Differential gene expression profiles of radioresistant pancreatic cancer cell lines established by fractionated irradiation

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Abstract. Identification of the genes that are differentially-expressed between radiosensitive and radioresistant cancer cells is important to the ability to predict the clinical effectiveness of radiotherapy. We established radioresistant human pancreatic cancer cell lines using fractionated irradiation in order to identify genes that are differentially-expressed between parental lines and radioresistant cell sublines. Six pancreatic cancer cell lines (PK-1, PK-8, PK-9, T3M4, MiaPaCa2 and PANC-1) were treated with 10 Gy fractionated irradiation at approximately two-week intervals (total dose 150-180 Gy). Five radioresistant sublines (PK-1, PK-8, PK-9, T3M4, and MiaPaCa2) were successfully established. Using oligonucleotide microarrays containing 17,086 genes, we identified 73 up-regulated genes and 55 down-regulated genes common to radioresistant sublines. Subsequent analysis by quantitative RT-PCR confirmed the reliability of our microarray strategy. Up-regulated genes were associated with growth factor (example, *amphiregulin*), cell-cycle check point (*MAPKAPK2*), intracellular signaling pathway (*regucalcin*), and angiogenesis stimulation (*angiopoietin 2*). Down-regulated genes were associated with apoptosis (*caspase 8*), retinoid esterification (*lecithin retinol acyltransferase*), and electron transport (*calcium-activated chloride channel 1*). Some of these genes have known association with response to radiation, such as *caspase 8* and *MAPKAPK2*, but others are novel. Global gene analysis of radioresistant sublines may provide new insights into the mechanisms underlying clinical radioresistance and to improving the efficacy of radiotherapy for pancreatic cancer.

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction, MAPKAPK2, MAPK-activated protein kinase 2

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Introduction

Radiotherapy is one of the major adjuvant treatments for many malignant tumors, and it has been frequently applied for patients with pancreatic cancer. The general rationale for radiotherapy is based on the findings that radiation can inhibit cell proliferation or induce apoptotic cell death *in vitro* and inhibit tumor growth *in vivo* (1). Pancreatic cancer has one of the poorest prognoses among malignant tumors, with the 5-year survival rate of patients treated with both surgery and chemoradiotherapy ranging from 1 to 2% (2,3). One of the reasons for this low survival rate is the insensitivity of pancreatic cancer to radiotherapy, which decreases the ability to cure or delay progression of disease in these patients (4-6). Therefore, it is necessary to elucidate the mechanisms underlying radioresistance to improve prognosis.

Recently, a relationship between radioresistance and expression of several genes has been reported, with candidate genes including *p53* (7), *ras* (8), *raf-1* (9), *bcl-2* (5), and *survivin* (6). Although such discoveries have helped develop a partial understanding of the molecular mechanisms responsible for cellular radiosensitivity, the entire process remains to be elucidated. The advent of microarray gene expression technology permits simultaneous analysis of the expression levels of thousands of genes (10-12). Therefore, a study on molecular genetic events related to radiosensitivity can be conducted (13-16). This research may also lead to identification of gene regulatory pathways that result in development of cell resistance to therapeutic procedures. To date, differential gene expression profiles of radioresistant cancer and cancer cell lines including breast cancer, esophageal cancer and uterine cervical cancer have been reported (13-16). However, to the authors' knowledge, there has been no information regarding the global gene analyses of radioresistant pancreatic cancer or cancer cell lines.

In the current study, we tried to identify differentially-expressed genes between radiosensitive and radioresistant pancreatic cancer cell lines. Radioresistant sublines were established by fractionated irradiation, and we applied oligonucleotide microarrays containing 17,086 genes to parent and radioresistant sublines in order to investigate the differential expression of genes between parent and radioresistant cells.

Materials and methods

Cells and cell culture. The human pancreatic cancer cell line PANC-1 was provided by H. Iguchi, Clinical Research Institute, National Kyushu Cancer Center, Fukuoka, Japan. The other human pancreatic cancer cell lines, PK-1, PK-8, PK-9, T3M4, and MiaPaCa2 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. The MiaPaCa2 cell line was cultured in DMEM medium containing 10% fetal bovine serum in an environment of 5% CO_2 at 37˚C. The other cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum in an environment of 5% CO_2 at 37˚C.

Establishment of radioresistant cell lines. The method for establishing radioresistant cell lines by fractionated irradiation has been described previously (17). Briefly, on day 1, cells were counted and passaged. On day 2, cells were treated with 10 Gy X-ray irradiation (100 kV and 3.5 mA for 5 min) using an X-ray generator (MBR-1505R; Hitachi Medical Co., Tokyo, Japan); cells were cultured in conditioned medium before the next passage on approximately day 15. This challenge was repeated every 2 weeks until radioresistant cell sublines were established.

Assay for radiosensitivity. Cell survival after X-ray irradiation was measured by clonogenic assay. Either $1x10^3$ cells (PK-8, T3M4, and PANC-1) or $1x10^4$ cells (PK-1, PK-8, and MiaPaCa2) were plated in each 60-mm tissue culture dish (Iwaki Glass, Chiba, Japan), and were irradiated at different doses ranging from 0 to 10 Gy. These cells were incubated at 37˚C for 10-14 days (three plates in each radiation dose). After fixation with formalin and staining with 0.1% crystal violet, colonies consisting of 50 cells or more were counted under a light microscope, and the surviving fraction was determined. All survival curves represent at least three independent experiments.

