Abstract
A major early event in papillary thyroid carcinogenesis is constitutive activation of the mitogen-activated protein kinase signaling pathway caused by alterations of a single gene, typically rearrangements of the RET and NTRK1 genes or point mutations in the BRAF and RAS genes. In childhood papillary thyroid cancer, regardless of history of radiation exposure, RET/PTC rearrangements are a major event. Conversely, in adult-onset papillary thyroid cancer among the general population, the most common molecular event is BRAFV600E point mutation, not RET/PTC rearrangements. To clarify which gene alteration, chromosome aberration, or point mutation preferentially occurs in radiation-associated adult-onset papillary thyroid cancer, we have performed molecular analyses on RET/PTC rearrangements and BRAFV600E mutation in 71 papillary thyroid cancer cases among atomic bomb survivors (including 21 cases not exposed to atomic bomb radiation), in relation to radiation dose as well as time elapsed since atomic bomb radiation exposure. RET/PTC rearrangements showed significantly increased frequency with increased radiation dose ($P_{\text{trend}} = 0.002$). In contrast, BRAFV600E mutation was less frequent in cases exposed to higher radiation dose ($P_{\text{trend}} < 0.001$). Papillary thyroid cancer subjects harboring RET/PTC rearrangements developed this cancer earlier than did cases with BRAFV600E mutation ($P = 0.03$). These findings were confirmed by multivariate logistic regression analysis. These results suggest that RET/PTC rearrangements play an important role in radiation-associated thyroid carcinogenesis.

Introduction
Thyroid cancer is, as is well-known, associated with exposure to external or internal ionizing radiation, such as from the atomic bombings (1) or the Chernobyl nuclear power plant accident (2, 3). The excess relative risk of thyroid cancer per Gy weighted thyroid dose was 1.15 in the Life Span Study (LSS) of atomic bomb (A-bomb) survivors (4), and a strong relationship between thyroid cancer and radiation exposure was indicated from the data of the Chernobyl accident (3). A histopathologic study has revealed that the thyroid cancers found in A-bomb survivors were largely conventional papillary in nature, and this is also the case of spontaneous thyroid cancer in the Japanese population at large. Solid variant papillary thyroid cancer (PTC) has not been found in A-bomb survivors yet, although this cancer has been frequently observed among post-Chernobyl children (5, 6).

Gene alterations that lead to constitutive activation of the mitogen-activated protein kinase (MAPK)-signaling pathway are frequently found in PTC. These alterations are mutually exclusive, nonoverlapping events that involve rearrangements of the RET and neutrotrophic tyrosine kinase receptor 1 (NTRK-1) genes and point mutations in the RAS and BRAF genes (7–9). Alteration of one of these genes can be detected in >70% of PTC, suggesting that the constitutive activation of the MAPK-signaling pathway is a major early event in papillary thyroid carcinogenesis.

RET proto-oncogene is normally expressed in a subset of cells derived from the neural crest as well as from the kidney and the enteric nervous system (10, 11). In PTC, the RET proto-oncogene is activated by fusion of the RET TK domain with the 5' terminal sequence of one of different heterologous genes via rearrangements that generate a series of chimeric-transforming oncogenes collectively described as RET/PTCs. To date, at least 12 rearranged forms of the RET gene have been isolated, of which RET/PTC1 and RET/PTC3 are by far the most common (12). RET/PTC rearrangements were commonly found in childhood PTC regardless of radiation history (13–15). Among the childhood PTC from areas contaminated by the Chernobyl nuclear accident in 1986, RET/PTC3 rearrangement seemed to be strongly associated with solid variant-type PTC and with a short latency period after exposure (15, 16).

On the other hand, in the Japanese general adult population, typical frequency of RET/PTC seems to be of the magnitude of 10% to 40%, although a wide variation, ranging from 2.6% to 70%, has been observed in different geographic areas (17–19). RET/PTC rearrangements, especially RET/PTC1, was reported as being detected at higher frequency in PTC from adult patients with a
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).

BRAFV600E 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 BRAFV600E 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 BRAFV600E mutations in this PTC has been reported (range, 0–12%; refs. 27–31). However, prevalence of BRAFV600E 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 BRAFV600E mutation. On the other hand, in adult-onset PTC among A-bomb survivors, we have previously reported that prevalence of BRAFV600E 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 BRAFV600E 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 BRAFV600E 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 DNA, 71 cases were analyzable for both RET/PTC and BRAFV600E 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 BRAFV600E mutation.** Reverse transcription-PCR (RT-PCR) with BCR as internal control was

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**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.**

**Fishers 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**

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Exposed patients (&gt;0 mGy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RET/PTC</td>
<td>Wild-type RET</td>
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<tr>
<td>Gender</td>
<td>Male (n)</td>
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<tr>
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<td>Female (n)</td>
<td>11</td>
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<tr>
<td>Histology</td>
<td>Conventional PTC (n)</td>
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<tr>
<td></td>
<td>Follicular variant (n)</td>
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<tr>
<td>Median age ATB †</td>
<td>Years</td>
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<td>Range</td>
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<tr>
<td>Median age at diagnosis</td>
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<td></td>
<td>Range</td>
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<tr>
<td>Median time after exposure</td>
<td>Years</td>
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<td>Range</td>
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</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0–2,304)</td>
</tr>
</tbody>
</table>

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

**Conduct**

A novel gene 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 High Fidelity (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 Mg2+ 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.[1]

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.

