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Classification of Sensitivity or Resistance of Cervical Cancers to Ionizing Radiation According to Expression Profiles of 62 Genes Selected by cDNA Microarray Analysis[1]

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Abstract

To identify a set of genes related to radiosensitivity of cervical squamous cell carcinomas and to establish a predictive method, we compared expression profiles of 9 radiosensitive and 10 radioresistant tumors obtained by biopsy before treatment, on a cDNA microarray consisting of 23,040 human genes. We identified 121 genes whose expression was significantly greater in radiosensitive cells than in radioresistant cells, and 50 genes that showed higher levels of expression in radioresistant cells than in radiosensitive cells. Some of these genes had already known to be associated with the radiation response, such as aldehyde dehydrogenase 1 (*ALDH1*) and X-ray repair cross-complementing 5 (*XRCC5*) ($P<.05$, Mann-Whitney test). The validity of the total of 171 genes as radiosensitivity related genes were certified by permutation test ($P<.05$). Furthermore, we selected 62 genes on the basis of a clustering analysis, and confirmed the validity of these genes with cross-validation test. The cross-validation test also indicates the possibility of making prediction of radiosensitivity for discriminating radiation-sensitive from radiation resistant biopsy samples by predicting score (PS) values calculated from expression values of 62 genes in 19 samples, because the prediction successfully and unequivocally discriminated the radiosensitive phenotype from the radioresistant phenotype in our test panel of 19 cervical carcinomas. The extensive list of genes identified in these experiments provides a large body of potentially valuable information for studying the mechanism(s) of radiosensitivity, and selected 62 genes opens the possibility of providing appropriate and effective radiotherapy to cancer patients.

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Keywords: cervical cancer, radiosensitivity, cDNA microarray, gene expression profiles, classification.

Introduction

Although proper diagnosis and effective treatments for cervical cancers are widely available now [1], this disease is still a leading cause of death for women worldwide [2]. Radiotherapy is a generally effective therapeutic method, particularly for patients with cancers at an advanced stage. However, individual patients may show quite different patterns of response against radiotherapy; some can be cured, but others cannot, and the latter may therefore suffer needlessly from severe side effects. Hence, if treatment is to become more patient specific, the molecular mechanism(s) of radiosensitivity need to be clarified.

Several molecular markers that reflect radiosensitivity have been proposed as the result of studies that have involved, for example, transfection of oncogenes such as *N-ras*, *v-myc* with *H-ras*, and v-*fos* into cultured cells to induce a radioresistant phenotype [3,4]. Activation of c-*raf*-1 has been positively correlated with radioresistance of head and neck squamous cell carcinomas [5], and certain cell cycle- and apoptosis-related genes also have been correlated with radiosensitivity; e.g., loss or dysfunction of p16 renders melanoma cells resistant to ionizing radiation, whereas expression of exogenous wild-type p16 and p21 in glioblastoma cells can induce radiosensitivity [6,7]. Transfection- or radiation-induced expression of Bcl-2 proteins, which regulate apoptosis, into pro-myeloid cells has introduced a radioresistant phenotype [8,9]; furthermore, expression of Bax, when induced by gamma irradiation, confers radiosensitivity on lymphoid cells, small intestinal epithelial cells [10] and cervical cancer cells [9]. However, although such discoveries have brought partial understanding of the molecular mechanisms responsible for cellular radiosensitivity, the whole picture remains to be clarified.

Because the complex mechanism of radiosensitivity cannot be explained by a small number of genes, we need to collect genome-wide information about all the genes involved. To that end, we recruited a newly developed

Abbreviations: PS, predictive scoring; aRNA, amplified RNA; CR, complete response; NC, no change; *ALDH1*, aldehyde dehydrogenase; *XRCC5*, X-ray repair cross-complementing 5; *RBP1*, retinol-binding protein 1; DSB, double-strand break

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technique, cDNA microarray [11], which provides high-throughput analysis of expression profiles by means of small-array slides spotted with cDNAs [12-15].

Here we report a genome-wide cDNA microarray analysis of 23,040 human cDNAs, in biopsy samples from 19 cervical squamous carcinomas (9 of them radiosensitive and 10 radioresistant, classified according to tumor-suppression ratios). We identified 171 genes that were differentially expressed between the two groups; of those, 121 showed elevated expression and 50 showed decreased expression in radiosensitive tumor cells relative to their expression levels in radioresistant cells. In addition, further selected 62 genes showed feasibility of predicting the radiosensitivity of cervical squamous cell carcinomas. These results not only disclose the complex nature of radiosensitivity as regards the response of cervical squamous cell carcinomas to ionizing radiation, but also provide information that should identify novel targets for efforts to expand the effectiveness of radiotherapy.

Materials and Methods

Tissue Samples

Cervical squamous cell carcinoma tissues were obtained with informed consent from 19 patients who underwent biopsy before radiotherapy at Kansai Medical University, and snap-frozen at -80°C. All cases were at stages IIB to IVB, and were papillomavirus (HPV)-positive. We observed the population of tumor cells in all biopsy samples was over 90% by hematoxylin and eosin staining. Methods for typing of HPV and establishing p53 status were described previously [9].

Radiation Treatments

All 19 patients were treated with radiotherapy after sampling. A total of 30.6 Gy was provided to the whole pelvis, plus an additional dose to parametria with central shielding to complete 52.2 Gy, along with ^{192}Ir high dose-rate intracavitary brachytherapy. Details have been described elsewhere [9]. Effects of the therapy, including local failure, were checked a month after treatment. Nine patients revealed 100% reduction in tumor size and the remaining 10 showed 0% to 40% reduction (Table 1). The former were classified as a radiosensitive group (complete response; [CR]) and the latter as a radioresistant group (no change [NC]).

