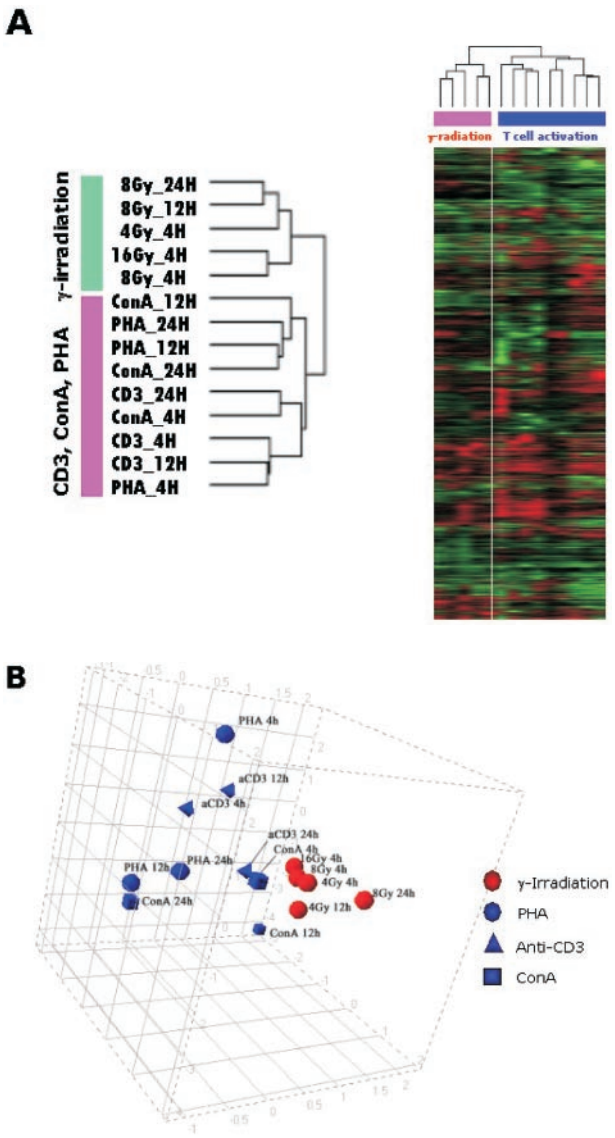

Figure 2 Hierarchical clustering of gene expression data. The expression patterns of 14 independent experiments using γ-irradiated or T-cell activated cells were categorized into two main clusters by unsupervised hierarchical clustering (**a**) and MDS (multidimensional scaling) method (**b**)

activation fold using log2 values (Table 1). Among the transcripts significantly changed by radiation treatment, a number of genes were previously known to be radiation inducible, and another set of genes were newly identified as being IR regulated. T-cell activation by PHA, ConA, or anti-CD3 induced a class of genes related to immune or anti-pathogenic functions. In this category, one could include genes like TCP1 (t-complex 1), SFTPD (surfactant, pulmonary-associated protein D), PRG2 (proteoglycan 2), and TMSB4X (thymosin, beta 4). However, these genes were unchanged in γ-irradiated cells.

Several categories of genes like protein synthesis and signal transduction genes were altered only in γ-irradiated cells. Protein translation was selectively down-regulated after 12 h of IR (Table 1). Although T cell activators did not induce any significant change, IR did induce the down-regulation of translation machinery like EEF1G (eukaryotic translation elongation factor 1 gamma), EIF3S10 (eukaryotic translation initiation factor 3), and several ribosomal protein genes. Phospholipases like PLCG2 (phospholipase Cγ2), PLCD1 (phospholipase Cδ1), and PLA2G2A (phospholipase A2, group IIA) were increased by γ-radiation, but not by T-cell activation. Small G protein-related genes like Rab1B and ARHGDIB (Rho GDP dissociation inhibitor (GDI) beta) were also increased by IR. Among those listed genes, several genes were already known to be regulated in a p53-independent manner. However, the expression of another two genes, thymosin beta 4 and microsomal epoxide hydrolase have been known to be dependent on p53 status. The remainder of the genes were not reported on their dependency of p53. From these results, we found that most of radiation-related genes identified were p53-independent.

