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Gene Expression Profiling Distinguishes Between Spontaneous and Radiation-induced Rat Mammary Carcinomas

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Ionizing radiation/Breast cancer/Sprague-Dawley rats/Radiation signature/Expression microarray.

The ability to distinguish between spontaneous and radiation-induced cancers in humans is expected to improve the resolution of estimated risk from low dose radiation. Mammary carcinomas were obtained from Sprague-Dawley rats that were either untreated (n = 45) or acutely γ-irradiated (1 Gy; n = 20) at seven weeks of age. Gene expression profiles of three spontaneous and four radiation-induced carcinomas, as well as those of normal mammary glands, were analyzed by microarrays. Differential expression of identified genes of interest was then verified by quantitative polymerase chain reaction (qPCR). Cluster analysis of global gene expression suggested that spontaneous carcinomas were distinguished from a heterogeneous population of radiation-induced carcinomas, though most gene expressions were common. We identified 50 genes that had different expression levels between spontaneous and radiogenic carcinomas. We then selected 18 genes for confirmation of the microarray data by qPCR analysis and obtained the following results: high expression of *Plg*, *Pgr* and *Wnt4* was characteristic to all spontaneous carcinomas; *Tnfsf11*, *Fgf10*, *Agtr1a*, *S100A9* and *Pou3f3* showed high expression in a subset of radiation-induced carcinomas; and increased *Gp2*, *Areg* and *Igf2* expression, as well as decreased expression of *Ca3* and non-coding RNA *Mg1*, were common to all carcinomas. Thus, gene expression analysis distinguished between spontaneous and radiogenic carcinomas, suggesting possible differences in their carcinogenic mechanism.

INTRODUCTION

Cancers arise from various types of cells via multiple oncogenic pathways that may involve various genetic and epigenetic alterations. Ionizing radiation is a well-known cause of human cancers. Humans are constantly exposed to cosmic radiation and naturally occurring radioactivity (*e.g.*, from radon gas and its decay products), but the largest component of radiation exposure is from medical sources.[1)] Whereas increased risks of cancers from exposure to large doses are unquestionable, the risk estimates at low doses are difficult because the estimated excess risk is much smaller than the background risk, which can be easily confounded by other factors.[2)] Currently, low dose radiation exposure risks are estimated by extrapolating high-dose risks, albeit with large uncertainty. Thus, it is anticipated that, if molecular fingerprints of radiation-related cancer could be established, it would help improve the risk estimations at low doses.[3)] However, investigation of genetic changes in cancer-related genes have not produced information on radiation-associated alterations with only a few exceptions.[4–6)] Recent evidence indicates that radiation induces persistent genetic instability in the progeny of irradiated cells, and the spectrum of these resulting mutations is very similar to that of spontaneously arising mutations, which implies that radiation increases the rate of spontaneous cancer incidence by enhancing accumulation of mutations.[7–9)] Alternatively, it is hypothesized that the carcinogenic effect of radiation is mediated by induction of clonal expansion of cells which already harbor spontaneously-arising mutations of cancer-related genes.[10)]

Analysis of gene expression profiles for tumors is a powerful tool in cancer biology. It has been utilized to classify cancer subtypes, to predict therapeutic outcomes and to choose appropriate targeted therapies.[11–13)] Recently, rat mammary cancers induced by several chemical carcinogens have been compared based on their gene expression profiles,

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and the profiles of these cancers reflect, to some extent, differences among etiological agents.[14–16] Rat mammary cancer is an important experimental model of human breast cancer due to similarities in both pathology and hormone dependence.[17,18] Several epidemiological studies have shown that breast cancer is one of the most prevalent cancers after radiation exposure.[19,20] Thus, the rat model of mammary cancer has been a useful tool to analyze radiation-induced breast cancer.[21–23] Animal cancer models are also advantageous because they are induced by defined carcinogenic agents in contrast to human cancers, which are rarely ascribed to a single etiological factor.

Thus, comparison of gene expression profiles should provide evidence of molecular characteristics that distinguish between radiation-induced and spontaneously developed rat mammary carcinomas. In the present study, we analyzed gene expression profiles of mammary carcinomas from irradiated and non-irradiated rats using oligonucleotide microarrays in combination with quantitative polymerase chain reaction (qPCR) and showed that ionizing radiation–induced and spontaneous cancers can be distinguished based on their gene expression profiles.

MATERIALS AND METHODS

Mammary carcinomas and normal mammary glands

Mammary carcinomas were collected in our previous study.[24,25] Briefly, 7-week-old Sprague Dawley rats were either treated with γ rays (1 Gy, $n = 20$) or left untreated ($n = 45$). All rats were fed a high corn-oil diet, and rats with palpable tumors were sacrificed at 1 year of age, or earlier in case of moribundity, for tissue collection. As reported previously, the multiplicities of palpable mammary carcinoma that developed in untreated and irradiated rats before one year of age were 0.067 and 0.506, respectively.[25] We refer to them as spontaneous and radiation-induced carcinomas, hereafter. Their first ages of detection were 32.0 ± 5.9 and 29.6 ± 11.2 weeks of age (mean ± standard deviation), respectively, and were not significantly different.[25] For molecular analyses, we added one spontaneous carcinoma that developed later at 70 weeks of age and used in total 4 spontaneous and 10 radiation-induced adenocarcinomas that were histologically uniform (papillotubular and tubular types) and contained no identifiable necrotic region. Normal mammary tissues were collected from rats of the untreated group (1 year of age) that did not develop mammary carcinoma. Tissues were snap-frozen in liquid nitrogen and stored at –80°C until use.

