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Ret/PTC Activation in Benign and Malignant Thyroid Tumors Arising in a Population Exposed to Low-Dose External-Beam Irradiation in Childhood

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Ionizing radiation is the strongest risk factor known for the development of thyroid neoplasia. Although ret/PTC rearrangements have been identified in both spontaneous and radiation-induced papillary thyroid cancer, they seem more frequent among radiation-associated tumors.

We studied the frequency of ret/PTC activation in a group of sporadic and radiation-induced thyroid carcinomas (n = 49) and adenomas (n = 13) among 44 individuals treated for Tinea Capitis with low-dose external irradiation as well as in 18 nonirradiated subjects.

Total RNA recovered from paraffin-embedded thyroid cancer surgical specimens was analyzed for ret/PTC 1, 2, and 3 mutations using RT-PCR with Southern blotting to maximize detection sensitivity.

Ret/PTC rearrangements were identified in 42.9% of thyroid carcinoma and 46.2% of adenoma subjects. Among the positive carcinoma specimens, three were follicular carcinomas. Ret/PTC 1, the predominant rearrangement, was more prevalent in nonirradiated compared with irradiated carcinomas (66.7 *vs.* 27.0%; $P = 0.04$). Ret/PTC activation was associated with male gender.

The strengths of this study included analysis of age-, gender-, and ethnicity-matched groups; molecular analysis using two techniques; and a complete blinding of laboratory analysis from clinical features. The differences seen between these and other published results may be related to differences in radiation doses to the thyroid, latency period between time of radiation exposure and development of clinically apparent thyroid cancer, and ethnic background of the study populations. (*J Clin Endocrinol Metab* 89: 2281–2289, 2004)

THE ROLE OF ionizing radiation in the causation of thyroid cancer is well established. Furthermore, exposure to ionizing radiation early in life is the strongest risk factor known for the subsequent development of thyroid neoplasia (1).

Nevertheless, the molecular mechanisms underlying this association remain to be well understood. The most common molecular abnormality found in radiation-induced thyroid neoplasia is oncogenic activation of the ret protooncogene, a transmembrane tyrosine kinase receptor. Intrachromosomal inversions or translocations result in loss of the extracellular ligand binding domain and constitutive activation of the mutant receptor. When initially described, ret/PTC activation was thought to be specific for papillary thyroid cancer. However, recent studies have documented the presence of ret/PTC mutations in Hashimoto's thyroiditis (2), benign adenomas (3–7), hyperplastic nodules (5), Hurthle cell carcinoma (8, 9), and in hyalinizing trabecular tumors of the thyroid (10, 11). Because most of these studies were based on the detection of ret/PTC by RT-PCR, using total mRNA recovered from thyroid surgical specimens, the precise cell of origin of ret/PTC transcripts could not be determined with certainty. Therefore, it can be hypothesized that the cells of origin of ret/PTC transcripts detected in specimens of benign thyroid disease or follicular thyroid neoplasia were in fact the cells of clinically and histologically undetected microfoci of papillary thyroid cancer. Unlike RT-PCR studies, fluorescence *in situ* hybridization can be used to determine the presence of ret/PTC mutations in individual cells. Using this approach, ret/PTC mutations have been directly identified in both papillary thyroid cancers and follicular adenoma samples (7). These reports of ret/PTC mutations in histological samples other than papillary thyroid cancer have challenged the original assumption that ret/PTC mutations were specific for papillary thyroid cancer and have caused us to reexamine the role of this important oncogene in the pathogenesis of many benign and malignant thyroid conditions.

So far, ret/PTC rearrangements have been identified in both spontaneous and radiation-induced papillary thyroid cancers and have been observed in both childhood and adult cases of the disease, although they appear to occur more frequently in childhood cases and in radiation-associated rather than spontaneous cases (12). Although at least eight different ret/PTC-activating mutations have been realized, the most commonly described mutations are ret/PTC 1 or ret/PTC 3, and, to a smaller extent, ret/PTC 2. The other ret/PTC rearrangements are rare events, often described in single patients (13, 14). Ret/PTC 1 appears to be the most common rearrangement in spontaneous adult and childhood thyroid cancers, whereas both ret/PTC 1 and ret/PTC 3

Abbreviations: dUTP, Deoxyuridine triphosphate; TC, Tinea Capitis.
* B.M. is deceased.