RNA Extraction and Amplification

Total RNAs were extracted from each specimen by TRIZOL (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The extracted RNAs were treated for 1 hour at 37°C with 10 U of DNase I (Nippon Gene, Japan) in the presence of 1 U of RNase inhibitor (TOYOBO, Osaka, Japan), to remove any contaminating genomic DNA. After inactivation of DNase at 70°C for 10 minutes, the RNAs were purified with phenol–chloroform–isoamyl alcohol (Gibco BRL, Grand Island, NY) and then precipitated by ethanol. Next, all DNase I–treated RNAs were subjected to T7-based RNA amplification as described previously [16]. Two rounds of amplification yielded 65 to 152 μg of amplified RNA (aRNA) from each sample. As a control for comparing gene expression profiles between the CR and NC groups on the microarray, we performed two rounds of T7-RNA amplification using a mixture of poly A^+ RNAs from tissues of 12 normal human organs (brain, heart, liver, skeletal muscle, small intestine, spleen, placenta, thyroid, fetal brain, fetal kidney, fetal lung, and fetal liver) purchased from Clontech (Palo Alto, CA) as a control.

Microarray Design, Production, and Hybridization

We selected 23,040 cDNA clones from the UniGene database of the National Center for Biotechnology Information (Bethesda, MD) (build #131). Our cDNA microarray was constructed essentially as described previously [13]; 2.5 μg of aRNA from each cervical carcinoma was labeled with Cy5-dCTP and the control aRNA was labeled with Cy3-dCTP by a protocol described elsewhere [13]. Hybridization, washing, and scanning were carried out according to published methods [13].

Data Analysis and Selection of Differentially Expressed Genes

Signal intensities of Cy3 and Cy5 from the 23,040 spots were quantified by the Array vision software (Amersham Biosciences, Piscataway, NJ) and normalized as described previously [12]. In the quantification step, local background correction method was adopted. Because the data were unreliable when intensities fell below 2.5×10^5 relative fluorescent units or signal to noise ratios were below 3.0

Table 1. Clinical Characteristics of 19 Cervical Squamous Carcinoma Samples.

Sample No.	Stage at Diagnosis*	Initial Response†	Tumor Suppression Ratio in Size (%)	Age	Status at p53‡
16	IIIB	CR	100	69	WT
17	IIIB	CR	100	65	WT
23	IIIB	CR	100	67	WT
47	IVA	CR	100	67	WT
74	IIIB	CR	100	77	WT
75	IIIB	CR	100	32	WT
81	IVB	CR	100	55	WT
83	IIIB	CR	100	59	WT
89	IIIB	CR	100	63	WT
31	IIIB	NC	40	53	WT
35	IIIB	NC	19	47	WT
39	IIIB	NC	0	70	WT
45	IIIB	NC	40	59	WT
52	IVB	NC	5	56	WT
53	IVB	NC	5	63	WT
55	IVB	NC	0	76	WT
85	IVB	NC	0	52	WT
87	IVB	NC	0	84	WT
96	IVB	NC	10	53	WT

*Tumors were staged according to International Federation of Gynecology and Obstetrics criteria.
†*CR*, tumor suppression ratio of 100%. *NC*, 50% suppression to +25% growth a month after radiotherapy.
‡Mutational status of p53. *WT*, wild type.

for both Cy3 and Cy5, genes corresponding to those spots were not investigated any further. To investigate genes that were clearly expressed differently between CR and NC tumors, the Mann-Whitney test was applied based on gene-expression values of X, where X=the Cy5/Cy3 signal intensity ratio for each gene and for each sample. U values for Mann-Whitney test were calculated for each gene. Genes with U values lower than 20 or greater than 70 were selected ($P<.05$ for comparing 9 CR samples vs. 10 NC samples). Because the U values were calculated for each sample in the CR group against each sample in the NC group for each gene based on each X value, genes that have U value lower than 20 indicate upregulated in the CR group compared to the NC group. However, genes that have U value more than 70 indicate upregulated in the NC group compared to the CR group. 297 genes were upregulated in the CR group and 132 genes were upregulated the in NC group. However, because more than half of these genes have small differences in expression level between CR and NC group, the difference might be caused by data fluctuation. Therefore, genes showing differences more than double the median expression value between the two groups ($\mu X_{CR}/\mu X_{NC} \leqq 0.5$ or $\geqq 2.0$, where μX_{CR} and μX_{NC} indicate median X values for the CR or NC group, respectively) were defined as radiosensitivity (or radioresistance) related genes. A total of 171 genes were selected (121 were significantly greater in radiosensitive cells than in radioresistant cells, and 50 were higher levels of expression in radioresistant cells than in radiosensitive cells).

Permutation Test

To further evaluate the validity of 171 genes selected by Mann-Whitney tests, permutation test was performed as described previously [18], and the probabilities of the genes to be correlated to group distinction, Ps, were also estimated. When each gene was represented by expression vector $v(g)=(X_1, X_2, \ldots, X_{19})$, where X_i denotes the expression level of gene of the ith sample in the initial set of samples, idealized expression patterns were represented by $c=(c_1, c_2, \ldots, c_{19})$, where $c_i=+1$ or 0 according to whether the ith sample belongs to the CR or NC group. The correlation between a gene and a group distinction P_{gc} was defined as follows: i.e., $P_{gc}=(\mu_{CR}-\mu_{NC})/(\sigma_{CR}+\sigma_{NC})$, where μ_{CR} (μ_{NC}) and σ_{CR} (σ_{NC}) indicate the means and the standard deviations of $\log_2 X$ of the gene "g" for each sample in newly defined CR (NC) group. Permutation test was conducted by permuting the coordinates of c 10,000 times. During every permutation, the correlation values, P_{gc}s, were calculated. These procedures were performed 10,000 times, repeatedly. On the hypothesis that these obtained 10,000 A_g values show ideal normal distribution, P values, which imply probability of the genes to classify the two groups by chance was estimated for each selected 171 gene.