Evaluation of apoptosis by Annexin and propidium iodide staining. Sensitivity to radiation was confirmed by apoptosis after a bolus dose of 10 Gy X-ray irradiation. After 48 h of bolus irradiation, cells were harvested with 0.05% trypsin, re-suspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$], and then incubated with FITC-conjugated Annexin V and 5 μg/ml propidium iodide (PI) (Annexin V-FITC kit; Bender Medsystem) as described previously (18). Cells were then analyzed using an EPICS XL flow cytometer (Beckman Coulter Corp.). Annexin V-positive cells with or without PI-positive cells were considered to be apoptotic. Cells that were not treated with the bolus of 10 Gy X-ray irradiation were used as the negative controls. This experiment was repeated three times for each parent line and radioresistant cell subline.

Total RNA extraction. For cultured cell lines, total RNA samples were extracted from each pancreatic cancer cell line into 350 μl buffer RLT (Qiagen, Hilden, Germany) with added ß-mercaptoethanol to a concentration of 1% as described previously (19). Total RNA was then purified with RNeasy Tissue Kit (Qiagen) according to the manufacturer's protocols.

Oligonucleotide microarray analysis (labeling, hybridization and scanning). We used the commercially available Agilent Human 1A oligonucleotide microarray containing 17,086 genes, a listing of which can be found online at http://www.chem.agilent.com/scripts/generic.asp?lpage=5175&indcol=Y&prodcol=Y. Cyanine-labeled cRNA was prepared using a T7 linear amplification as described in the Agilent Low RNA Input Fluorescent Linear Amplification Kit Manual (Agilent). Briefly, 50 ng of purified total RNA was reverse-transcribed to generate double-stranded cDNA using oligo(dT) T7 promoter primer and MMLV reverse transcriptase. Next, cRNA was synthesized using T7 RNA polymerase, which simultaneously incorporated Cy3 or Cy5 labeled CTP. During this process, the sample of each radioresistant cell line was labeled with Cy5 whereas that of each parent cell line was labeled with Cy3 as control. Quality of the cRNA was again checked using the Agilent 2100 Bioanalyzer. One microgram aliquot each of Cy3 labeled cRNA and Cy5 labeled cRNA were combined, and fragmented in a hybridization cocktail (Agilent). Then, the labeled cRNAs were hybridized to 60 mer probe oligonucleotide microarrays and incubated for 17 h at 60˚C. The fluorescent intensities were determined by an Agilent DNA Microarray Scanner and were analyzed by G2567AA Feature Extraction Software version A.7.5.1 (Agilent), which used the LOWESS (locally weighted linear regression curve fit) normalization method (20).

Oligonucleotide microarray data analysis. After subtraction of local and global background signals, the expression values were calculated as the log ratio of the dye-normalized red (Cy5) and green (Cy3) channel signals. Data flagged as being of poor quality by the Agilent data extraction software were removed from the analysis. All data calculated by the data extraction software were imported to the Rosetta Luminator System version 2.0 (Rosetta Biosoftware, Kirkland, WA). Sequences that were 2.0-fold up-regulated or down-regulated in radioresistant cells compared to parent cells were defined as differentially regulated.

Real-time qantitative RT-PCR. Real-time monitoring of PCR reactions was performed using the LightCycler™ system (Roche Applied Science, Indianapolis, IN), and SYBR Green I dye (Roche Diagnostics). Monitoring was performed according to the manufacturer's instructions, as described previously (21,22). In brief, a master mixture was prepared on ice, containing 1 μl cDNA of each gene, 2 μl LC DNA Master SYBR Green I mix, 50 ng primers and 2.4 μl 25 mM $MgCl_2$. The final volume was adjusted to 20 μl with water. After the reaction mixture was loaded into the glass capillary tube, PCR was carried out under the cycling conditions listed in Table I. After amplification, products were subjected to a temperature gradient from 68˚C to 95˚C at 0.2˚C/sec under continuous fluorescence monitoring to produce a melting curve of the products. Only one peak was observed for each sample.

We determined expression levels of target genes and expression of *GAPDH* mRNA by comparisons with cDNA from Human Universal Reference Total RNA (Clontech, Palo Alto, CA). After proportional baseline adjustment, the fit point method was employed to determine the cycle in which the log-linear signal was first distinguishable from baseline,

Table I. Quantitative real-time PCR amplification programs.

Gene		Primers from 5' to 3'	Denaturation annealing extension	Cycle no.
Quantitative real-time PCR				
AREG	f	GCCGCTGCGAAGGACCAA	94˚C for 30 sec	40
	r	TCACTCACAGGGGAAATCTCACTC	58˚C for 20 sec	
			72˚C for 20 sec	
ANGPT	f	AGCCGGCAAAATAAGCAGCATC	94˚C for 30 sec	40
	r	GGTTGTGGCCTTGAGCGAATAGC	56˚C for 30 sec	
			72˚C for 20 sec	
CASP8	f	CCCCAGAGCAGCACTGACG	94˚C for 30 sec	40
	r	CAGCTGAGCTGGGTCGACC	65˚C for 30 sec	
			72˚C for 30 sec	
LRAT	f	GGCGCGGAGGGCTGAAAAG	94˚C for 30 sec	40
	r	ACGCCAATCCCAAGACTGCTGAAG	65˚C for 30 sec	
			72˚C for 30 sec	
GAPDH	f	TTGGTATCGTGGAAGGACTCA	95˚C for 10 sec	40
	r	TGTCATCATATTTGGCAGGTT	60˚C for 10 sec	
			72˚C for 10 sec	

AREG, amphiregulin; ANGPT, angiopoietin 2; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase 2; CASP8, caspase 8; LRAT, lecithin retinoal acyltransferase; CaCC1, calcium-activated chloride 1.

