Rearranged Anaplastic Lymphoma Kinase (*ALK*) Gene in Adult-Onset Papillary Thyroid Cancer Amongst Atomic Bomb Survivors

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Background: We previously noted that among atomic bomb survivors (ABS), the relative frequency of cases of adult papillary thyroid cancer (PTC) with chromosomal rearrangements (mainly *RET/PTC*) was significantly greater in those with relatively higher radiation exposure than those with lower radiation exposure. In contrast, the frequency of PTC cases with point mutations (mainly *BRAF*^{V600E}) was significantly lower in patients with relatively higher radiation exposure than those with lower radiation exposure. We also found that among ABS, the frequency of PTC cases with no detectable gene alterations in *RET*, neurotrophic tyrosine kinase receptor 1 (*NTRK1*), *BRAF*, or *RAS* was significantly higher in patients with relatively higher radiation exposure than those with lower radiation exposure. However, in ABS with PTC, the relationship between the presence of the anaplastic lymphoma kinase (*ALK*) gene fused with other gene partners and radiation exposure has received little study. In this study, we tested the hypothesis that the relative frequency of rearranged *ALK* in ABS with PTC, and with no detectable gene alterations in *RET*, *NTRK1*, *BRAF*, or *RAS*, would be greater in those having relatively higher radiation exposures.

Methods: The 105 subjects in the study were drawn from the Life Span Study cohort of ABS of Hiroshima and Nagasaki who were diagnosed with PTC between 1956 and 1993. Seventy-nine were exposed (>0 mGy), and 26 were not exposed to A-bomb radiation. In the 25 ABS with PTC, and with no detectable gene alterations in *RET*, *NTRK1*, *BRAF*, or *RAS*, we examined archival, formalin-fixed, paraffin-embedded PTC specimens for rearrangement of *ALK* using reverse transcription–polymerase chain reaction and 5′ rapid amplification of cDNA ends (5′ RACE).

Results: We found rearranged *ALK* in 10 of 19 radiation-exposed PTC cases, but none among 6 patients with PTC with no radiation exposure. In addition, solid/trabecular-like architecture in PTC was closely associated with *ALK* rearrangements, being observed in 6 of 10 PTC cases with *ALK* rearrangements versus 2 of 15 cases with no *ALK* rearrangements. The six radiation-exposed cases of PTC harboring both *ALK* rearrangements and solid/trabecular-like architecture were associated with higher radiation doses and younger ages at the time of the A-bombing and at diagnosis compared to the other 19 PTC with no detectable gene alterations.

Conclusion: Our findings suggest that *ALK* rearrangements are involved in the development of radiation-induced adult-onset PTC.

Introduction

THYROID CANCER IS ONE OF THE MALIGNANCIES most closely associated with exposure to ionizing radiation in humans (1), such as the atomic bombs in Hiroshima and Nagasaki and the Chernobyl nuclear power plant accident (2,3). Radiation Effects Research Foundation (RERF) epidemiology studies of atomic bomb survivors (ABS) have found that an

excess relative risk for papillary thyroid cancer (PTC) per Gy is remarkably high among survivors (4,5). The data from the studies after the Chernobyl accident also indicate a strong relationship between thyroid cancer and radiation exposure (3).

Gene alterations that lead to constitutive activation of the mitogen-activated protein kinase (MAPK)-signaling pathway—such as alterations of *RET*, neurotrophic tyrosine kinase receptor 1 (*NTRK1*), *BRAF*, and *RAS* genes—are

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frequently found in PTC (6–8). These gene alterations can be detected in >70% of PTC cases, so the constitutive activation of the MAPK-signaling pathway appears to be a major early event in papillary thyroid carcinogenesis.