Hierarchical Clustering

These 171 genes were subjected to a hierarchical-clustering protocol using "Cluster" and "Tree view" software written by M. Eisen [17]. Before applying the clustering algorithm, gene-expression values (X) for each gene in each of the 19 samples were log-transformed ($\log_2 X$). After that all values in each row and/or column of data were multiplied by scale factor S, so that the sum of the squares of the values in each row and column was 1.0 (a separate S is computed for each row/column). Next, row-wise and column-wise median values were subtracted from the values in each row and/or column data, so that the median value of each row and/or column is 0. Hierarchical clustering was performed using distance metrics based on Pearson correlation and adopting Average Linkage Clustering method.

Cross-Validation Test

The selected 62 genes from 171 genes by clustering experiment were subjected to cross-validation test. Among the total 19 samples tested above, one sample was withheld as test sample and the other 18 samples were used for building predictor according to the method as described previously [18]. Next, predictive score (PS) for test sample was calculated as follow; $PS=\sum V_g$, where $V_g=A_g'(X_g-B_g)$, $A_g'=(\mu_{CR}'-\mu_{NC}')/(\sigma_{CR}'+\sigma_{NC}')$, and $B_g=(\mu_{CR}'+\mu_{NC}')/2$; μ_{CR}' (μ_{NC}') and $\sigma_{CR}'(\sigma_{NC}')$ indicates the means and standard deviations of $\log_2 X$ of the gene "g" for each sample in the CR (NC) group defined as predictor samples. X_g denotes the $\log_2 X$ of the gene "g" for test sample. Finally, the predictor sample and test sample were changed and then PS for new test sample was calculated. This process was performed 19 times, repeatedly.

Results

Identification of Genes Responding to Radiation

We performed cDNA microarray analysis of gene expression in 19 cervical-cancer materials, of which 9 were radiation sensitive and 10 were radiation resistant on clinical grounds. By means of the Mann-Whitney test ($P<.05$) and subsequent procedures (see Materials and Methods section), we selected a total of 171 genes (including 74 ESTs) as being differently expressed between CR (complete response) and NC (no change) groups. Of those 171 genes, 121 (including 62 ESTs) revealed increased expression, and 50 (including 12 ESTs) showed decreased expression, in carcinomas belonging to the CR group compared with the NC group (Table 2, A and B). Genes involved in adipogenesis and in the MAP kinase pathway were significantly upregulated in the CR group compared to the NC group; the former included aldehyde dehydrogenase 1 (*ALDH1*), and retinol-binding protein 1 (*RBP1*; Figure 1*A*). The latter included mitogen-activated protein kinase kinase kinase 2 (*MAP3K2*), G protein beta subunit-like (*GBL*), and *RAB5C* (a member of the RAS oncogene family). However, genes that are considered to be associated with repair of breaks in double-stranded DNA, including X-ray repair cross-complementing 5 (*XRCC5*; Figure 1*B*) were downregulated in the CR group relative to

Table 2. Genes Showing Different Expression Between CR and NC Groups.

