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Review

Combined Effect of Ionizing Radiation and Alkylating Agents on Cancer Induction

Yoshiya Shimada[1,3], Mayumi Nishimura[1], Shizuko Kakinuma[1], Kazumi Yamauchi[1], Tatsuhiko Imaoka[1], Yoshiko Amasaki[2], Yi Shang[1], Isao Kawaguchi[2] and Masahiro Doi[2]

[1]Experimental Radiobiology for Children's Health Research Group and [2]Regulatory Sciences Research Group, National Institute of Radiological Sciences, Chiba, Japan

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Human beings are exposed to numerous natural and man-made agents that are potentially carcinogenic. Therefore, cancer risk by ionizing radiation (IR) should be assessed as a result of combined exposures with other agents. These agents include genotoxic and non-genotoxic chemical carcinogens such as, tobacco, hormones, viruses, metals etc. Carcinogenesis is a multi-step process that accumulates several genetic and epigenetic changes of oncogenes and tumor suppressor genes. For agents having similar biological function and affecting the same step of carcinogenesis, additivity is generally expected, while for agents acting at different rate-limiting step, combined exposure is expected to be deviated from additivity. Conceptually, carcinogens are classified as initiator and promoter. IR could function at several steps as initiator, promoter or both. In order to predict the mode of combined action of IR with other agents, the sequence and time interval of the exposures, the dose, and the type of exposure (acute or chronic) are the critical factors. In this review, we focus on the combined effect of IR and alkylating agents. The data in the literatures and in our laboratory on mouse thymic lymphomas indicate that combined effect of these two genotoxic agents is synergistic, additive or antagonistic, depending on the dose and the sequence. Mechanistic approach determining frequency and spectrum of cancer-related genes and loss of heterozygosity (LOH) shows that role of IR differs in combined exposures depending on the dose. At low dose range, in general, the combined effect may not deviate from additivity. More information on the mode and the mechanism of low-level exposures, which occasionally encountered in environmental and occupational situation, are required for reaching a unifying concept.

Key words: combined effect, ionizing radiation, alkylating agent, carcinogenesis

Introduction

Human beings are exposed to numerous natural and man-made agents that have potent carcinogenic activity. The increase in number of these agents has given rise to growing concerns about the cumulative risks of mixed exposures. Historically, national and international regulatory agencies have set standards for individual hazardous substances. Recognizing that this approach may not be appropriate, U.S. Environmental Protection Agency (EPA) published general guideline for the risk assessment of chemical mixtures (Guidelines for the health risk assessment of chemical mixtures, 51 Fed. Reg. 34014–34025, 1986). Currently, EPA uses dose-additive and response-additive model in chemical mixture risk assessment. Ionizing radiation (IR) is now of great concern because of an increased prevalence of medical and industrial use. Since the number of man-made agents is rapidly increasing, the effect of IR should be assessed as a result of combined exposures with these agents. The combined effect may be greater or smaller than the sum of the effect of single exposure. This review attempts to summarize the combined effect of IR with genotoxic chemicals, especially alkylating agents, as an example of evaluation for the cancer risk of IR in the environment with numerous chemicals mixtures.

The Mode of Combined Effect of Carcinogens

One of the basic concerns for the combined effect in our life surrounded by numerous carcinogens is whether the effect of combined exposure is simple sum of the effect of each carcinogen. In case of combined exposure of two agents whose dose response curves are linear, the mode of combined effect could be classified into additive, synergistic (or supra-additive) and antagonistic (or sub-additive) effects. These modes reflect a combined effect equal to, greater and smaller than the sum, respectively. For the agents with non-linear dose response, the identification of interaction is more complicated. For an upward bending dose response or dose response with a

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[3]Correspondence to: Yoshiya Shimada, Experimental Radiobiology for Children's Health Research Group, National Institute of Radiological Sciences, 4-9-1, Anagawa, Inage-ku, Chiba, 263-8555, Japan. Tel: +81-43-206-3221, Fax: +81-43-206-4138, E-mail: y_shimad@nirs.go.jp

threshold, additional increment per the second dose of the same agent would be larger than that of the first dose. Thus, the term of "envelope of additivity", which covers the range of additivity, is defined. The greater effect of "envelop of additivity" could be considered as synergism and the smaller effect of envelope could be considered as antagonism (Fig. 1). Therefore, the dose effect relationship is critical to judge the existence of interactions. Epidemiological study on A-bomb survivors, the dose response for solid cancers after exposure to radiation is linear (L) and that for leukemia is linear quadratic (LQ) (Fig. 2) (1). Dose response relationship shows threshold exceptionally for skin cancer at around 1 Gy (2). Several tumor models in animals, such as mouse skin tumors, bone tumors, ovarian tumors and thymic lymphoma, and rat kidney tumors also give threshold (3). Thus, the mode of combined effect may be tissue dependent.