Table II. Comparisons of surviving fraction of pancreatic cancer cells exposed to various radiation doses between parent cells and radioresistant cells.

	Radiation dose			
Cell line	2 Gy	5 Gy	10 Gy	p-value
PK-1				0.00011
Parent	0.506±0.049	0.151±0.050	0.021±0.010	
Radioresistant	0.760±0.079	0.406±0.075	0.063±0.040	
PK-8				0.00212
Parent	0.300±0.086	0.017±0.007	0.001±0.001	
Radioresistant	0.070±0.101	0.286±0.078	0.021±0.009	
PK-9				0.00492
Parent	0.483±0.057	0.158±0.040	0.005±0.002	
Radioresistant	0.770±0.044	0.410±0.090	0.033±0.023	
T3M4				0.00401
Parent	0.453±0.084	0.090±0.036	0.004±0.002	
Radioresistant	0.723±0.075	0.263±0.060	0.017±0.006	
MiaPaCa2				0.00439
Parent	0.393±0.050	0.050±0.026	0.002±0.001	
Radioresistant	0.703±0.085	0.407±0.095	0.060±0.056	
PANC-1				NS (0.1489)
Parent	0.610±0.100	0.226±0.075	0.023±0.006	
Radioresistant	0.733±0.058	0.317±0.076	0.033±0.021	

Values reflect mean ± standard deviation (SD).

and that cycle number (threshold cycle) was used as a crossing-point value. The standard curve was produced by measuring the crossing point for each dilution of the reference standard (4-fold serially diluted cDNAs of Human Universal Reference total RNA) and plotting these crossing point values as a function of concentration on a logarithmic scale. Concentrations

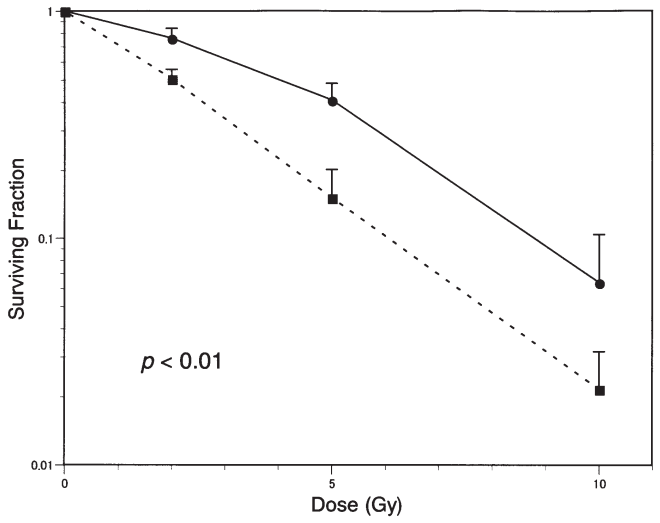

Figure 1. Radiation cell survival curves for parent (square) and radioresistant (circle) PK-1 cells. The colony formation assay was described in Materials and methods. Data represent means with standard deviation (SD) from three independent experiments. There was a significant difference in survival fraction between parent and radioresistant cells by repeated measure ANOVA (p=0.00011).

for each sample were calculated by plotting their crossing points against the standard curve, and these concentrations were then divided by the endogenous reference (*GAPDH*) concentration to obtain a normalized value for expression of each gene. Each assay was performed three times to verify the results, and mean mRNA expression was used for subsequent analysis.

Statistical analysis. Differences between groups were estimated using the χ^2 test, and the Student's t-test. When comparing the survival fractions between control and radioresistant cells, repeated measure analysis of variance (ANOVA) method was carried out. A probability level of 0.05 was chosen for statistical significance.

Results

Establishment of cell sublines resistant to irradiation. All six human pancreatic cancer cell lines were treated with fractionated irradiation, and all lines survived. Cell populations surviving fractionated irradiation were analyzed for their radiosensitivity using a clonogenic assay to assess survival after a bolus dose of 10 Gy irradiation. Surviving fractions for all cell lines are shown in Table II. There was a significant increase in radioresistance in the PK-1, PK-8, PK-9, T3M4 and MiaPaCa2 sublines compared with their parental cell lines. Fig. 1 shows the survival curves of parent and radioresistant PK-1 cells. The survival curves for PK-8, PK-9, T3M4 and MiaPaCa2 cell lines were similar to that for PK-1 cells. On the other hand, there was not a significant increase in radioresistance in PANC-1 sublines compared with the parental cell line.

Evaluation of apoptosis by Annexin V also indicated that there was a significant decrease in apoptosis in the PK-1, PK-8, PK-9, T3M4, and MiaPaCa2 sublines but not in the PANC-1 subline compared with the six parental cell lines (Table III). Therefore, we considered five sublines (e.g., PK-1, PK-8, PK-9, T3M4 and MiaPaCa2) as radioresistant. All radioresistant sublines maintained a radioresistant phenotype for at least two months after cessation of fractionated irradiation (data not shown).

Table III. Comparative analysis of induced apoptosis after bolus 10 Gy irradiation in six treated cell lines.