A. Genes Showing Relatively Higher Expression in Radiosensitive Carcinoma Cells than in Radioresistant Cells

Category	Unigene ID (build #131)	Gene Symbol	Gene Name	U	CR/NC	P	Locus
DNA repair	Hs.3248	MSH6	mutS (E. coli) homolog 6	13	2.0	0.003	2p16
Signal transduction	**Hs.76578**	**PIAS3**	**protein inhibitor of activated STAT3**	**10**	**4.5**	**0.000**	**1q21**
	Hs.29203	GBL	G protein beta subunit-like	11	2.8	0.044	16
	Hs.479	RAB5C	RAB5C, member RAS oncogene family	12	2.8	0.001	17q21.2
	Hs.28827	MAP3K2	mitogen-activated protein kinase kinase kinase 2	12	2.4	0.002	2
	Hs.85155	BRF1	butyrate response factor 1 (EGF-response factor 1)	14	2.4	0.003	14q22–q24
	Hs.74615	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	14.5	3.3	0.023	4q11–q13
	Hs.77439	PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta	15	5.9	0.009	7q22–q31.1
	Hs.83070	GRB14	growth factor receptor-bound protein 14	17	9.8	0.042	2q22–q24
Transcription	Hs.66394	RNF4	ring finger protein 4	6	5.1	0.000	4p16.3
	Hs.8858	**BAZ1A**	**bromodomain adjacent to zinc finger domain, 1A**	**9**	**5.0**	**0.031**	**14q12–q13**
	Hs.155321	SRF	serum response factor (c-fos serum response element-binding transcription factor)	9	2.0	0.038	6pter–6q15
	Hs.289068	**TCF4**	**transcription factor 4**	**15**	**2.2**	**0.048**	**18q21.1**
	Hs.760	GATA2	GATA-binding protein 2	15	2.0	0.011	3q21
	Hs.316	**DDX6**	**DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 6**	**16**	**3.3**	**0.009**	**11q23.3**
	Hs.301963	HOXD8	homeo box D8	17	3.3	0.038	2q31–q37
	Hs.228059	TIF1B	KRAB-associated protein 1	19	2.0	0.029	5
Adipogenesis	**Hs.76392**	**ALDH1**	**aldehyde dehydrogenase 1, soluble**	**15.5**	**14.1**	**0.018**	**9q21**
	Hs.101850	**RBP1**	**retinol-binding protein 1, cellular**	**19**	**2.1**	**0.018**	**3q23**
Cytoskeleton	Hs.7645	FGB	fibrinogen, B beta polypeptide	9	4.4	0.018	4q28
	Hs.75279	LAMA2	laminin, alpha 2 (merosin, congenital muscular dystrophy)	10	5.2	0.040	6q22–q23
	Hs.75445	SPARCL1	SPARC-like 1 (mast9, hevin)	15	4.0	0.004	7
	Hs.97266	PCDH18	protocadherin 18	16	5.9	0.006	
	Hs.11494	FBLN5	fibulin 5	16	4.0	0.008	14q32.1
	Hs.6441	TIMP2	tissue inhibitor of metalloproteinase 2	17	2.8	0.033	17q25
	Hs.79914	**LUM**	**lumican**	**17**	**2.2**	**0.035**	**12q21.3–q22**
	Hs.108896	LOC51084	lambda-crystallin	18	4.7	0.022	13cen3q14.2
	Hs.20072	MIR	myosin regulatory light-chain interacting protein	19	2.4	0.015	6p23–p22.3
Immune system	Hs.1244	CD9	CD9 antigen (p24)	8	2.2	0.001	12p13
	Hs.74631	BSG	basigin	8	2.2	0.001	19p13.3
	Hs.502	ABCB3	ATP-binding cassette, subfamily B (MDR/TAP), member 3	11	2.1	0.009	6p21.3
	Hs.24395	SCYB14	small inducible cytokine subfamily B (Cys-X-Cys), member 14 (BRAK)	17	5.0	0.007	5q31
Proteolysis	**Hs.173091**	**UBL3**	**ubiquitin-like 3**	**15**	**2.5**	**0.016**	**13q12–q13**
	Hs.75275	**UBE4A**	**ubiquitination factor E4A (homologous to yeast UFD2)**	**19**	**3.8**	**0.047**	**11**
Tumor related	Hs.81988	DAB2	disabled (Drosophila) homolog 2 (mitogen-responsive phosphoprotein)	16	2.4	0.003	5p13
	Hs.75462	BTG2	BTG family, member 2	16	2.1	0.028	1q32
Peptide hormone	Hs.134932	UCN	urocortin	9	11.3	0.017	2p23–p21
Others and ESTs	Hs.150926	FPGT	fucose-phosphate guanylyltransferase	10	2.1	0.009	1
	Hs.74566	DPYSL3	dihydropyrimidinase-like 3	18	2.6	0.015	5q32
	Hs.101735	DKFZP564J102	DKFZP564J102 protein	19	3.0	0.042	4
	Hs.74571	ARF1	ADP-ribosylation factor 1	19	2.9	0.015	1q42
	Hs.56874	HSPB7	heat shock 27-kDa protein family, member 7 (cardiovascular)	4	2.5	0.005	1p36.23–p34.3
	Hs.74376	NOE1	olfactomedin-related ER localized protein	7	22.4	0.028	9
	Hs.112569	GAN	giant axonal neuropathy (gigaxonin)	12	2.6	0.001	16q24.1
	Hs.7535	LOC55871	COBW-like protein	13	2.3	0.001	2
	Hs.20597	LCP	host cell factor homolog	13	2.0	0.002	
	Hs.24948	SNCAIP	synuclein, alpha interacting protein (synphilin)	14	7.0	0.003	5q23.1–q23.3
	Hs.108725	LOC51660	HSPC040 protein	15	3.0	0.005	6
	Hs.49912	LOC55895	22-kDa peroxisomal membrane protein-like	15	2.7	0.015	
	Hs.78103	NAP1L4	nucleosome assembly protein 1-like 4	17	7.0	0.009	11p15.5
	Hs.111779	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	17	2.2	0.020	5q31.3–q32
	Hs.75887	COPA	coatomer protein complex, subunit alpha	17	2.0	0.028	1q23–q25
	Hs.129872	**SPAG9**	**sperm-associated antigen 9**	**17**	**2.9**	**0.001**	**17**
	Hs.62041	NID	nidogen (enactin)	17	11.3	0.032	1q43
	Hs.83834	CYB5	cytochrome b-5	18	3.6	0.040	18q23
	Hs.29981	**SLC26A2**	**solute carrier family 26 (sulfate transporter), member 2**	**19**	**2.8**	**0.001**	**5q31–q34**

Table 2. (*continued*)