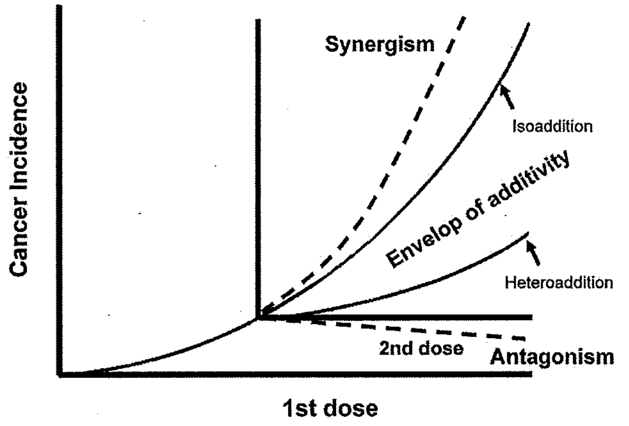

Fig. 1. Combined effect of two agents having non-linear dose-response relationship. Isoaddition is given when two agents have function, and heteroaddition is given when these act independently.

It is generally accepted that carcinogenesis is a multi-step process. It consists of initiation, which is defined as genomic alteration of oncogenes and tumor suppressor genes, promotion with clonal expansion of initiated cells, which leads to further accumulation of mutations, and progression, which is characterized by the acquisition of malignancy. Multi-stage cancer model was proposed by Armitage and Doll, which was the first attempt to develop a biological model of carcinogenesis (4). Then, Knudson, Moolgavkar and Venzon proposed a two stage stochastic model, considering clonal expansion of initiated cells, cell death and differentiation (5,6). We have recently applied two-stage model for the combined exposures of two agents, which are presumed to act at both stages (7). On a mechanistic level, synergism can be seen when each agent acts at different rate-limiting step of multi-step process or at different molecular target corresponding to rate-limiting step (8). When both agents affect the same step, combined effect is expected to be additive. Antagonistic effect could be observed when the agent could enhance the capacity for DNA repair or biological defense system against oxidative stress or induce apoptosis of initiated cells.

The Risk Factors that Interact with Radiation in Cancer Induction

Human epidemiological data have demonstrated several examples of combined effect of radiation and other physical, chemical and biological factors such as smoking, diet, ultraviolet (UV) radiation, virus and exogenous hormones (Fig. 3) (9).

A large body of information on uranium miners has provided an estimation of lung cancer risk in combined exposure to radon and smoking (10). It should be of

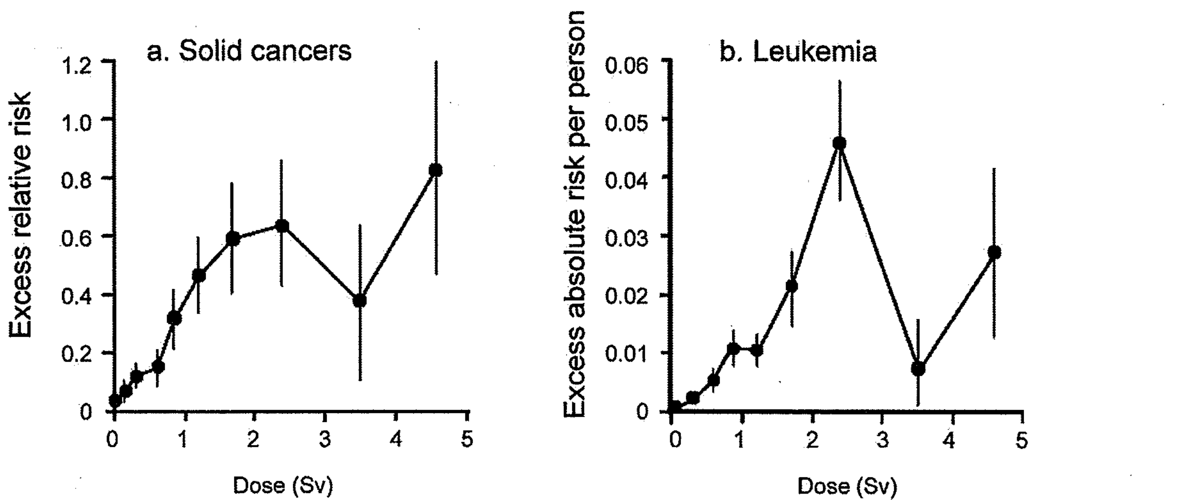

Fig. 2. Dose response for mortality of solid tumors and leukemia in A-bomb survivors. A linear relationship with no threshold is fitted for solid tumors (a), while a linear-quadratic model with upward curvature is best described for leukemia (b). Exceptionally, dose response of non-melanoma skin cancer shows curvilinearity with a possible threshold of 1Sv. Redrawn from the data in reference (1).