Common responses to γ-radiation in various types of cell

We selected 384 radiation-specific genes out of 2400 human cDNAs and fabricated a 'RadChip'. Using this chip, we could reduce the dimension and easily test different cells for their responses to γ-irradiation. Two neuroblastoma cell lines, SK-N-SH and SK-N-AS with wild type p53 were examined their expression patterns upon γ-radiation. The early responses of these tumor cells at 4 h were very similar regardless of their p53 status (Figure 3). Based on this result, we suggest that the 384 genes on RadChip represent general radio-responsive genes. PBMCs were also isolated from healthy volunteers, irradiated with 2 Gy and their responses screened at 4 h. Although normal PBMC's were clustered into a discrete class, the overall patterns were similar to those of the tumor cell lines. From these data, we could conclude that the genes in RadChip could detect radiation exposure in every type of cells even with wild type p53.

Distinct expression profile from genotoxic stress

RadChip contained 384 genes related to IR-related genes, but any other genotoxic stress can induce those genes, either. To test the specificity of RadChip, we compared the expression pattern of γ-irradiated cells with other genotoxic stimuli like cisplatin, UV and doxorubicin. As shown in Figure 3, the expression patterns were quite similar in between cell lines regardless of their p53 status, but the gene expression pattern of γ-irradiated cells were definitely different from other stimuli in cluster analysis (Figure 4). While UV and anti-cancer drugs might show similar responses, we could conclude that 384 genes in RadChip were highly specific to ionizing radiation.

Induction of phospholipase Cγ2 and cytoplasmic epoxide hydrolase by γ-irradiation

We found the induction of several phospholipase genes in RadChip after γ-irradiation. As shown in Table 1,

Table 1 Meaningful expression data was extracted and categorized from a 2.4K cDNA microarray

Category	*Locus ID*	*CONA_12*	*PHA_12*	*aCD3_12*	*8GY_4*	*8GY_12*	*8GY_24*
Apoptosis	BCL2L2	0.299	0.465	1.528	−0.997	−0.631	−1.163
	CST3	2.026	−0.565	−0.432	−0.465	−0.930	−1.262
	AP3K10	−1.229	−0.100	−0.266	0.830	1.296	1.329
	CD63	−0.166	0.066	0.033	1.096	0.963	0.963
Cell proliferation	CDK9	3.222	1.196	2.990	−1.296	0.598	−1.462
	GH1	4.983	−0.565	−0.365	3.554	−1.030	−1.262
	PTHLH	0.830	0.963	−0.465	0.532	0.731	0.997
	EWSR1	0.299	−0.565	−0.565	0.930	1.129	1.595
	RPL10	0.532	0.930	−0.432	0.532	0.830	0.399
Defense	TCP1	0.963	1.362	1.196	−0.498	−0.897	−1.063
	SFTPD	1.096	−0.498	−0.532	−0.664	−1.329	−1.296
	PRG2	7.076	−0.233	1.727	0.000	0.299	0.000
	TMSB4X	0.698	0.698	0.963	−0.698	−0.532	−1.329
	EPHX2	0.000	−1.030	−2.990	2.126	0.432	1.163
DNA	H3F3A	−0.233	0.233	0.199	−0.498	−0.532	−1.462
	RPA2	0.000	0.033	0.033	−0.664	−0.797	−1.129
Metabolism	SLC2A1	−0.100	−0.797	2.890	0.000	3.123	0.000
	MTCYB	0.233	−0.365	−0.066	−0.664	−1.129	−0.864
	GLUD2	0.565	0.631	0.066	−1.163	−0.830	−0.332
	COX6C	1.129	0.465	0.199	−0.930	−0.797	−1.362
	AMPD2	−3.521	0.199	−1.063	4.020	0.664	1.927
	ATP5B	−1.561	−0.897	−0.631	0.598	0.997	1.096
	PGAM1	−1.893	−0.864	−0.698	1.063	1.462	0.897
Signal transduction	CLTC	0.000	0.864	−1.262	2.857	3.023	0.864
	RABIB	−3.853	−0.100	−0.797	0.166	1.794	0.997
	IL3RA	−1.628	0.033	0.532	0.166	1.262	0.465
	AIP	0.100	0.033	−1.063	0.598	0.498	1.096
	ARHGDIB	−1.163	−1.794	0.930	3.853	−0.166	2.890
	PLCG2	0.000	0.000	0.000	0.000	4.451	0.000
	PLCD1	−2.658	0.000	−1.129	1.727	0.000	−1.860
	PLA2G2A	0.000	−1.561	0.000	1.827	−1.329	−1.096
Translation	RPL24	1.262	0.299	0.332	−0.498	−0.930	−1.296
	RPL37A	0.664	−0.299	−0.332	−0.698	−0.797	−1.296
	RPL23A	0.000	0.797	−0.930	−0.532	−0.864	−1.030
	RPS15	−0.133	−0.066	−0.565	−0.532	−0.532	−1.262
	RPL38	1.296	0.432	0.498	−0.631	−1.063	−1.229
	EEF1G	0.000	1.063	−0.332	−0.399	−0.631	−0.664
	EIF3S10	−0.299	0.399	0.133	−0.365	−0.365	−1.229