Cell line	Apoptosis rate		p-value
	Parent (%)	Resistant (%)	
PK-1	28.3±6.8	13.2±2.8	0.01
PK-8	35.0±6.5	18.1±4.4	<0.01
PK-9	35.5±7.8	21.5±5.5	<0.01
T3M4	36.2±4.3	16.7±1.3	0.01
MiaPaCa2	45.2±3.3	23.8±1.4	<0.01
PANC-1	18.1±2.2	14.4±1.2	0.07

Values reflect mean ± standard deviation (SD).

Gene selection from oligonucleotide microarray data analysis. We performed global expression analysis of 17,086 genes using an oligonucleotide microarray and compared the five profiles of parent lines and derived radioresistant sublines. We identified 128 genes as differentially-expressed in at least four of the five radioresistant sublines; 73 genes up-regulated (defined as a 2.0-fold or greater increase, Table IV) and 55 down-regulated (defined as a 2.0-fold or greater decrease, Table V). Up-regulated genes were associated with growth factor (example, *amphiregulin*), cell-cycle check point (*MAPKAPK2*), intracellular signaling pathway (*regucalcin*), and angiogenesis stimulation (*angiopoietin 2*). Down-regulated genes were associated with apoptosis (*caspase 8*), retinoid esterification (*lecithin retinol acyltransferase*), and electron transport (*calcium-activated chloride channel 1*). Some of these genes were already known to be associated with responsiveness to radiation, such as *caspase 8* and *MAPKAPK2*, but others were novel.

Real-time RT-PCR assay. In order to verify the microarray data, we arbitrarily selected four genes and performed two sets of quantitative real-time PCR studies using five pancreatic cancer cell lines: one set of PCR studies using parent cells, and the other set using radioresistant cells. These results corresponded very well with those for the microarray data for all four genes, strongly supporting the reliability and rationale of our strategy (Figs. 2 and 3).

Discussion

In the current study, radioresistant sublines were obtained by exposing parental cell lines to repeated fractions of 10 Gy, with an approximately 14-day recovery period between each fraction. This resulted in a statistically significant decrease in the radiosensitivity of the five exoposed sublines as measured

Table IV. Identification of 73 genes up-regulated by fractionated irradiation in pancreatic cancer.