Our molecular analysis on rearrangements of *RET*, *NTRK1*, and *BRAF* genes, and also point mutations of *BRAF* and *RAS* genes in adult-onset PTC cases from the Life Span Study (LSS) cohort of ABS, found that the relative frequency of PTC cases with *RET*/PTC or *NTRK1* rearrangements (mainly *RET*/PTC) in all available PTC cases was significantly increased with an increased radiation dose, whereas PTC cases with *BRAF* or *RAS* point mutations (mainly *BRAF*^{V600E}) were significantly decreased (9,10). Apart from those PTC cases with known gene alterations, we found that the relative frequency of PTC cases with so-called nondetected gene alterations (i.e., no alterations in the *RET*, *NTRK1*, *BRAF*, or *RAS* gene) tended to increase with an increased radiation dose. The prevalence of the selected PTC cases peaked between 1956 and 1962, and rapidly decreased thereafter (10).

We postulated that some of the cases of PTC among ABS for which we had been unable to find gene alterations might have gene alterations that had previously not been looked for. Therefore, we initiated further molecular analyses of these cases by determining if some of them had rearrangements of anaplastic lymphoma kinase (*ALK*).

The *ALK* gene was first identified as a fusion partner of nucleophosmin in anaplastic large-cell lymphoma (ALCL) with the t(2;5) chromosomal rearrangement (11,12). Translocation of *ALK* with multiple fusion partner genes was subsequently identified in ALCL as well as in other inflammatory myofibroblastic tumors (13). One novel type of *ALK* rearrangement was an echinoderm microtubule-associated protein-like 4 (*EML4*)-*ALK* fusion gene, which was recently detected in nonsmall-cell lung cancer (14). Numerous *EML4*-*ALK* fusion variants have been identified to date (15–18). The *EML4*-*ALK* fusion variants were also detected in breast and colon cancers (19), but to date, there has been no report on *ALK* rearrangements in thyroid cancer.

In this study, we report for the first time the finding that *ALK* rearrangement selectively occurred in radiation-exposed PTC cases that carried no known gene alterations, and that about half of the PTC cases with rearranged *ALK* developed solid/trabecular-like architecture in the cancer tissue. The PTC cases harboring both *ALK* rearrangements and solid/trabecular-like architecture were related to higher radiation doses and younger ages at the time of the bombings and at diagnosis compared to the other cases, implying a key role of *ALK* rearrangements in the development of radiation-induced thyroid cancer.

Methods

Study subjects and tissue specimens

The study subjects were 105 adults with PTC who were members of the LSS cohort of ABS of Hiroshima and Nagasaki diagnosed in selected hospitals in the two cities between 1956 and 1993. Of these, 79 were exposed (>0 mGy) and 26 were not exposed to A-bomb radiation. Of these 105 patients, 71 had been a part of our previous study on *RET*/PTC rearrangements (10). The 26 nonradiation-exposed subjects were either those with a radiation dose estimated to be 0 mGy or those who were not in the city of Hiroshima or Nagasaki at

the time of the bombing. Study subjects who were not in these cities at the time of the bombing were assigned to the non-exposed group in this study, consistent with our previous article (10). This study was conducted with approval of the Human Investigation Committee and the Ethics Committee for Genome Research at the RERF.

Histological examination

Examination of histology was done by one of the authors (Y.H.) according to histopathological typing established by the WHO (20). All study specimens were nonbuffered, formalin-fixed, and paraffin-embedded PTC tissue specimens surgically resected from 1956 to 1993. Since amounts of tissue materials were limited, histological examination was conducted on one hematoxylin and eosin-stained tissue section per case.

RNA preparation and cDNA synthesis

RNA was extracted from microdissected noncancer or cancer regions using the High Pure RNA Paraffin Kit (Roche Diagnostics GmbH), as described previously (21). Reverse transcription was performed with random primers (9-mer) using 100 ng total RNA as template, as described previously (21).

Reverse transcription–polymerase chain reaction

Reverse transcription–polymerase chain reaction (RT-PCR) was carried out with the Fast Start High Fidelity PCR system (Roche Diagnostics GmbH) for the *ALK* gene, with AccuSure DNA polymerase (BIOLINE) for the *EML4* (exon 13)/*ALK* (exon 20) fusion and with Phusion Hot Start II (New England BioLabs) for the *EML4* (exon 20)/*ALK* (exon 20) fusion, using primer sets shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/thy) and as previously described (22).