Microarray analysis

Three spontaneous and four radiation-induced carcinomas, as well as three normal mammary glands, were randomly selected for microarray analysis. GeneChip Rat Genome 230 2.0 arrays carrying 31,024 probe sets, where each probe set corresponds to one gene sequence, were purchased from Affymetrix (Santa Clara, CA, USA). The procedures for complementary RNA (cRNA) labeling, hybridization and image scanning were essentially identical to those described.[14] Briefly, total RNA was isolated by the acid guanidine phenol chloroform method (Isogen; Nippon Gene, Tokyo, Japan) and further purified with a silica-gel membrane (RNeasy Mini kit; Qiagen Inc., Valencia, CA, USA). The quality of all RNA samples was assessed by formalin-containing agarose gel electrophoresis. Total RNA (8 μg) was used for the first-strand cDNA synthesis with a T7-$(dT)_{24}$ primer (Proligo, Kyoto, Japan) and SuperScript III reverse transcriptase (Invitrogen Co., Carlsbad, CA, USA). Double-stranded cDNA was then synthesized with *E. coli* RNase, *E. coli* DNA polymerase and *E. coli* DNA ligase (Toyobo, Tokyo, Japan). Biotin-labeled fragmented cRNA was subsequently prepared with a BioArray HighYield RNA Transcript Labeling kit (Enzo, Farmingdale, NY, USA). Labeling was confirmed by formalin-containing agarose gel electrophoresis. Labeled cRNA was placed in a hybridization mixture containing control biotinylated probes according to manufacturer's instructions. GeneChip arrays were hybridized with labeled cRNA for 16 h at 45°C with constant rotation (60 rpm). The arrays were washed and then stained with streptavidin-phycoerythrin conjugate (Molecular Probes, Tokyo, Japan) in a Fluidics Station 450 (Affymetrix) and subsequently scanned with the GeneChip Scanner 3000 (Affymetrix). The scanned images were processed using Affymetrix GeneChip Analysis Suite software. Each data set was scaled such that the average intensity of all probe sets was adjusted to 500. Data were exported to flat text files and used for statistical analysis.

Data analysis

Probe sets with fluorescent intensity values less than 1,000 for all carcinoma arrays were excluded in order to confine analysis to quantitatively reliable data. Before conducting clustering analysis, the fold change values, in comparison with the average intensity values for normal mammary glands, were transformed to base-2 logarithms. Average linkage clustering of an uncentered Pearson correlation similarity matrix was applied with the Cluster software, and the figures were generated with the TreeView program.[26] Similarities among expression profiles were assessed by the Pearson correlation coefficient.[26] Welch's *t*-test was used to calculate *P* values for gene selection.

qPCR analysis

First-strand cDNA was synthesized from purified total RNA as described.[27] The qPCR reaction was performed on an Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA). The expression of a housekeeping gene, *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), was first measured as an internal standard by qPCR (TaqMan Rodent

Table 1. Primers and their annealing temperatures for quantitative PCR

Gene symbol [a]	Gene name	Forward primer Reverse primer [b]	T_a [c]
Plg	Plasminogen	TGTGCAACCGCGCTGAGTAT	60
		AGCACAGCCAAGACCCCAAG	
Rn.160502	EST	AGGAGGGCCCAGAGTCCAAG	60
		AGGCGAGACAGCGAGAAGGA	
Pgr	Progesterone receptor	GGGTGGTCCCCAGTTCACAA	60
		CCGGAAATTCCACAGCCAGT	
Wnt4	Wingless-related MMTV integration site 4	ACAACGAGGCTGGCAGGAAG	60
		TTAGTGCGTGGCCAACCTGA	
Rn.20273	EST	GGTTCCAGCACGTTGGTCCT	60
		TGTAATCGTTCTCCTCTTGGGACA	
Kit	v-kit oncogene homolog	TGCCGGTCGATTCCAAGTTT	60
		TTGGCCTTTTCAGGGGATCA	
Tnfsf11	Tumor necrosis factor superfamily member 11	GGAAGGTTCGTGGCTCGATG	60
		GCCCAGCCTCGATCATGGTA	
Fgf10	Fibroblast growth factor 10	GGGAGATGTCCGCTGGAGAA	60
		CGGCAACAACTCCGATTTCC	
Agtr1a	Angiotensin II receptor type 1	TGGCTGGCATTTTGTCTGGA	60
		CCTTGGGGCAGTCATCTTGG	
Pou3f3	POU domain class 3 transcription factor 3	GGCGCAGGAGATCACCAACT	60
		GGTCCCCACCTGCGAGTAGA	
S100a9	S100 calcium binding protein A9	TGGACATCCTGACACCCTGAA	64
		GGTTTGTGTCCAGGTCCTCCA	
Rn.177404	EST	CCTCCCAGGCTTTCCCACTT	60
		GAGTGCCACCGGATCTTTGG	
Ptges	Prostaglandin E synthase	ACGCGTTGAAACGTGGAGGT	60
		AGAGGGTTGGGTCCCAGGAA	
Gp2	Glycoprotein 2	TCGCAGTAGTGAACCAGCCATC	60
		GCCAGGAAGACAGGCAGGAA	
Areg	Amphiregulin	CGTCGCAGCTATTGGCATCA	60
		TGGCTTGGCAGTGACTCGAC	
Igf2	Insulin-like growth factor 2	GGACCGCGGCTTCTACTTCA	60
		CACGTCCCTCTCGGACTTGG	
Ca3	Carbonic anhydrase 3	GGACGGGAGAAAGGCGAGTT	60
		CCAATAGTCCCGGCAAGCAG	
RGD:727910	Mg1 protein	CAGTGCTGCCAAGACCCTGA	60
		CCACCATCCCTCACACTCACA	