BCL2L2, BCL2-like 2; CST3, cystatin C (amyloid angiopathy and cerebral hemorrhage); MAP3K10, mitogen-activated protein kinase kinase kinase 10; CD63 antigen (melanoma 1 antigen); CDK9, cyclin-dependent kinase 9 (CDC2-related kinase); GH1, growth hormone 1; PTHLH, parathyroid hormone-like hormone; EWSR1, Ewing sarcoma breakpoint region 1; RPL10, ribosomal protein L10; TCP1, t-complex 1; SFTPD, surfactant, pulmonary-associated protein D; PRG2, proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein); TMSB4X, thymosin, beta 4, X chromosome; EPHX2, epoxide hydrolase 2, cytoplasmic; H3F3A, H3 histone, family 3A; RPA2, replication protein A2 (32 kD); SLC2A1, solute carrier family 2 (facilitated glucose transporter), member 1; MTCYB, cytochrome B; GLUD2, glutamate dehydrogenase 2; COX6C, cytochrome c oxidase subunit Vic; AMPD2, adenosine monophosphate deaminase 2 (isoform L); ATP5B, ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide; PGAM1, phosphoglycerate mutase 1 (brain); CLTC, clathrin, heavy polypeptide (Hc); RAB1B, Rab1B, member RAS oncogene family; IL3RA, interleukin 3 receptor, alpha (low affinity); AIP, aryl hydrocarbon receptor interacting protein; ARHGDIB, Rho GDP dissociation inhibitor (GDI) beta; PLCG2, phospholipase C, gamma 2 (phosphatidylinositol-specific); PLCD1, phospholipase C, delta 1; PLA2G2A, phospholipase A2, group IIA (platelets, cynovial fluid); RPL24, ribosomal protein L24; RPL37A, ribosomal protein L37A; RPL23A, ribosomal protein L23A; RPS15, ribosomal protein S15; RPL38, ribosomal protein L28; EEF1G, eukaryotic translation elongation factor 1 gamma; EIF3S10, eukaryotic translation initiation factor 3, subunit 10 (theta, 150/170 kD)

PLA2, PLCG2, and PLCB4 were induced in irradiated cells by more than twofold. We confirmed the induction of PLCG2 in irradiated cells by RT–PCR (Figure 5a). The induction started at 12 h and sustained until 24 h after γ-radiation. Another gene induced by γ-radiation in Jurkat T cells is EPHX2, which normally scavenges epoxide radicals *in vivo*. The induction of cytosolic epoxide hydrolase was detected from 12 h after γ-radiation by RT–PCR (Figure 5a). The induction of these two genes was conserved in different types of cells with wild type p53. As shown in Figure 5b, PBMC from healthy donor could induce the expression of PLCG2 as well as EPHX2 upon γ-irradiation.

The sensory mechanism of those expressions was supposed to be mediated by NF-κB in p53 null cells. We treated TPCK, a potent inhibitor of NF-κB before γ-irradiation to check the regulation of these two genes. In Figure 5b, IR-induced expression of PLCG2 and EPHX2 was blocked by TPCK in both of Jurkat T cells as well as PBMCs. From the transcript analysis using RT–PCR, we could confirm the up-regulation of two novel radiation-related genes PLCG2 and EPHX2 by NF-κB.