The statistical analysis of the data was carried out with SPSS software (version 12.0). 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 BrafV600E mutation, to assess differences between PTC patients with RET/PTC rearrangement and those with BrafV600E mutation, in terms of pathologic and epidemiologic variables, including radiation dose, histology, gender, and time-related factors [Table 1]. All statistical analyses were performed with SPSS software (version 12.0).

**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/PTCf) 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).

While 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 BrafV600E mutation was marginally lower in exposed patients than that in nonexposed ones ($P = 0.06$; Table 1).

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**Submitted.**
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 BRAFV600E mutation. Pathologic and epidemiologic characteristics of study patients were shown by BRAFV600E mutation status (Table 3). Close association of BRAFV600E mutation status with radiation dose and time since exposure remained unchanged from our previous results (32): PTC patients with BRAFV600E 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 BRAFV600E mutation than those without BRAFV600E 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, BRAFV600E 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 BRAFV600E mutation frequency with increased radiation dose. To examine the relationship between RET/PTC and BRAFV600E 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 (Ptrend = 0.002; Fig. 1A). 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. 1A: 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/PTC3 (1.5 Gy).

On the other hand, prevalence of BRAFV600E mutation significantly decreased with radiation dose (Ptrend = 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. 1A).

Frequency of RET/PTC and BRAFV600E alterations in PTC patients grouped by time elapsed since atomic radiation exposure. RET/PTC rearrangements and BRAFV600E mutation were further studied in relation to time since radiation exposure (Fig. 1B). BRAFV600E mutation significantly increased with increased time since exposure (Ptrend = 0.001), whereas unidentified alterations in PTC having wild-type RET and BRAF significantly decreased with increased time since exposure (Ptrend = 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 BRAFV600E mutation. As was the case for PTC among the general population (7–9), RET/PTC rearrangements and BRAFV600E 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 BRAFV600E mutation status |
|-------------------------------------------------|-------------------------------|-----------------|
| All patients | Exposed patients (>0 mGy) | |
| BRAFV600E (n = 45) | Wild-type BRAF (n = 26) | P |
| (n = 28) | Wild-type BRAF (n = 22) | P |

| Gender | Male (n) | 5 | 3 | 0.7* | 3 | 3 | 0.5* |
| Female (n) | 40 | 23 | | |
| 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) | — | (18–9) | (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) | — | (0–2,304) | (0–2,304) | — |

*Fisher’s exact test.
†ATB: at the time of atomic bombing.
‡Mann-Whitney’s U test.
RET/PTC rearrangements and 28 patients having BRAF<sub>V600E</sub> mutation. PTC patients with RET/PTC rearrangements revealed past exposure to significantly higher radiation dose (P = 0.001; Fig. 2A), shorter time elapsed since radiation exposure (P = 0.03; Fig. 2B), and younger age ATB (P = 0.06; Fig. 2C), compared with the patients with BRAF<sub>V600E</sub> mutation.

Subsequent logistic regression analysis for mutually exclusive occurrence of RET/PTC rearrangements or BRAF<sub>V600E</sub> 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<sub>V600E</sub> 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 rearrangements and 28 patients having BRAF<sub>V600E</sub> mutation. PTC patients with RET/PTC rearrangements revealed past exposure to significantly higher radiation dose (P = 0.001; Fig. 2A), shorter time elapsed since radiation exposure (P = 0.03; Fig. 2B), and younger age ATB (P = 0.06; Fig. 2C), compared with the patients with BRAF<sub>V600E</sub> mutation.

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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 BRAFV600E mutation. Noting that 17 (81%) and 1 (5%) of 21 nonexposed PTC patients having BRAFV600E 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. 1A 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, BRAFV600E mutation significantly decreased frequency with increased radiation dose (Fig. 1A). 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 BRAFV600E (Fig. 2; Table 4). Taken together, our findings imply that RET/PTC rearrangements, not BRAFV600E 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. 1B: 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 BRAFV600E. Because RET/PTC and BRAFV600E 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

![Figure 2. Comparison of RET/PTC and BRAFV600E alterations in PTC patients. A, radiation dose; B, time since exposure; C, age at the time of atomic bombing. RET/PTC PTC rearrangement (□) and BRAFV600E mutation (○), respectively, are shown.](#)

### Table 4. Logistic regression analysis of 39 exposed PTC patients with RET/PTC rearrangements or BRAFV600E mutation

<table>
<thead>
<tr>
<th>Variables</th>
<th>β*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiation dose (mGy)</td>
<td>0.002</td>
<td>0.012</td>
</tr>
<tr>
<td>Age at the time of atomic bombing (y)</td>
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<td>0.031</td>
</tr>
<tr>
<td>Year since exposure (y)</td>
<td>−0.192</td>
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</tr>
<tr>
<td>Gender, male vs. female</td>
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<td>Histology, conventional vs. follicular variant</td>
<td>0.157</td>
<td>0.927</td>
</tr>
</tbody>
</table>

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 might 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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RET/PTC Rearrangements Preferentially Occurred in Papillary Thyroid Cancer among Atomic Bomb Survivors Exposed to High Radiation Dose

Kiyohiro Hamatani, Hidetaka Eguchi, Reiko Ito, et al.


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