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rearrangements are observed to have a high prevalence in radiation-induced thyroid cancers (12). Ret/PTC 2 mutations occur much less frequently in both spontaneous and radiation-induced thyroid cancer at similar frequencies (12). Published clinical studies examining the rate of ret/PTC activation after radiation exposure are derived from either subjects treated during childhood with relatively high doses of external-beam irradiation or children exposed to radioactive iodine after the Chernobyl (Ukraine) accident. Higher rates of ret/PTC activation are seen in the most heavily contaminated regions of Gomel and Brest in Belarus, suggesting that a dose-response relationship may exist (15). Furthermore, papillary thyroid cancers with a short latency period between radiation exposure and development of clinically evident thyroid cancer are more likely to harbor ret/PTC 3 mutations (15). The cohort described in the current study is unique in that the development of thyroid cancer followed low-dose external-beam irradiation with latency periods that are 10–20 yr longer than those described in the post-Chernobyl experience.

Between 1948 and 1960, about 20,000 Israeli individuals, particularly children, were treated with ionizing radiation to the head area for Tinea Capitis (TC) , a benign fungal disease of the scalp. This population was composed mostly of newly arrived immigrants from North Africa, with a smaller number coming from the Middle East.

In 1968, our group initiated a comprehensive follow-up of a cohort belonging to this population and two control groups to determine possible delayed radiation effects (TC cohort). During the same period, an unknown number of children were also irradiated abroad, mainly in Morocco, en route to their arrival in Israel. These children were not included in the TC cohort (16–19).

Based on all thyroid neoplasms diagnosed in Israel between 1950 and 1980, an estimated thyroid dose of 9 cGy was linked to a 2-fold (95% confidence interval, 1.3–3.0), and 4-fold (95% confidence interval, 2.3–7.9) increase of benign and malignant thyroid tumors, respectively (19).

According to a law established in Israel in 1994, compensation is given to irradiated people who developed specific delayed effects proven to result from exposure to this radiation (*i.e.* mainly head and neck neoplasms) (20).

The aim of this study was to investigate the association between ret/PTC activation and radiation-induced thyroid neoplasms in individuals exposed to low doses of external-beam radiation compared with nonirradiated individuals of same origin in the unique population of the Israeli TC cohort. Based on previous reports, our hypotheses were that papillary thyroid carcinoma that develops after an exposure to ionizing radiation treatment would have higher rates of ret/PTC activation than nonirradiated subjects and that benign thyroid nodules that developed after ionizing radiation would have very low rates of ret/PTC activation.

Subjects and Methods

The TC cohort includes 10,834 irradiated subjects, an equal number of nonirradiated population controls, individually matched to the cases by age (±2 yr), gender, country of birth, and year of immigration, and 5392 nonirradiated sibling controls (16).

The therapeutic procedure followed the Adamson-Kienbock technique. The hair was shaved and the scalp irradiated over 5 consecutive days, and the remaining hair was removed through a waxing process. About 10% of children received more than one course of treatment due to relapse.

The irradiation was performed with a 75- to 100-kVp superficial therapy x-ray machine. The children were exposed to 3.5–4 Gy per field, at a focus-skin-distance of 25–30 cm. Dosimetric studies estimated that the upper layers of the brain received radiation doses between 1.2 and 1.4 Gy, whereas the doses at the lower layer (2.5 cm deep) were smaller by 8–20%. For children receiving one course of therapy, the average dose to the thyroid was 8.4 cGy (range, 4.5–16.5), whereas the mean dose for all irradiated patients was 9.3 cGy (range, 4.5–49.5). The dose was highly and negatively correlated with age at exposure (17). Detailed reports of the treatment techniques, dosimetry, and methodology of this study have been published earlier (16, 17).