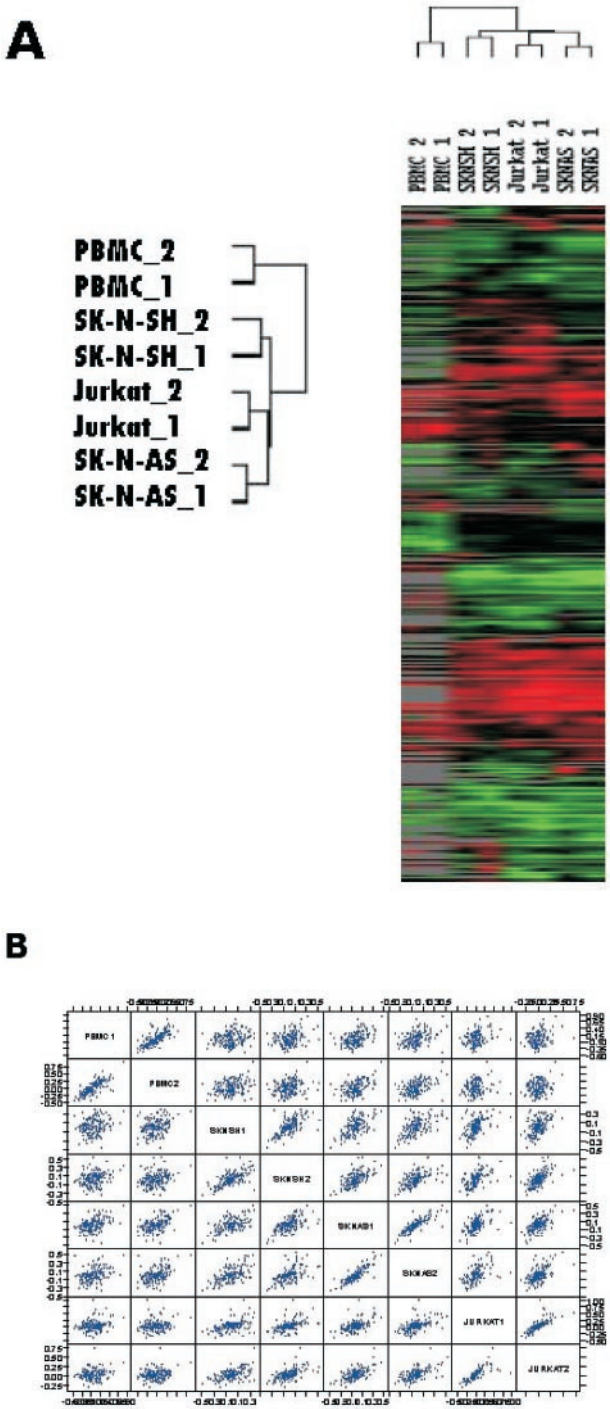

Figure 3 Cluster analysis of four different cell lines after 2 Gy γ-irradiation. Expression patterns of three different types of cancer cells or peripheral blood mononuclear cells (PBMC) after the exposure to 2 Gy γ-irradiation were analysed by unsupervised hierarchical clustering (**a**). The same sets of data were transformed into pairwise plots for four different cell lines (**b**)

