history of radiotherapy than in those without radiation history (20), but another report disputed such findings (21). Interestingly, we found that *RET/PTC1* rearrangements were induced in human thyroid cells by X-irradiation *in vitro* and *in vivo* as tissue transplants in severe combined immunodeficient mice (22). These findings may provide supporting evidence that activation of the *RET* oncogene via rearrangements plays a crucial role in radiation-associated papillary thyroid carcinogenesis.

The *BRAF* gene encodes a serine/threonine kinase responsible for transduction of signals in the MAP-kinase cascade (23). *BRAF* somatic mutations were first discovered in several types of human cancers, including malignant melanomas (24). Except for very rare instances, the *BRAF* mutation identified in thyroid cancer is thus far almost exclusively thymine-to-adenine transversion at nucleotide 1799, resulting in substitution of glutamate with valine at residue 600 (V600E; ref. 25). The V600E substitution is thought to convert BRAF inactive conformation into its active form by disrupting the residue-residue interaction between the activation loop and the ATP binding site (26).

BRAF^{V600E} mutation has thus far been described as occurring with frequency ranging from 29% to 83% in PTC among an adult general population (25). Regarding the relationship with radiation exposure, the *BRAF*^{V600E} gene mutation was studied in post-Chernobyl PTC, which is believed to have developed in those exposed to radiation in childhood. A very low frequency of *BRAF*^{V600E} mutations in this PTC has been reported (range, 0–12%; refs. 27–31). However, prevalence of *BRAF*^{V600E} mutation was originally low (range, 0–6%) in PTC among children, unrelated to their history of radiation exposure (27, 28, 31). Therefore, it may be difficult to assess the relationship between radiation exposure and childhood PTC in terms of *BRAF*^{V600E} mutation. On the other hand, in adult-onset PTC among A-bomb survivors, we have previously reported that prevalence of *BRAF*^{V600E} mutation was very low in adult-onset PTC among A-bomb survivors exposed to high radiation dose (>0.5 Gy), in contrast to high prevalence in nonexposed survivors or in the general population (32).

These findings lead us to a hypothesis that *RET/PTC* rearrangements in the MAPK-signaling pathway might play a major role in development of adult-onset radiation-associated PTC among A-bomb survivors. Therefore, to examine this hypothesis, this article analyzed pathologic and epidemiologic characteristics of adult-onset PTC in A-bomb survivors in terms of *RET/PTC* rearrangements and *BRAF*^{V600E} mutation.

Materials and Methods

Patients and tissue specimens. Study patients comprised 71 adult-onset PTC cases diagnosed from 1956 to 1993, consisting of 50 exposed and 21 nonexposed patients found among A-bomb survivors in Hiroshima and Nagasaki; 54 of these 71 cases were those used in our previous study on *BRAF*^{V600E} mutation (32). In the LSS (4), a total of about 250 PTC cases were identified in a cohort of LSS among A-bomb survivors during the aforementioned period. To date, we have obtained thyroid tissue specimens from 90 cases of these pathologically confirmed 250 cases, This number covered only about 36% of PTC found in the LSS cohort among A-bomb survivors during 1958 to 1993. After examining quality of RNA, 71 cases were analyzable for both *RET/PTC* and *BRAF*^{V600E} in this study.

Classification of histology was done by one of the authors (T.H.) according to histopathologic typing established by the WHO (33). All study materials were formalin-fixed and paraffin-embedded PTC tissue specimens surgically resected during 1956 to 1993. This study was conducted under approval of the Human Investigation Committee and the Ethics Committee for Genome Research at the Radiation Effects Research Foundation (RERF).

RNA preparation and cDNA synthesis. RNA was extracted from microdissected noncancerous or cancerous regions using the High Pure RNA Paraffin kit (Roche Diagnostics GmbH), as described previously (34). Reverse transcription was performed with random primers (9 mer) using 100 ng total RNA as template, as described previously (34).

Identification of *RET/PTC* rearrangements and *BRAF*^{V600E} mutation. Reverse transcription-PCR (RT-PCR) with *BCR* as internal control was

Table 1. Pathologic and epidemiologic characteristics of patients by radiation exposure status

		Exposed (dose > 0 mGy; n = 50)	Nonexposed* (n = 21)	P
Gender	Male (n)	6	2	1†
	Female (n)	44	19	
Histologic subtype	Conventional PTC (n)	47	21	0.6†
	Follicular variant (n)	3	0	
Median age ATB‡ (y, range)		22 (1–47)	20 (0–50)	0.3§
Median age at diagnosis (y, range)		50 (18–89)	48 (24–84)	0.9§
Median time after exposure (y, range)		24 (11–46)	—	—
Median radiation dose (mGy, range)		203 (0.4–2,758)	0	—
RET/PTC rearrangement	Absence (n)	39	20	0.09†
	Presence (n)	11	1	
	Frequency (%)	22	5	
BRAF^{V600E} mutation	Absence (n)	22	4	0.06†
	Presence (n)	28	17	
	Frequency (%)	56	81	

*The nonexposed patients were either those with radiation dose estimated to be 0 mGy or those who were not in the city of Hiroshima or Nagasaki at the time of bombing.
† Fisher's exact test.
‡ ATB: at the time of atomic bombing.
§ Mann-Whitney's U test.

Table 2. Pathologic and epidemiologic characteristics of patients by *RET/PTC* rearrangement status

		All patients			Exposed patients (>0 mGy)		
		RET/PTC (*n* = 12)	Wild-type *RET* (*n* = 59)	*P*	*RET/PTC* (*n* = 11)	Wild-type *RET* (*n* = 39)	*P*
Gender	Male (*n*)	1	7	0.6*	1	5	0.6*
	Female (*n*)	11	52		10	34	
Histology	Conventional PTC (*n*)	11	57	0.9*	10	37	0.9*
	Follicular variant (*n*)	1	2		1	2	
Median age ATB†	Years	15	21	0.2‡	13	26	0.1‡
	Range	(3–41)	(0–52)		(3–41)	(1–47)	—
Median age at diagnosis	Years	39	51	0.1‡	39	54	0.05‡
	Range	(21–59)	(18–89)	—	(21–59)	(18–89)	—
Median time after exposure	Years	—	—	—	20	24	0.3‡
	Range	—	—	—	(15–36)	(11–46)	—
Median radiation dose	mGy	943	12	0.001‡	960	151	0.005‡
	Range	(0–2,304)	(0–2,758)	—	(67–2,304)	(0.4–2,758)	—

*Fisher's exact test.
† ATB: at the time of atomic bombing.
‡ Mann-Whitney's *U* test.

conducted to confirm whether RNA extracted from archival tissue specimens was available for RT-PCR. The samples were examined for expression of *RET* TK domain by RT-PCR. RNA with detectable expression of the TK domain was further analyzed for determination of rearrangement types. cDNA derived from 10 ng of total RNA was used as an RT-PCR template. RT-PCR was performed with 0.5 U of Platinum Taq DNA polymerase (Invitrogen) for BCR, the TK domain, *RET/PTC1* and *RET/PTC3*, or 0.5 U of Platinum Taq DNA polymerase High Fidelity (Invitrogen) for *TRK-T2* and novel *RET/PTC* in 25 μL volume containing 1× PCR buffer, 200 μmol/L each of deoxynucleotide triphosphate mixture, and 0.4 μmol/L of each primer. RT-PCR conditions consisted of initial denaturation (95°C for 3 min), followed by 40 cycles (36 cycles for TK domain of *RET*) of denaturation at 95°C for 30 s, annealing for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Primer sets, oligonucleotides, annealing temperature, and Mg^{2+} concentration are summarized in Supplementary Table S1.

For samples that showed expression of *RET* gene TK domain but not assigned as *RET/PTC1* or *RET/PTC3*, rearrangement types were examined by an improved SMART RACE method, which was developed by us.[11] Briefly, after completion of cDNA synthesis, the reaction solution was further incubated at 42°C for 60 min in the presence of SMART adaptor. This SMART-PCR was conducted using FastStart High Fidelity PCR system (Roche Diagnostics GmbH), and primers RET-Ex12PR9 and S-RACE 1, followed by nested RT-PCR using primer RET-Ex12A4 and SMART adaptor. SMART-PCR conditions were as described above, except for the cycle numbers (45 cycles for 1st PCR and 25 cycles for nested PCR). All target bands in RT-PCR were confirmed by digestion of restriction enzyme, *BamH* I (TaKaRa) for *RET/PTC1* and *RET/PTC3*, *Alu* I for *BCR*, and *Hae* III for the TK domain, which existed within each amplified target fragment. Other *RET/PTC* rearrangement types identified by improved SMART RACE were confirmed by sequencing using a CEQ8000 DNA sequencer (Beckman Coulter, Inc.).

BRAF gene mutation causing amino acid substitution of glutamic acid for valine at codon 600 ($BRAF^{V600E}$) was determined by RFLP using *TspR* I (New England Biolabs) and direct sequencing, as described previously (32).

[11] Submitted.

Statistical analysis. Mann-Whitney's *U* test was used for nonparametric two-sample comparisons of continuous variables. Fisher's exact test was used for categorical variables. The Cochran-Armitage test was used for nonparametric trend analysis. Logistic regression analysis was carried out among 39 A-bomb survivor exposed patients who had either *RET/PTC* rearrangement or $BRAF^{V600E}$ mutation, to assess differences between PTC patients with *RET/PTC* rearrangement and those with $BRAF^{V600E}$ mutation, in terms of pathologic and epidemiologic variables, including radiation dose, histology, gender, and time-related factors [Note that age at diagnosis = age at the time of A-bombing (ATB) + the time since exposure]. All statistical analyses were performed with SPSS software (version 12.0).