Gene name	Sequence code	Fold change PK-1	PK-8	PK-9	T3M4	MIA	Average
Five cells up-regulated							
1. Inositol polyphosphate-5-phosphatase ii	Q14642	6.65	3.02	3.68	3.61	3.57	4.11
2. Protein containing a SAM domain	AK093571.1	3.33	5.68	2.54	2.78	3.33	3.53
3. Calcium-binding tyrosine-(Y)-phosphorylation	NM_153770.1	2.81	2.77	6.14	3.00	2.43	3.43
4. Protein phosphatase 4 regulatory subunit 2	CAB93534.1	4.02	2.96	3.57	3.22	3.00	3.35
5. Myeloid cell nuclear differentiation antigen	P41218	4.21	3.57	2.16	2.17	3.64	3.15
6. Protein containing six ankyrin repeats	NM_024708.1	2.58	3.27	4.30	2.89	2.73	3.15
7. Protein of unknown function	NM_015594.1	2.93	2.28	4.47	2.02	3.88	3.12
8. Regenerating islet-derived 1 ß	P48304	2.68	3.57	2.88	3.57	2.66	3.07
9. 5(3)-deoxyribonucleotidase	CAD34856.1	2.68	2.21	2.69	4.11	3.57	3.05
10. Uncoupling protein 3	P55916	3.22	2.44	3.35	2.79	3.44	3.05
11. Protein containing a Kazal-type serine protease inhibitor domain	AK001520.1	3.84	2.33	2.74	3.21	2.98	3.02
12. WAP four-disulfide core domain 1	Q9HC57	3.37	3.07	3.04	2.80	2.70	3.00
13. Hypothetical protein dJ434O14.3	NM_025228.1	2.74	2.69	3.74	3.21	2.54	2.98
14. Helicase-like protein	NM_173082.1	2.14	4.25	3.20	2.57	2.68	2.97
15. Hypothetical protein FLJ31614	NM_152573.1	2.33	2.58	3.30	3.67	2.80	2.94
16. Member of the C. elegans putative membrane protein family 8	O76090	3.67	2.98	2.36	2.68	2.58	2.85
17. Hypothetical protein FLJ37953	NM_152382.1	2.77	3.57	2.21	2.41	3.21	2.83
18. Protein containing a kringle domain	AAH11049.1	2.69	2.04	2.89	2.37	3.68	2.73
19. Protein containing four WD domains	AAH35512.1	2.01	2.12	2.68	3.99	2.74	2.71
20. Protein of unknown function	AL834409.1	2.36	3.21	3.00	2.68	2.22	2.69
21. Pallidin homolog	NM_012388.1	2.68	2.99	2.41	2.90	2.31	2.66
22. Protein with high similarity to rab4-interacting protein	AAL67520.1	2.47	2.64	2.22	2.68	2.31	2.46
23. PR domain containing 1	NM_001198.1	2.68	2.33	2.36	2.01	2.88	2.45
24. ADAMTS-like 1	NM_139264.1	2.08	2.57	2.11	2.07	2.63	2.29
Four cells up-regulated							
25. Afadin	BAA32485.1	4.57	3.14	1.88	5.12	3.33	3.61
26. Cytohesin 3	O43739	4.15	4.04	3.36	1.90	4.12	3.51
27. Interleukin 1 receptor accessory protein	NM_134470.2	5.66	1.70	2.45	3.78	3.10	3.34
28. Wingless-related MMTV integration site 7B	P56706	4.47	2.54	3.36	4.18	1.98	3.31
29. Putative mitochondrial space protein 32.1	NM_014472.1	5.12	2.68	3.74	2.88	1.69	3.22
30. Protein of unknown function	NM_030806.1	1.12	3.83	5.55	2.86	2.53	3.18
31. NY-REN-41 antigen	NM_080654.1	4.24	3.73	3.42	1.05	2.67	3.02
32. Protein of unknown function	NM_031269.1	3.45	4.10	3.31	1.95	2.31	3.02
33. Protein with high similarity to BRCA1-interacting protein	AAH05368.1	3.38	2.21	3.64	1.81	3.71	2.95
34. Putative cGMP-specific cone phosphodiesterase 6C α	NM_006204.2	3.33	4.25	1.25	2.27	3.53	2.93
35. MAPKAPK2	NM_004759.2	2.98	3.56	1.88	2.68	3.00	2.82
36. Hypothetical protein LOC222967	NM_173565.1	4.21	1.67	2.47	2.61	3.08	2.81
37. Chromosome 20 open reading frame 96	NM_153269.1	2.23	3.10	1.15	2.78	4.64	2.78
38. Member of the AMP-binding enzyme family	AAH09317.1	2.48	1.74	4.14	2.57	2.98	2.78
39. Prolactin	P01236	3.24	2.07	3.68	1.88	2.47	2.67
40. Hypothetical protein FLJ30317	NM_172136.1	3.57	2.84	1.87	2.74	2.31	2.67
41. Protein of unknown function	I_962912	1.79	2.17	3.71	3.01	2.61	2.66
42. Regucalcin	Q15493	2.18	1.54	3.36	2.80	3.23	2.62
43. Protein containing a caspase recruitment domain	Q9Y2G2	2.75	3.94	1.65	2.47	2.31	2.62
44. Gonadotropin-releasing hormone receptor	P30968	3.00	3.31	1.34	3.06	2.25	2.59
45. Protein containing a type 1 thrombospondin domain	NM_018676.1	4.27	2.92	2.13	2.16	1.48	2.59
46. Amphiregulin	P15514	1.05	3.99	2.35	3.13	2.40	2.58
47. Chemokine-like receptor 1	Q99788	3.57	2.00	2.57	1.49	3.20	2.57
48. Hypothetical protein FLJ37464	NM_173815.1	2.99	2.67	2.47	1.69	2.77	2.52
49. Plexin C1	NM_005761.1	2.58	3.14	1.64	2.87	2.10	2.47
50. Protein containing two pleckstrin homology domains	BAA86514.1	2.45	2.69	1.45	3.00	2.68	2.45
51. Protein containing an IQ calmodulin-binding domain	NM_022784.1	2.74	2.66	2.34	3.46	1.07	2.45
52. Cytochrome P450 subfamily IIC polypeptide 8	P10632	2.10	2.51	2.97	2.83	1.70	2.42
53. KIAA1383 protein	NM_019090.1	2.77	2.18	1.22	3.17	2.63	2.39
54. Purinergic receptor P2X	P51575	2.45	2.13	3.47	1.42	2.45	2.38
55. Zinc finger protein 254	NM_004876.1	4.29	2.00	2.29	1.23	2.03	2.37
56. Sarcolemmal-associated protein	NM_007159.1	3.30	1.66	2.28	2.05	2.56	2.37
57. Chromosome 21 open reading frame 94	NM_145180.2	1.00	2.75	1.74	3.41	2.88	2.36
58. Protein of unknown function	NM_031929.1	2.00	2.70	1.91	2.14	2.99	2.35
59. Protein containing a CXXC zinc finger	AAC37520.1	2.56	2.14	1.57	2.47	2.98	2.34
60. Protein of unknown function	NM_053002.2	2.45	2.69	1.22	2.56	2.69	2.32
61. Neurotrimin	Q9P121	2.57	1.47	2.39	2.46	2.60	2.30
62. Nuclear receptor subfamily 0 group B member	P51843	2.41	2.74	1.54	2.23	2.60	2.30
63. Protein with high similarity to copine III	AAO21123.1	2.49	2.68	1.69	2.47	2.11	2.29
64. Lipoma-preferred-partner	CAB59169.1	2.99	2.41	2.68	2.13	1.17	2.28
65. Hypothetical protein MGC34725	NM_173637.1	2.13	2.64	2.66	1.69	2.30	2.28
66. Synovial sarcoma X breakpoint 3	Q99909	2.40	2.31	2.81	2.59	1.26	2.27
67. Sodium channel voltage gated type II α 2	Q99250	2.17	2.57	3.01	1.14	2.47	2.27
68. Protein with high similarity to ATP-binding cassette subfamily B	P21448	2.82	2.25	3.05	2.02	1.11	2.25
69. Hypothetical protein FLJ25791	NM_173559.1	2.04	1.74	2.68	2.37	2.44	2.25
70. Retired, was Protein of unknown function	NM_032173.1	0.88	2.14	2.98	2.73	2.47	2.24
71. Angiopoietin 2	O15123	3.06	2.12	1.02	2.75	2.07	2.20
72. Membrane-spanning four-domains subfamily A	AAK37417.1	3.15	2.13	2.77	0.84	2.07	2.19
73. ATP-binding cassette, subfamily A	NM_080284.2	2.47	2.39	1.78	2.11	2.00	2.15

MIA, MiaPaCa2; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase 2.

Table V. Identification of 55 genes down-regulated by fractionated irradiation in pancreatic cancer.