Category	Unigene ID (build #131)	Gene Symbol	Gene Name	U	CR/NC	P	Locus
	Hs.17930	**BING4**	**BING4**	**19**	**2.9**	**0.000**	**6p21.3**
	Hs.6113	STAU	staufen (Drosophila, RNA-binding protein)	19	2.0	0.002	20q13.1
	Hs.11951	ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1	19.5	>50	0.047	6q22–q23
	Hs.172870		ESTs	4	>50	0.000	
	Hs.29664		Human DNA sequence from clone 682J15 on chromosome 6p11.22.3.	6	15.1	0.001	
	Hs.12365	**KIAA1427**	**KIAA1427 protein**	**7**	**>50**	**0.004**	
	Hs.107515		ESTs	7	3.5	0.000	
	Hs.40583		***Homo sapiens* clone TCBAP1028 mRNA sequence**	**7**	**4.3**	**0.004**	
	Hs.115315		ESTs	7	2.2	0.037	
	Hs.176092		**ESTs, moderately similar to myosin-binding protein H [H. sapiens]**	**7**	**7.5**	**0.000**	
	Hs.125291		**ESTs**	**7**	**10.0**	**0.000**	
	Hs.85053		*H. sapiens* clone 24440 mRNA sequence	8	11.2	0.000	
	Hs.188228		*H. sapiens* cDNA FLJ11003 fis, clone PLACE1002851	9	4.0	0.002	
	Hs.296772		**Human DNA sequence from clone RP1-292B18**	**10**	**4.1**	**0.001**	
	Hs.83724		**Human clone 23773 mRNA sequence**	**10**	**6.8**	**0.033**	
	Hs.124558		**EST**	**10**	**2.8**	**0.008**	
	Hs.120399		**ESTs**	**10**	**8.2**	**0.016**	
	Hs.116464		**ESTs**	**11**	**3.4**	**0.014**	
	Hs.281434		***H. sapiens* cDNA FLJ14028 fis, clone HEMBA1003838**	**11**	**2.9**	**0.031**	
	Hs.191271		**ESTs**	**11**	**5.5**	**0.003**	
	Hs.26676	FLJ10850	hypothetical protein FLJ10850	11	4.8	0.004	20pter–20q12
	Hs.292162		ESTs	11	>50	0.007	
	Hs.181304	13CDNA73	putative gene product	11	>50	0.000	13
	Hs.98265		ESTs	11	10.9	0.000	
	Hs.126857		H. sapiens cDNA FLJ12936 fis, clone NT2RP2005018	11	20.5	0.014	
	Hs.95071		ESTs	11.5	>50	0.001	
	Hs.110373		ESTs	12	4.7	0.007	
	Hs.114453		**ESTs**	**12**	**2.6**	**0.005**	
	Hs.15725	LOC51278	hypothetical protein SBBI48	12	2.7	0.003	1p36.13q41
	Hs.291979		**ESTs, Highly similar to pre-mRNA splicing SR protein rA4**	**12**	**4.6**	**0.019**	
	Hs.113082	KIAA0443	KIAA0443 gene product	13	6.3	0.019	X
	Hs.116117		EST	13	2.2	0.003	
	Hs.24391		*H. sapiens* cDNA FLJ13612 fis, clone PLACE1010833	13	5.9	0.034	
	Hs.23120		*H. sapiens* cDNA: FLJ21421 fis, clone COL04123	13	2.1	0.007	
	Hs.116585		ESTs	13	2.0	0.014	
	Hs.49476		*H. sapiens* clone TUA8 Cri-du-chat region mRNA	14	10.8	0.010	
	Hs.112745		EST	14	2.1	0.029	
	Hs.21851		*H. sapiens* cDNA FLJ12900 fis, clone NT2RP2004321	14	2.7	0.001	
	Hs.13809		**ESTs**	**14**	**2.8**	**0.007**	
	Hs.61268		ESTs	14	>50	0.032	
	Hs.8469		**ESTs**	**15**	**2.0**	**0.049**	
	Hs.11365		*H. sapiens* cDNA FLJ12145 fis, clone MAMMA1000395	15	2.1	0.006	
	Hs.107812		ESTs, Weakly similar to SPOP [H. sapiens]	15	2.8	0.027	
	Hs.25329		ESTs	15	4.4	0.033	
	Hs.178730		ESTs	16	2.3	0.000	
	Hs.112607		ESTs	16	2.6	0.023	
	Hs.27497		*H. sapiens* cDNA FLJ11756 fis, clone HEMBA1005595	16	2.5	0.002	
	Hs.29356		ESTs	16	2.0	0.024	
	Hs.158688	**IF2**	**KIAA0741 gene product**	**16**	**4.6**	**0.042**	**2**
	Hs.23617	FLJ20531	hypothetical protein FLJ20531	17	2.8	0.041	
	Hs.44159	**LOC51105**	**CGI-72 protein**	**17**	**13.7**	**0.003**	**8**
	Hs.23650		ESTs, Weakly similar to AAB47496 NG5 [H. sapiens]	17	3.3	0.041	
	Hs.133081		ESTs, Weakly similar to hypothetical protein [*H. sapiens*]	17	24.6	0.011	
	Hs.191379		**ESTs**	**17**	**6.1**	**0.003**	
	Hs.72363		*H. sapiens* mRNA for FLJ00116 protein, partial cds	17.5	>50	0.038	
	Hs.173094		*H. sapiens* mRNA; cDNA DKFZp564H142 (from clone DKFZp564H142)	17.5	6.0	0.006	
	Hs.179891		ESTs, Weakly similar to prolyl 4-hydroxylase alpha subunit [*H. sapiens*]	18	2.5	0.005	
	Hs.22860		ESTs	18	2.3	0.011	

(continued on next page)

Table 2. (*continued*)

Category	Unigene ID (build #131)	Gene Symbol	Gene Name	U	CR/NC	P	Locus
	Hs.12867		ESTs	18	2.3	0.005	
	Hs.200332	**FLJ20651**	**hypothetical protein FLJ20651**	**18**	**3.8**	**0.004**	**9p24.1–9q22.33**
	Hs.273186	LOC56997	hypothetical protein, clone Telethon(Italy_B41)_Strait02270_FL142	18	2.8	0.041	1
	Hs.30643		ESTs	18	5.7	0.018	
	Hs.11805		ESTs	19	3.1	0.038	
	Hs.22505	FLJ10159	hypothetical protein FLJ10159	19	2.7	0.025	6
	Hs.127407		**ESTs**	**19**	**5.6**	**0.011**	