[a] The Unigene ID is shown for unidentified expressed sequence tags (ESTs).
[b] Base sequences are indicated in the order of 5' to 3'.
[c] Annealing temperature (°C).

GAPDH Control Reagent, VIC Probe; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The concentration of cDNA in samples was adjusted so that the fluctuation of *Gapdh* expression was less than two-fold between samples. Then qPCR analysis on genes of interest was performed with a commercial mixture of Taq DNA polymerase and a fluorescent dye (SYBR Premix Ex Taq; Takara Bio Inc., Otsu, Japan). The PCR program consisted of denaturation at 95°C for 10 sec and 45 subsequent amplification cycles of denaturation at 95°C for 5 sec and annealing/elongation at the temperature indicated in Table 1 for 20 sec. The primer sequences are listed in Table 1. Relative gene expression was calculated by the $2^{-\Delta\Delta C_T}$ method.[28]

RESULTS

Gene expression profile of mammary carcinomas

Three spontaneous and four radiation-induced mammary adenocarcinomas, as well as three normal mammary tissues, were firstly analyzed by expression microarrays carrying 31,024 probe sets, each of which corresponds to one gene sequence. We selected data from 6,926 probe sets for cluster analysis excluding those with an intensity value less than 1,000. Unsupervised hierarchical clustering separated a cluster of spontaneous carcinomas from radiation-induced cancers, whereas the expression profiles of radiation-induced cancers did not form a single cluster (Fig. 1). Changing the threshold intensity value for probe set selection between 50 and 3,000 did not affect the topology of the dendrogram (data not shown), indicating the robustness of the clustering result. Spontaneous and radiation-induced cancers were thus distinguishable based on their global expression profiles.

Genes with differential expression

We then analyzed the intensity data of 6,926 genes to select those that exhibited differential expression between these two types of cancers. First, we searched for genes that had P values < 0.05 by Welch's t-test and simultaneously showed > 2-fold difference in the average intensity between the two groups. We obtained 33 genes that fulfill this criterion, but none of them were increased specifically in radiogenic tumors as compared to normal tissues (Table 2). Because the global gene expression of radiogenic carcinomas were heterogeneous (Fig. 1A), we speculated that radiogenic cancer-specific gene expression would be confined to a subset of radiogenic carcinomas and we might have overlooked such genes using the cutoff value of $P < 0.05$. We therefore searched for genes that had P values ≥ 0.05 but exhibited > 4-fold higher expression in radiogenic carcinomas as compared to spontaneous ones, identifying 18 additional genes that fulfill this criterion (Table 3).

Clustering analysis (Fig. 1) indicates that most genes were commonly altered in all of seven carcinomas. We examined

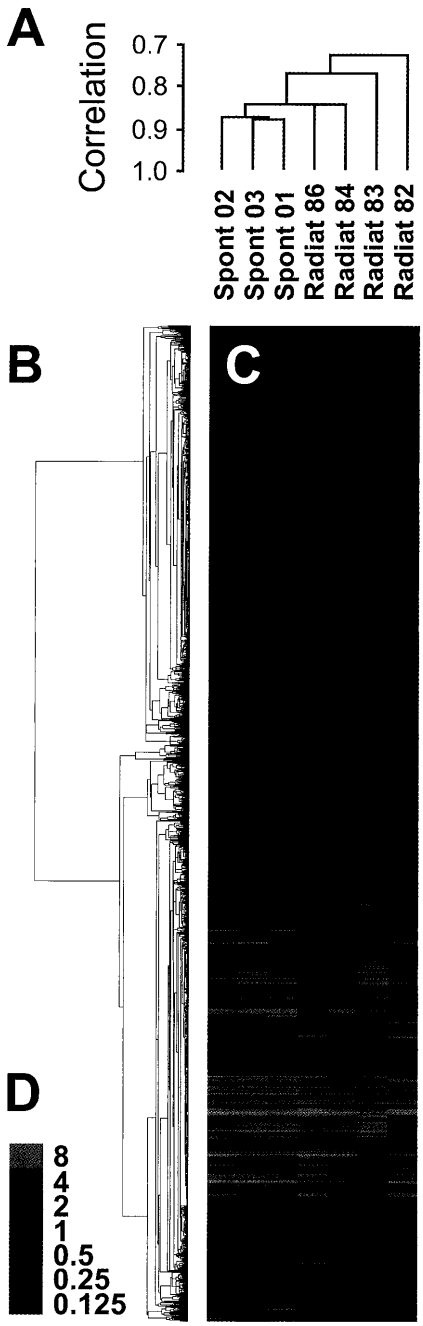

Fig. 1. Hierarchical clustering of global gene expression profiles for rat mammary carcinomas. *A*, Clustering of three spontaneous (*Spont*) and four ionizing radiation–induced (*Radiat*) carcinomas, showing the degree of similarity between tumors. The Pearson correlation coefficient is indicated on the left. *B*, Clustering of 6,926 genes. *C*, Overall expression profiles of 6,926 genes across the seven rat mammary carcinomas. *D*, Color scale for panel *C*.