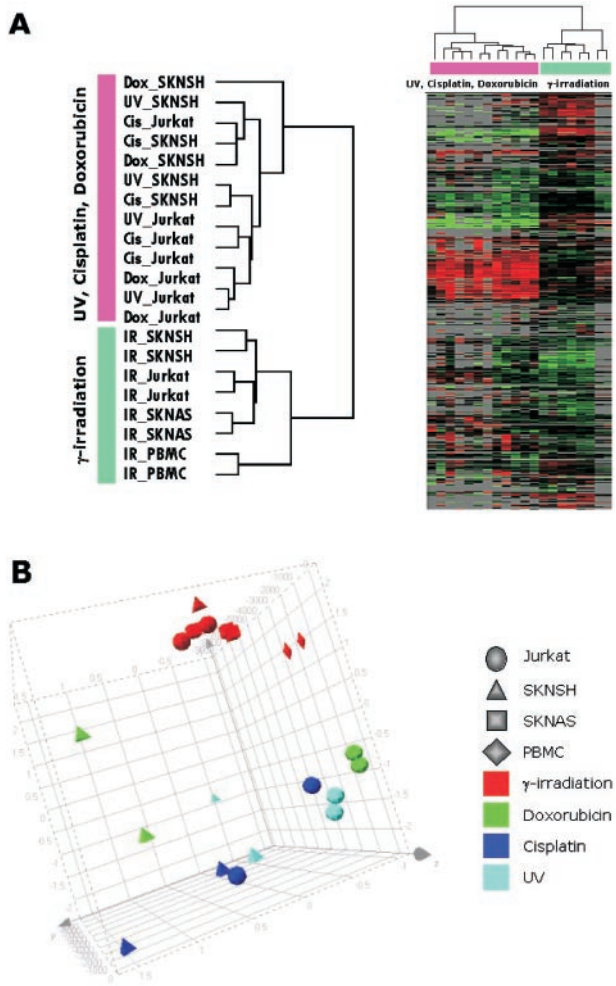

Figure 4 Distinct pattern of responses to γ-irradiation from other types of genotoxic stress. Expression patterns from γ-irradiated cells (Jurkat, SJ-N-SH, SK-N-AS and PBMC) were compared with UV-, doxorubicin- and cisplatin-treated cells (Jurkat and SK-N-SH) by unsupervised hierarchical clustering (**a**) and multidimensional scaling (**b**)

Discussion

In this study, we focused upon drawing a molecular portrait of responses to γ-radiation. We found that the expressions of many genes related to signal transduction and protein synthesis, had changed differentially versus other nonspecific stimuli upon T-cells. From the results obtained, we selected 384 genes and fabricated a RadChip, which contained the radio-responsive genes. The expressions of these genes were very similar in every cell types with different p53 status used in this study. Moreover, this chip could discriminate responses to γ-radiation from other genotoxic stress response. Among those changes, we focused two novel genes induced by γ-radiation, phospholipase Cγ2 and cytoplasmic epoxide hydrolase, which might be regulated by NF-κB.

In vivo effects at low doses to whole organisms cannot be detected before we notify the change in the numbers of white blood cells in the peripheral blood. Amundson *et al*. (2001) recently reported upon gene

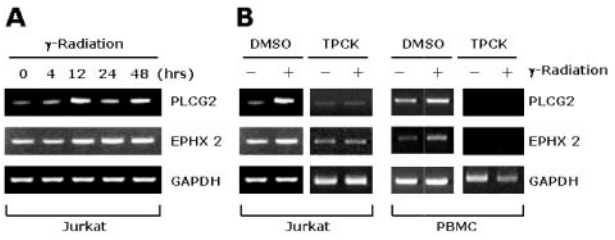

Figure 5 RT–PCR analysis of gene expression in γ-irradiated T cells. The expressions of EPHX2 and PLCG2 after γ-irradiation were checked by RT–PCR in Jurkat T cells (**a**). After exposing cells to γ-radiation, total RNAs from Jurkat T cells were isolated at the indicated times and subjected to reverse transcription to synthesize first strand cDNA (Superscript II, Stratagene). Using specific primers for PLCG2 (sense 5′-cctgccctcaacaagatgttttg-3′ and antisense 5′- tcaaagctgttccgtcccag-3′), EPHX2 (sense 5′-ggaacttcgactttgtcaccgag-3′ and antisense 5′- gaacctctcattcaaccgcctag-3′), GAPDH (sense 5′-ccacccatggcaaattccatggca-3′ and antisense 5′-tctagacggcaggtcaggtccacc-3′) Specific bands of the corresponding size were analysed by agarose gel electrophoresis. Jurkat T cells or PBMCs were also pretreated with DMSO or TPCK prior to γ-irradiation and the expression of two gene were checked (**b**)

profiles in peripheral blood cells after exposure to ionizing radiation using the cDNA microarray technique. Using our list of genes, we were able to select the biological markers for radiation hazard. We tested RadChip in *ex vivo* experiment using γ-irradiated blood samples from several healthy donors, and the pattern was exactly matched with that of other cell lines (data not shown). Although the list of genes was different from previous data (Amundson *et al*., 2001), we could build an assay system for checking exposure to IR using cDNA microarray. The application and further refinement of this RadChip have the potential to advance our understanding of the fields of stress gene response and of radiation biology. Moreover, this technology can be extended beyond simple pair-wise comparisons to applications such as the screening of radiation biohazards, checking radiation susceptibility and for monitoring the effects of radiotherapy.