Gene name	Sequence code	Fold change PK-1	PK-8	PK-9	T3M4	MIA	Average
Five cells down-regulated							
1. Phenylalanine hydroxylase	P00439	0.26	0.29	0.25	0.30	0.32	0.28
2. Protein with high similarity to odorant receptor 83	CAD69427.1	0.39	0.31	0.15	0.45	0.32	0.32
3. Glypican 3	P51654	0.41	0.09	0.27	0.42	0.46	0.33
4. Protein of unknown function	I_1109501	0.34	0.26	0.35	0.47	0.29	0.34
5. Protein of unknown function	AAK13488.1	0.44	0.33	0.48	0.30	0.16	0.34
6. Fatty acid binding protein 1	P07148	0.25	0.41	0.28	0.35	0.42	0.34
7. Member of the phospholipid and glycerol acyltransferase family	Q9NRZ7	0.26	0.14	0.44	0.43	0.42	0.34
8. Mitochondrial folate transporter	Q9H2D1	0.39	0.28	0.47	0.39	0.37	0.38
9. Protein with high similarity to melalloprotease thrombospondin 2	O15072	0.41	0.39	0.44	0.37	0.27	0.38
10. Protein with strong similarity to heparan sulfate 6-O-sulfotransferase 2	BAB55322.1	0.41	0.38	0.38	0.41	0.37	0.39
11. Protein containing a C-type lectin domain	NM_138337.1	0.33	0.35	0.48	0.46	0.37	0.40
12. Protein of unknown function	NM_032908.1	0.47	0.35	0.44	0.35	0.41	0.40
13. Retired, was Protein containing three EF hand domains	NM_032606.1	0.39	0.32	0.44	0.41	0.42	0.40
14. Calcium-activated chloride channel 1	AAD25487.1	0.45	0.45	0.35	0.42	0.37	0.41
15. SH2-containing inositol phosphatase	AAB53573.1	0.38	0.37	0.47	0.41	0.49	0.42
Four cells down-regulated							
16. Protein with high similarity to ß-2 tubulin	AAH33679.1	0.17	0.19	0.51	0.34	0.19	0.28
17. Protein containing a cold-shock nucleic acid binding domain	NM_024674.1	0.22	0.12	0.34	0.54	0.31	0.31
18. Hemogen	NM_018437.2	0.27	0.54	0.34	0.36	0.35	0.37
19. Protein of unknown function	NM_024976.1	0.33	0.31	0.20	0.48	0.51	0.37
20. G protein-coupled receptor 86	AAL26484.1	0.26	0.15	0.75	0.44	0.30	0.38
21. Caspase 8	BAB32555.1	0.27	0.21	0.34	0.70	0.44	0.39
22. Tumor necrosis factor superfamily member 18	Q9UNG2	0.38	0.63	0.24	0.44	0.31	0.40
23. Dermatan sulfate proteoglycan 3	Q99645	0.25	1.00	0.19	0.35	0.23	0.40
24. Protein of unknown function	Q8TDI7	0.55	0.44	0.21	0.41	0.37	0.40
25. Ryanodine receptor 3	NM_001036.1	0.43	0.63	0.41	0.28	0.28	0.41
26. ATP-binding cassette, subfamily C, member 4	NM_005845.1	0.54	0.41	0.28	0.41	0.39	0.41
27. Protein with high similarity to ras-related GTP-binding protein 7	AAH17092.1	0.57	0.47	0.21	0.44	0.41	0.42
28. Defensin, ß 106	NM_152251.1	0.26	0.19	0.48	0.66	0.50	0.42
29. Diacylglycerol kinase epsilon	P52429	0.50	0.60	0.47	0.24	0.29	0.42
30. Hypothetical protein FLJ30194	NM_152541.1	0.45	0.44	0.34	0.61	0.27	0.42
31. Member of the rhodopsin family of G protein-coupled receptors	CAD69041.1	0.53	0.45	0.41	0.44	0.34	0.43
32. ETS variant 1 protein	P50549	0.48	0.23	0.33	0.44	0.69	0.43
33. Prothrombin (coagulation factor II)	P00734	0.34	0.43	0.52	0.42	0.44	0.43
34. A disintegrin metalloprotease with a thrombospondin type 1 motif 1	Q9UHI8	0.48	0.27	0.39	0.55	0.45	0.43
35. Hypothetical protein FLJ32871	NM_144674.1	0.44	0.32	0.71	0.22	0.48	0.43
36. Protein of unknown function	I_929744	0.47	0.42	0.61	0.41	0.25	0.43
37. Protein containing a guanylate-binding protein N-terminal domain	BAC04709.1	0.44	0.32	0.25	0.88	0.33	0.44
38. Polycystic kidney and hepatic disease 1	NM_138694.2	0.37	0.46	0.30	0.62	0.47	0.44
39. Protein containing a phorbol ester or diacylglycerol binding domain	BC036472.1	0.33	0.61	0.48	0.50	0.33	0.45
40. Proteoglycan 4	AAB09089.1	0.48	0.36	0.40	0.47	0.54	0.45
41. Member of the rhodopsin family of G protein-coupled receptors	Q9Y5P0	0.44	0.52	0.41	0.50	0.45	0.46
42. TAF1 RNA polymerase II TATA box binding protein-associated factor	NM_004606.2	0.44	0.51	0.37	0.49	0.47	0.46
43. Hypothetical protein MGC39830	NM_152756.1	0.36	0.66	0.46	0.33	0.47	0.46
44. Protein containing eight leucine rich repeats	AAG28019.2	0.33	0.41	0.74	0.38	0.47	0.47
45. Lecithin retinol acyltransferase	AAD13529.1	0.53	0.44	0.49	0.49	0.41	0.47
46. Immunity associated protein 1 (IMAP1)	NM_130759.2	0.41	0.50	0.66	0.38	0.42	0.47
47. NYD-SP16 protein	AAH47333.1	0.44	0.36	0.42	0.47	0.68	0.47
48. Carboxypeptidase A metalloprotease family of zinc carboxypeptidases	NM_032785.1	0.42	0.40	0.49	0.61	0.45	0.47
49. Hypothetical protein MGC39497	NM_152436.1	0.63	0.45	0.47	0.41	0.41	0.47
50. Microtubule-associated protein 2	NM_031846.1	0.59	0.49	0.49	0.34	0.44	0.47
51. Member of the endonuclease V family	NM_173627.1	0.38	0.45	0.47	0.66	0.41	0.47
52. Terminal deoxynucleotidyl transferase	NM_004088.1	0.48	0.67	0.47	0.33	0.44	0.48
53. Cyclophilin type peptidyl-prolyl cis-trans isomerase family	AAH38716.1	0.47	0.46	0.58	0.41	0.47	0.48
54. Protein of unknown function	I_957288	0.50	0.69	0.44	0.41	0.48	0.50
55. Protein containing two C2 domains	AAB39720.1	0.41	0.87	0.44	0.46	0.47	0.53

