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Differential Expressions of Apoptosis-related Genes in Lung Cancer Cell Lines Determine the Responsiveness to Ionizing Radiation

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Abstract

Radiotherapy would be the choice of treatment for human cancers, because of high cost-effectiveness. However, a certain population of patients shows a resistance to radiotherapy and recurrence. In an effort to increase the efficacy of radiotherapy, many efforts were driven to find the genes causing the unresponsiveness to ionizing radiation. In this paper, we compared the gene expression profiles of two lung cancer cell lines, H460 and H1299, which showed differential responses to ionizing radiations. Each cell were irradiated at 2 Gy, and harvested after 0, 2, 4, 8, 12 and 24 hours to examine the expressions. Two-way ANOVA analysis on time-series experiments of two cells could select 2863 genes differentially expressed upon ionizing radiation among 32,321 genes in microarray ($p < 0.05$). We classified these genes into 21 clusters by SOM clustering according to the interaction between cell types and time. Two SOM clusters were enriched with apoptosis-related genes in pathway analysis. One cluster contained higher levels of phosphatidyl inositol 3-phosphate kinase (PI3K) subunits in H1299, radio-resistant cells than H460, radiosensitive cells. TRAIL receptors were expressed in H460 cells while the decoy receptor for TRAIL was expressed in H1299 cells. From these results, we could characterize the differential responsiveness to ionizing radiation according to their differential expressions of apoptosis-related genes, which might be the candidates to increase the power of radiotherapy.

Keywords: apoptosis, ionizing radiation, lung cancer, radio-sensitivity, radiotherapy

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Introduction

Radiotherapy would play an important role in cancer treatment for long times and widely used to have more than one million patients received radiotherapy every year (Wu *et al*., 2002). In some type of cancers at prostate and cervix, radiotherapy achieved high performance in cure rate in comparison to radical surgery. Moreover, it can be applied to organ conservation surgery in breast cancers and rectal cancers. However, a certain proportion of patients do not respond to radiotherapy, and the mechanism of radiation resistance has been the main target of research to increase the efficacy of radiotherapy.

Although single molecules were targeted to modulate the radiation responses at pre-clinical models, it was hard to be applied to patients. It might be due to the complexity of radiation responses as shown in gene expression profiling experiments (Park *et al*., 2002). Recently miRNA also has been proposed to be important regulator of radiation responses (Weidhaas *et al*., 2007). Gene expression profiling using microarray provided valuable tools for the clinical oncology to determine the prognosis of patients (Lossos *et al*., 2004; Pomeroy *et al*., 2002), the molecular diagnosis (Golub *et al*., 1999) as well as the responsiveness to therapeutics (Snyder and Morgan, 2004).

There have been many reports on the molecular pattern analysis using microarray to understand the chemo- and radio-resistance in cervical cancer (Achary *et al*., 2000; Tewari *et al*., 2005; Wong *et al*., 2006), rectal cancer (Kim *et al*., 2007) and esophageal cancer (Fukuda *et al*., 2004). Most of the studies are to identify differentially expressed genes in patients with different clinical outcomes, which can be applied to the evaluation of prognosis more accurately. Although the conventional parameters like tumor stage and grade can be used to decide optimal cancer therapy, molecular markers would provide valuable information to make clinical decisions (Klopp and Eifel, 2006). Genome-wide analysis on gene expression can predict the clinical consequences more accurately. In addition, the information from gene expression profiling can facilitate the development of biological target for therapeutics by identifying pathways and determining steps contributing to the phenotype.

In this study, we examined the expression profiles of two lung cancer cell lines, which showed differential re-

sponses to ionizing radiation. Especially the time-series data of two cell lines revealed radiation response-related genes from constitutively up- or down-regulated genes in two cells. Especially we focused on the apoptosis pathway in different clusters of radiation response to explain the differential responses upon ionizing radiations.