The molecular context of each cell is critical in determining the fate of IR-exposed cells (Zhan *et al*., 1996). We speculate that the IR-response follows multiple signaling pathways, and that it is likely that the patterns of transcriptional responses usually involve p53, NF-κB, and AP-1 (Liu *et al*., 1996; Smith and Fornace, 1996). p53 seems to be the major actor for DNA damage response, but we focused on the p53-independent pathway because the majority of tumors lost functional p53. While molecular approaches in radiation biology have mainly focused on p53-dependent gene induction (Madden *et al*., 1997; Polyak *et al*., 1997), exclusive focus on such approaches would probably overlook many IR-response genes. Accordingly, we listed and tested the expression of p53-independent signals. As shown in Figure 4, the overall pattern of gene expression was similar in every type of cells even with wild type p53. Although the expression pattern of primary cells was clustered into somewhat distinct group, we could not discriminate between the profiles of p53-wild type and p53-mutant cells. Although doxorubicin, cisplatin and UV can induce DNA damage, expression pattern in RadChip could dissect IR-specific responses among these genotoxic stress. These IR-specific signatures would be used for an important discriminator of stress-response against other types of DNA damage. In addition, our data demonstrates that the mechanisms of cellular responses to chemotherapeutic drugs and radiotherapy are independent, which might support well-known synergistic effects of the combined chemo- and radiotherapy in advanced malignancies (Green *et al*., 2001).

Many lipids or lipid-derived products generated by phospholipases acting on phospholipids in membranes are implicated as mediators and second messengers in signal transduction. Ionizing radiation is known to induce the expression of cytokine receptors and G proteins (Fuks *et al*., 1993). The activation of G proteins, cytokine receptors could be explained in terms of mitogenic/proliferative signaling to promote cellular survival after genotoxic stress. At downstream to those receptors and G proteins signaling, phospholipase C-γ (PLC-γ), protein kinase C (PKC)/Ras/Raf network would probably be involved in the proliferative response (Dent *et al*., 1995; Marais *et al*., 1995; Ghosh *et al*., 1996). In our experiment, we found elevation of phospholipase transcripts after ionizing radiation treatment in Jurkat T cells as well as other cell lines. The upstream promoter of PLCG2 contains SP1, NF1, AP2, SRE and EBF (Kang *et al*., 1996), while our data in Figure 5c suggest the regulation of PLCG2 by NF-κB as well. We presume that the induction of the PLC gene is a kind of adaptive response to increase the proliferative potential (Denekamp, 1973; Kavanagh *et al*., 1995).

Epoxide compounds are xenobiotics produced by the monooxygenase system like cytochrome P450 and metabolic intermediates. These are highly reactive and very unstable in aqueous solution, and might initiate oncogenic mutations. Epoxide hydrolase can hydrate these epoxides and make them soluble in the aqueous phase. Microsomal epoxide hydrolase (EPHX1) exhibits a broad substrate specificity, and soluble epoxide hydrolase might work complementarily to other substartes (Seidegard and Ekstrom, 1997). The transcription regulation of EPHX1 is dependent on p53 (Madden *et al*., 1997), while the transcription of EPHX2 is not clearly known yet. In this study, we suggest that NF-κB might mediate the expression of EPHX2. Recently polymorphisms and mutations of the EPHX2 gene have been highlighted in the context of tumor susceptibility (Kim *et al*., 1997). The genetic variability of expression level has already been reported upon EPHX2. Here, we report for the first time upon the increased expression of the EPHX2 gene in response to radiation exposure, which might confer individual susceptibility to ionizing radiation and other types of genotoxic stress.

We used the protease inhibitor TPCK, a serine protease inhibitor, which blocks activation of NF-κB

by preventing degradation of I-κB (Miyamoto *et al*., 1994). TPCK may not be so specific in its mechanism of action, but it is widely used to inhibit the action of NF-κB. In our experiment, TPCK-treatment could suppress the expression of EPHX2 and PLCG2 upon γ-irradiation (Figure 5b). From these results, we suggested that the expression of EPHX2 and PLCG2 might be regulated in NF-κB-dependent pathway. Previously many reports demonstrated that oxidative stress like ionizing radiation and UV can activate NF-κB by the production of ROS (Schreck *et al*., 1992). When we suppress the production of ROS by NAC, IR could not activate NF-κB. While ROS can be produced in process of T cell activation (Cemerski *et al*., 2002; Pahl, 1999), the expression of PLCG2 an EPHX2 was not detected in our system (Table 1). From this notion, we can raise the possibility that other signaling molecule(s) might work in concerted manner with NF-κB in γ-irradiated cells. To close the complete story for the differential expression of these genes, we need more experiments on this issue using ROS scavengers or signaling modifiers.