B. Genes Showing Relatively Higher Expression in Radioresistant Carcinoma Cells than in Radiosensitive Cells

Category	Unigene ID (build #131)	Gene Symbol	Gene Name	U	CR/NC	P	Locus
DNA repair	**Hs.84981**	**XRCC5**	**X-ray repair complementing defective repair in Chinese hamster cells 5**	**73**	**0.39**	**0.034**	**2q35**
Signal transduction	**Hs.155924**	**CREM**	**cAMP responsive element modulator**	**71**	**0.48**	**0.020**	**10p12.1–p11.1**
	Hs.118520	**LOC55970**	**G-protein gamma2 subunit**	**73**	**0.40**	**0.015**	**1**
	Hs.34780	DCX	doublecortex; lissencephaly, X-linked (doublecortin)	74	0.48	0.012	Xq22.3–q23
	Hs.7138	**CHRM3**	**cholinergic receptor, muscarinic 3**	**77**	**0.50**	**0.034**	**1q41–q44**
	Hs.250857	**CAMK2G**	**calcium/calmodulin-dependent protein kinase II gamma**	**85**	**0.41**	**0.009**	**10q22**
Transcription	**Hs.168005**	**TIF1GAMMA**	**transcriptional intermediary factor 1 gamma**	**72**	**0.38**	**0.000**	**1p13.1**
	Hs.21771	WHSC2	Wolf-Hirschhorn syndrome candidate 2	73	0.47	0.021	4p16.3
	Hs.172280	**SMARCC1**	**SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin**	**74**	**0.38**	**0.005**	**3p23–p21**
	Hs.110457	WHSC1	Wolf-Hirschhorn syndrome candidate 1	78	0.45	0.005	4p16.3
	Hs.78580	DDX1	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 1	73	0.32	0.032	2p24
Translation	Hs.129673	EIF4A1	eukaryotic translation initiation factor 4A, isoform 1	78	0.43	0.001	17p13
Glycolysis	**Hs.2795**	**LDHA**	**lactate dehydrogenase A**	**75**	**0.39**	**0.036**	**11p15.4**
Cytoskeleton	Hs.821	BGN	biglycan	71	0.24	0.026	Xq28
	Hs.172928	COL1A1	collagen, type I, alpha 1	74	0.46	0.007	17q21.3–q22
	Hs.90408	**NEO1**	**neogenin (chicken) homolog 1**	**78**	**0.48**	**0.006**	**15q22.3–q23**
Immune system	**Hs.516**	**CCR1**	**chemokine (C–C motif) receptor 1**	**71**	**0.28**	**0.039**	**3p21**
	Hs.198253	**HLA-DQA1**	**major histocompatibility complex, class II, DQ alpha 1**	**72.5**	**0.32**	**0.007**	**6p21.3**
	Hs.75498	**SCYA20**	**small inducible cytokine subfamily A (Cys–Cys), member 20**	**73**	**0.18**	**0.041**	**2q33–q37**
	Hs.833	**ISG15**	**interferon-stimulated protein, 15 kDa**	**78**	**0.25**	**0.042**	**1**
Proteolysis	**Hs.61153**	**PSMC2**	**roteasome (prosome, macropain) 26S subunit, ATPase, 2**	**73**	**0.49**	**0.032**	**7q22.1–q22.3**
Apoptosis	**Hs.93213**	**BAK1**	**BCL2-antagonist/killer 1**	**73**	**0.36**	**0.006**	**6p21.3**
	Hs.278602	API5	apoptosis inhibitor 5	74	0.46	0.013	11
Others and ESTs	Hs.75593	UROS	uroporphyrinogen III synthase	71	0.13	0.041	10q25.2–q26.3
	Hs.108196	**LOC51659**	**HSPC037 protein**	**71**	**0.37**	**0.027**	**16**
	Hs.64595	AASDHPPT	aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	71	0.44	0.033	11q22
	Hs.286049	PSA	phosphoserine aminotransferase	72	0.47	0.009	9
	Hs.93659	**ERP70**	**protein disulfide isomerase-related protein**	**73**	**0.26**	**0.036**	**10**
	Hs.2281	CHGB	chromogranin B (secretogranin 1)	75	0.31	0.011	20pter–p12
	Hs.110099	CBFA2T3	core-binding factor, runt domain, alpha subunit 2; translocated to, 3	75	0.33	0.009	16q24
	Hs.4747	**DKC1**	**dyskeratosis congenita 1, dyskerin**	**75**	**0.40**	**0.003**	**Xq28**
	Hs.83848	TPI1	triosephosphate isomerase 1	77	0.43	0.040	12p13
	Hs.75799	**PRSS8**	**protease, serine, 8 (prostasin)**	**77**	**0.13**	**0.045**	**16p11.2**
	Hs.143600	GPP130	type II Golgi membrane protein	77	0.35	0.024	3
	Hs.13565	T-STAR	Sam68-like phosphotyrosine protein, T-STAR	78	0.44	0.013	8q24.2
	Hs.169476	GAPD	glyceraldehyde-3-phosphate dehydrogenase	80	0.47	0.042	12p13
	Hs.114366	**PYCS**	**pyrroline-5-carboxylate synthetase**	**87**	**0.24**	**0.006**	**10q24.3**
	Hs.43445	**PARN**	**poly(A)-specific ribonuclease (deadenylation nuclease)**	**90**	**0.35**	**0.003**	**16p13**
	Hs.137556		**H. sapiens mRNA; cDNA DKFZp434A132**	**71**	**0.18**	**0.004**	
	Hs.65403	**LOC51323**	**hypothetical protein**	**71**	**0.50**	**0.003**	**6pter–6q15**
	Hs.164285		**ESTs, Weakly similar to Afg1p [S. cerevisiae]**	**72**	**0.44**	**0.026**	
	Hs.26675		**ESTs**	**74**	**0.32**	**0.012**	
	Hs.11641		***H. sapiens* cDNA: FLJ21432 fis, clone COL04219**	**74**	**0.50**	**0.000**	

Table 2. (*continued*)

Category	Unigene ID (build #131)	Gene Symbol	Gene Name	U	CR/NC	P	Locus
	Hs.14846		***H. sapiens* mRNA; cDNA DKFZp564D016**	**75**	**0.38**	**0.009**	
	Hs.283127		**ESTs**	**75**	**0.36**	**0.032**	
	Hs.63224		ESTs	75	0.16	0.038	
	Hs.227591		**ESTs, Weakly similar to AF1488561 unknown [*H. sapiens*]**	**76**	**0.50**	**0.017**	
	Hs.11156	**LOC51255**	**hypothetical protein**	**76**	**0.21**	**0.005**	**2**
	Hs.133207		*H. sapiens* mRNA for KIAA1230 protein, partial cds	77	0.42	0.011	
	Hs.201925		*H. sapiens* cDNA FLJ13446 fis, clone PLACE1002968	80	0.47	0.015	

U, indicates Mann-Whitney statistics. *CR/NC*, difference ratio between median expression values for each group. *P*, permutational *P* value. Genes used for calculating predictive scores are noted in bold type.

the NR group. In addition, a number of genes related to DNA repair, transcription, signal transduction, cell skeleton, and adhesion were among those expressed differently in the two groups.