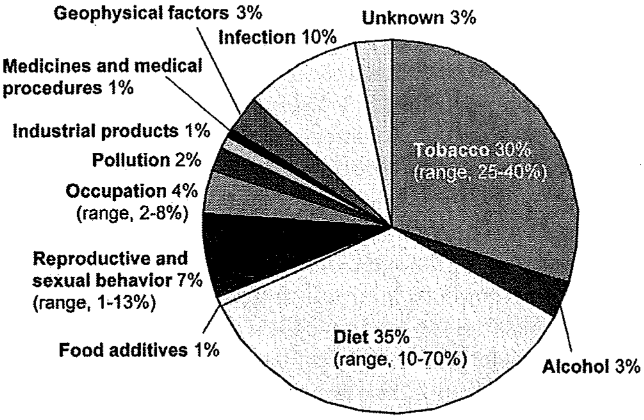

Fig. 3. Proportion of cancer deaths attributable to various environmental factors. Redrawn from the data in the reference (9).

note that tobacco smoke itself is a mixture of more than 4,000 genotoxic and non-genotoxic substances and some natural radionuclides such as ^{210}Po and ^{210}Pb. Importantly, nitrosamine and polycyclic aromatic hydrocarbons may function as initiators and catechols, phenols and terpenes may do as promoting agents. Reduction of reduces risk of cancer development with increasing time after quitting smoking is understood because of the reduction of promotion (11). Lubin *et al.* (12) reviewed 11 studies of underground miners and concluded that combined effect of radon and smoke on lung cancers was more than additive but less than multiplicative. The absolute risk of lung cancer in smokers was about three times higher than that in non-smokers.

UV radiation is recognized as an important initiator of human skin cancer. Skin color and age at exposure are the critical determinants for this cancer. The skin cancer risk of IR was particularly pronounced on the face, where there would be more UV exposure. These findings suggest that UV exposure levels or sensitivity to such exposure interact with ionizing radiation (13). Not only exogenous but also endogenous hormones are potent growth stimulators and play a role as promoters in breast carcinogenesis. These include estradiol-17 beta (E2), prolactin, diethylstilbestrol (DES) and androgens. Virus may also have a role in the development of human cancers. Statistically significant interaction between A bomb radiation and hepatitis C virus infection is demonstrated in the etiology of hepatocellular carcinoma (14).

Although epidemiological data are important to identify the combined effects, they have limited potential to dissect the interactions completely because human being are exposed numerous known and unknown mixture of agents. Therefore, knowledge on combined effect has been confirmed and provided by animal experiments. Although animal experiments have disadvantage to extrapolate the data to humans, they have advantage over epidemiological studies in that they retain control of the dose and population (age, gender, genetic background and so on). So far, numerous chemical agents have been examined using mice and rats if they interact with radiation to induce cancers. Chemical carcinogens may be classified into either genotoxicants or non-genotoxicants. Genotoxicants directly act on DNA molecules, thereby forming small or bulky adducts, strand breaks, and DNA-protein cross-links. Non-genotoxic chemicals may affect cell proliferation, differentiation and senescence. The experimental data on combined exposures are mostly accumulated for alkylating agents such as *N*-methyl-*N*-nitrosourea (MNU), *N*-ethyl-*N*-nitrosourea (ENU), 1,2-dimethylhidrazine (DMH), diethylnitrosamine (DEN) etc (15–22).

Combined Effect of IR and Alkylating Agents

Murine T-lyphomagenesis is one of the most extensively studied model for research of combined effect of IR and alkylating agents. Weekly lower doses (12 × 0.25 Gy; 4 Gy in total) concurrently combined with butylnitrosourea (BNU) enhanced lymphoma development, while intermediate doses (12 × 0.5 Gy) had no effect and high doses (12 × 0.75 Gy) delayed it (16). There appeared an inverse relationship between lymphomagenesis and dose, which might be ascribed to cell killing. Another study revealed that the incidence of lymphoma increased to 92% after ENU was preceded by 4 Gy from whole body irradiation, whereas single treatment with ENU induced lymphomas in 20% of mice and 4 Gy irradiation alone had little effect (18). Cell kinetics analysis indicated that 4 Gy irradiation was followed by regeneration of cells within a few days and maximum induction of lymphomas was given at the peak of DNA synthesis. This suggested that the role of IR was to provide susceptible subpopulation for the subsequent ENU treatment. Urethane has been also used for the combined treatments. X-rays (11 × 0.4 or 0.8 Gy; 4.4 or 8.8 Gy in total) every 4 days induced lymphomagenesis, which was enhanced by simultaneous treatment with urethane even at non-effective doses by itself in C57BL mice. Urethane also augmented the induction of lymphoma by X-rays in BALB-c mice (19). We recently examined T-lymphomagenesis of mice after weekly exposure of IR at doses of 0.2–1.0 Gy for 4 times followed by ENU in drinking water for 4 weeks (Kakinuma unpublished data). Combined exposure to ENU and high doses (4 × 1.0 Gy; 4 Gy in total) enhanced and accelerated T-lymphoma development compared to ENU alone. Surprisingly, low doses (4 × 0.2 Gy) reduced and delayed it, suggesting a protective role of low-dose IR for ENU-induced lymphomagenesis. Similarly, incidence of brain tumors, which were induced by ENU treated *in utero*, decreased after combined treatment with pre-exposed X-rays (1 and 2 Gy), showing antagonistic effect. The reduction of tumor incidence corresponded with the inductive effect of X-irradiation on O^6-alkylguanine-DNA alkyltransferase (ATase), suggesting a protective role of IR by inducing ATase for subsequent ENU treatment (20). Induction of ATase by IR has been frequently observed in several tissues *in vivo*. Interestingly, small but significantly higher increase in ATase activity was achieved when mice were exposed at a low dose rate (0.015 Gy/min) compared to a high dose rate (0.5 Gy/min), suggesting a protective effect of chronic exposure for alkylating agents (21). Collectively, these studies point out that the dose and dose rate are critical determinants for the mode of combined effect.