Radiation dose. A-bomb radiation doses used in this analysis were shielded organ dose to the thyroid estimated by the recently implemented DS02 system (35).

Results

Pathologic and epidemiologic characteristics of PTC among A-bomb survivors. Pathologic and epidemiologic characteristics of study patients are shown in Table 1. All tumors were well-differentiated PTC including three cases of follicular variant. When comparing exposed and nonexposed patients, no differences were found based in gender, histologic subtypes, age ATB, and age at diagnosis.

Of 71 patients, we detected *RET/PTC* rearrangements in 12 patients: 9 with only *RET/PTC1*, 1 with both *RET/PTC1* and *RET/PTC3*, 1 with *RET/PTC8*, and 1 with a novel *RET* rearrangement. This novel *RET/PTC* (*RET/PTCX*) was regarded as one *RET* rearrangement, whose partner gene, acyl-CoA binding domain containing 5 (*ACBD5*, located on chromosome 10p12.1), had at least one coiled-coil domain, expression of which was confirmed by RT-PCR (Supplementary Fig. S1). Although the exposed patients showed a higher frequency of *RET/PTC* rearrangements than did nonexposed ones, this difference was not statistically significant (Table 1). On the other hand, frequency of $BRAF^{V600E}$ mutation was marginally lower in exposed patients than that in nonexposed ones ($P = 0.06$; Table 1).

Pathologic and epidemiologic characteristics by *RET/PTC* rearrangement status. Pathologic and epidemiologic characteristics of study patients were shown by *RET/PTC* rearrangement status in Table 2, where nonexposed patients (0 mGy) were excluded ("exposed patients") or included ("all patients"). Significant difference was found in radiation dose between all patients with and without *RET/PTC* rearrangement ($P = 0.001$; median dose, 943 versus 12 mGy), and also between exposed patients with and without *RET/PTC* rearrangement ($P = 0.005$; median dose, 960 versus 151 mGy; Table 2). Presence or absence of *RET/PTC* rearrangement revealed marginal association with age at diagnosis in exposed patients ($P = 0.05$), although no significant association was found in all patients ($P = 0.1$). No significant relationship was observed between *RET/PTC* rearrangement status and age ATB, histologic subtype, or gender in both all patients and only exposed patients. Furthermore, no significant association was found in exposed patients with time elapsed since A-bomb exposure to diagnosis.

Pathologic and epidemiologic characteristics by *BRAF*^{V600E} mutation. Pathologic and epidemiologic characteristics of study patients were shown by *BRAF*^{V600E} mutation status (Table 3). Close association of *BRAF*^{V600E} mutation status with radiation dose and time since exposure remained unchanged from our previous results (32): PTC patients with *BRAF*^{V600E} mutation showed significantly lower radiation dose ($P = 0.0001$ or 0.0002 in all patients or exposed patients, respectively) and significantly longer time since exposure ($P = 0.0003$ in exposed patients), compared with those without *BRAF* mutation. Age at diagnosis was found to be significantly older in patients with *BRAF*^{V600E} mutation than those without *BRAF*^{V600E} mutation ($P = 0.001$ or 0.0002 in all patients or exposed patients, respectively), although this association did not reach significance in our previous study (32) based on a smaller number of patients. In addition, in only exposed patients, *BRAF*^{V600E} mutation status revealed a significant association with age ATB, but this was not significant in all patients. Furthermore, no significant association was found between *BRAF* mutation status and histology or gender as was also the case in our previous study (32).

Increased *RET/PTC* rearrangements and decreased *BRAF*^{V600E} mutation frequency with increased radiation dose. To examine the relationship between *RET/PTC* and *BRAF*^{V600E} mutation and radiation dose, exposed PTC patients were divided into three groups by dose tertiles. *RET/PTC* rearrangements were more frequently found in patients with increased radiation dose ($P_{trend} = 0.002$; Fig. 1*A*). Specifically, *RET/PTC* rearrangements were found in 50% (8 of 16) of PTC patients who were exposed to high doses (>0.5 Gy) in Fig. 1*A*: 5 with only *RET/PTC1*, 1 with both *RET/PTC1* and *RET/PTC3* (2.2 Gy), 1 with *RET/PTC8* (2.3 Gy), and 1 with *RET/PTCX* (1.5Gy).

On the other hand, prevalence of *BRAF*^{V600E} mutation significantly decreased with radiation dose ($P_{trend} = 0.00006$). In addition, PTC patients having wild-type *RET* and *BRAF* showed a marginally significant increasing trend with radiation dose ($P = 0.08$; Fig. 1*A*).

Frequency of *RET/PTC* and *BRAF*^{V600E} alterations in PTC patients grouped by time elapsed since atomic radiation exposure. *RET/PTC* rearrangements and *BRAF*^{V600E} mutation were further studied in relation to time since radiation exposure (Fig. 1*B*). *BRAF*^{V600E} mutation significantly increased with increased time since exposure ($P_{trend} = 0.001$), whereas unidentified alterations in PTC having wild-type *RET* and *BRAF* significantly decreased with increased time since exposure ($P_{trend} = 0.001$). In contrast, *RET/PTC* rearrangements showed a peak at time since exposure 18 to 27 years, suggesting that unidentified alterations other than *RET/PTC* may also play an important role in PTC occurred in relatively short time since the exposure.

Radiation-related factors underlying occurrence of *RET/PTC* rearrangements versus *BRAF*^{V600E} mutation. As was the case for PTC among the general population (7–9), *RET/PTC* rearrangements and *BRAF*^{V600E} mutation were found to be mutually exclusive among exposed PTC patients (Supplementary Table S2). On the basis of this result, pathologic and epidemiologic characteristics were compared between 11 PTC patients having

Table 3. Pathologic and epidemiologic characteristics of patients by *BRAF*^{V600E} mutation status

		All patients			Exposed patients (>0 mGy)		
		BRAF^{V600E} ($n = 45$)	Wild-type *BRAF* ($n = 26$)	*P*	BRAF^{V600E} ($n = 28$)	Wild-type BRAF ($n = 22$)	*P*
Gender	Male (n)	5	3	0.7*	3	3	0.5*
	Female (n)	40	23		25	19	
Histology	Conventional PTC (n)	44	24	0.3*	27	20	0.4*
	Follicular variant (n)	1	2		1	2	
Median age ATB†	Years	22	17	0.09‡	31	17	0.04‡
	Range	(0–52)	(1–47)	—	(1–47)	(1–47)	—
Median age at diagnosis	Years	54	39	0.001‡	55	38	0.0002‡
	Range	(20–89)	(18–62)	—	(20–89)	(18–59)	—
Median time after exposure	Years	—	—	—	29	18	0.0003‡
	Range	—	—	—	(15–46)	(11–36)	—
Median radiation dose	mGy	8	538	0.0001‡	69	859	0.0002‡
	Range	(0–2,758)	(0–2,304)		(0.4–2,758)	(12–2,304)	—

*Fisher's exact test.
†ATB: at the time of atomic bombing.
‡Mann-Whitney's *U* test.