Methods

Cell culture and ionizing radiation

H460 and H1299 lung cancer cells were purchased from ATCC and maintained in high-glucose Dulbecco's Eagle's medium (DMEM; GIBCO/BRL, Gaithersburg, Md., USA) containing 10% heat-inactivated fetal bovine serum (FBS, GIBCO/BRL) and 50 U/ml gamma-interferon (Gemzyme, Cambridge, MA, USA).

After the exposure to ionizing radiation generated by 4MV linear accelerator (Clinac 4/100, Varian, Palo Alto, CA), cells were harvested at the indicated time. We repeated sets of experiment three times to collect biological triplicates in every sample.

Microarray and data analysis

Samples in each group were harvested in triplicates and total RNAs were extracted by dissolving in TriZol and the purification using Qiagen RNAeasy column (Park *et al.*, 2002). We used GeneChip Human Gene 1.0 ST array from Affymetrix which includes 32,000 human genes. Fluorescence intensity was processed and measured using Exon Microarray Analyzer. Intensity data were imported to an in-house microarray database as described previously (Lee *et al.*, 2006).

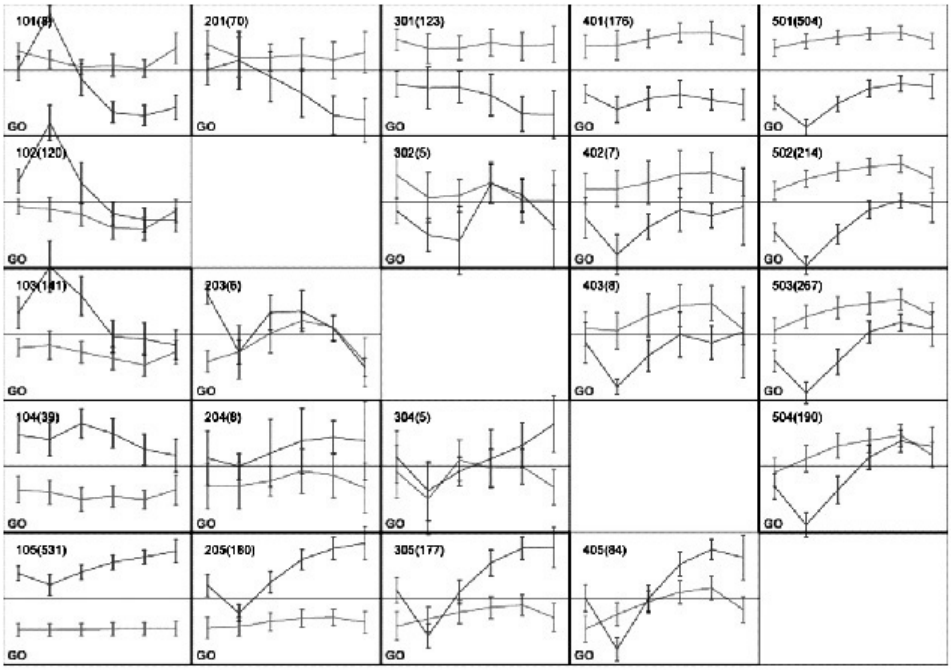

Fig. 1. SOM cluster analysis on 2863 differentially expressed genes.

Table 1. Classification of SOM clusters according to their response patterns upon ionizing radiation

Group	Response pattern*	Cluster (gene No.)
Radio-sensitive (RS), higher in radio-sensitive H460 cells (H460 > H1299)	Constitutive (RSC)	104 (39), 105 (531)
	Early (RSE)	102 (120), 103 (141)
	Late (RSL)	205 (180), 305 (177), 405 (84)
Radio-resistant (RR), higher in radio-resistant H1299 cells (H1299 > H460)	Constitutive (RRC)	301 (123), 401 (176)
	Early (RRE)	501 (504), 502 (214), 503 (267), 504 (190)
	Late (RRL)	201 (70)

*"Response pattern" represents the patterns of transcriptional regulation in H460 cells upon ionizing radiation.

Gene Ontology (GO) and pathway analysis

Differentially expressed genes were further analyzed using DAVID for GO analysis as well as pathway analysis (Dennis *et al*., 2003).