Information on cancer development among the TC cohort was obtained from the Israel Cancer Registry updated to December 1996. This registry was established in 1960 and is notified by law on information of all malignant tumors. Pathological records verified each cancer case. Among the TC cohort, 101 irradiated patients and 44 nonirradiated individuals with the diagnosis of thyroid tumor were identified.

As mentioned above, since 1994, claims for compensation are being submitted by a subset of the irradiated population in the framework of the Israeli compensation law. This subset includes patients who were irradiated either in Israel or abroad, and it is composed of patients who are included in the TC cohort as well as others who are not included in this unique follow-up. Irradiation treatment for TC as a basis for inclusion in the framework of this law is being determined by a special expert committee who decides on the validity of the irradiation of each individual. Sixteen irradiated patients for this study were identified via these claim files.

For each thyroid cancer patient, demographic data, date of irradiation (for irradiated cases), pathological report, and date and hospital of surgery were available. Altogether, 161 patients were identified who were diagnosed with thyroid cancer and thyroid adenomas and were treated in one of the 21 different hospitals in Israel in the yr 1959–2000. The hospital's institutional review board committees approved this study protocol. Consent for collaboration was accepted from 15 hospitals including 122 patients.

All available paraffin blocks of each patient were collected from the pathological departments of these participating hospitals, and all pathological reports were reviewed. The presence of thyroiditis was determined according to these pathological reports. We validated the diagnosis of follicular carcinoma of three patients and the diagnosis of poorly differentiated carcinoma of one patient by another independent pathologist.

Molecular analysis was performed in a blinded fashion with no knowledge of the associated clinical data. Paraffin-embedded tissue sections (20 μm in thickness) immediately adjacent to the diagnostic slides were rehydrated using techniques previously described (21). RNA was extracted from the rehydrated tissue pellets using the Qiagen RNeasy Mini kit (Qiagen, Inc., Valencia, CA) with only a single modification. Cell lysis was done for 12 h at room temperature in Buffer RLT rather than the 30 min the manufacturer suggested.

RT-PCR for each of the ret/PTC oncogenes and β-actin was performed as previously described (21), including the addition of 1 μM digoxigenin-11-deoxyuridine triphosphate (dUTP) (Roche Applied Science, Indianapolis, IN) to the PCR to facilitate chemiluminescent detection of the amplified PCR products after Southern blotting (22). The amplified product was separated in a 2% agarose gel and detected with ethidium bromide (10 mg/ml, aqueous solution, Sigma Chemical Co., St. Louis, MO). The DNA was then transferred from the agarose gel to a solid support by the method of Southern (23). The digoxigenin-11-dUTP PCR products were detected by chemiluminescence using anti-digoxigenin antibody conjugated with alkaline phosphatase as previously described (22). To verify our findings, RT-PCR for ret/PTC 1, ret/PTC 2, and ret/PTC 3 were repeated for each sample (without direct incorporation of digoxigenin-11-dUTP). These products were also analyzed by gel electrophoresis and transferred to a positively charged membrane as described above. The membrane was then hybridized with previously published mutation-specific oligonucleotide probes (Table 1) (24) that had been 3′ end-labeled with digoxigenin-11-dUTP using a digoxigenin oligonucleotide 3′-end labeling Kit (Roche Applied Sci-

ence). The specific labeling was detected by chemiluminescence using antidigoxigenin antibody conjugated with alkaline phosphatase as previously described (22). Reverse transcriptase-negative and template-negative controls were included along with each amplification. Table 1 shows the primer sequences for ret/PTC 1, ret/PTC 2, ret/PTC 3, and β-actin with expected amplification product sizes. Positive control clones for ret/PTC 1, ret/PTC 2, and ret/PTC 3 had been previously provided by Dr. C Jhiang, (Ohio State University, Columbus, OH) and were used as previously described (21).