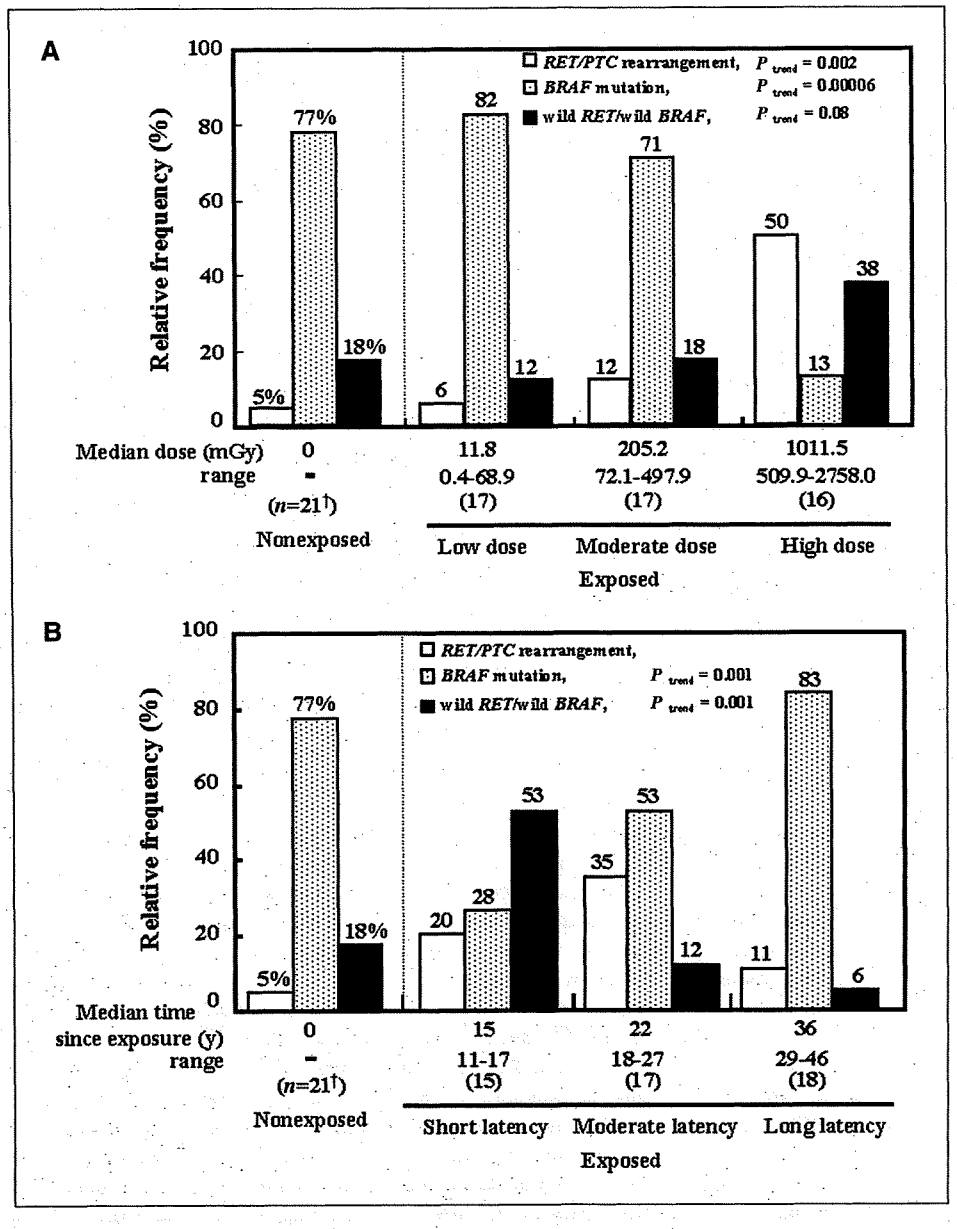

Figure 1. *A*, relative frequency of *RET/PTC* and $BRAF^{V600E}$ alterations in PTC patients grouped by radiation exposure dose levels (nonexposed and dose tertiles). Exposed PTC patients were divided into three groups by dose tertiles. *B*, relative frequency of *RET/PTC* and $BRAF^{V600E}$ alterations in PTC patients grouped by time elapsed since atomic radiation exposure (nonexposed and tertiles of time since exposure). Exposed PTC patients were divided into three groups by tertiles of time since exposure. †, one case in the nonexposed group had both *RET/PTC* and $BRAF^{V600E}$. Relative frequency of genes in the nonexposed group was calculated by using 22 for number of gene alterations. PTC with *RET/PTC* rearrangement (*open bars*), with $BRAF^{V600E}$ mutation (*dotted bars*), or with other unknown alterations (*closed bars*), respectively, are shown.

RET/PTC rearrangements and 28 patients having $BRAF^{V600E}$ mutation. PTC patients with *RET/PTC* rearrangements revealed past exposure to significantly higher radiation dose ($P = 0.001$; Fig. 2*A*), shorter time elapsed since radiation exposure ($P = 0.03$; Fig. 2*B*), and younger age ATB ($P = 0.06$; Fig. 2*C*), compared with the patients with $BRAF^{V600E}$ mutation.

Subsequent logistic regression analysis for mutually exclusive occurrence of *RET/PTC* rearrangements or $BRAF^{V600E}$ mutation confirmed these findings, using "age ATB" and "time since exposure" as independent time-related explanatory variables (Note that "age at diagnosis" = "age at exposure" + "time since exposure"). Radiation dose, age at exposure, and time elapsed since exposure were significantly associated with which alteration type of *RET/PTC* rearrangements or $BRAF^{V600E}$ mutation occurred in the development of PTC among A-bomb survivors ($P = 0.012$, 0.031, and 0.034, respectively; Table 4).

Rearrangements of *NTRK1* and *BRAF* genes. *NTRK1* rearrangements and the *AKAP9-BRAF* fusion gene were also examined in the 71 cases. The *TRK-T2* gene was detected in only one exposed case with wild-type *RET* and *BRAF*. However, five *NTRK1*-derived nucleotides were deleted in this amplified fragment. On the other hand, no *AKAP9-BRAF* fusion gene was detected in these 71 cases.

Discussion

In papillary thyroid carcinogenesis, constitutive activation of the MAPK-signaling pathway, namely rearrangements of *RET* and *NTRK* genes and mutations in *RAS* and *BRAF* oncogenes, seems to be required for transformation (36). Recent *in vitro* and *in vivo* experiments have also shown the requirement of activation of the RET/PTC-RAS-BRAF-MAPK pathway in thyroid tumorigenesis (37–39). Interestingly, mutual exclusion of these genetic alterations in the MAPK-signaling pathway was reported; one event among *BRAF* mutation, *RAS* mutations, and *RET/PTC* rearrangements (7, 8, 29) or one among *BRAF* mutation, *RET/PTC*

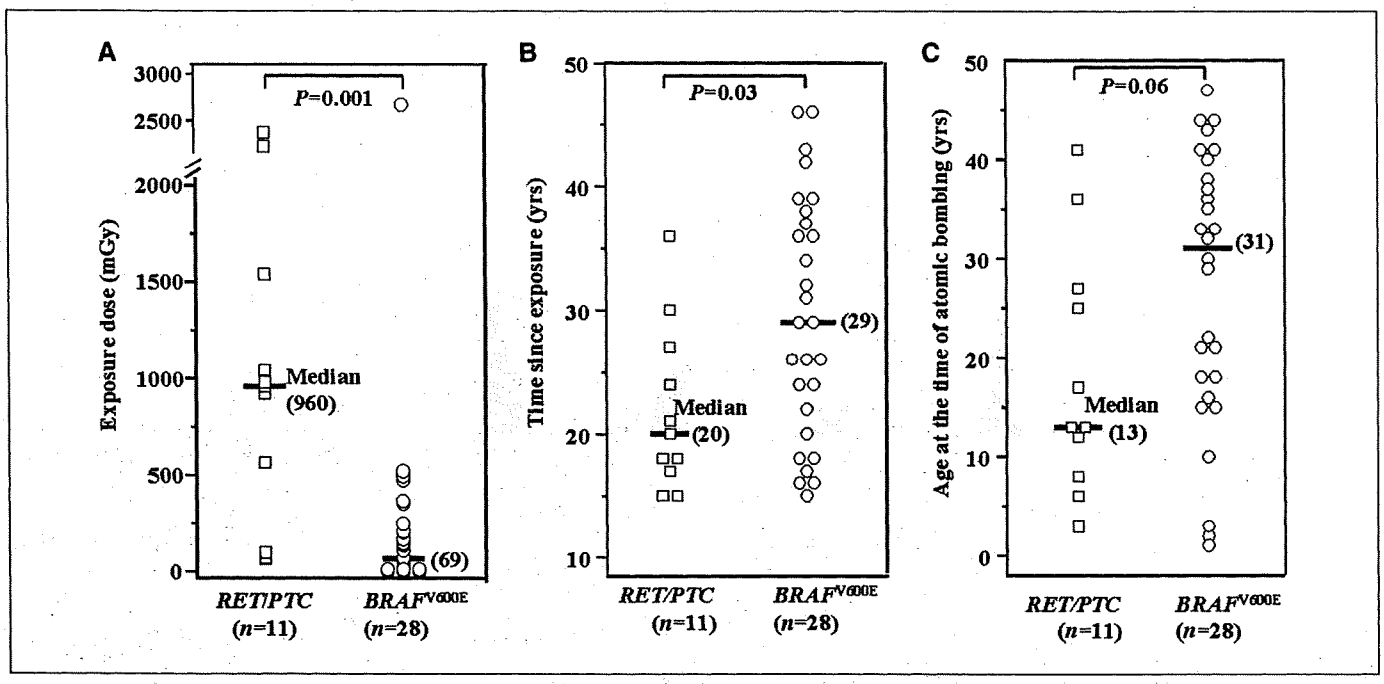

Figure 2. Comparison of *RET/PTC* and $BRAF^{V600E}$ alterations in PTC patients. *A*, radiation dose; *B*, time since exposure; *C*, age at the time of atomic bombing. *RET/PTC PTC* rearrangement (□) and $BRAF^{V600E}$ mutation (○), respectively, are shown.

rearrangements, and *NTRK1* rearrangements (9) was singularly found, indicating that one such gene alteration is an important early event in development of PTC. Furthermore, a recently identified *AKAP9-BRAF* rearrangement did not coexist with *BRAF* mutation in radiation-associated PTC (30). These data suggest that a single genetic event in the MAPK-signaling pathway may be sufficient for thyroid cell transformation and tumorigenesis.

In this study, pathologic and epidemiologic characteristics, specifically radiation-related ones, of PTC having *RET/PTC* rearrangements contrasted clearly with those of PTC having $BRAF^{V600E}$ mutation. Noting that 17 (81%) and 1 (5%) of 21 nonexposed PTC patients having $BRAF^{V600E}$ mutation and *RET/PTC* rearrangement in this study, respectively, are in agreement with other data on nonexposed adult-onset Japanese PTC (18, 25, 40–42), we for the first time have shown that the frequency of *RET/PTC* rearrangements significantly increased with increased radiation dose as well as shorter time elapsed since radiation exposure and younger age at the time of bombing (Figs. 1*A* and 2; Table 4). *RET/PTC* rearrangements were detected in 50% (8 of 16) of adult-onset PTC patients who were exposed to radiation dose of >0.5 Gy, although this frequency was somehow lower than that (about 80%) reported for French thyroid cancer patients who had received external radiotherapy (18). This difference in frequency of *RET/PTC* rearrangements may be due to the different radiation conditions (i.e., single or repeated irradiation and dose). On the other hand, $BRAF^{V600E}$ mutation significantly decreased frequency with increased radiation dose (Fig. 1*A*). This finding seems to be consistent with our parallel observations, shorter time elapsed since exposure, and younger age at the time of bombing in PTC patients with *RET/PTC*, compared with those in the patients with $BRAF^{V600E}$ (Fig. 2; Table 4). Taken together, our findings imply that *RET/PTC* rearrangements, not $BRAF^{V600E}$ mutation, are closely associated with radiation-associated adult-onset PTC.