the average intensity values of seven carcinomas and three normal glands, in which Welch's t-test identified 2,407 genes from the 6,926 probe-set data ($P < 0.05$). We listed the top 30 genes, including 15 of those most increased and 15

Table 2. Genes that fulfilled the criteria of $P < 0.05$ and > 2-fold difference between spontaneous and radiation-induced rat mammary carcinomas in the microarray analysis

Gene symbol	Gene name	*P* value [a]	Log fold change (vs. normal) [b]	
			Spontaneous	Radiation
Plg	Plasminogen	0.02	4.0	0.8
RT1-Ba	RT1 class II, locus Ba	0.02	2.0	–0.6
Igfals	Insulin-like growth factor binding protein, acid labile subunit	0.02	6.8	4.4
Rn.160502 [c]	EST	<0.01	1.6	–0.4
Pgr	Progesterone receptor	0.04	3.6	1.7
Scamp1	Secretory carrier membrane protein 1	<0.01	3.8	2
LOC363320	Similar to Discs large homolog 5 (Placenta and prostate DLG)	0.03	2.2	0.5
Rn.42977 [c]	EST	<0.01	3.2	1.5
LOC306096	Similar to Dachshund homolog 1 (Dach1)	<0.01	2.5	0.9
LOC685462	Similar to EMI domain containing 1	0.04	3.3	1.8
Rn.173547 [c]	EST	0.02	1.1	–0.4
Spon2	Spondin 2, extracellular matrix protein	0.02	1.7	0.3
Eiih	Hepatic protein EIIH	0.03	2.4	1
Sytl2_predicted	Synaptotagmin-like 2 (predicted)	<0.01	3.2	1.8
Gpr37	G protein-coupled receptor 37	<0.01	5.4	4
Rn.39113 [c]	EST	0.02	4.3	3.1
Cd200	Cd200 antigen	<0.01	2.7	1.6
Scnn1a	Sodium channel, nonvoltage-gated 1 alpha	0.01	2.1	1
RGD1308221 predicted	Similar to TBC1 domain family, member 8 (with GRAM domain); vascular Rab-GAP/TBC-containing (predicted)	0.02	2.1	0.9
Wnt4	Wingless-related MMTV integration site 4	0.02	4.3	3.1
Rn.40510 [c]	EST	0.04	2.9	1.8
RGD1562168 predicted	Similar to retinoid binding protein 7 (predicted)	0.01	–1.7	–3.1
Slpi	Secretory leukocyte peptidase inhibitor	0.03	0.1	–1.2
Ptges	Prostaglandin E synthase	0.03	–1.0	–2.2
Scnn1g	Sodium channel, nonvoltage-gated 1 gamma	<0.01	–0.3	–1.3
Rn.20273 [c]	EST	0.03	–3.1	–0.9
C3	Complement component 3	<0.01	–3.1	–1.0
RT1-Aw2 [e]	RT1 class Ib, locus Aw2	0.05	–1.7	0.3
Hba-a1 [e]	Hemoglobin alpha, adult chain 1	0.05	–1.3	0.3
RT1-CE5	RT1 class I, CE5	0.03	–1.1	0
Rn.33382 [c]	EST	0.05	–0.8	0.3
Kit	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	0.05	–1.1	–0.1
RT1-CE16	RT1 class I, CE16	0.02	–1.0	1.1

[a] Intensity values of spontaneous and radiation-induced carcinomas were subjected to Welch's *t*-test.
[b] Base-2 logarithm for the ratio of the average intensity value of carcinomas to that of normal tissues.
[c] The Unigene ID is indicated for unidentified expressed sequence tags (ESTs) and genes without gene symbols.

Table 3. Genes that showed $P \geq 0.05$ but > 4-fold higher expression in radiation-induced rat mammary carcinomas than spontaneous carcinomas in the microarray analysis

Gene symbol	Gene name	*P* value [a]	Log fold change (vs. normal) [b]	
			Spontaneous	Radiation
Rn.133430 [c]	EST	0.27	–2.4	3.3
Tnfsf11	Tumor necrosis factor (ligand) superfamily, member 11	0.28	0.1	5.7
Rn.177404 [c]	Transcribed locus, moderately similar to NP_620608.1 protein LOC207121	0.20	–0.9	2.7
Rn.47673 [c]	EST	0.38	0.5	4
Fgf10	Fibroblast growth factor 10	0.31	–1.3	1.9
Agtr1a	Angiotensin II receptor, type 1 (AT1A)	0.08	0.1	3.1
Cyp26b1	Cytochrome P450, family 26, subfamily b, polypeptide 1	0.43	0.2	3.1
Lpo_predicted	Lactoperoxidase (predicted)	0.35	–3.8	–0.9
Pou3f3	POU domain, class 3, transcription factor 3	0.21	–0.4	2.5
LOC689064	Beta-globin	0.18	–1.1	1.4
RT1-Aw2	RT1 class Ib, locus Aw2	0.05	–2.0	0.1
RT1-Bb	RT1 class II, locus Bb	0.11	–1.1	1
RT1-Ba	RT1 class II, locus Ba	0.06	–1.6	0.5
S100a9	S100 calcium binding protein A9 (calgranulin B)	0.27	–0.8	1.2
RGD1305645 predicted	Similar to RIKEN cDNA 1500015O10 (predicted)	0.18	0.0	2.1
Rn.54456 [c]	Polymeric immunoglobulin receptor AATTAA-containing 3'UTR Group 1 mRNA sequence	0.26	–4.7	–2.7
Tmem2_predicted	Transmembrane protein 2 (predicted)	0.14	0.2	2.2
Nhn1 or Flt4	Conserved nuclear protein Nhn1 *or* Fms-related tyrosine kinase 4	0.11	2.2	4.2