Screening of rearranged *ALK*

The primer sets were made against the *ALK* kinase domain (K region) and the region spanning the boundary of exons 19 and 20 of *ALK* (W region) as shown in Figure 1A. PCR amplifications, for the K region with cDNA derived from 10 ng of total RNA of in-house control PTC and for the W region with cDNA from 5 ng of the same total RNA, were conducted at 36 cycles and 40 cycles, respectively. These PCR amplifications were repeated four times. The K/W ratios of 1.9 at 40 cycles or 2.9 at 36 cycles were drawn from an average of four experiments plus 2× the standard deviation as shown in Figure 1C. When the intensity ratio of the K region to W region after a 40-cycle RT-PCR amplification was >1.9 (Fig. 1B; samples II, III, and IV) based on the calculation shown in Figure 1C, samples were further analyzed by 36-cycle PCR amplification to determine whether the samples were positive for *ALK* rearrangements. We assumed that the samples with an intensity ratio >2.9 at 36-cycle PCR amplification were positive for *ALK* rearrangements (Fig. 1B; samples II, III, and IV). We used only the K/W ratio for screening, and intensities of individual amplified DNA fragments were not taken into account for screening the candidates. This screening was conducted only when clear and detectable K-region bands were observed in the first PCR products. Subsequently, cDNA fragments of

FIG. 1. (A) Diagram of the rearranged *ALK* gene. W and K regions indicate the regions spanning boundary of exons 19 and 20, and of exons 26 and 27 (kinase domain) in *ALK*, respectively. (B) Expression levels of W and K regions in *ALK*. The 5 μ L of 40-cycle PCR products were electrophoresed on an 8% acrylamide gel. Sample I (lanes 2 and 3) indicates RT-PCR products of in-house control PTC. Samples II–V (lanes 4–11) reveal RT-PCR products of four exposed PTC with nondetected gene alterations; lanes with even numbers are cDNA fragments derived from the W region, and those with odd numbers are fragments derived from the K region. Lane 1 indicates pUC19-*Msp*I digest for DNA size marker. (C) Screening of the rearranged *ALK* gene. The intensity ratio for selection of rearranged *ALK* was calculated using RT-PCR amplification with cDNA derived from 10 ng of total RNA of in-house control PTC for the K region, and with cDNA derived from 5 ng of total RNA of the same PTC for the W region as template. (D) Detection of expression of *EML4-ALK* fusion genes by RT-PCR. Lanes 3–7 indicate cDNA fragments of the *EML4-ALK* fusion gene in PTC cases from which the 3'-end fragments of exon 13 of *EML4* were isolated by the SMART RACE method. Lanes 6 and 7 correspond to PTC in samples IV and III of (B), respectively. Lane 9 indicates cDNA fragments (49 bp) derived from the chimera gene that was formed by fusion of exon 20 of *EML4* and exon 20 of *ALK*. Lanes 2 and 8 show H₂O for negative control, and Lane 1 shows pUC19-*Msp*I digest for DNA size marker. *ALK*, anaplastic lymphoma kinase; PTC, papillary thyroid cancer; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends.

rearranged *ALK* genes were isolated and analyzed by the SMART rapid amplification of cDNA end (RACE) method, using primer sets shown in Supplementary Table S1 as described previously (22).

Statistical analysis

The Fisher's exact test was used for categorical variables. The Mann-Whitney *U* test was used for nonparametric two-sample comparison of continuous variables. SPSS software

(version 15.0) was used for the statistical analyses. *p*-value was calculated by using a two-sided test. The Cochran-Armitage test for nonparametric trend analysis was carried out with Excel Statistics 2006 software.

Radiation dose

A-bomb radiation dose used in the analyses were shielded-organ doses to the thyroid as estimated by the recently implemented DS02 system (23), except for those cohorts who were not in the city of Hiroshima or Nagasaki at the time of the bombing.