Permutation Test

To evaluate the validity of the 171 genes selected as radiosensitivity-related genes, permutation test was performed as described in the Materials and Methods section. Expression levels of each 19 samples in both groups for each gene were permuted (randomly scrambled) 10,000 times. P_{gc} values were calculated using μ_{CR}, μ_{NC}, σ_{CR}, and σ_{NC} values derived from newly classified CR group and NC group during every permutation. Large absolute values of P_{gc} indicate a strong correlation between the gene expression and the class distinction, whereas the sign of P_{gc} being positive or negative corresponds to gene "g" being more highly expressed in the CR or NC group. After the 10,000 times permutation, the probabilities of the genes to be correlated to group distinction, *P*s, were estimated under the hypothesis that these 10,000 P_{gc} values show ideal normal distribution (Table 2, A and B). As a result, all of the selected 62 genes showed *P* values >.05 without exception. Hence, it was proved that these selected 171 genes were to be radiosensitivity predictive gene under the confidence of $P < .05$.

Hierarchical Clustering

Of these 171 genes were subjected to hierarchical clustering as described in the Materials and Methods section. This procedure clearly separated the two groups from each other, except for tumor No. 47 (data not shown). To achieve complete separation, we selected 62 genes that showed greater than 2.0 standard deviations of expression values among all 19 samples. Cluster analysis using these 62 genes achieved complete separation of the groups (Figure 2).

Cross-Validation Test

Cross-validation test was performed to examine whether the 62 genes were crucial for classifying CR and NC groups and whether they could predict the group for test samples. Among the 19 samples, 18 samples were used for group predictor and 1 sample was used as the test sample. The

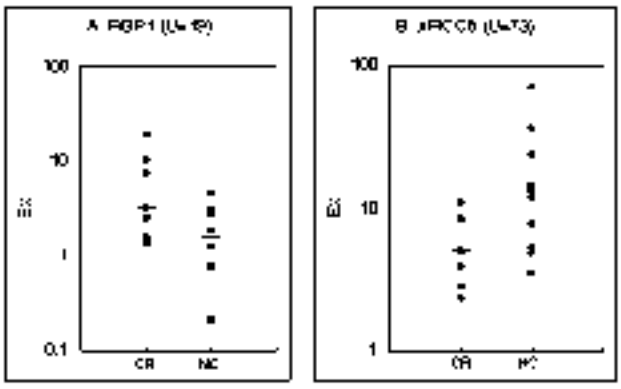

Figure 1. *Differential gene expression between the radiosensitive group (CR; 9 samples) and the radioresistant group (NC; 10 samples) with significant difference (P < .05). U indicates the Mann-Whitney test statistic. Expression levels (Ex = Cy5 signal intensity from cancer sample/Cy3 signal intensity from control), of two genes are plotted here. Median Ex values for each group of tumors are denoted by horizontal lines. (A) Retinol-binding protein 1 (RBP1; U = 19); (B) X-ray repair cross-complementing 5 (XRCC5; U = 73).*

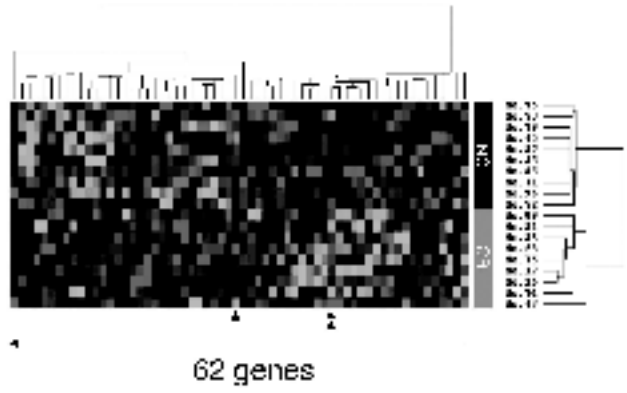

Figure 2. *Expression patterns of 62 genes across 19 samples of cervical squamous cell carcinoma. Red or green colors indicate higher or lower expression, respectively, relative to the mean signal intensity of a given gene across 19 tumor samples; black, same expression level with mean value; gray, no expression detected (intensities of both Cy3 and Cy5 were below cut-off values). Each row represents each gene and each column a cervical squamous cell carcinoma sample. Single and double triangles indicate the gene-expression profiles of TCF4 and BAK1, respectively.*