The existence of a molecular mechanism other than *RET/PTC* rearrangement is suggested from Fig. 1*B*: *RET/PTC* rearrangements showed a peak at 20 to 30 years since radiation exposure and relatively low frequency of 20% in <20 years since exposure, in contrast to 53% of unidentified alterations other than *RET/PTC* and $BRAF^{V600E}$. Because *RET/PTC* and $BRAF^{V600E}$ account for 82% of nonexposed PTC and about 60% to 70% of PTC in the Japanese general population (18, 25, 40–42), this increase of unidentified alterations in <20 years is thought to be caused by radiation. This unidentified mechanism may be involved in radiation-associated PTC, which occurred earlier after radiation exposure than did PTC having *RET/PTC*. However, regarding *NTRK1* rearrangements and the *BRAF* fusion gene, the *TRK-T2* gene lacking five nucleotides was

Table 4. Logistic regression analysis of 39 exposed PTC patients with *RET/PTC* rearrangements or $BRAF^{V600E}$ mutation

Variables	β*	*P*
Radiation dose (mGy)	0.002	0.012
Age at the time of atomic bombing (y)	−0.113	0.031
Year since exposure (y)	−0.192	0.034
Gender, male vs. female	2.674	0.204
Histology, conventional vs. follicular variant	0.157	0.927

NOTE: A dependent variable was defined as follows: rearranged *RET* and wild *BRAF* = 1; wild *RET* and mutated *BRAF* = 0.
*Regression coefficients in the logistic regression model.

detected in only one exposed case. Therefore, the unidentified alterations may be involved in pathways other than the MAPK-signaling pathway.

We need to confirm our findings with an increased number of study patients, given that the present study covered only about 36% of PTC found in the LSS cohort among A-bomb survivors during 1958 to 1993 for whom tissue specimens could be obtained. Toward this end, an efficient system to collect archival specimens from A-bomb survivors, which are dispersed over a number of hospitals in Hiroshima and Nagasaki, will be necessary in cooperation with the institutions concerned (it took 3 years to collect 90 PTC specimens, 71 of which were used in the present study). Because the specimens deteriorate as time goes by, it is urgent that our collection and analyses be conducted soon to increase our knowledge, which in turn might lead to improved treatment and prevention of radiation-associated cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Epidemiology

DNA methylation status is inversely correlated with green tea intake and physical activity in gastric cancer patients

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ABSTRACT

Epigenetic silencing of genes by aberrant DNA methylation is recognized as a crucial component of the mechanism underlying tumorigenesis. However, the relationship between DNA methylation and the past lifestyle in cancer patients remains largely unknown. We examined the methylation statuses of 6 tumor-related genes, *CDX2* (homeobox transcription factor), *BMP-2* (bone morphogenetic protein 2), *p16 (INK4A)*, *CACNA2D3* (calcium channel-related), *GATA-5* (transcription factor) and *ER* (estrogen receptor), in 106 primary gastric carcinomas by methylation-specific PCR and compared them with the past lifestyles of the patients. The methylation frequencies of the genes were 23.6, 21.7, 9.4, 32.4, 40.8 and 59.1%, respectively. Significant association was found between a decreased intake of green tea and methylation of *CDX2* and *BMP-2*. More physical activity was correlated with a lower methylation frequency of *CACNA2D3*. Of these 6 genes, the methylation statuses of *CDX2*, *BMP-2* and *p16* revealed a significant interrelationship and those of *CACNA2D3*, *GATA-5* and *ER* did likewise. Thus, some epidemiological factors, such as green tea intake, could be important as to determination of the methylation statuses of selected genes and may influence the development of cancer, including that of the stomach. © 2008 Wiley-Liss, Inc.

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ARTICLE TEXT

Epigenetic changes, particularly methylation of cytosine in CpG dinucleotides in gene promoters, are found in almost every type of human neoplasm and are associated with transcriptional gene silencing.[1][2] Such promoter hypermethylation is as common as the disruption of tumor-suppressor genes in human cancer by mutation. Unlike irreversible genetic changes, epigenetic changes are thought to possibly be reversible by the environment, diet or pharmacological intervention. For example, monozygotic twins are considered genetically identical and are thus ideal for studying the effects of environmental and dietary factors on human health and diseases. In a study of a large cohort of identical twins, the patterns of DNA methylation across the genome were found to be very similar in young monozygotic twins in several cell types, but in older twins the patterns diverged.[3] This strongly suggests that 1 or more environmental factors affect individuals throughout life, modifying gene expression through epigenetic mechanisms that have important implications for health.

Dietary factors are important determinants of cancer risk.[4] Aberrant DNA methylation is associated with dietary factors and other lifestyle factors and may underlie carcinogenesis. The prevalence of promoter hypermethylation of 6 genes, such as *APC*, *p14*^{ARF}, *p16/INK4a* (hereafter *p16*) and *hMLH1*, was higher in colorectal cancers derived from patients with a low folate/high alcohol intake than in ones with a high folate/low alcohol intake, but the differences were not statistically significant.[5] The incidences of hypermethylation of *D17S5* and *p16* ir lung cancer are significantly higher in cigarette smokers than in those who have never smoked.[6-8] However, the relationship between DNA methylation and the past lifestyle in cancer patients remains largely unknown.

In 2000, gastric cancer was the second most frequent cause of cancer death worldwide.[9] Infection with *Helicobacter pylori* is a strong risk factor for gastric cancer but is not a sufficient cause for its development.[10] Epidemiological studies have strongly suggested that the risk may be increased with a high intake of salt and salt-preserved foods and decreased with a high intake of fruit and vegetables.[11] The aberrant methylation of many genes has been reported in gastric cancer.[12-14] We previously reported that *CDX2* methylation in men was correlated with a decreased intake of green tea, suggesting that diet could be an important factor determining the methylation status of genes such as *CDX2* and the resultant aberrant expression of genes involved in carcinogenesis.[15] However, these effects may not be universal but gene-specific, and female patients have not been examined. Thus, we analyzed the methylation states of 6 genes in more gastric cancer patients. Five of the 6 genes, that is, *CDX2*,[15] *BMP2*,[16] *p16*,[17] *CACNA2D3*[18] and *GATA5*,[19] were often methylated in gastric cancers but rarely in noncancerous epithelia. We, then, compared the relationship between DNA methylation and the past lifestyle in cancer patients including female ones.

Material and methods

Study population

Cancer tissue specimens were collected from 106 consecutive patients with primary gastric carcinoma in a hospital affiliated to Tokyo Medical and Dental University during 2000-2005. Informed consent was obtained from all patients, and the study was approved by the institutional review committee of Tokyo Medical and Dental University. A self-administered questionnaire was used in this study to assess the lifestyle before cancer onset, covering the disease history, familial history of cancer, medication, cigarette smoking, alcohol consumption, physical activity, intake frequencies of selected food groups and food items, daily consumption of tea (green tea, oolong tea and black tea), regularity of sleep and meals, eating quantity, bowel motion, height and body weight. The food groups were beef, pork, chicken, ham/sausage/bacon, grilled meat, all meat, grilled fish, salted/dried/other processed fish products, pickled vegetables, green leaf vegetables, yellow colored vegetables, cruciferous vegetables, all vegetables, fruits and probiotics-fermented milk. The intake frequencies of these food groups were categorized into not eaten, 1-2 times/month, 1-2 times/week, 3-4 times/week, almost every day and almost every meal. Most lifestyle factors in this questionnaire were selected from those which had previously been reported to be risk or preventive factors for gastric and colon cancers on epidemiological observation.

Tumors were reviewed by a pathologist and microdissected prior to DNA extraction. Histological classification was performed according to the general rules established by the Japanese Gastric Cancer Association[20] and Laurén's classification.[21]

Methylation analyses by the methylation-specific PCR procedure

We extracted genomic DNA from paraffin-embedded tissues by the phenol-chloroform method, and then carried out bisulfite modification and the methylation-specific PCR (MSP) procedure as previously described.[22] The primer sequences of the *CDX2, BMP-2, p16, CACNA2D3, GATA5, and estrogen receptor (ER)* genes for the MSP analyses are shown in Table I. The PCR reaction was performed for 35 cycles in a 25 μl mixture comprising bisulfite-modified DNA (~50 ng), 2.5 μl of 10 ラ PCR buffer, 1.25 μl of 25 mM dNTP, 10 pmole of each primer and 1 U of JumpStart Red Taq polymerase (Sigma, St. Louis, MO). Each PCR cycle consisted of 95−C for 30 sec, 58−C for 30 sec and 72−C for 30 sec, followed by final extension at 72−C for 5 min. The PCR products were electrophoresed in 2.5% agarose gels. All the MSP procedures were repeated more than twice. The methylation statuses of *CDX2* and *CACNA2D3* in several gastric cancer samples were also analyzed by LightCycler real-time PCR using bisulfite-modified DNA and methylation-specific primers, and the results were concordant with the MSP results.