Results and Discussion

To examine the molecular changes upon ionizing radiation, we used H460 and H1299 lung cancer cells, which show different responses to ionizing radiation in the clo-

Table 2. Gene ontology (GO) analysis on the genes in RS clusters

Response pattern	Cluster (gene No.)	GO term	Count	%	p value	Fold change
RSE	102 (120)	Signal transduction	27	25.00	0.00362	1.703
		Inflammatory response	6	5.56	0.00368	5.746
		Response to biotic stimulus	14	12.96	0.01004	2.162
	103 (141)	G-protein coupled receptor protein signaling pathway	17	13.49	0.00093	2.504
		Response to stimulus	28	22.22	0.00485	1.678
		Phosphoinositide-mediated signaling	4	3.17	0.01234	8.234
RSC	104 (39)	Regulation of progression through cell cycle	5	13.16	0.00731	6.096
		Regulation of physiological process	11	28.95	0.04775	1.808
	105 (531)	Regulation of cell proliferation	23	4.60	4.3E-06	3.146
		Humoral immune response	13	2.60	0.00069	3.246
		Enzyme linked receptor protein signaling pathway	13	2.60	0.00203	2.866
		Cell adhesion	30	6.00	0.00232	1.815
		Apoptosis	26	5.20	0.00258	1.907
		Cell differentiation	22	4.40	0.01247	1.781
		Response to stress	38	7.60	0.02858	1.414
		Dephosphorylation	9	1.80	0.02973	2.470
		Amino acid and derivative metabolism	14	2.80	0.03143	1.921
		Secretion	12	2.40	0.03187	2.064
RSL	205 (180)	Catabolism	18	10.40	2.4E-05	3.329
		Cellular lipid metabolism	15	8.67	6.9E-05	3.584
		Cellular carbohydrate metabolism	10	5.78	0.00323	3.298
		Phospholipid biosynthesis	4	2.31	0.01089	8.635
		Positive regulation of protein kinase activity	4	2.31	0.01317	8.050
		Protein biosynthesis	14	8.09	0.01934	2.030
		Glycoprotein metabolism	5	2.89	0.03631	3.984
		Cytokine biosynthesis	3	1.73	0.04197	9.134
		Response to stress	16	9.25	0.04482	1.705
	305 (177)	Vesicle-mediated transport	9	5.17	0.01121	2.943
		Transport	35	20.11	0.01181	1.488
		Small GTPase mediated signal transduction	7	4.02	0.02899	2.995
		Regulation of cell proliferation	7	4.02	0.03906	2.787
		Protein transport	10	5.75	0.04377	2.132
		Wnt receptor signaling pathway	4	2.30	0.05179	4.731
		Carboxylic acid metabolism	9	5.17	0.05512	2.163
		Ubiquitin cycle	9	5.17	0.06438	2.092
		Lipid metabolism	10	5.75	0.07580	1.912
		Intracellular signaling cascade	15	8.62	0.08165	1.597
	405 (84)	Intracellular signaling cascade	11	13.25	0.01106	2.462
		Regulation of transcription	17	20.48	0.01421	1.841
		Negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	4	4.82	0.03354	5.573
		Negative regulation of cellular metabolism	4	4.82	0.04624	4.900
		Protein ubiquitination	4	4.82	0.07009	4.123

nogenic assay to determine the radiation sensitivity (data not shown). As previously reported (Nishizaki *et al*., 2001), H1299 showed higher clonogenic survival upon ionizing radiation. Using these two lung cancer cells, we examined the transcriptomes of each cell at 0, 2, 4, 8, 12 and 24 hours after the exposure using