Results

Detection of *ALK* rearrangements in PTC cases with previously nondetected gene alterations

Molecular epidemiological characteristics of 105 PTC cases examined thus far are shown in Supplementary Table S2, with 34 PTC cases that were newly added after our previous article (10). When comparing 79 radiation-exposed subjects and 26 nonexposed ones, no significant differences were found in distribution of gender, city, age at the time of the bombing (ATB), age at diagnosis, or presence or absence of *RET/PTC* rearrangements, *NTRK1* rearrangements, and *BRAF* or *RAS* point mutations. However, relative frequencies of *RET/PTC* rearrangements and *BRAF* mutation in radiation-exposed PTC cases showed significantly increasing or decreasing trends depending on the radiation dose ($P_{\text{trend}}=0.01$ and 0.0006), as shown in Supplementary Table S2. Furthermore, when comparing nonexposed PTC cases with those exposed to relatively a high radiation dose (the 3rd tertile, >355 mGy) for *RET/PTC* rearrangements, the relative frequency was significantly higher in exposed PTC cases (>355 mGy) than in nonexposed PTC cases (1/26 cases vs. 9/26 cases, respectively; $p=0.01$). On the other hand, the relative frequency of the *BRAF* mutation was significantly lower in PTC cases exposed to the radiation dose (>355 mGy) than in nonexposed PTC cases (19/26 cases vs. 7/26 cases, respectively; $p=0.002$). Among 105 PTC cases examined thus far for *RET*, *NTRK1*, and *BRAF* rearrangements and *BRAF* and *RAS* point mutations, 25 PTC cases were found to have no alterations in these genes, which tended to increase with an increased radiation dose, though this is not statistically significant (Supplementary Table S2). It should be noted that these cases showed a temporal change with a peak a short time after exposure, which then rapidly decreased (i.e., median time after exposure was 23 years for this group vs. 32 years for cases with *BRAF* and *RAS* point mutations, $p=0.014$). These findings are consistent with those in our previous article (10).

Of 25 PTC cases with previously nondetected gene alterations (also referred to as nondetected gene alterations) that carried no alterations in *RET*, *NTRK1*, *BRAF*, or *RAS* genes, we found *ALK* rearrangements in 10 of 19 radiation-exposed cases, but no *ALK* rearrangements in any of the six nonexposed cases (Table 1). The median dose in PTC cases with rearranged *ALK* was significantly higher than in grouped radiation-exposed and nonradiation-exposed PTC cases with no rearranged *ALK* (Fig. 2). However, there was no significant difference in the radiation dose between radiation-exposed PTC cases with rearranged *ALK* and with non-rearranged *ALK*. SMART 5' RACE followed by sequencing analysis

TABLE 1. REARRANGED *ALK* IN PAPILLARY THYROID CANCER CASES WITH NONDETECTED GENE ALTERATIONS

PTC case	ALK rearrangements		p ^a
	Present	Absent	
Nonexposed (n=6)	0	6	0.051
Exposed (>0 mGy, n=19)	10 ^b	9	

^aFisher's exact test.

^bFive cases were *EML4*(exon 13)/*ALK*(exon 20); one case was *EML4*(exon 20)/*ALK*(exon 20); *ALK* partner genes in four cases have not been determined yet.

PTC, papillary thyroid cancer.

revealed that five PTC cases with rearranged *ALK* were a fusion of *EML4* (exon 13) and *ALK* (exon 20), and that one case was a fusion of *EML4* (exon 20) and *ALK* (exon 20) (Table 1). Expression of cDNA fragments of chimeric *EML4-ALK* in the PTC cases was confirmed by RT-PCR (Fig. 1D, lanes 3–7, and 9). The counterpart genes in the remaining four cases are currently being studied.

Morphological characteristics in PTC with rearranged *ALK*

Histological review of the 10 PTC cases with rearranged *ALK* found that 6 of the 10 had solid/trabecular-like architecture comprising either several small sites or several sites of greater total area within the cancer regions, but did not have insular architecture. Necrosis or mitotic figures were not observed in the single tissue section examined for each case. Supplementary Figure S1A and B show pathological photographs of two PTC cases with rearranged *ALK*, with solid/trabecular-like architecture in more than 50% of the cancers. Supplementary Figure S1C and D reveal a typical papillary

TABLE 2. ASSOCIATION BETWEEN REARRANGED *ALK* AND SOLID/TRABECULAR-LIKE ARCHITECTURE

	Rearranged <i>ALK</i>		p ^a
	Present (n=10)	Absent (n=15)	
Solid/trabecular-like architecture ^b			
Present	6 ^c	2	0.028
Absent	4	13	

Data represent 25 PTC cases with nondetected gene alterations.