Statistical analysis

The distributions of the study covariates (gender, birth year, and year and age at diagnosis) were described and compared between irradiated and nonirradiated cases as well as between ret/PTC-positive and ret/PTC-negative patients for carcinoma patients and adenoma patients separately. The latent period (time since irradiation until date of diagnosis) was described for irradiated cases only. Ret/PTC activation rates of thyroid cancer diagnosed less than 30 yr after irradiation were compared with those diagnosed at 30 yr or more, the median latency period. Pearson's χ^2 test was used to compare categorical variables, and Fisher's exact test was used when appropriate. Exact χ^2 tests for expected variables less than five were calculated with StatExact version 4 software (Cytel Statistical Software, Cambridge, MA). The differences between two groups in continuous variables were analyzed with Student's *t* test. The level for statistical significance was set at 0.05. All statistical tests were two-sided. All data were coded and entered into an SPSS database (version 10.1.0; SPSS, Chicago, IL).

Results

Of the target population of 122 patients, a total of 72 specimens (59%) were successfully retrieved from the pathology departments. Significantly more specimens were allocated from the more recent period compared with the earlier one (71.7% in 1981–2000 *vs.* 46.8% in 1959–1980; $P = 0.005$).

RNA was successfully recovered in 62 of the 72 cases (86%) in which paraffin-embedded thyroid tissue samples were available (Fig. 1). Of these 62 cases, 54 were from the original TC cohort. The characteristics of these cases were further analyzed.

Both irradiated and nonirradiated cases demonstrated the same origin distribution predominated by North Africans, mainly Moroccan origin (data not shown).

Table 2 describes the characteristics of the study population by type of tumor and exposure to radiation. At least two thirds of both the irradiated and the nonirradiated patients were females. For both thyroid carcinoma and adenoma, irradiated cases were diagnosed at similar age and year of diagnosis as nonirradiated subjects. The mean age at diagnosis was younger for adenoma patients compared with carcinoma patients (31.15 *vs.* 36.47 yr; $P = 0.015$). Also, the mean latent period for the adenoma patients was 24.6 yr compared with 29.4 yr for the carcinoma patients; however, this difference was not statistically significant.

Ret/PTC analysis

Altogether, ret/PTC rearrangements were identified in 43.5% of the specimens (n = 27) (Fig. 2). Surprisingly, a similar rate of ret/PTC rearrangements was found among carcinoma specimens and adenoma specimens (42.9%, n = 21 and 46.2%, n = 6, respectively). The most frequent rearrangement was ret/PTC 1, which was found in 26 specimens. Adenoma patients had only ret/PTC 1 activation, two of the carcinoma patients had both ret/PTC 1 and ret/PTC 2 rearrangements, and one carcinoma patient had ret/PTC 2 mutation alone. None of the patients in our series had ret/PTC 3 rearrangement (Table 3).

Ret/PTC 1 as the only rearrangement was significantly more prevalent in the nonirradiated carcinoma patients compared with the exposed group (66.7 *vs.* 27.0%, respectively;

TABLE 1. Ret/PTC primer design: RT-PCR analysis and Southern blotting mutation-specific oligonucleotide probes

	Sense (5′–3′)	Antisense (5′–3′)	Expected size (bp)	Oligoprobes (5′–3′)
ret/PTC1	CAAAGCCAGCGTTACCATCG	CCTTCTCCTAGAGTTTTTCC	81	GGGCACTGCAGGAGGAGAACCGCGA
ret/PTC2	GAAATTGTGGGGCATCGACC	CCTTCTCCTAGAGTTTTTCC	108	GACCGAGACAGCTATAGAAGAATC
ret/PTC3	CAAGCTCCTTACATACC	CCTTCTCCTAGAGTTTTTCC	134	GGTCGGTGCTGGGTATGTAAGGA
β-Actin	TCATCACCATTGGCAATGAG	CACTGTGTTGGCGTACAGGT	154	