[a] Intensity values of spontaneous and radiation-induced carcinomas were subjected to Welch's *t*-test.
[b] Base-2 logarithm for the ratio of the average intensity value of carcinomas to that of normal tissues.
[c] The Unigene ID is indicated for unidentified expressed sequence tags (ESTs) and genes without gene symbols.

of those most down-regulated, respectively (Table 4).

Validation of microarray results by qPCR

From genes listed in Tables 2 and 3, which were specific to either spontaneous or radiation-induced carcinomas, we chose 10 probe sets for known genes of importance in regard to their biological functions. We also randomly selected 3 probe sets for unidentified expressed sequence tags (EST). Furthermore, 5 additional genes were selected from Table 4 that exhibited altered expression in all carcinomas as compared to normal tissue. Their expression was analyzed by qPCR for validation of microarray results. Microarray and qPCR results for the same set of tumors showed significant correlation for 16 of 18 genes (Table 5). To exclude the possibility that the above results held true only for this particular set of tumors, we analyzed additional tissue samples including one spontaneous and five radiation-induced mammary carcinomas and one normal tissue that were available from the same experiment but not previously examined by microarrays. Together with these additional data, we observed that three spontaneous cancer-specific up-regulated genes (*Plg, Pgr* and *Wnt4*) still maintained their statistical significance, whereas the differences turned out to be non-significant for three other genes (EST Rn.160502, *Ptges* and EST Rn.20273; Table 5, *rightmost column*). Likewise, regarding five radiation-associated genes (*Tnfsf11, Fgf10, Agtr1a, Pou3f3* and *S100a9*), some radiation-induced carcinomas showed a larger value than the mean value of four spontaneous carcinomas (Fig. 2), in which the difference was more than three times larger than the standard deviation of four values of spontaneous carcinomas. Moreover, statistically significant difference was observed in the expression of *Tnfsf11* and *Agtr1a* between radiogenic and spontaneous carcinomas ($P < 0.05$, Table 5, *rightmost column*). Thus, qPCR analysis confirmed that eight genes were specifically up-regulated in either spontaneous or radiation-induced

Table 4. The top 15 genes, either up-regulated or down-regulated, in rat mammary carcinomas compared to normal mammary glands in the microarray analysis

Gene symbol	Gene name	*P* value [a]	Log fold change [b]
i) High expression in carcinomas			
Mup5	Major urinary protein 5	0.04	8.0
Mcpt10	Mast cell protease 10	0.02	6.2
Igfals	Insulin-like growth factor binding protein, acid labile subunit	0.03	5.9
Gp2	Glycoprotein 2 (zymogen granule membrane)	<0.01	5.4
Cdkn2a	Cyclin-dependent kinase inhibitor 2A	0.03	5.4
Cited1	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	<0.01	4.9
Gpr37	G protein-coupled receptor 37	<0.01	4.8
Pgf	Placental growth factor	<0.01	4.7
Areg	Amphiregulin	<0.01	4.6
Tcfap2b_predicted	Transcription factor AP-2 beta (predicted)	<0.01	4.0
Rn.81000 [c]	EST	<0.01	3.9
Mmp3	Matrix metallopeptidase 3	0.02	3.9
Col2a1	Procollagen, type II, alpha 1	0.02	3.9
Igf2	Insulin-like growth factor 2	0.01	3.8
Id4	Inhibitor of DNA binding 4	<0.01	3.8
ii) Low expression in carcinomas			
Ca3	Carbonic anhydrase 3	0.02	–5.1
Cd36	Cd36 antigen	<0.01	–4.0
Acsl1	Acyl-CoA synthetase long-chain family member 1	0.01	–3.6
Angptl4	Angiopoietin-like 4	<0.01	–3.6
Thrsp	Thyroid hormone responsive protein	0.02	–3.6
Rn.54456 [c]	Polymeric immunoglobulin receptor AATTAA-containing 3'UTR Group 1 mRNA sequence	<0.01	–3.2
IgG-2a	Gamma-2a immunoglobulin heavy chain	<0.01	–3.1
LOC316122	CGI-58-like protein	0.02	–3.1
Slc34a2	Solute carrier family 34 (sodium phosphate), member 2	0.03	–3.0
Rn.17804 [c]	EST	<0.01	–2.7
LOC287004	Mg1	0.03	–2.6
Igha_mapped	Immunoglobulin heavy chain (alpha polypeptide) (mapped)	0.03	–2.4
Tf	Transferrin	0.04	–2.4
Ndrg1	N-myc downstream regulated gene 1	0.02	–2.4
Rn.19106 [c]	EST	0.02	–2.4

[a] Intensity values of carcinoma and normal tissue were subjected to Welch's *t*-test.
[b] Base-2 logarithm for the ratio of the average intensity value of carcinomas to that of normal tissues.
[c] The Unigene ID is indicated for unidentified expressed sequence tags (ESTs) and genes without gene symbols.