Table 3. Gene ontology analysis on the genes RR clusters

Response pattern	Cluster (gene No.)	GO term	Count	%	p value	Fold change
RRE	501 (504)	Regulation of metabolism	85	17.71	4.09E-05	1.522
		Transcription, DNA-dependent	74	15.42	1.90E-04	1.515
		Vesicle-mediated transport	20	4.17	4.35E-04	2.503
		Cell cycle	29	6.04	0.00205	1.852
		Ubiquitin cycle	23	4.79	0.00207	2.046
		Chromosome organization and biogenesis	15	3.12	0.00762	2.234
		Protein amino acid phosphorylation	24	5.00	0.01227	1.726
	502 (214)	Cell cycle	17	8.17	9.46E-04	2.549
		Vesicle-mediated transport	11	5.29	0.00211	3.232
		Protein kinase cascade	8	3.85	0.01596	3.043
		Positive regulation of I-kappaB kinase/NF-kappaB cascade	4	1.92	0.03703	5.421
	503 (267)	Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	74	28.35	3.51E-08	1.803
		Ubiquitin cycle	17	6.51	2.67E-04	2.883
		Response to DNA damage stimulus	11	4.21	7.50E-04	3.723
		Protein localization	17	6.51	0.00134	2.481
		Establishment of cellular localization	17	6.51	0.00175	2.417
		Cell cycle	17	6.51	0.00784	2.069
	504 (190)	Protein transport	15	8.15	1.22E-04	3.389
		Ubiquitin cycle	13	7.07	6.95E-04	3.203
		Phosphatidylinositol biosynthesis	2	1.09	0.04564	42.624
RRC	301 (123)	M phase	13	11.30	1.45E-09	11.219
		Chromatin assembly or disassembly	10	8.70	1.10E-07	12.231
		Response to DNA damage stimulus	11	9.57	2.61E-06	7.193
		Cell cycle	17	14.78	3.57E-06	3.998
		Response to endogenous stimulus	11	9.57	4.59E-06	6.751
		Second-messenger-mediated signaling	7	6.09	9.55E-04	6.103
		Regulation of transcription from RNA polymerase II promoter	6	5.22	0.02329	3.656
	401 (176)	M phase	15	9.15	1.12E-09	8.970
		Organelle organization and biogenesis	20	12.20	9.38E-05	2.759
		Transcription from RNA polymerase II promoter	11	6.71	0.00868	2.634
		DNA repair	7	4.27	0.01457	3.509
		Chromosome organization and biogenesis	8	4.88	0.01594	3.040
		Regulation of protein kinase activity	5	3.05	0.02793	4.331
		Response to endogenous stimulus	7	4.27	0.02978	2.977
		Cell proliferation	10	6.10	0.04077	2.162
RRL	201 (70)	Establishment and/or maintenance of chromatin architecture	7	10.77	1.76E-04	8.220
		Cell cycle	10	15.38	6.70E-04	3.979
		Regulation of transcription, DNA-dependent	16	24.62	0.00502	2.104
		Organelle organization and biogenesis	9	13.85	0.00782	3.032
		Regulation of cellular metabolism	16	24.62	0.01734	1.835
		Apoptosis	6	9.23	0.04261	3.055
		DNA repair	4	6.15	0.04591	4.897

microarray. Because two lung cancer cells originated from two different individuals with different genetic backgrounds, there should be lots of discrepancy in transcription profiles. However, if we can compare the time-series expression patterns in parallel, it would be possible to detect the genes related to differential responses to ionizing radiation.

Differentially expressed genes (DEGs) were selected by two-way ANOVA on two time-series data ($p < 0.05$). Then these 2863 DEGs were further classified into 21 clusters according to their expression patterns using SOM (Fig. 1). Fourteen out of 21 clusters containing more than 10 genes could be divided into two groups like RS and RR (Table 1). The level of transcripts in RS clusters are higher in radio-sensitive H460 cells than in H1299 cells, while RR cluster genes are expressed at higher levels in H1299 cells. RS group contained 7 clusters with 1272 genes, which were up-regulated in H460