^aFisher's exact test.

^bThe samples were counted as positive for solid/trabecular-like architecture when that architecture was found in several areas within cancer regions.

^cMore than 50% of cancer regions revealed such architecture in two cases.

structure for two exposed PTC cases with no rearranged *ALK*. In contrast, only 2 of 15 PTC cases with no rearranged *ALK* had such architecture. Analysis of 25 PTC cases with nondetected gene alterations revealed a close association between *ALK* rearrangement and solid/trabecular-like architecture ($p=0.028$, Table 2).

Association of PTC harboring both rearranged *ALK* and solid/trabecular-like architecture with radiation dose and age at A-bombing

Since there were only two PTC cases with no rearranged *ALK*, but having solid/trabecular-like architecture, we compared six PTC cases harboring both rearranged *ALK* and solid/trabecular-like architecture with the remaining 19 PTC cases in terms of radiation dose and ages at A-bombing and diagnosis (Fig. 3). These six PTC cases received significantly greater radiation doses and were or younger age at the time of A-bombing and at the time of diagnosis compared to the other 19 PTC cases (Fig. 3A–C, $p=0.005$, 0.008, and 0.016, respectively). Furthermore, comparison of 6 PTC cases harboring both rearranged *ALK* and solid/trabecular-like architecture with the remaining exposed 13 PTC cases by Mann-Whitney test also showed significant differences for radiation ($p=0.036$), age at A-bombing ($p=0.021$), and age at diagnosis ($p=0.020$).

Discussion

We report here the first evidence that rearrangements of *ALK* in adult-onset PTC cases develop in subjects exposed to radiation. Furthermore, the median radiation dose in PTC cases with rearranged *ALK* was significantly higher than in PTC cases with no rearranged *ALK* (Fig. 2), indicating involvement of rearranged *ALK* in radiation-induced papillary thyroid carcinogenesis. However, since only six nonexposed PTC cases were examined for rearranged *ALK*, it is necessary to examine a larger number of sporadic PTC cases to clarify possible presence of *ALK* rearrangements. We are thus unable at present to exclude the possibility that *ALK* rearrangements also occur in sporadic PTC. Although partner genes in four PTC cases have not yet been identified, PCR amplification in these cases was found in the K region, but not in the W region (Fig. 1), and therefore these PTC cases were judged to have rearranged *ALK*. We are attempting to identify the partner genes.

FIG. 2. Comparison of radiation dose distribution in PTC cases with (+, O) and without (-, □) rearranged *ALK*.

FIG. 3. Epidemiological characteristics of PTC cases harboring both the rearranged *ALK* gene and solid/trabecular-like architecture, in relation to radiation dose (A), age at the time of atomic bombing (B), and age at the time of diagnosis (C).

Some of the 10 PTC cases with rearranged *ALK* revealed weak, but observable, PCR-amplified fragments as shown in Figure 1C. Those bands may be in part due to amplified fragments derived from another allele with wild-type *ALK*. Another plausible interpretation is that the rearrangement did not occur in all tumor cells. It is important to clarify whether *ALK* rearrangement is a driver mutation or a passenger mutation to better understand radiation-related carcinogenesis of PTC. Furthermore, although rearrangements of *ALK* are involved in the pathogenesis of many malignancies, including lymphoma and lung cancer, few *ALK* rearrangements have been well defined relative to their pathogenic role in cancer. Recently, constitutive *ALK* activation in several cancers was reported to cause the activation of selected downstream pathways, including MAPK and STAT (24,25), but no reports are available regarding a putative pathogenic role of rearranged *ALK* in PTC. To assess biological significance of rearranged *ALK* in radiation-induced PTC, *in vitro* and *in vivo* comparative experiments with *RET/PTC* rearrangements and *BRAF* point mutation need to be performed to shed light on these issues.