The sequence of exposure of two agents is also important. C57BL/6 mice were received X-irradiation (5 × 0.9 Gy with every 5 day) followed by urethane (5 × 20 mg, intraperitoneally injection) or urethane followed by radiation (22). Augmentation of lymphomagenesis by urethane was only obtained when urethane followed X-irradiation, but not when the sequence was reversed. This indicated that IR acted as initiator and urethane as promoter in lymphomagenesis. This is in good contrast to that urethane acts as an initiator in skin carcinogenesis, while for the lungs it is a complete carcinogen (23,24). The same agent plays different roles in carcinogenesis depending on the tissues.

DNA Damage and Molecular Signature Induced by IR and Alkylating Agents

DNA is a principal target of IR. IR induces several types of DNA damages including single- and double-strand breaks (dsb), base damage, and cross-links with protein. IR is considered to initiate carcinogenesis through generating DNA deletion and/or rearrangement caused by DNA dsb. On the other hand, molecular studies of induced somatic mutation show that majority of alkylating agents act through inducing point mutations. It is therefore expected that distribution of molecular changes in DNA differs between tumors induced by IR and those by alkylating agents.

Inactivation of TP53 is strongly suspected to contribute to the early development of human cancers. There are evidences that exogenous genotoxic agents are associated with the specific mutation spectrum of the *TP53* (*p53*) gene in human cancers. For instance, aflatoxin B1 (AFB1), a fungal derived contaminant of grain and peanuts, induces human hepatocellular carcinomas, acting with HBV (25). A specific *TP53* mutation is reported in hepatocellular carcioma from hepatitis B virus positive patients having AFB1 contaminated food in certain areas of China (26). This is the AGG to AGT transversion at codon 249. The evidence for an increased amount of AFB1-N7-guanine adduct in urine support the targeting the last nucleotide of codon 249 by AFB1 (27). Another example of *TP53* fingerprint is CC to TT double mutation in UV-induced skin cancer. C to T transition is also associated with UV irradiation (28). These mutations correspond to the two major types of DNA damages induced by UV radiation; cyclobutane pyrimidine dimmers and (6–4) photoproducts.

Radon is a carcinogen of lung cancer. Lung squamous cell carcinomas in uranium miners showed a *TP53* hotspot mutation in codon 249; 16 out of 52 tumors harbored AGG to ATG mutation, suggesting radon-associated mutation (29). However, the following study could not confirm such specific mutation (30). This discrepancy is possibly ascribed to the difference in cancer histology and the exposure to mycotoxins in the former study. On current knowledge, it is considered unlikely that unique *TP53* mutation exists in radon-induced lung cancer.

Animal experiments have an advantage for the finding distinct molecular signature associated with the cause of cancer because of the ability to exclude the exposure to other carcinogens. UV-induced skin tumors have been confirmed to characteristic to harbor C to T and CC to TT mutation in the *p53* gene in hairless mice (31). Treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) induces a signature mutation of G deletion from GGGA sequence in rat colon tumors (32). Nitroso-compounds such as MNU or ENU are good inducers of point mutations. MNU efficiently induces rat mammary tumors and mouse thymic lymphomas. All mammary tumors, which were induced by MNU, contained *H-ras* mutation at codon 12 GGA to GAA (17). Eighty percent of mice developed lymphomas after MNU treatment, and they harbored *K-ras* gene mutation in codon 12 GGT > GAT (33). Likewise, *K-ras* point mutation was found in a half of ENU-induced lymphomas in B6 mice, most of which were GGT to GAT at codon 12 (34). In contrast, only 13% of X-ray-induced lymphomas contained *K-ras* mutation. The G to A transition mutation may result from the formation of O^6-methyl- or O^6-ethyl-guanine, which are extremely mutagenic (35). Mutation spectrum of ENU-induced tumors, however, differed among the genes examined. Although G to A transition was main spectrum of mutation in *K-ras* gene, T to A was dominant for *p53* and T to C for *Ikaros* (Table 1). The cells with these type of mutations in these genes may be selected because of their advantage for survival and growth (36). It is to be mentioned that mutation spectrum is also highly dependent upon the balance of repair capacity. The mutation of *Ikaros* in radiation-induced lymphomas induced in *Mlh1* deficient mice was completely different from that in wild-type mice. Almost all mutations in *Mlh1* deficient lymphomas were frameshift

Table 1. Mutations of the *Ikaros*, *p53* and *K-ras* genes in X-ray- and ENU-induced thymic lymphomas in B6C3F1 mice

		Ikaros		*p53*	*K-ras*
		X-rays	ENU	ENU	ENU
Null mutation		5			
Alternative splicing		4	1*		
Insertion		1			
Point mutation	G>T				4
	G>A	2		2	4
	T>C	5	4		
	T>A			4	
	others	1	1	2	1
Number of lymphomas examined	37	27	27	27	

*: The point mutation at splice-donor site within intron 4.
Thymic lymphomas were induced by repeated exposure of X-rays (4 × 1.6 Gy) or by ENU (200 ppm in drinking water).
The data were taken from the references (34,36).