Table 4. Pathway analysis on DEGs in two-way ANOVA analysis

Response pattern	Cluster	Pathway term	Count	%	p value
RSC	105	HSA00330:Arginine and proline metabolism	8	1.60	0.0079
		HSA04010:MAPK signaling pathway	19	3.80	0.0322
		HSA05060:Prion disease	4	0.80	0.0459
		HSA04610:Complement and coagulation cascades	7	1.40	0.0613
		HSA02010:ABC transporters - general	7	1.40	0.0083
		HSA04512:ECM-receptor interaction	9	1.80	0.0332
		HSA00380:Tryptophan metabolism	8	1.60	0.0701
		HSA04210:Apoptosis	8	1.60	0.0844
		HSA04060:Cytokine-cytokine receptor interaction	18	3.60	0.0308
RSE	103	HSA04060:Cytokine-cytokine receptor interaction	6	4.76	0.0117
RSL	305	HSA00120:Bile acid biosynthesis	3	1.72	0.0591
		HSA00520:Nucleotide sugars metabolism	3	1.72	0.0119
	205	HSA04910:Insulin signaling pathway	6	3.47	0.0658
		HSA00600:Glycosphingolipid metabolism	5	2.89	0.0045
		HSA00512:O-glycan biosynthesis	3	1.73	0.0778
RRE	502	HSA00280:Valine, leucine and isoleucine degradation	5	2.40	0.0011
		HSA04350:TGF-beta signaling pathway	4	1.92	0.0327
		HSA00072:Synthesis and degradation of ketone bodies	2	0.96	0.0640
		HSA00650:Butanoate metabolism	4	1.92	0.0100
		HSA04510:Focal adhesion	6	2.88	0.0305
	501	HSA04620:Toll-like receptor signaling pathway	6	1.25	0.0614
		HSA00770:Pantothenate and CoA biosynthesis	3	0.62	0.0986
		HSA04930:Type II diabetes mellitus	4	0.83	0.0816
		HSA04360:Axon guidance	7	1.46	0.0926
		HSA04910:Insulin signaling pathway	10	2.08	0.0038
		HSA04210:Apoptosis	7	1.46	0.0167
		HSA04120:Ubiquitin mediated proteolysis	4	0.83	0.0775
		HSA04662:B cell receptor signaling pathway	6	1.25	0.0179
		HSA04660:T cell receptor signaling pathway	6	1.25	0.0637
		HSA04650:Natural killer cell mediated cytotoxicity	8	1.67	0.0266
		HSA04070:Phosphatidylinositol signaling system	7	1.46	0.0211
RRC	401	HSA04512:ECM-receptor interaction	4	2.44	0.0619
		HSA04110:Cell cycle	6	3.66	0.0048
	301	HSA01510:Neurodegenerative disorders	3	2.61	0.0330
		HSA04310:WNT signaling pathway	5	4.35	0.0339
		HSA04120:Ubiquitin mediated proteolysis	3	2.61	0.0522
		HSA04110:Cell cycle	7	6.09	0.0003
RRL	201	HSA04010:MAPK signaling pathway	4	6.15	0.0743
		HSA04110:Cell cycle	3	4.62	0.0617

cells. When we examine the responses in H460 cells in detail, RS group could be classified into three classes according to the gene expression patterns like constitutive (RSC), early (RSE) and late (RSL) up-regulation by ionizing radiation. RR group also could be classified as RRC, RRE and RRL, too.

We have characterized each clusters according to their GO terms over-represented in each cluster significantly ($p < 0.05$). As listed in Table 2, RS clusters were enriched with GO terms related to the response to radiation, signal transduction, apoptosis and metabolism. For RR clusters, we could find the GO terms related to cell cycle, DNA damage and apoptosis (Table 3). Using DAVID web-accessible program, we examined the KEGG pathways related to each cluster to understand the time-series data on the cellular responses to ionizing radiation (Table 4). As GO analysis showed the enrichment of terms on biological process, apoptosis