Materials and methods

Cell culture and γ-irradiation

Human peripheral blood mononuclear cells were isolated from volunteers as previously described. Briefly, within 30–60 min of drawing the components were separated by centrifugation on a Histopaque (Sigma) density gradient. The buffy coat layers were washed in phosphate-buffered saline and resuspended at a density of 2×10^6/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics at 37°C in a humidified, 5% CO_2 atmosphere. PBMCs were allowed to equilibrate to culture conditions for 45–60 min before treating the γ-irradiation using a ^{137}Cs γ-ray source (J. L. Shepherd and Associates, Inc.) with lead attenuators. Jurkat and SK-N-SH, human tumor cell lines were kept in 10-cm plates or 75 cm^2 flasks in DMEM supplemented with 10% fetal bovine serum and antibiotics and subcultured at a ratio of 1 : 10. Cells were maintained to 5×10^5/ml or 2×10^6/10-cm dish at 16 h prior to γ-irradiation. After equilibrium at 37°C with 5% CO_2 for the indicated periods, total RNAs were extracted for analysis.

Treatment of drugs

Jurkat T cells were incubated with anti-CD3 antibody-coated plates. Phetohemaglutinin (PHA) and concanavalin A (ConA) were added to media at 5 μg/ml. SK-N-SH and Jurkat cells were also treated with doxorubicin (0.34 μM) cisplatin (5 μg/ml). We used Stratalinker (Stratagene) for UV radiation at 100 J/m^2. TPCK was treated at 10 μM concentration in complete media for 12 h prior to γ-radiation.

MTS assay

Cells were divided into 1×10^4/well in 96-well plate and maintained at 37°C in an incubator in 5% CO_2. MTS reagent (CellTiter96R Aqueous Non-Radioactive Cell Proliferation Assay, Promega) was added to each well in PMS solution. After 4 h, absorption at 490 nm was measured.

Cell cycle analysis

Cultured cells were collected and washed with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS). Cell pellets were fixed with 70% ethanol and stained with propidium iodide (PI) solution (PI 5 pg/μl, Rnase 1 μg/ml in PBS). The cell cycle was analysed using a fluorescence-assistant cell sorter (FACS).

cDNA microarray

A total of 40 μg of whole-cell RNA was labeled and hybridized to 2400 element microarrays as described previously (Park *et al*., 2001). In brief, probes were prepared by the PCR amplification of Macrogen clones and arrayed on poly-L-lysine-coated glass slides. Fluorescently labeled cDNA was prepared from control, and γ-irradiated whole-cell RNA was obtained by a single round of labeling using a Macrogen kit in the presence of fluorescent dNTP (Cy3 dUTP or Cy5 dUTP, Amersham). Probes and targets were hybridized together for 16 h in 3 × SSC at 65°C. Hybridized slides were washed at room temperature once in 0.5 × SSC, 0.01% SDS for 5 min, and again in 0.06 × SSC for 5 min. Cy3 and Cy5 fluorescences were scanned using a laser confocal microscope, and images were analysed using an ImaGene v3.0 program to calibrate relative ratios and confidence intervals used for significance determinations. Log activation fold ratios were normalized by nonlinear regression (Quackenbush, 2001).

Data analysis

Fluorescence intensity was processed and measured by ImaGene v4.0 software (BioDiscovery Ltd., Swansea, UK), and data were imported into an Excel (Microsoft) database, with the corresponding gene names, for analysis and normalized by nonlinear regression (Park *et al*., 2001). Hierarchical clustering (Gene Cluster v2.11) and display programs (Tree View v1.50) developed by Eisen *et al*. (1998) were also used for analysis (http://rana.stanford.edu/software). Data was also processed by GeneCluster v1.1 (http://www-genome.wi.mit.edu/mpr/software.html) as developed by Tamayo *et al*. (1999).

RT–PCR

First strand cDNA was synthesized from 1 μg of total RNA with reverse transcriptase and 1 μM of olgo-dT primer. Each cDNA sample was amplified by PCR using specific primers against EPHX2 (sense: 5′-CGT GAC TTG GGA ATG GTC AC-3′, antisense: 5′-CT GTT CCA GTT CAG CCT CAG-3′), PLCG2 (sense: 5′-GAC TCT TCA TCA AAC TAC GAC CC-3′ and antisense: 5′-GG AGT AAA GTT CCT CTT CGC-3′), and GAPDH (sense: 5′-ACC ACA GTC CAT GCC ATC AC-3′ and antisense: 5′-TCC ACC ACC CTG TTG CTG TA-3′). Specific bands with corresponding sizes were analysed by agarose gel electrophoresis.

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