Table 5. Quantitative PCR (qPCR) validation of differential gene expression identified by microarray analysis

Gene symbol	Gene name	Correlation	Log fold [b]		Difference
		P value [a]	Array	qPCR	*P* value [c]
i) $P < 0.05$ between spontaneous and radiation in microarrays (Table 2)					
Plg	Plasminogen	<0.01	3.2	4.1	<0.01
Rn.160502 [d]	EST	<0.01	2.0	3.1	0.08
Pgr	Progesterone receptor	<0.01	1.7	1.5	<0.01
Wnt4	Wingless-related MMTV integration site 4	<0.01	1.1	0.7	0.03
Ptges	Prostaglandin E synthase	<0.01	1.2	1.3	0.42
Rn.20273 [d]	EST	<0.01	–2.3	–2.7	0.09
Kit	v-kit oncogene homolog	0.08	–1.0	–1.2	0.25
ii) $P \geq 0.05$ between spontaneous and radiation in microarrays (Table 3)					
Tnfsf11	Tumor necrosis factor superfamily member 11	<0.01	–4.1	–6.9	0.03
Fgf10	Fibroblast growth factor 10	0.01	–3.2	–8.8	0.08
Agtr1a	Angiotensin II receptor type 1	<0.01	–2.8	–4.1	0.01
Pou3f3	POU domain class 3 transcription factor 3	<0.01	–2.9	–4.0	0.07
S100a9	S100 calcium binding protein A9	<0.01	–2.1	–2.0	0.15
Rn.177404 [d]	EST	0.07	–3.7	–1.1	0.07
iii) Common to all carcinomas (Table 4)					
Gp2	Glycoprotein 2	0.01	5.4	7.3	<0.01
Areg	Amphiregulin	<0.01	4.6	3.9	0.02
Igf2	Insulin-like growth factor 2	<0.01	3.8	5.8	0.02
Ca3	Carbonic anhydrase 3	0.02	–5.1	–8.2	<0.01
RGD:727910	Mg1	<0.01	–2.6	–4.2	<0.01

[a] Expression values of each carcinoma, relative to the average expression in normal tissues, were transformed into base-2 logarithms and then compared between microarrays and qPCR.
[b] Base-2 logarithm for the ratio of average expression in spontaneous carcinomas to that in radiation-induced ones for the top two categories (i and ii) or the ratio of average expression in carcinomas to that in normal mammary glands for the last category (iii).
[c] qPCR values, relative to the average expression in normal tissues, were transformed into base-2 logarithms and compared between spontaneous and radiation-induced carcinomas (i and ii) or between carcinomas and normal mammary glands (iii) by Welch's *t*-test. The qPCR analysis incorporated additional samples that were not analyzed with microarrays.
[d] The Unigene ID is indicated for unidentified expressed sequence tags (ESTs).

mammary carcinomas. The analysis also confirmed that the three genes (*Gp2, Areg* and *Igf2*) showed increased expression in both spontaneous and radiogenic carcinomas, whereas two genes (*Ca3* and *RGD:727910*) exhibited decreased expression (Table 5).

The expression of five possibly radiation-associated genes (*Tnfsf11*, *Fgf10*, *Agtr1a*, *Pou3f3* and *S100a9*) varied widely between nine radiation-induced carcinomas examined. Therefore, we searched for correlation of these gene expressions with some of parameters such as tumor weight and latency. The expression of *S100a9* showed a significant ($P < 0.05$) inverse correlation with the latent period of radiation-induced tumors, whereas those of other genes did not (Fig. 3). No correlation was found between either of these five gene expressions and tumor weight (data not shown). Moreover, significant correlation existed between expression levels (log-transformed value relative to the average of those for normal tissues) of *Agtr1a* and *Tnfsf11* ($P < 0.01$) and between those of *Agtr1a* and *Fgf10* ($P < 0.002$) and the levels of *Tnfsf11* and *Fgf10* were indicative of correlation ($P = 0.06$); no correlation existed for other combinations (data not shown).