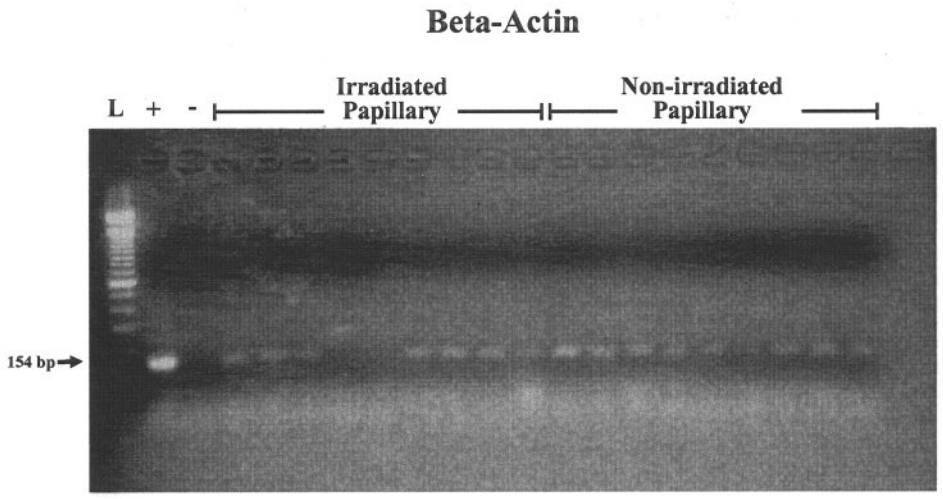

FIG. 1. β-Actin RT-PCR products detected by 3% agarose gel electrophoresis documenting recovery of viable mRNA from paraffin-embedded papillary thyroid cancer samples. The expected 154-bp RT-PCR product is visible in 15 of the 18 samples shown on this gel. L, Ladder; +, β-actin-positive control; −, reverse transcription-negative control; papillary, papillary thyroid cancer.

TABLE 2. Characteristics of the study population by type of tumor and study group

	Irradiated cases	Nonirradiated cases	Total
Thyroid carcinoma patients			
Total	37	12	49
Gender, n (%)			
Males	10 (27.0)	4 (33.3)	14 (28.6)
Females	27 (73.0)	8 (66.7)	35 (71.4)
Birth year (mean ± SD)	1948 ± 4	1948 ± 5	1948 ± 4
Year of diagnosis (mean ± SD)	1984 ± 10	1985 ± 10	1985 ± 10
Age at diagnosis (yr) (mean ± SD)	36.24 ± 10.56	37.17 ± 10.21	36.47 ± 10.3
Latency (yr) (mean ± SD)	29.37 ± 10.53		
Morphology, n (%)			
Papillary	34 (91.9)	10 (83.3)	44 (89.8)
Follicular	3 (8.1)	1 (8.3)	4 (8.2)
Poorly differentiated	0	1 (8.3)	1 (2.0)
Thyroid adenoma patients			
Total	7	6	13
Gender n (%)			
Males	0	2 (33.3)	2 (15.4)
Females	7 (100.0)	4 (66.7)	11 (84.6)
Birth year (mean ± SD)[a]	1952 ± 1	1946 ± 4	1949 ± 4
Year of diagnosis (mean ± SD)	1982 ± 6	1979 ± 1	1981 ± 5
Age at diagnosis (yr) (mean ± SD)	29.86 ± 6.23	32.67 ± 4.13	31.15 ± 5.35
Latency (yr) (mean ± SD)	24.57 ± 6.32		

[a] $P = 0.013$.