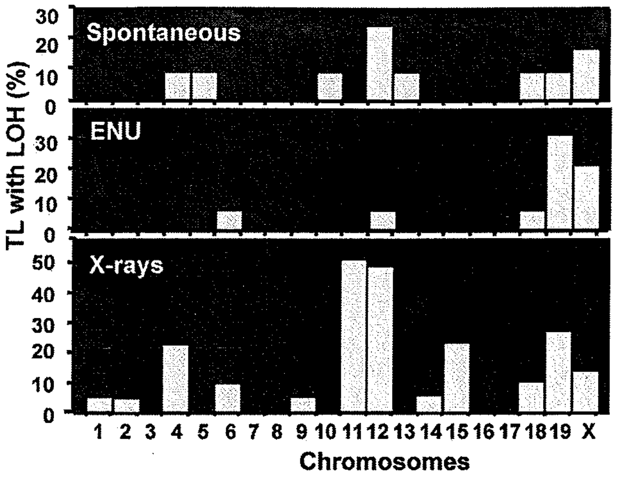

Fig. 4. Distribution of LOH in spontaneously developed, ENU-induced and X-ray-induced thymic lymphoma in B6C3F1 mice. The data are derived from the reference (39).

mutation at the mononucleotide repeat, which was rarely observed in radiation-induced lymphomas in wild-type mice (37). Therefore, one-to-one correspondence between chemical exposure and mutation spectrum is not always observable.

Loss of heterozygosity (LOH) has been examined in many cancers as a possible localization of tumor suppressor genes. Since LOH could be generated by deletions and recombination, radiation-induced tumors were considered to harbor frequent LOH. Others and we have extensively studied the distribution of LOH in radiation-induced lymphomas (38,39). We found a significant increase in the frequency of LOH in the centromeric region of chromosome 11 in radiation-induced lymphomas, compared to spontaneous or ENU-induced lymphomas in B6C3F1 mice (Fig. 4). We mapped the *Ikaros* gene in this region and found numerous aberrations of *Ikaros* sequence and expression (39,40). Mice with heterozygous *Ikaros* point mutation or dominant negative isoform are reported prone to the development of IR-induced thymic lymphoma (Table 1) (41,42). Thus, *Ikaros* is a critical tumor suppressor gene for the genesis of thymic lymphomas. Interestingly, ENU-induced lymphoma also harbors *Ikaros* point mutation (T to C transition), but it did not accompany the loss of wild-type allele (36). Further accumulation of data on LOH in radiation-induced tumors is required to conclude that existence of radiation-induced molecular signature.

Mechanism of Combined Effect of IR and Alkylating Agents

The interaction of combined exposure takes place at molecular, cellular and tissue levels. At low dose ranges, damages induced by IR may accumulate independently of those by alkylating agents. Therefore, the biological effect of combined exposure to IR and other agents is generally expected to be additive. In some cases as described above, IR induces several repair enzymes, some of which have protective activities for chemically-induced damages (21). High dose IR can kill the target cells or provide the environment to expand the preneoplastic subpopulation. Several growth factors or growth promoting cytokines are induced by high dose radiation. High dose X-rays enhance the expression of IL-1 beta and IL-7 in normal spleen cells (43) and fetal thymus (44), respectively. It is known that thymic lymphoma is developed in the unirradiated thymus transplanted into thymoectomized, irradiated mouse (45,46). This evidence is interpreted as that IR provides the tumor-promoting microenvironment for pre-existing pre-lymphoma cells in unirradiated thymus (47). Transforming growth factor-beta (TGF-beta) is the most potent known inhibitor of the proliferation of normal epithelial cells, and TGF-beta can act as an anti-tumor promoter. Advanced breast cancer cells, contrarily, are mostly refractory to TGF-beta-mediated growth inhibition. Recent observations indicate that IR can cause stromal fibroblasts to activate TGF-beta, thereby providing growth advantage for malignant cells over normal cells (48).