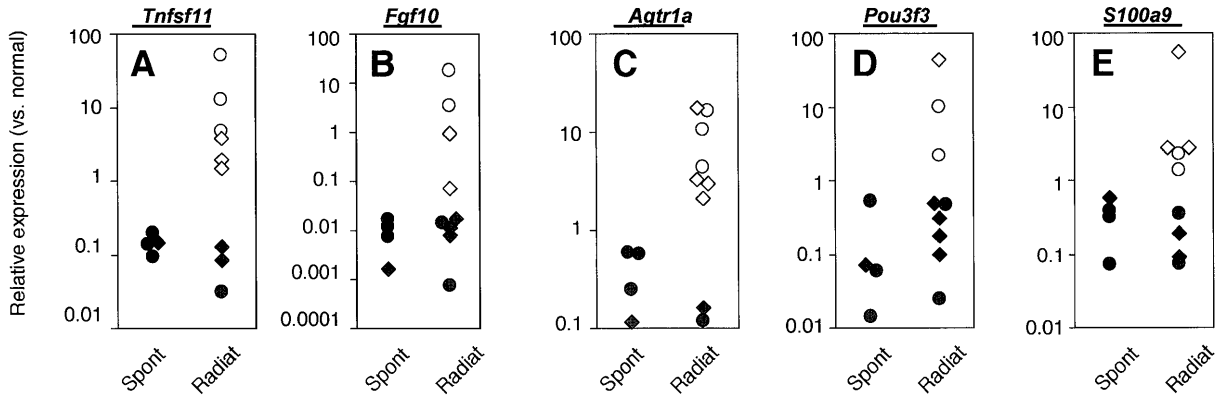

Fig. 2. Expression levels of five genes in each carcinoma as determined by quantitative PCR (qPCR). Four spontaneous (*Spont*) and nine radiation-induced (*Radiat*) carcinomas were analyzed for expression of *Tnfsf11, Fgf10, Agtr1a, Pou3f3* and *S100a9* (panels A–E, respectively), and data are indicated as relative expression values compared to the average of four normal mammary glands. *Circles* indicate tumors that were used for the microarray analysis and *diamonds* indicate additional samples examined only by qPCR. *Gray symbols* represent tumors whose expression levels were within ± 3 standard deviations of spontaneous tumor levels; other tumors are represented by *white symbols*.

DISCUSSION

The present study was undertaken to clarify whether tumor gene expression correlates with tumor etiology (spontaneous development versus induction by ionizing radiation) in a rat mammary cancer model. We showed that three spontaneous mammary cancers could be distinguished from four radiation-induced cancers based on their global gene expression profiles. We then focused on 18 differentially expressed genes and confirmed by qPCR that eight of them were differentially expressed even after new tissue samples were added into the analysis. Thus, we show for the first time that radiation-induced rat mammary cancer is distinguishable from spontaneous one according to their gene expression.

It is not well understood why cancers of different etiological origin can be distinguished using gene expression profiles. In accordance with our data, rat mammary cancers induced by different chemical carcinogens have been successfully classified by their causative agents based on their gene expression profiles[14,16]; however, the rationale for such classification has not been provided. In our study, gene expression profiles of spontaneous cancers were homogenous, whereas radiation-induced cancers comprised a heterogeneous population. The following hypothesis, although speculative, might account for this observation: During the initiation step of spontaneous mammary tumor development, rare mutations must accumulate over time, which should only occur in long-lived, stem-like cells. Given that the target cell type is thus limited, the resulting cancers would resemble one another. In contrast, acute exposure to ionizing radiation may produce a large number of mutations in a cell in a short period; therefore, relatively short-lived partially differentiated cells could also be the origin of radiation-induced mammary carcinoma. The various states of differentiation among these cells might reflect the heterogeneity of the resulting cancers. Actually, genes such as *Pgr*, *Wnt4* and *Plg* are involved in ductal branching,[29,30] which is a relatively early step of the mammary gland development, and were specifically up-regulated in spontaneous cancers. In contrast, radiation-associated genes such as *Tnfsf11* and *Fgf10* encode secreted factors that are thought to direct alveolar morphogenesis,[31,32] a rather later step of mammary gland differentiation. Furthermore, though the function of *Agtr1a* in the mammary gland is unknown, the correlation between the expression levels of *Tnfsf11*, *Fgf10* and *Agtr1a* suggests the link in their role in radiation-induced development of rat mammary carcinoma.

As gene expression profiles have previously been investigated for chemically-induced rat mammary carcinomas,[14–16] comparison of our present result with these previous data inform us whether above genes are specific to spontaneous or radiation-induced tumors or are also expressed in chemically-induced cancers. In one of these previous studies,[14] although the number of genes on the microarrays were small therein, two spontaneous tumor-specific (*Plg* and *Pgr*) and three radiation-specific (*Fgf10, Pou3f3* and *S100a9*) genes did not show high expression levels in chemically-induced carcinomas. This ascertains that high expressions of these genes are specific to spontaneous and radiogenic mammary cancers, respectively. *Agtr1a* were upregulated in most of chemically-induced carcinomas, indicating its possible relevance to exogenously (regardless of radiation- or chemically) induced, but not spontaneous, cancer development.

In addition, an inverse correlation existed between *S100a9* expression and tumor latency. This relationship suggests that *S100a9*-overexpressing cancer develops more rapidly than