MIA, MiaPaCa2.

by clonogenic assay. One explanation for the observed increase in radioresistance might be an adaptive response to radiation, a phenomenon that has been observed in human lymphocytes (23). However, we observed that the radioresistant sublines maintained a radioresistant phenotype for at least two months after cessation of irradiation exposure. Russell *et al* (24) reported that the selective pressure of repeated irradiation favors the emergence of radioresistant clones. Comparing the radioresistant sublines with their parental cell lines, we could truly examine genes associated with radioresistance.

DNA microarray analysis is a powerful tool for obtaining comprehensive information concerning expression of thousands of genes in cancer cells (15,19). In the current study, we identified 73 up-regulated (Table IV) and 55 down-regulated (Table V) genes common to at least four of the five radioresistant sublines. To the authors' knowledge, this is the first

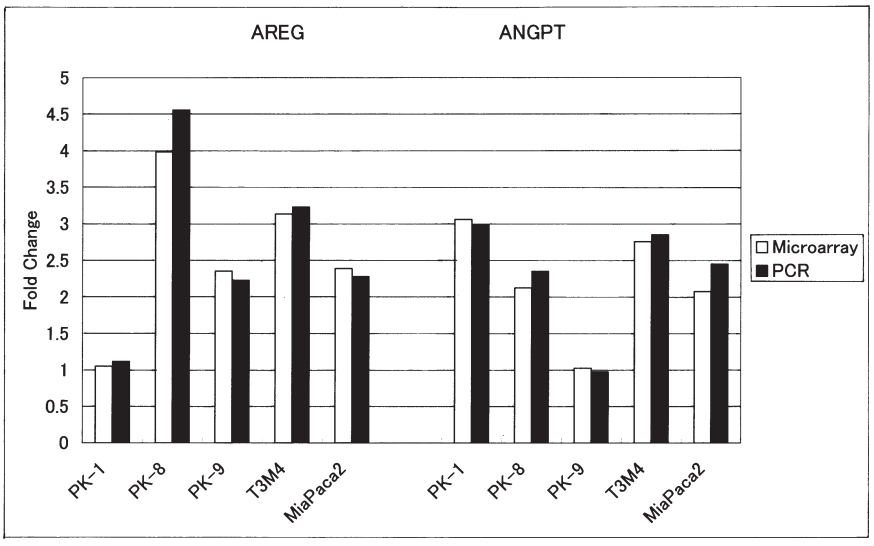

Figure 2. Real-time quantitative PCR analysis and microarray data for up-regulated genes: *amphiregulin*, and *angiopoietin 2*. For each sample, the left side bar (black) indicates the expression ratio of a cancer cell line by microarray while the right side bar (white) indicates the expression ratio by quantitative real-time PCR. All quantitative real-time PCR data were consistent with microarray data.

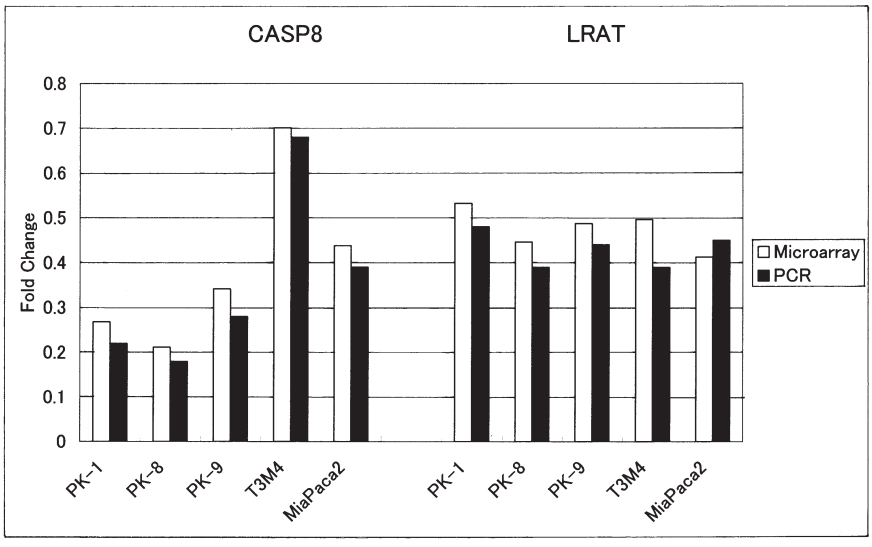

Figure 3. Real-time quantitative PCR analysis and microarray data for down-regulated genes: *caspase 8*, and *lecithin retinol acyltransferase*. For each sample, the left side bar (black) indicates the expression ratio of a cancer cell line by microarray while the right side bar (white) indicates the expression ratio by quantitative real-time PCR. All quantitative real-time PCR data were consistent with microarray data.