The available data on the mutations in tumors developed after combined exposures to carcinogens are quite limited. Rat mammary tumors induced by MNU harbor *H-ras* codon 12 mutation, while DMBA-induced tumors show activation of *H-ras* codon 61. The mammary tumors induced by both MNU and DMBA show predominantly G to A mutation in *H-ras* codon 12 (49). When MNU is combined with IR, mammary tumors with *H-ras* mutation are more frequent and develop

Table 2. Effects of IR and/or MNU treatment on the development of mammary adenocarcinomas carrying *H-ras* mutation in rats

Group	Tumors examined	Adenocarcinoma with *H-ras1* mutation			Adenocarcinoma without *H-ras1* mutation		
		Number (%)	Number per rat	Frequency [×10^{-3}; per rat per week]	Number (%)	Multiplicity	Frequency [×10^{-3}; per rat per week]
Control	3	0 (0%)	0	0	3 (100%)	0.08±0.05	2±1
γ-Rays	23	0 (0%)	0	0	23 (100%)*	1.15±0.27*	31±7*
MNU	54	29 (54%)*,†	1.61±0.56*,†	42±14*,†	25 (46%)*	1.39±0.37*	37±12*
Combined	76	47 (62%)*,†	2.24±0.74*,†	82±33*,†	29 (38%)*	1.38±0.36*	45±13*

*,†$p < 0.001$ vs. control and γ-rays, respectively.
The data were taken from the reference (50).

significantly earlier than those when MNU is administered alone (Table 2) (50). *H-ras* mutation is not seen in IR-induced tumors. We have now analyzing the LOH and mutation of *ras* or *Ikaros* in the mouse thymic lymphomas and rat mammary tumors induced by IR and alkylating agents. The result will add further information on the role of these two agents in carcinogenesis.

Recent mouse models for detection of mutation *in vivo* give important results for consideration of the mode and the mechanism of combined exposures. Combined action of B[*a*]P and amosite (asbestos) caused a synergistic increase in mutation rate in the lung of *lambda-lacI* transgenic rats (51). In combination of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone(NNK), chronic IR did not result in any obvious combined effect in the *gpt* selection, while the combined exposure suppressed large deletions in *Spi*-selection (52). We have recently found that combined exposures of ENU with high dose exposures increased mutation rate in a synergistic manner, while low dose IR decreased mutation rate (Yamauchi *et al.*, unpublished). Time course dependent changes in the mutant rate and its spectrum in the tissues and tumors induced by combined exposures with those by single exposure will shed light on the mechanism of combined effect. The descriptive approaches must be supplemented by the use of mechanism based cancer model (8).

Extrapolation to Low Dose

For risk assessment for human health, combined effect of numerous carcinogens at low doses is particularly relevant. However, many experiments have used acute, high doses of IR and other agents. It is not available how these data could be extrapolated to low and chronic exposure conditions. It is occasionally observed that many genotoxic agents have non-linear dose response. For low dose and dose rate, the linear term of dose response tends to remain. Under these conditions, the interaction of two agents decreases, and additivity results. At low doses, the interaction associated with compensatory cell proliferation, which usually occurs after high dose exposure, is unlikely to take place. Non-genotoxic substances, which act as tumor promoter, have threshold dose and the effect at less than threshold dose will not be manifested. Low dose IR might have capability to induce repair system for other carcinogens, resulting in antagonistic response. Recently, it is reported that irradiation of non-transformed cells with low doses lead to stimulation of intercellular induction of apoptosis of neighboring transformed cells *via* reactive oxygen species, which was induced by TGF-beta (53). These results suggested that low dose radiation had potential on anticancer defense mechanism. Taken together, although synergistic effect of combined exposure might be common at high dose and dose rate, large deviation from additivity cannot be expected at low dose and dose rate relevant in occupational and environmental condition.

Combined Effect on Fetal and Infant Animals

For several decades, evidences have been accumulated that young children are more susceptible to cancer-causing agents than adults. The risk from childhood exposures to environmental chemicals and IR is thought to be heightened for the following two reasons. First, children's rapidly growing organs are vulnerable to carcinogen-induced changes. Secondly, children's behavior makes them prone to high exposures; they crawl on the ground, and they inhale more air per unit body than adults. Therefore, EPA has recently assumed that children under age 2 are 10 times more susceptible to carcinogens, and children aged 2–15 are 3 times more vulnerable than adults (Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens. EPA/630/R-03/003F, 2005).

In the viewpoint of combined effect, it is a concern whether or not the early-life exposure to one carcinogen may have impact on the subsequent exposure to another carcinogen. Hoyes-KP *et al.* examined the effect of early-life exposure to IR on the development of adulthood cancer (54). They showed that exposure to IR at the fetal, neonatal and juvenile stages of development induced residual haematopoietic damage and increased oncogenic susceptibility to adulthood exposure to MNU. It was of interest that the neonatal stage was the most sensitive for induction of lymphoid malignancy while fetal stage was the most sensitive for the induction of myeloid leukemia. Mice irradiated on day 15 of gestation with 0.2 or 0.4 Gy X-rays in combination with postnatal exposure to ENU showed the greater-than-additive effect (55). This suggests that low-level of prenatal and perinatal X-irradiation leads to a lasting sensitivity towards a subsequent carcinogenic stimulus.