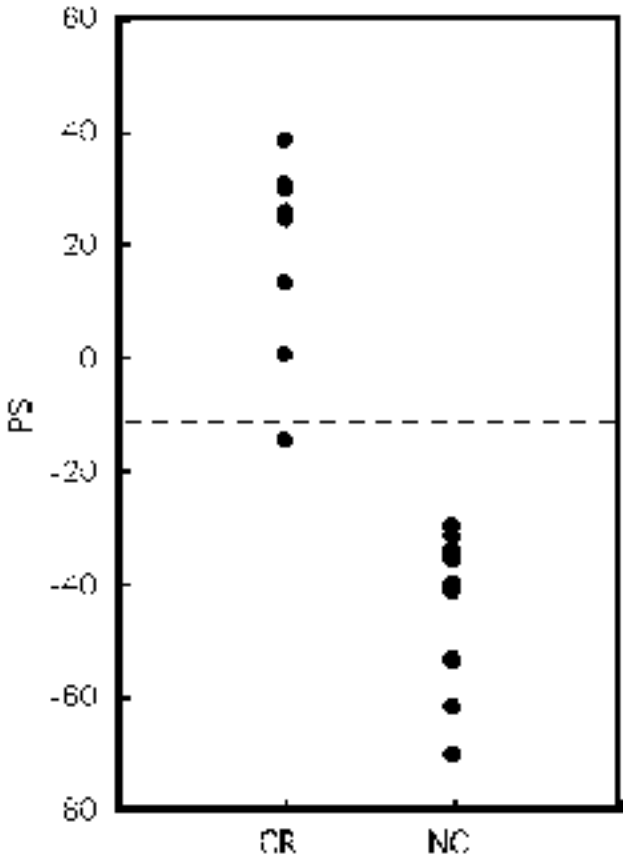

Figure 3. *Predictive score (PS) for radiosensitive and radioresistant groups by cross-validation test. Details of the calculation method were noted in the Materials and Methods section. A dashed line indicates the threshold.*

sample sets of predictor and test sample were changed 19 times and PS for each 19 samples was calculated as described in the Materials and Methods section (Figure 3 and Table 3). Threshold line for PSs to discriminate CR or NC group were settled at the half point between average PS values of the CR group and that of the NC group: −12. As shown in Figure 3, PSs for samples in CR and NC groups were clearly separated, except for sample No.74 (error ratio was 5.3% (=1 sample/19 samples)).

Discussion

cDNA Microarray analysis is a powerful tool for obtaining comprehensive information about expression of thousands of genes in cancer cells [12-15]. By combining this technology with statistical analysis, we identified 171 genes that showed different expression patterns between two distinct clinical groups and, therefore, were likely to reflect differences in the response of cervical cancer cells to radiotherapy. To examine the validity of 171 genes selected as radiosensitivity-related genes, random permutation test was performed by calculating P_{gc}s, and P values for each gene were evaluated. After 10,000 times permutation test, P values for all of the 171 genes were lower than .05, indicating that these genes were significantly correlated to radiosensitivity. Furthermore, in a clustering analysis, the expression profiles of these 171 genes were able to classify each of 19 tumor samples, except for one, to the appropriate group (radiosensitive or radioresistant). However, when the cluster analysis was limited to 62 genes having greater than 2.0 standard deviations of expression level across the 19 samples, all tumors were properly classified into their respective groups. To further evaluate the validity of 62 genes selected as radiosensitivity-related genes, we carried out cross-validation test. After 19 times cross-validation test, each PS value for each sample that belonged to the CR (or NC) group showed higher (or lower) value than threshold line. This study not only supports the feasibility of these 62 genes as radiosensitivity-related genes, but also indicates the possibility of predicting radiosensitivity for discriminating radiation-sensitive from radiation-resistant biopsy samples by PS values calculated from expression values of 62 genes. However, further study with additional tumor samples would be required to apply these genes for predicting radiosensitivity of tumors in patients before therapy begins.

Because all of the cervical cancer samples we used in this study were human papillomavirus (HPV)-positive, p53 as well as RB functions were likely to be eliminated by the viral protein [19]. Hence, the set of genes listed here may be associated with a cell-death pathway independent of p53. Radiation kills tumor cells mainly as a result of double-strand breaks (DSBs) in DNA [20]. If cells are defective in their DNA-repair systems, especially as regards DSB repair, they should be more susceptible to cell death. The gene product of *XRCC5*, Ku80, a protein that binds double-stranded DNA and a component of DNA-dependent protein kinase holo enzyme, is involved in DSB repair [21]. *XRCC5*-deficient cells and Ku80-knockout mice are hypersensitive to ionizing

Table 3. Predictive Scores to Classify Radiosensitive Group and Radio Resistant Group.

Sample No.	PS
CR	
16	36
17	30
23	38
47	0.6
74	−15
75	31
81	25
83	26
89	13
NC	
31	−48
35	−40
39	−54
45	−35
52	−62
53	−34
55	−70
85	−32
87	−42
96	−30

PS, predictive score. *CR*, radiosensitive group. *NC*, radioresistant group.

radiation [22,23]. Therefore, the higher expression of this gene we observed in radioresistant cancer cells accords with its physiological function. *XRCC5* would be one of the most crucial genes for determining the fate of cells under the genotoxic stress caused by irradiation. We also observed a relatively higher level of expression of lactate dehydrogenase (*LDHA*), which aids glycolysis under hypoxic conditions [24] in radioresistant cells. Cells in fact become radioresistant under hypoxic conditions [25], and a high level of *LDHA* expression could be an important mechanism conferring radioresistance.

In radiosensitive cells, we found elevated expression of adipogenesis-related genes including *ALDH1* and *RBP1*. The product of *ALDH1* is involved in retinoic acid (RA) synthesis [26] and *RBP1* is a transporter of retinol. Cervical carcinoma cells treated with RA before irradiation are reported to become radiosensitive [27]; furthermore, RA induces TRAIL expression and causes apoptosis [28]. Therefore, elevated expression of these genes may induce RA synthesis and, thereby, encourage apoptosis after radiation.

Our list of 171 genes should be useful not only as an aid to understanding the mechanism of radiosensitivity, but also as a means to expand the possibilities for effective radiotherapy. For example, if some novel drugs could block gene products that are involved in radioresistance, or if genes that induce apoptotic signals after radiation could be exogenously introduced, the effectiveness of radiotherapy would be increased. In addition, the 62 selected genes might prove of great benefit for diagnosing radiosensitivity of individual cervical cancers, to provide opportunities for selecting appropriate treatment (personalized medicine) for each patient.

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