Table I. PCR Primer Sequences Used for MSP

	Sense	Antisense
CDX2		
U	GAAGTTGTTGGTTTGGGGTTTTGTAT	CCCACAATACTCCACTAACTCCTCACA
M	CGTCGGTTTGGGGTTTCGTAC	GATACTCCGCTAACTCCTCGCG
BMP2		
U	GGATGGTTGTTTTGAGTTATGGGTTGT	CCTTAAAAACCAACACCCAAAAAACACA

M GGTTGTTTCGAGTTATGGGTCGC AAAACCAACGCCCGAAAAACGCG

p16

U TTATTAGAGGGTGGGGTGGATTGT CAACCCCAAACCACAACCATAA

M TTATTAGAGGGTGGGGCGGATCGC GACCCCGAACCGCGACCGTAA

CACNA2D3

U GGATATTGGAGTTTTTGAGTTTTTGTTTGT ACAACAACCACCCAACCCCACCTCA

M ATATTGGAGTTTTCGAGTTTTCGTTCGC ATATTGGAGTTTTCGAGTTTTCGTTCGC

GATA5

U TGGAGTTTGTTTTTAGGTTAGTTTTTGGT AACTTCATAAACCCCAAAAAATCAAACA

M AGTTCGTTTTTAGGTTAGTTTCGGC TTCGTAAACCCCGAAAAATCGAACG

ER

U GGTGTATTTGGATAGTAGTAAGTTTGT CCATAAAAAAAACCAATCTAACCA

M GTGTATTTGGATAGTAGTAAGTTCGTC CGTAAAAAAAACCGATCTAACCG

Statistical analysis

The promoter methylation status of specific genes, clinico-pathological parameters and lifestyle variables in the patients were computed. Differences in frequency by methylation status were tested using the χ^2 test, and differences in mean values were tested using the *t* test. The association between the methylation status and dietary variables was also analyzed using a nonparametric test (Mann-Whitney U test). We further studied the association using the backward elimination (Wald test) method of logistic regression analysis. In this analysis, the intake frequencies of food groups were dichotomous as follows: ≤6 cups/day *vs.* ≥7 cups/day for green tea, ≤twice/week *vs.* ≥3 times/week for pickled vegetables and ≤twice/week *vs.* ≥3 times/week for drinking. Physical activity was defined as "recreational and voluntary physical exercise for health promotion" and primarily divided into 4 categories in questionnaire as follows: Never, 1-2 hr/week, 3-4 hr/week and ≥5 hr/week. However, in the logistic regression analysis, we combined these categories into 2 groups, ≥1 hr/week *vs.* never. Pearson's contingency coefficients for methylation status of an every pair of the 6 genes were calculated in 106 gastric carcinomas. *p* for trend was calculated by the Cochran-Armitage test. The statistical software used was SPSS software (version 14.0).

Results

Methylation statuses of CDX2, BMP-2, p16, CACNA2D3, GATA5 *and* ER *in primary gastric carcinomas*

The methylation statuses of *CDX2, BMP-2, p16, CACNA2D3, GATA5* and *ER* were determined in 106 primary gastric carcinomas by MSP analyses using the specific primers shown in Table I. The methylation frequencies of these genes were 23.6, 21.7, 9.4, 32.4, 40.8 and 59.1% respectively. When we examined the *CDX2* methylation statuses of noncancerous gastric tissues in 12 patients with methylation-positive gastric cancer and in 13 patients with methylation-negative one, and the *CACNA2D3* methylation statuses of noncancerous tissues in 7 patients with methylation-positive gastric cancer and 19 patients with methylation-negative one, we found no methylation in any samples by MSP, indicating cancer-related methylation of these genes (data not shown).

The relationship between methylation frequencies of the 6 genes and clinicopathological parameters

The clinico-pathological characteristics of the studied patients by the methylation statuses of *CDX2, BMP-2, p16, CACNA2D3, GATA5* and *ER* are shown in Table II. The methylation of *p16* was significantly more frequent in diffuse type (8/49, 16.3%) than in intestinal (2/57, 3.5%) type gastric carcinomas ($p = 0.04$). *CACNA2D3* methylation was more frequently found in lymph node metastasis-positive cases (20/45, 44.4%) than in negative ones (14/60, 23.3%) ($p = 0.03$). In contrast, there was no statistically significant correlation between methylation of 4 of the genes, *CDX2, BMP-2, GATA5* and *ER*, and clinico-pathological parameters (Table II).

Table II. Clinicopathological Characteristics of Studied Patients According to the Methylation Statuses of Six Genes

	CDX2 (*n* = 106)			*BMP-2* (*n* = 106)			*p16* (*n* = 106)		
	Methylated (*n* = 25)	Unmethylated (*n* = 81)	*p* value	Methylated (*n* = 23)	Unmethylated (*n* = 83)	*p* value	Methylated (*n* = 10)	Unmethylated (*n* = 96)	*p* value
Age (mean ± SD)	66.0 ± 10.6	63.8 ± 10.3	0.35	63.6 ± 8.8	64.5 ± 10.8	0.73	59.4 ± 12.1	64.8 ± 10.0	0.12
Sex									
Male	22	57	0.11	17	62	1.00	9	70	0.45
Female	3	24		6	21		1	26	
Size (cm, mean ±	5.7 ± 4.3	5.6 ± 3.5	0.91	5.6 ± 4.6	5.7 ± 3.4	0.92	6.1 ± 4.0	5.6 ± 3.7	0.69

SD)									
Histology									
Intestinal	9	48	0.07	11	46	0.52	2	55	0.04
Diffuse	16	33		12	37		8	41	
Depth of tumor invasion									
m, ms	15	39	0.36	13	41	0.64	3	51	0.20
mp - si	10	42		10	42		7	45	
Lymph node metastasis									
-	16	44	0.49	17	43	0.10	3	57	0.10
+	9	37		6	40		7	39	

	CACNA2D3 (*n* = 105)			*GATA5* (*n* = 98)			*ER* (*n* = 93)		
	Methylated (*n* = 34)	Unmethylated (*n* = 71)	*p* value	Methylated (*n* = 40)	Unmethylated (*n* = 58)	*p* value	Methylated (*n* = 55)	Unmethylated (*n* = 38)	*p* value
Age (mean ± SD)	65.8 ± 11.2	63.8 ± 9.9	0.35	64.9 ± 9.8	64.1 ± 10.9	0.71	65.7 ±9.9	62.3 ± 10.3	0.12
Sex									
Male	25	54	0.81	27	46	0.24	42	25	0.35
Female	9	17		13	12		13	13	
Size (cm, mean ± SD)	5.9 ± 3.6	5.5 ± 3.7	0.61	6.4 ± 4.2	5.4 ± 3.4	0.23	5.5 ± 3.4	5.8 ± 3.8	0.72
Histology									
Intestinal	17	40	0.68	20	31	0.84	32	17	0.21
Diffuse	17	31		20	27		23	21	
Depth of tumor invasion									
m, ms	16	37	0.68	21	25	0.41	28	19	1.00
mp - si	18	34		19	33		27	19	
Lymph node metastasis									
-	14	46	0.03	22	32	1.00	28	25	0.20
+	20	25		18	26		27	13	

Clinicopathological characteristics between patients with and without methylation were compared using the χ^2 test for categorical data and the *t* test for comparison of mean.

The relationship between methylation frequencies of the 6 genes and epidemiological parameters in gastric carcinoma patients

As shown in Table III, the methylation frequencies of *CDX2* and *BMP-2* were lower in patients consuming 7 cups or more per day of green tea than those consuming 6 cups or less per day (2/25 (8%) *vs.* 22/80 (27.5%), $p = 0.06$ and 1/25 (4%) *vs.* 22/80 (27.5%), $p = 0.02$, respectively). Patients consuming more pickled vegetables exhibited a higher methylation frequency of *GATA5* than ones consuming less ($p = 0.04$). *CACNA2D3* methylation was more frequently found in patients with no physical activity (20/44, 45.5%) than in those with more physical activity (14/59, 23.7%) ($p = 0.03$). In contrast, there was no statistically significant correlation between methylation of 2 of the genes, *p16* and *ER*, and clinico-pathological parameters (Table III).

Table III. Relationships Between the Methylation Status and Lifestyle Factors

	CDX2 (*n* = 106)			*BMP-2* (*n* = 106)			*p16* (*n* = 106)		
	Menthylated (*n* = 25)	Unmethylated (*n* = 81)	Univariate *p* value	Menthylated (*n* = 23)	Unmethylated (*n* = 83)	Univariate *p* value	Menthylated (*n* = 10)	Unmethylated (*n* = 96)	Univariate *p* value
Green tea									
≥7 cups/day	2	23	0.06	1	24	0.02	1	24	0.45
≤6 cups/day	22	58		22	58		9	71	

Pickled vegetables									
≥3 times/week	14	40	0.5	13	41	0.62	5	49	1.00
≤twice/week	10	40		10	40		5	45	
Physical activity									
≥1 hr/week	12	47	0.49	13	46	0.80	5	54	0.74
Never	12	33		9	36		5	40	

	CACNA2D3 (*n* = 105)			*GATA5* (*n* = 98)			*ER* (*n* =93)		
	Menthylated (*n* = 34)	Unmethylated (*n* = 71)	Univariate *p* value	Menthylated (*n* = 40)	Unmethylated (*n* = 58)	Univariate *p* value	Menthylated (*n* = 55)	Unmethylated (*n* = 38)	Univariate *p* value
Green tea									
≥7 cups/day	7	18	0.63	10	15	1.00	15	8	0.63
≤6 cups/day	27	52		30	42		39	30	
Pickled vegetables									
≥3 times/week	19	34	0.54	26	24	0.04	29	19	0.68
≤twice/week	15	35		14	32		24	19	
Physical activity									
≥1 hr/week	14	45	0.03	20	35	0.40	29	23	0.67
Never	20	24		19	22		24	15	

***p* values for χ^2 test.**

When the intake of green tea was stratified, the prevalence of aberrant methylation of *CDX2* and *BMP-2* decreased significantly with a higher intake of green tea (Mann-Whitney U-test, both $p = 0.04$; Cochran-Armitage test, $p_{trend} = 0.03$ and 0.02, respectively) (Figs. 1*a* and 1*b*). A distinct distribution of patients with methylated and unmethylated *CACNA2D3* was also demonstrated for physical activity (Mann-Whitney U-test, $p = 0.03$; Cochran-Armitage test, $p_{trend} = 0.03$) (Fig. 1*c*). On the other hand, an increased intake of pickled vegetables was not associated with an increased methylation frequency of *GATA5* ($p = 0.11$).