Table 5. Apoptosis-related genes in three different clusters

Response pattern (cluster)	GenBank accession	Gene name
RSC (105)	NM_001621	Aryl hydrocarbon receptor
	NM_032977	Caspase 10, apoptosis-related cysteine peptidase
	NM_001228	Caspase 8, apoptosis-related cysteine peptidase
	NM_001831	Clusterin
	NM_014800	Engulfment and cell motility 1
	NM_003608	G protein-coupled receptor 65
	NM_005347	Heat shock 70kda protein 5 (glucose-regulated protein, 78kda)
	NM_006410	Hiv-1 tat interactive protein 2, 30kda
	NM_000875	Insulin-like growth factor 1 receptor
	NM_005531	Interferon, gamma-inducible protein 16
	NM_000575	Interleukin 1, alpha
	NM_000576	Interleukin 1, beta
	NM_000600	Interleukin 6 (interferon, beta 2)
	NM_000314	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
	NM_017542	Pogo transposable element with krab domain
	NM_005505	Scavenger receptor class b, member 1
	NM_004760	Serine/threonine kinase 17a (apoptosis-inducing)
	NM_002575	Serpin peptidase inhibitor, clade b (ovalbumin), member 2
	NM_003955	Suppressor of cytokine signaling 3
	NM_003238	Transforming growth factor, beta 2
	NM_003844	Tumor necrosis factor receptor superfamily, member 10a
	NM_003842	Tumor necrosis factor receptor superfamily, member 10b
	NM_002546	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
	NM_018647	Tumor necrosis factor receptor superfamily, member 19
	NM_006290	Tumor necrosis factor, alpha-induced protein 3
	NM_005157	V-abl abelson murine leukemia viral oncogene homolog 1
RRL (201)	NM_001168	Baculoviral iap repeat-containing 5 (survivin)
	NM_001924	Growth arrest and dna-damage-inducible, alpha
	NM_016639	Tumor necrosis factor receptor superfamily, member 12a
	NM_002128	High-mobility group box 1
	NM_002466	V-myb myeloblastosis viral oncogene homolog (avian)-like 2
	NM_001065	Tumor necrosis factor receptor superfamily, member 1a
RRE (501)	NM_006218	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
	NM_002736	Protein kinase, camp-dependent, regulatory, type ii, beta
	NM_007236	Calcium binding protein p22
	NM_001165	Baculoviral iap repeat-containing 3
	NM_014602	Phosphoinositide-3-kinase, regulatory subunit 4, p150
	NM_006219	Phosphoinositide-3-kinase, catalytic, beta polypeptide
	NM_016123	Interleukin-1 receptor-associated kinase 4

pathways were significantly changed in both of RS (RSC) and RR (RRE) clusters. In addition, metabolic and signaling pathways were selected in RS group, and cell cycle pathway in RR group.

RSC cluster contained 26 apoptosis-related genes (Table 2), which were over-expressed in H460 cells, but not changed upon ionizing radiation. Caspases (CASP8 and CASP10), interleukins (IL1A, IL1B and IL6), TRAIL receptors (TNFRSF10A and TNFRSF10B) might play an important role in cell death upon ionizing radiation in H460 cells (Table 5). These genes were present in lower levels, and not induced by irradiation in H1299. RRL cluster contained the genes which were up-regulated in H1299 cells, and induced in H460 cells after 8 hours of irradiation. Among 6 genes, Survivin (BIRC5) and GADD45A might be related to radio-resistance of H1299 cells. RRE clusters in RR group contained 7 apoptosis-related genes like PI3K subunits (PIK3CA, PIK3CB and PIK3R4) and IRAK4. Many genes related to apoptosis were selected in two-way ANOVA analysis, and their differential activity to determine the radiation responses could be sorted out by SOM and pathway analysis.

Radiation sensitivity is usually determined by classical clonogenic assay in radiation biology. But it is not easy to establish cell line from the individual patient's specimen and too slow to be used as a routine work. As a result, there is no available clinical method to predict radiation response to radiation therapy. If we can predict individual response rate to radiation therapy, we can modify total dose and fractionation schedule of radiation therapy individually so as to increase therapeutic ratio.

We have examined the gene expression profiles to understand the underlying molecular changes in cells with different radiation sensitivity. From the differentially expressed genes, we can select the biomarkers to discriminate the radio-resistant tumors from radio-sensitive one. Using those genomic biomarkers, we can develop the platform to check the radiation response in radiotherapy patients. Further study is undergoing with patient's tumor specimen.

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