The nonradiation-exposed subjects in this study were comprised of two distinct groups: subjects whose radiation dose levels were estimated to be zero by the DS02 system, and subjects who were not in Hiroshima or Nagasaki, and thus not a part of the DS02 dose estimation. Our analyses included two not-in-city subjects in the six nonexposed subjects with non-detected gene alterations, but even when we excluded these not-in-city subjects from the analysis, the results were substantially unchanged. Specifically, the six nonexposed subjects without rearranged *ALK* in Table 1 would become 4 ($p=0.1$ in this case); the median value of 2 mGy for 15 subjects with no rearranged *ALK* in Figure 2 would become 3 mGy for 13 subjects ($p=0.034$); and the 13 subjects (denoted by “◇”) in Figure 3 harboring neither rearranged *ALK* nor solid/trabecular-like architecture would become 11, and their median values of 1 mGy, 24 years, and 54 years in Figure 3A–C would be 2 mGy, 24 years, and 54 years ($p=0.009$, 0.012, and 0.023), respectively.

The solid/trabecular-like architecture appeared in one of three different types of groupings as (I) relatively small, clonal, multicellular clusters distributed throughout the cancer; (II) larger groupings (each composed of a few multicellular clusters) distributed throughout the cancer, or as (III) a few large groupings that occupied a major portion (>50%) of the cancer volume. Interestingly, 6 of 10 PTC cases with rearranged *ALK* had solid/trabecular-like architecture corresponding to types (I) and (II) above (numbering 3 and 1, respectively) in several areas within the cancers, and two cases had such architecture in more than 50% of the cancer (type III and shown in Supplementary Fig. 1A, B). However, only 2 of 15 PTC cases with no rearranged *ALK* had such architecture; they were small and found in several areas scattered throughout the cancer (type I). The 25 PTC cases with nondetected gene alterations showed no necrosis or mitotic figures, but did show PTC-specific nuclear features such as nuclear grooves, intranuclear cytoplasmic inclusions, and psammoma bodies. Taking into account the diagnostic criteria proposed by Volante *et al.* (26), it is reasonable to conclude that two PTC cases harboring solid/trabecular-like architecture in more than 50% of cancer regions are a mixed type of PTC and solid variant, or a solid variant of PTC rather than poorly differentiated thyroid cancer. This is true even though the size of one solid/trabecular nest was small and uniform compared with that of post-Chernobyl childhood PTC, and that the other six PTC cases (four and two with and without rearranged *ALK*, respectively) harboring such architecture in several cancer regions are typical PTC.

PTC cases harboring both rearranged *ALK* and solid/trabecular-like architecture were significantly associated not only with a greater radiation dose but also with younger age at the time of bombing and at diagnosis, compared with the remaining PTC cases. One plausible interpretation of this finding is that in the process of radiation-induced papillary thyroid carcinogenesis involving *ALK* rearrangements, unknown gene alterations may additionally occur and generate solid/trabecular-like architecture. Considering this, further analysis will be required to identify such gene alterations and

to assess the pathogenic role of *ALK* rearrangements and its potential link to solid/trabecular-like architecture.

Acknowledgments

The RERF, Hiroshima and Nagasaki, Japan, is a private, nonprofit foundation funded by Japan's Ministry of Health, Labour, and Welfare, and the U.S. Department of Energy (DOE), the latter in part through DOE Award DE-HS0000031 to the National Academy of Sciences. This publication was supported by RERF Research Protocols RP 5-02, and in part by a Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science, and Technology, and a Grant-in Aid for Cancer Research from Japan's Ministry of Health, Labour, and Welfare, as well as research grant for the Princess Takamatsu Cancer Research Fund (08-24015). We thank Dr. J. Cologne for his helpful statistical advice, Mr. K. Koyama for his excellent technical assistance, and Ms. M. Yonezawa for her good help with preparation of article. The views of the authors do not necessarily reflect those of the two governments.

Disclosure Statement

The authors declare that they have no commercial associations that might create a conflict of interest in connection with this article.

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