Figure 1. Frequencies of the presence (closed bars) or absence (open bars) of *CDX2* (*a*) and *BMP-2* (*b*) methylation in gastric cancers stratified as to intake of green tea and those of *CACNA2D3* methylation and physical activity (*c*).
[Normal View 11K | Magnified View 25K]

Since dietary factors are closely interrelated, we further performed the backward elimination (Wald) method of logistic regression analysis of the methylation status of each gene in gastric cancer patients including female ones (Table IV). A significant association was found between the intake of green tea and methylation of 2 of the genes, *CDX2* and *BMP-2*. Increased daily consumption of green tea (7 cups or more per day) showed a significant association with decreased methylation frequencies of *CDX2* and *BMP-2* after adjustment ($p = 0.04$ and $p = 0.049$, respectively). On the other hand, an increased methylation frequency of *CACNA2D3* was associated with less physical activity (negative *versus* positive), adjusting for confounding variables ($p = 0.06$) (Table IV). As for factors other than dietary ones, the logistic regression analysis also showed significant associations between *CDX2* methylation and gender or histology, *p16* methylation and histology, and *CACNA2D3* methylation and lymph node metastasis (Table IV).

Table IV. Standardized Partial Regression Coefficients of Variables Related with Methylation Status of Each Gene

	β	SE	*p*

CDX2			
Gender (men vs women)	-1.87	0.78	0.02
Histology (intestinal vs diffuse)	2.61	0.78	0.001
Green tea (≤6 cups/day vs. ≥7 cups/day)	-1.87	0.92	0.04
BMP-2			
Green tea (≤6 cups/day vs. ≥7 cups/day)	-2.08	1.06	0.049
p16			
Histology (intestinal vs. diffuse)	2.06	6.03	0.01
CACNA2D3			
Lymph node metastasis (negative vs. positive)	0.93	0.45	0.04
Physical activity (never vs. ≥1 hr/week)	-0. 86	0.45	0.06

These variables were selected using the backward elimination (Wald) method of logistic regression analysis for the methylation status of each gene.

Interrelationship of the 6 genes relative to their methylation statuses in gastric carcinomas

The methylation statuses of the 6 genes in 106 gastric carcinomas are shown in Figure 2. The methylation patterns of 3 genes, *CACNA2D3*, *GATA-5* and *ER*, were distinct from other 3 genes, *CDX2*, *BMP-2* and *p16*. To determine the relationship of the methylation statuses among the 6 genes, Pearson's contingency coefficients for methylation status of an every pair of the 6 genes were calculated (Table V). On the basis of the contingency coefficients, we found that the 6 genes were divided into 2 groups, Group I (*CDX2*, *BMP-2* and *p16*) and Group II (*CACNA2D3*, *GATA-5* and *ER*), where a statistically significant interrelationship within each group but no intergroup association was noted. The methylation frequencies in gastric carcinomas were lower for Group I genes (*CDX2*, 23.6%; *BMP-2*, 21.7% and *p16*, 9.4%) than Group II ones (*CACNA2D3*, 32.4%; *GATA-5*, 40.8% and *ER*, 59.1%).

Figure 2. Summary of the methylation statuses of the 6 genes in 106 gastric carcinomas. Each column represents a different gene indicated on the top. Each row represents a primary gastric carcinoma. Black squares, methylated alleles in the carcinoma. White squares, unmethylated alleles in the carcinoma. CACN, *CACNA2D3*; GA5, *GATA5*. NA, not amplified.
[Normal View 16K | Magnified View 32K]

Table V. Contingency Coefficients for Methylation Status Between Two Genes

	CDX2	*BMP2*	*p 16*	*CACNA2D3*	*GATA5*	*ER*
CDX2						
BMP-2	0.24[1]					
p16	0.33[2]	0.22[1]				
CACNA2D3	*0.09*	*0.08*	*0.31*[2]			
GATA5	0.10	0.12	0.09	0.39[3]		
ER	0.01	0.02	0.2	0.36[4]	0.36[4]	

p value for χ^2 test.
[1] $p < 0.05$.
[2] $p < 0.01$.
[3] $p < 0.0001$.
[4] $p < 0.001$.

As described earlier, aberrant methylation of *CDX2* and *BMP-2* was inversely correlated with green tea intake. The prevalence of *p16* methylation was also higher in patients with a lower green tea intake than those with a higher intake, although the difference was not significant. When we analyzed the relationship between green tea intake and the methylation of combinations of Group I genes by the multinominal logistic regression model, the odds ratios of methylation for any 1 gene and ≥2 genes *vs.* no methylation were 4.9 (confidence interval (CI) 1.0-24.3) and 14.8 (CI 1.1-206.7), respectively, in patients consuming 6 cups or less per day of green tea compared with those

consuming 7 cups or more per day. On the other hand, no lifestyle factors were associated with the methylation of combinations of Group II genes.

Discussion

The methylation frequencies of the 6 genes in 106 gastric carcinomas varied from 9.4 to 59.1%. The prevalence of promoter hypermethylation of *CDX2* and *BMP-2* was significantly higher in gastric carcinomas derived from patients with a low green tea intake than those with a high intake. When we analyzed the association between the methylation status and variables using a nonparametric test, increased intake of green tea was found to be significantly associated with decreased methylation frequencies of *CDX2* and *BMP-2*. In a previous study,[15] methylation of 1 of 3 genes, *CDX2*, was correlated with a decreased intake of green tea in 58 male gastric carcinoma patients. Since an inverse relationship with green tea intake was also found for *BMP-2* promoter methylation in 106 gastric carcinoma patients including female ones in this study, the effect of green tea on the decrease of gene promoter methylation might be more common for many genes.

The evidence derived from epidemiologic studies on the relationship between drinking of green tea and cancer-preventive effects is inconclusive; some indicated preventive effects[23][24] and some did not.[25][26] In a detailed study, consumption of green tea was found to be associated with a decreased risk of gastric carcinoma in Japanese women after adjustment for potential confounding factors, whereas no association was observed among Japanese men.[27] The difference between women and men might be explained by the much higher cigarette smoking rate in men than women in Japan, which may play a role as an effect modifier.[28]

Green tea contains several polyphenolic compounds, such as (-)-epigallocatechin-3-gallate (EGCG). A significant inhibitory effect of EGCG on chemical carcinogenesis in the rat stomach has been reported.[29] As for its action on methylation, it was reported that EGCG dose-dependently inhibited DNA methyltransferase activity in several cancer cells, resulting in reactivation of methylation-silenced genes, such as *retinoic acid receptor* β, *p16* and *hMLH1*.[30][31] Polyphenols are rapidly metabolized to forms with quite different bioactivities. But the epithelial surfaces of the gut, particularly those of the esophagus and the stomach, are exposed on the luminal side to high concentrations of tea polyphenols before they undergo metabolism. These characteristics may make gastrointestinal epithelial tissues particularly susceptible to what are probably the beneficial effects of DNA methyltransferase inhibitors.

In other studies, however, EGCG did not inhibit DNA methyltransferase activity or reactivate genes, whereas nucleoside analogue methylation inhibitors, such as 5-aza-2′-deoxycytidine, were far more effective.[32][33] We also analyzed the effect of EGCG on transcriptional levels of *CDX2* and *BMP-2* in 3 human gastric cancer cell lines. Upregulation of both genes was not found in any cell lines (Hashimoto et al., personal communication). Thus, further studies are necessary to determine how tea polyphenols act on DNA methylation.

CACNA2D3 methylation was more frequently found in gastric carcinoma patients with no physical activity than in those with physical activity. It is known from epidemiological studies that physical activity protects against cancers of the colon, breast (postmenopause) and endometrium.[4] As for gastric carcinoma, a population based case-control study in Canada and a prospective cohort study in Norway indicated that recreational physical activity might have a protective effect against gastric cancer.[34][35] To determine the association of physical activity with promoter hypermethylation of *APC* and *RASSF1A* in breast tissue, a cross-sectional study on 45 women without breast cancer was performed, which revealed that physical activity was inversely associated with promoter hypermethylation of *APC* but not *RASSF1A*.[36] It is, therefore, possible that physical activity may affect the methylation of genes, such as *CACNA2D3*, and gastric carcinogenesis.

There are 2 types of genes according to contingency coefficients for methylation status in gastric carcinomas. The methylation frequencies were lower for Group I genes (9.4-23.6%) than Group II ones (32.4-59.1%). The odds ratios of methylation for any 1 gene and ≥2 genes vs. no methylation among the Group I genes were much higher in patients consuming 6 cups or less per day of green tea than in those consuming 7 cups or more per day. These data suggest that green tea intake may be inversely related to the methylation of Group I genes, which may be involved in carcinogenesis. The logistic regression analysis also showed significant associations between histology and the methylation of *CDX2* and *p16*. But there was no association between *BMP-2* methylation and histology. Further investigations are required to clarify the significance of the 2 different types of genes as to methylation in gastric carcinomas.