report indicating the differential gene expression profiles of radioresistant pancreatic cancer cell lines established by fractionated irradiation. Up-regulated genes were associated with growth factors, cell-cycle check points, intracellular signaling pathways and angiogenesis stimulation, whereas down-regulated genes were associated with apoptosis, retinoid esterification and electron transport. Previous reports concerning breast, esophageal and uterine cervical cancer also indicated that growth factors and intracellular signaling pathways were up-regulated while apoptosis-related genes were down-regulated in the radioresistant cancer or cancer cell lines (13-16). In the current study, some of these genes were previously known to be associated with responsiveness to radiation, such as *caspase 8* and *MAPKAPK2*, but others were novel. These novel genes can be expected to be involved in radioresistance, but the precise function of each gene remains unclear; so further study is necessary to clarify the nature of associations.

Genes up-regulated in radioresistanct cells include *amphiregulin*, *MAPKAPK2*, *regucalcin* and *angiopoietin 2*. Amphiregulin is a secreted hepain-binding growth factor that is structurally and functionally related to epidermal growth factor (EGF) and transforming growth factor α (TGF-α) (25). Amphiregulin protein stimulates cell replication and may be a promoter of tumor progression in multiple human cancers, including pancreatic cancer (26). Funatomi *et al* have indicated

that amphiregulin antisense oligonucleotide inhibits the growth of T3M4 human pancreatic cancer cells and sensitizes the cells to EGF receptor-targeted therapy (27). The presence of amphiregulin in cancer cells is associated with an increased frequency of local lymph node involvement (28), and the concomitant expression of EGFR and either EGF or TGF-α correlates with a decrease in patient survival (29). MAPKAPK2 is one of the protein kinases downstream of p38 MAPK (30). Han *et al* indicated that p38/MAPK/MAPKAPK2 signaling pathways regulate urokinase plasminogen activator mRNA stability in breast cancer cells (31). Recent reports have indicated that MAPKAPK2 is a cell-cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation (32). Regucalcin has been demonstrated to play a multifunctional role as a regulatory protein of the intracellular signaling process in both cytoplasm and nuclei of cells (33). Recent study has demonstrated that overexpression of regucalcin has a preventive effect on cell death and apoptosis in the cloned rat hepatoma cells (34). Angiopoietin 2 has been shown to play an intrinsic role in angiogenesis. Both VEGF and tissue hypoxia have been shown to up-regulate expression of angiopoietin 2 (35). Durkin *et al* indicated that angiopoietin 2 is overexpressed in pancreatic cancer (36).

Genes down-regulated in radioresistant cells include *caspase 8*, *lethicin retinol acyltransferase*, and *calcium-activated chloride channel 1*. Caspase 8 is a member of the cysteine protease family that plays a critical role in death receptor-mediated apoptosis. Caspase 8 has been reported to be a key molecule in radiation-induced apoptosis, and several reports have suggested that radiation-induced apoptosis utilizes mitochondria-dependent signal transduction (37,38). Uchida *et al* indicated that transduction of the caspase 8 gene using a minimal dose of adenoviral vector remarkably enhanced radiosensitivity of human colorectal cancer cells (37). The esterification of retinoids to retinyl esters is an important mechanism for maintaining tissue retinoids in a form that is easily stored and readily mobilized for additional metabolic needs (39). Recent reports have indicated there is a significant reduction in lecithin retinol acyltransferase expression in cancer compared with normal tissues, as well as an inverse correlation of this expression with increasing tumor stage (40). Therefore, restoration of lecithin retinol acyltransferase expression may be a reasonable strategy for cancer therapy. Calcium-activated chloride channel 1, activated by elevated intracellular calcium, has been shown to functionally exist in a variety of cell types, including cardiac myocytes, vascular smooth muscle cells, and endothelial cells (41). Recent reports have indicated that calcium-activated chloride 1 may regulate cancer cell apoptosis associated with perturbation of intracellular calcium channels (41,42). Expression of calcium-activated chloride 1 in normal mammary epithelium is consistently lost in human breast cancers, and re-expression of calcium-activated chloride 1 in human breast cancer cells abrogates invasiveness of Matrigel *in vitro* and tumorigenicity in nude mice, both of which indicate that calcium-activated chloride 1 acts as a tumor suppressor in breast cancer (43).

In conclusion, our oligonucleotide microarray analysis indicated that gene expression changes related to apoptosis, cell-cycle check point, growth metabolism, and angiogenesis factors are likely to contribute to the complexity of radioresistance seen in pancreatic cancer. Identification of these genes could lead to new therapeutic targets for pancreatic cancer. However, it is unlikely that this phenomenon can be explained by altered expression of a single gene. Whether this combination of gene expression changes leads to radioresistance of pancreatic cancers is a matter to be investigated further.

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