Recent growing use of interventional and fluoroscopic imaging in children represents a great benefit for diagnosis and treatment of benign conditions. Along with an increase in medical use for children, however, comes concern about the late effect of IR, especially cancers development. Therefore, the information on the effect of childhood IR exposure combined with later exposure to other carcinogens such as tobacco smoke will be necessary for the risk assessment for children.

Conclusion

Combined exposures are characteristic of life. Synergistic combined effects are common at high dose exposure, but the deviation from additivity is not expected at low dose exposure of genotoxic and non-genotoxic agents. The idea supports for the current approach of the risk assessment for mixture of carcinogens, which is based on the linear dose response and additive model. However, the agents that are exposed at high dose and function at different carcinogenic step may show synergy when combined with IR. These include tobacco smoke and, possibly, daily diet Systematic quantitative assessment and mechanistic understanding of combined exposures is needed for reliable risk estimation.

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Regular Paper

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

Tatsuhiko IMAOKA[1*], Satoshi YAMASHITA[2], Mayumi NISHIMURA[1],
Shizuko KAKINUMA[1], Toshikazu USHIJIMA[2]
and Yoshiya SHIMADA[1†]

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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*Corresponding author: Phone: +81-43-206-3221,
Fax: +81-43-206-4138,
E-mail: t_imaoka@nirs.go.jp
†Corresponding author: E-mail: y_shimad@nirs.go.jp
[1]Experimental Radiobiology for Children's Health Research Group, Research Center for Radiation Protection, National Institute of Radiological Sciences;
[2]Carcinogenesis Division, National Cancer Center Research Institute.

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

[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).

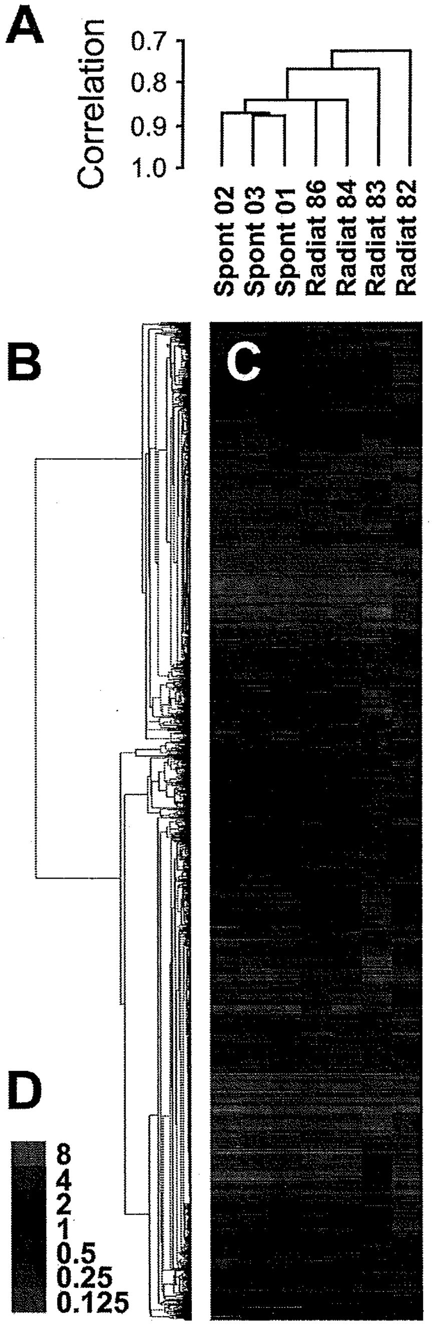

Fig. 1. Hierarchical clustering of global gene expression profiles for rat mammary carcinomas. *A*, Clustering of three spontaneous (*Spont*) and four ionizing radiation–induced (*Radiat*) carcinomas, showing the degree of similarity between tumors. The Pearson correlation coefficient is indicated on 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*.