In conclusion, there were inverse associations between the intake of green tea and the methylation of *CDX2* and *BMP-2*, and between physical activity and *CACNA2D3* methylation. We, therefore, hypothesized that some of the lifestyle factors, which have been reported to be preventive as to gastric cancer on epidemiological observation, may influence the development of gastric cancer through the demethylation or retaining of unmethylated status of selected genes. Because an epigenetic drift may contribute to the development of cancer, strategies involving changes in lifestyle including diet might be highly beneficial in preventing/reversing epigenetic alterations and counteracting cancer.

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Sixty years of follow-up of Hiroshima and Nagasaki survivors: Current progress in molecular epidemiology studies

by
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Review

Sixty years of follow-up of Hiroshima and Nagasaki survivors: Current progress in molecular epidemiology studies

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Abstract

This article provides an overview of the on-going molecular epidemiology studies among atomic-bomb survivors conducted at the Radiation Effects Research Foundation in Japan. The focus is on: (a) inter-individual variations in sensitivity to radiation-induced somatic mutations (*glycophorin A* (*GPA*) mutations) and their potential relevance to differences in susceptibility to radiation-related cancers and (b) the role of specific mutations/rearrangements in radiation-induced thyroid and colorectal cancers. The *glycophorin A* mutant fractions showed large differences between the survivors at each of the estimated bone marrow doses. Of note is the finding at doses ≥ 1 Gy; that the slope of the mutant fraction was significantly higher in the 'cancer group' than in the 'non-cancer group'. This study provided the basis for validating the use of γH2AX and reticulocyte micronucleus assays for evaluating radiosensitivity differences and genetic instability, respectively, in our studies in the coming years. Preliminary results from our molecular oncology studies on adult-onset papillary thyroid cancer provide evidence for the induction of *RET/PTC* rearrangements and *BRAF* point mutation (both known to be early stage events in adult-onset papillary thyroid cancer) but with a difference: cases associated with the rearrangements were more frequent at high doses, and developed sooner than those with *BRAF* mutation. In the case of colorectal cancer, the results suggest that radiation exposure might influence microsatellite instability (MSI) status through MSI-related epigenetic and genetic alterations—processes that might occur in the early stage of colorectal carcinogenesis.

Keywords: Radiation; Atomic-bomb survivors; Somatic mutation; Oxidative stress; Colorectal carcinogenesis; Microsatellite instability; Thyroid carcinogenesis

Contents

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1. Introduction

Molecular epidemiology studies among atomic-bomb survivors (A-bomb survivors) seek to deepen our understanding of the mechanisms by which ionizing radiation affects human health. Their importance will be evident in future prevention of radiation-associated diseases: not only confined to establishing safeguards against radiation exposure, but also in developing the ways and means to identify high-risk individuals and more efficient approaches to prevent radiation-associated diseases. The mechanistic study group in our laboratory comprises two subgroups and the following research themes: radiation effects on immune functions (specifically, T-cell mediated immunity) and aging-associated persistent inflammation [1], a genome approach relative to individual risks of radiation-associated diseases, somatic mutability in relation to cancer occurrence and molecular oncology analyses of radiation-associated cancers. These studies are based on analyses of invaluable biological resources obtained from the two cohort studies conducted at the Radiation Effects Research Foundation (RERF); the Life Span Study (LSS) with 120,000 survivors, and the Adult Health Study (AHS) with 20,000 survivors, which has conducted biennual medical examinations among a subcohort of the LSS.

In this article, we report on current progress in selected research themes of our laboratory relating to two critical issues: (1) inter-individual variations in sensitivity to radiation-induced somatic mutation and (2) relationship between radiation exposure and various gene alterations in carcinogenesis. The first issue was addressed using biomarkers of individual radiation sensitivity and presumed radiation-induced genetic instability, among A-bomb survivors, based on our previous findings in the *glycophorin A* (*GPA*) mutant fraction (Mf) study, as well as biomarkers of inflammation and production of reactive oxygen species (ROS). In this *GPA* Mf study, we demonstrated large inter-individual variation in the *GPA* Mf response of survivors exposed to atomic radiation, as well as a significantly higher sloped *GPA* Mf dose–response curve for doses $\geq$1 Gy, when the cancer group was compared with the non-cancer group [2]. This study prompted two questions: (1) are individual sensitivities to radiation-induced genetic damage responsible for the inter-individual variation noted in dose-dependent *GPA* Mf response? And (2) is radiation-induced genetic instability involved in the dose-dependent increase of *GPA* Mf? To address these questions, we considered several biomarkers and assessed and verified the suitability of two of them, namely the γH2AX and reticulocyte micronucleus endpoints. Both of these biomarkers appear applicable to evaluation of individual sensitivity to radiation-induced genetic damage and instability [3,4]. In parallel with the somatic mutation study, we have observed dose-dependent increase of various inflammatory biomarkers among A-bomb survivors [5]. This enhanced and persistent inflammation needs to be examined in relation to the endogenous production of reactive oxygen species (ROS), linking inflammation and somatic mutations.

The second issue can be further broken down into two questions: which types of genetic alterations e.g., chromosome aberration or point mutation, preferentially occurred in radiation-associated cancer, and whether or not radiation influenced epigenetic alterations during carcinogenesis. Adult-onset papillary thyroid cancer seems to be a good model for examining and contrasting chromosome aberrations and point mutations, since the major initial event of this cancer is either *RET/PTC* rearrangements or *BRAF* point mutation, which appear to occur in a mutually exclusive manner. It is well recognized that colorectal cancer can be phenotyped according to microsatellite stability. Accordingly, we have begun to analyze microsatellite instability (MSI), along with methylation status of *MLH1*, using cancer tissue specimens from A-bomb survivors. Although these studies are still preliminary and continuing, we report here our interim results [6].

2. Radiation-induced genetic damage and inflammation

2.1. GPA Mf study in the AHS cohort

We have conducted a prospective study among a total of 1723 MN heterozygous AHS participants who were cancer free and without cancer history at the time (1988–1996) of *GPA* Mf measurements, in which mutated erythrocytes, namely hemizygous Mφ or Nφ cells, were counted by flow cytometry [2]. During a follow-up period that lasted until 2000, a total of 186 cancer cases were identified. The major findings from this study were: (1) a radiation dose-dependent increase of *GPA* Mf was observed in the total population, as well as in the cancer and cancer-free participants in Hiroshima and Nagasaki, and (2) the slope of the *GPA* Mf dose-response above 1 Gy was significantly higher in the Hiroshima cancer group than in the cancer-free group within the same high dose region (Fig. 1). These findings imply that inter-individual variations in *GPA* Mf might indicate individual differences in somatic mutability response to radiation exposure, and that individuals with higher mutability in *GPA* Mf response could have increased risk of radiation-associated cancer. It also suggests that the inter-individual variation in *GPA* Mf might involve differences in hematopoietic stem cell repair capacity of DNA double-strand breaks induced by high-dose irradiation. Alternatively, differences might exist between individuals in terms of persistent radiation-induced genetic instability within the hematopoietic system.

Another important message of Fig. 1 is that the wide variation in *GPA* Mf among individuals exposed to high radiation doses was not merely the result of random errors in measurement, or in dose dosimetry. It was also thought to primarily reflect inter-individual differences in sensitivity to radiation: higher responders to given radiation doses had higher probability of developing radiation-associated cancer. Of course, unidentified factors other than radiation sensitivity are possibly involved in this inter-individual variation, specifically considering the long period elapsed since atomic radiation exposure and other environmental factors influencing *GPA* Mf [7]. The large reduction in size of the stem-cell pool as a consequence of cell destruction by A-bomb irradiation might cause stochastic fluctuation of *GPA* Mf derived from a limited

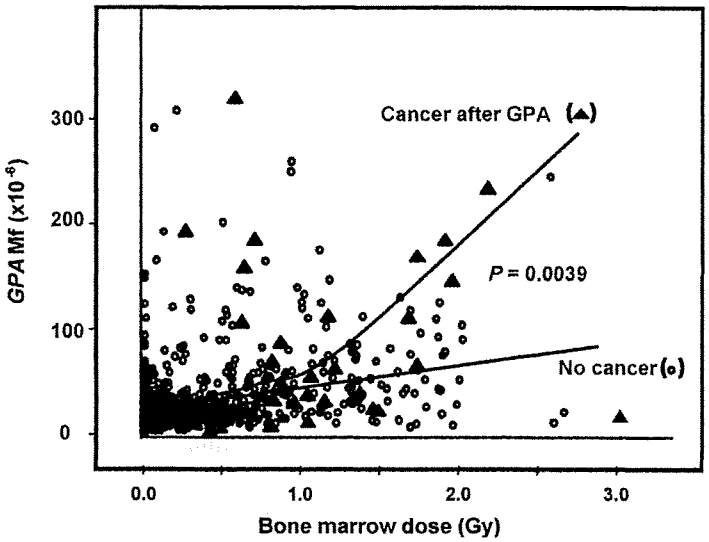

Fig. 1. Dose–response of *GPA* Mf to radiation dose (Gy) among MN heterozygous AHS participants in Hiroshima (modified from reference [1]). Non-parametric curve fitting for cancer (being identified by a follow-up study after *GPA* Mf measurement) and non-cancer groups revealed a significantly higher slope of *GPA* Mf dose–response found in the cancer group than in the cancer-free group in high dose region of ≥ 1 Gy (regression $P < 0.01$).

number of mutant stem cells [8]. Therefore, a new study will be required with use of biomarkers more specific to acute and delayed effects of radiation.