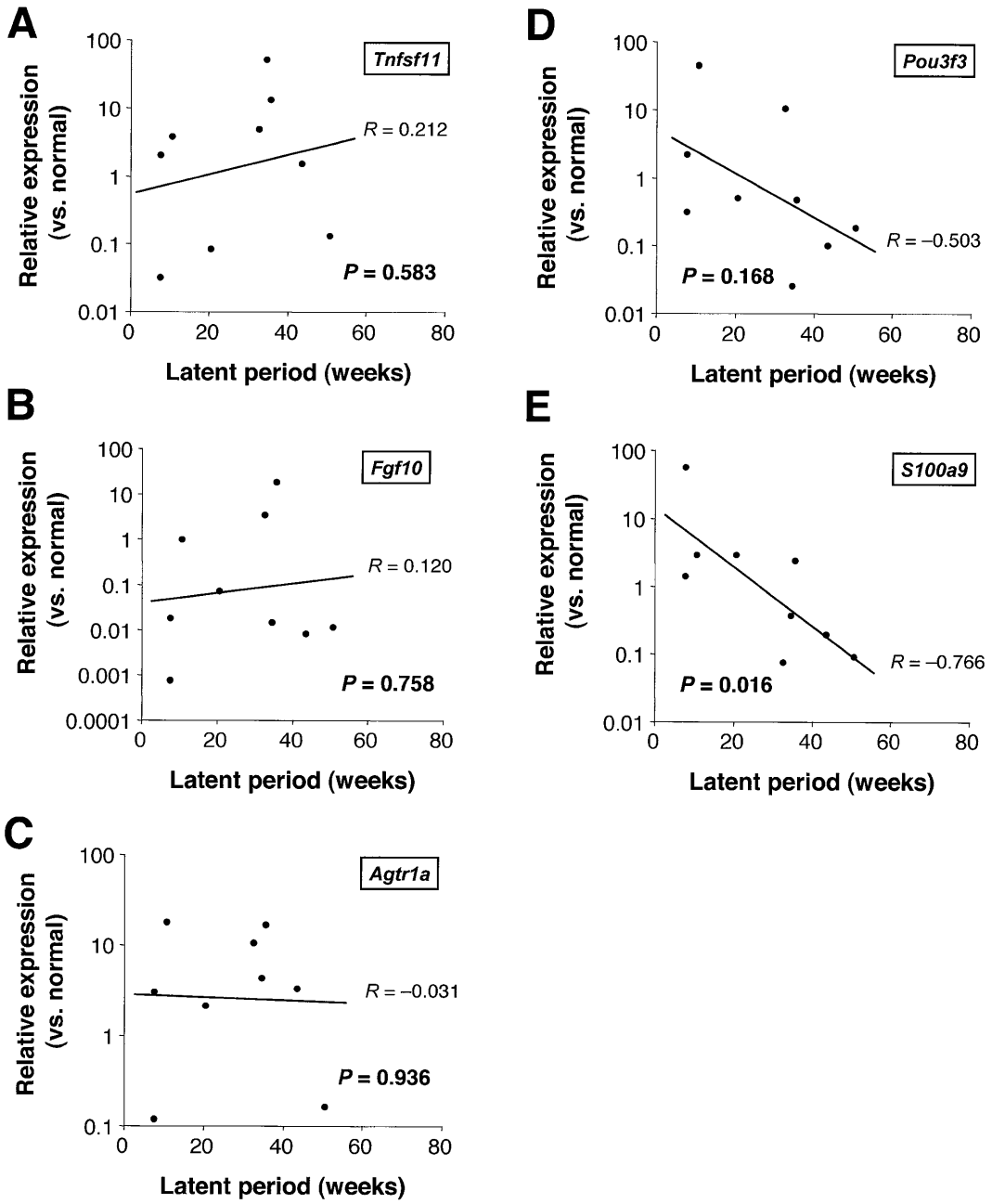

Fig. 3. Scatter plots showing the relationship between the latent period and expression of various genes for radiation-induced rat mammary carcinomas. The expression values of *Tnfsf11*, *Fgf10*, *Agtr1a*, *Pou3f3* and *S100a9* (panels *A–E*, respectively) in each tumor were determined by quantitative PCR and expressed as relative values compared to the average of four normal mammary glands. Each dot indicates one tumor sample. The least square method was applied to give linear approximation to the relationship between the latent period and logarithm-transformed expression values (lines). Pearson's correlation coefficient (R) and the P value for its significance are also indicated.

that without its overexpression, implying association between its expression and accelerated tumor progression. In fact, S100A9 immunopositivity has been reported to be a marker of poor differentiation in human breast cancers.[33]

We also found many changes in gene expression that were common to both types of carcinomas. Because rat mammary cancers are mostly of ductal origin, the search for spontaneous or radiogenic cancer-specific gene expression may have neglected important genes for ductal elongation, which should be up-regulated in both spontaneous and radiogenic mammary cancers. Some of these genes, for example *Areg*, *Cited1* and *Mmp3*,[34–36] showed increased expression in all

examined tumors. We also found several genes that showed decreased expression in carcinomas. Most of these (*e.g., Ca3*, *Cd36* and *Acsl1*) were adipocyte-associated genes and may merely reflect the adipocyte-rich composition of normal mammary tissue. Nevertheless, a previous study has shown that the non-coding RNA *Mg1*, which is encoded by the *RGD:727910* locus, is associated with hormone-induced protection against mammary cancer development in rats.[37] Our result is the first to show that this gene product is down-regulated in mammary carcinomas and supports the possibility that this non-coding RNA is a tumor suppressor.

In summary, our results indicate that spontaneous and radiogenic rat mammary cancers are distinguishable based on global and specific gene expression patterns, even though most gene expression changes were common to both cancers. The data indicate that spontaneous and radiogenic mammary cancer development involves distinct molecular and cellular mechanisms. When applied to human cancers, the distinction between radiogenic and spontaneous cancers will be helpful in assessing the risk of cancer from low doses of radiation.

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