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

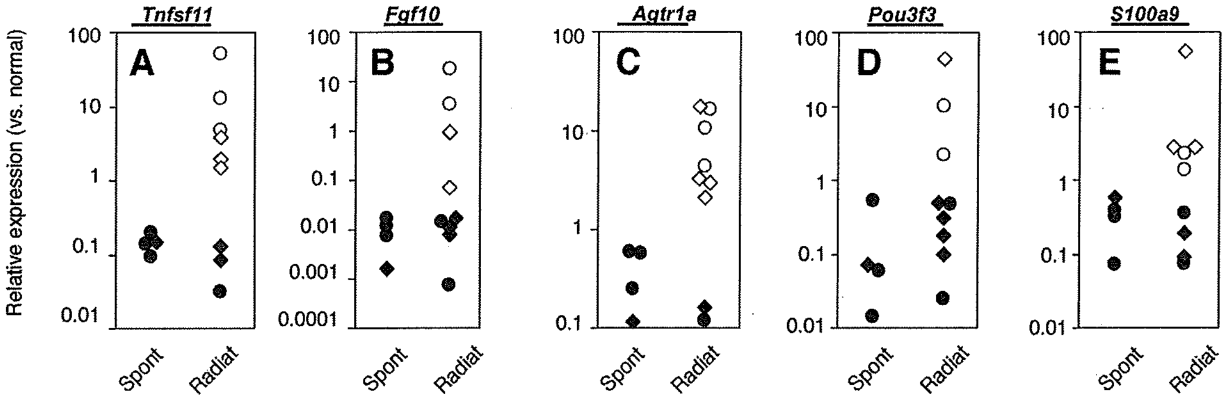

Fig. 2. Expression levels of five genes in each carcinoma as determined by quantitative PCR (qPCR). Four spontaneous (*Spont*) and nine radiation-induced (*Radiat*) carcinomas were analyzed for expression of *Tnfsf11, Fgf10, Agtr1a, Pou3f3* and *S100a9* (panels A–E, respectively), and data are indicated 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

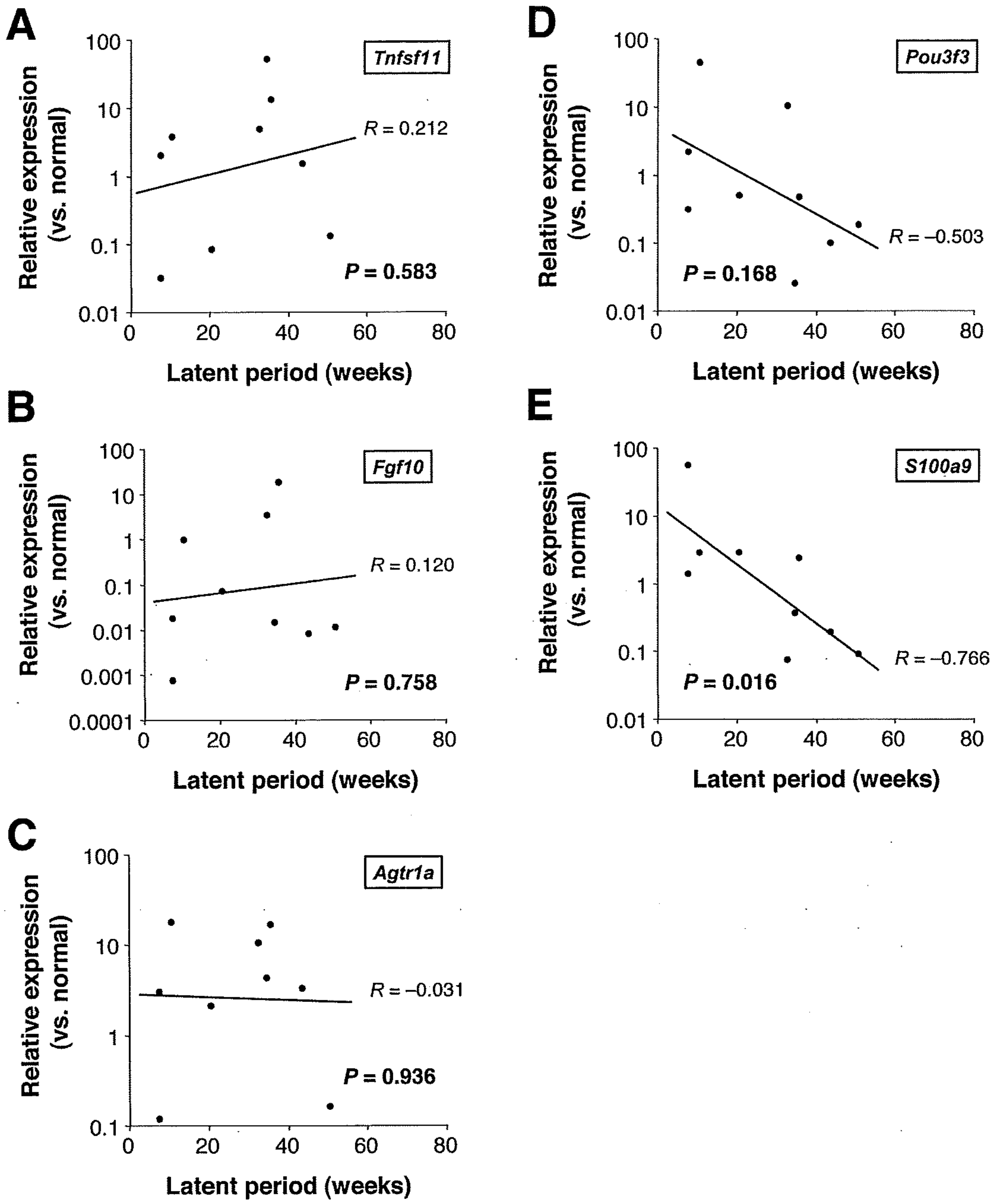

Fig. 3. Scatter plots showing the relationship between the latent period and expression of various genes for radiation-induced rat mammary carcinomas. The expression values of *Tnfsf11*, *Fgf10*, *Agtr1a*, *Pou3f3* and *S100a9* (panels *A–E*, respectively) in each tumor were determined by quantitative 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