These results provided the basis to ask two further questions. One question is whether or not individual sensitivity to radiation-induced genetic damage was reflected by the noted inter-individual variation in *GPA* Mf as a function of radiation dose. The other is whether genetic instability was induced and contributed to the noted dose-dependent increase of *GPA* Mf. To answer these questions, we recently developed a γH2AX assay system to evaluate individual sensitivity to the immediate effects of set doses of radiation, as well as a reticulocyte micronucleus assay system to investigate delayed effects of radiation, namely genetic instability.

2.2. *Biomarkers for radiation sensitivity and genetic instability*

Various biomarkers measuring radiation-induced cellular damage, such as DNA strand-breaks, chromosomal damage and lethality, have been studied to assess individual sensitivity to radiation exposure in vitro. However, these bioassays require considerable labor and technical skill to achieve reliable and reproducible results. Thus, assay platforms that can more easily and reliably measure radiation-induced cellular damage need to be identified, especially to facilitate biomarker assays and associated risk estimation in a large study population. For this purpose, we thought that flow cytometry was the most applicable method because of its performance characteristics: i.e., high throughput and high-sensitivity.

It is known that histone H2AX, a subfamily of histone H2A, is phosphorylated and forms foci (γH2AX foci) at the sites of DNA double-strand breaks induced by ionizing radiation [9]. The number of γH2AX foci closely corresponds to that of DNA double-strand breaks in cells [10], and counting γH2AX foci has frequently been used as a more sensitive DNA damage marker than more conventional assays, such as pulse-field gel electrophoresis, neutral single cell electrophoresis (Comet assay), or DNA elution assay [11]. We recently have established a flow cytometry system for detecting γH2AX induced by in vitro irradiation, using cultured T lymphocytes, which are readily available from healthy individuals, and attempted to validate its suitability for analysis of individual radiosensitivities in human populations [3]. Because γH2AX focus formation appears not only in genomically damaged cells, but also in cells undergoing DNA synthesis and mitosis [12,13], improved assays to detect level of radiation-induced γH2AX foci were required. Toward this end, we simultaneously analyzed γH2AX expression and DNA content, and γH2AX levels were determined in cell fractions accurately gated for G0/G1 phase cells. Such cell-cycle-specific analysis is feasible for flow cytometry, but not for conventional fluorescence microscopy. Short-term (7 days) cultured T lymphocytes, but not uncultured, freshly isolated lymphocytes from peripheral blood, exhibited significant inter-individual differences in level of γH2AX induced by in vitro X-irradiation (Fig. 2). The reason why no obvious inter-individual differences were detected in the assay using uncultured lymphocytes remains elusive, but substantial differences in metabolic status, such as differences in the levels of radical scavenger proteins, between cultured and uncultured lymphocytes might result in differences in radiation sensitivity (discussed in reference [3]). Our assay system also provides good reproducibility, as well as a capacity to detect significant inter-individual differences between the responses of T lymphocytes from six healthy donors 6 h after 4 Gy of X-irradiation [3]. Variation in the level of γH2AX in cultured T lymphocytes of these individuals was about 1.5-fold. Differences in lymphocyte subsets either before or after culture were not responsible for this noted variance in individual radiosensitivity. Thus, our γH2AX assay system using cultured T lymphocytes appears to be useful for the rapid and reliable assessment of individual radiation sensitivity. We have already

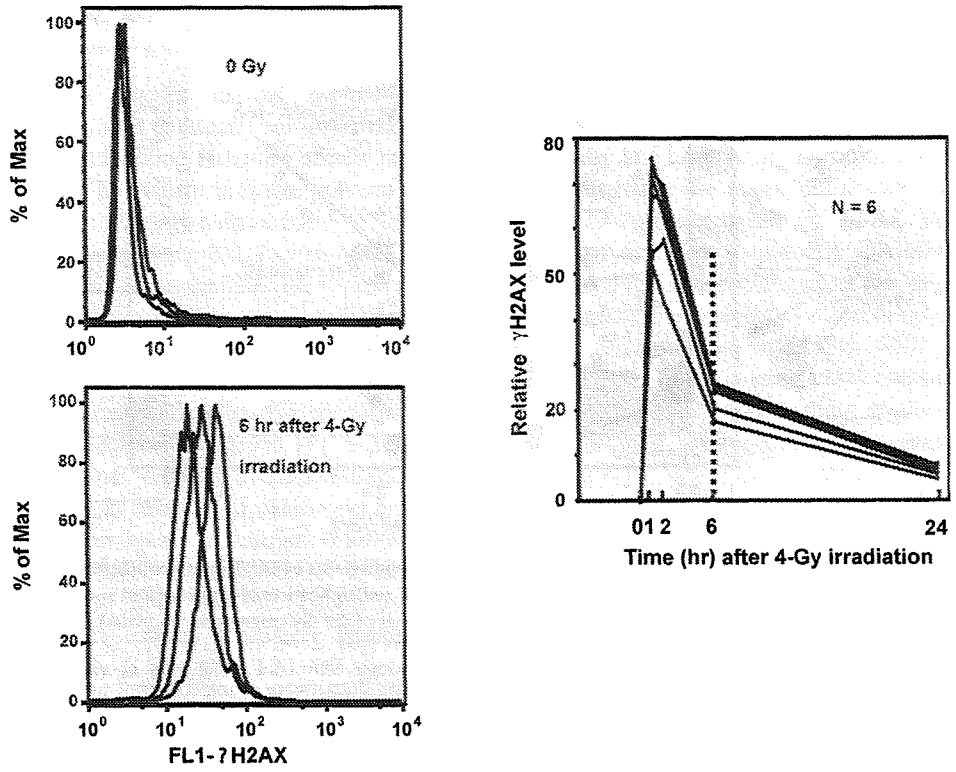

Fig. 2. Flow cytometry analysis of phosphorylated H2AX (γH2AX) in cultured T lymphocytes from three typical healthy individuals 6 h after 0 or 4 Gy of X-irradiation (left panels). Radiation-induced γH2AX expression levels in cultured T lymphocytes from six healthy individuals, which were measured 1, 2, 6 and 24 h after 4-Gy irradiation (right panel). There were significant inter-individual, but not inter-experimental, differences in the γH2AX levels 6 h after 4-Gy irradiation in three independent experiments using peripheral blood lymphocytes from six healthy donors (ANOVA, $P < 0.01$) [3].

confirmed that T lymphocytes cultured from cryopreserved lymphocytes are also useful for this radiation sensitivity assay. Evaluation of radiation sensitivity using cryopreserved lymphocytes from A-bomb survivors for whom *GPA* Mf has been analyzed is underway.

It has been recently reported that chromosomal alterations are indicated not only in cells that have been directly irradiated, but also in unirradiated neighboring cells, or descendants of irradiated cells [14]. Such delayed radiation effects, termed radiation-induced genetic instability effects, are thought to represent adverse cellular consequences, such as linked bystander and occasionally malignant transformation [14]. Although radiation-induced genetic instability is well characterized in vitro, evidence from in vivo studies has been limited due to lack of reliable bioassays capable of detecting and distinguishing "delayed type" chromosomal alterations induced by direct radiation effects versus those induced indirectly. Thus, to ensure identification of both delayed and direct radiation effects in vivo, we applied a flow cytometry-based reticulocyte micronucleus assay [15–17]. Because micronuclei in reticulocytes are chromosomal segments separated from entire chromosomes during the enucleation stage of erythrocyte maturation, and because the estimated lifespan of reticulocytes in vivo is as short as a few days [18], reticulocyte micronuclei observed more than 1 month after irradiation are not those that have been directly induced by radiation but those that have arisen in the course of normal erythopoiesis (Fig. 3). By utilizing this post-irradiation time discrepancy in the appearance of reticulocyte micronuclei, we were able to analyze delayed radiation effects separately from direct effects in whole-body X-irradiated mice [4]. In irradiated mice, we detected an acute effect from radiation dose as small as 0.1 Gy 2 days after irradiation, and a significant difference in the radiation-dose response between BALB/c and C57BL/6 mice (regression $P < 0.001$). As for delayed radiation effects, we also observed significantly increased frequencies of reticulocyte micronuclei in both BALB/c and C57BL/6 mice (1.6- and 1.3-fold increases compared with age-matched controls, Mann–Whitney, $P = 0.035$ and 0.039, respectively), 1 year after irradiation with 2.5 Gy of X-rays. Interestingly, there was also a significant mouse strain difference in the delayed radiation effect (Mann–Whitney, $P = 0.028$). It was therefore concluded that delayed effects of radiation on the murine hematopoietic system can persist in vivo for prolonged periods and that there were differences by mouse strain in susceptibility to such delayed radiation effects.

Two possibilities that are not mutually exclusive have been proposed for radiation-induced genetic instability [14]. One possibility is that cells not directly irradiated, but descended from irradiated cells, exhibit genetic instability. The other is that mediators, such as ROS and inflammatory cytokines, are released from irradiated cells and enhance DNA damage in unirradiated but neighboring cells to the irradiated cells (bystander effect). Our previous study indicated that low-grade inflammation may persist for more than 50 years after irradiation in A-bomb survivor populations [5]. It is intriguing to speculate that the reticulocyte micronucleus response, an