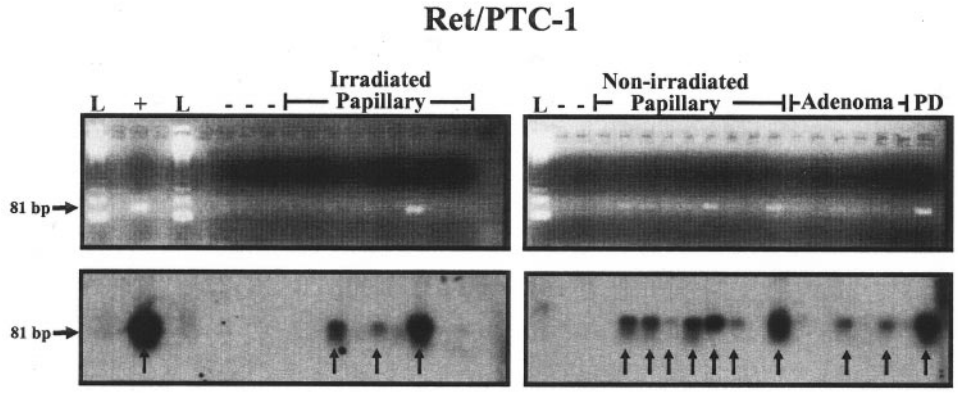

FIG. 2. Ret/PTC 1 mutations detected by 3% agarose gel electrophoresis (*top panels*) and Southern blot using digoxigenin-dUTP-labeled mutation-specific oligonucleotide probes (*bottom panels*). The Southern blot confirmed all visible RT-PCR products seen on the agarose gel and, as would be expected, identified several additional positive samples that could not be clearly visualized on the gel. L, Ladder; +, ret/PTC 1-positive control; −, three separate negative controls including reverse transcription-negative control, template-negative control, and ribonuclease-treated negative control; PD, poorly differentiated thyroid cancer sample; Papillary, papillary thyroid cancer. The *arrows* indicate samples positive for ret/PTC 1.

$P = 0.04$). The three cases found with ret/PTC 2 (as the only rearrangement as well as in combination with ret/PTC 1) were of irradiated carcinoma patients. Three of the six ret/PTC-positive adenoma patients were irradiated for TC.

Thyroiditis was found in 27.8% of the ret/PTC 1-positive thyroid cancer specimens compared with only 10.3% among the ret/PTC 1-negative patients (nonsignificant; data not shown).

Among the carcinoma patients, age at diagnosis was not associated with the presence of ret/PTC activation (mean age 37.90 ± 10.66 yr *vs.* 35.39 ± 10.21 yr, ret/PTC positive *vs.* negative, respectively); however, ret/PTC rearrangements were significantly more frequent in males compared with females (ret/PTC activation in 71.4% of males compared with 31.4% of females; $P = 0.01$) (Fig. 3). No statistically significant association was found between ret/PTC activation and latency period, with mean latent period of ret/PTC-positive and ret/PTC-negative carcinoma patients of 31.3 and 28.4 yr, respectively.

The analysis of ret/PTC by morphology shows that only 38.6% of the papillary thyroid carcinomas were positive for ret/PTC. Positive ret/PTC was found in specimens of three follicular thyroid carcinoma patients and one poorly differentiated carcinoma patient. It is important to mention that the histological diagnosis of four of these cases was confirmed by independent review of these histology slides; no adenomas were found in these specimens, and only one patient demonstrated thyroiditis in addition to follicular carcinoma. Paired samples of normal and tumor tissue were available in five of the patients who were positive for ret/PTC mutations. Ret/PTC mutations were not detected in any of these normal thyroid tissue samples.

All positive results of the RT-PCR were in accordance with the positive results found by the Southern blot technique; the

TABLE 3. Number and prevalence of samples positive for Ret/PTC by type of tumor, type of rearrangement, genetic assay (RT-PCR/Southern blots),[a] and study group

	Total		Irradiated cases		Non-Irradiated cases		*P* value
	n	%	n	%	n	%	
Thyroid carcinoma patients							
Total	49		37		12		
Ret/PTC 1 only (Southern blot)	18	36.7	10	27.0	8	66.7	0.04
Agarose gel	15	30.6	7	18.9	8	66.7	0.004
Ret/PTC 2 only	1	2.0	1	2.7	0	0	NS
Ret/PTC 3 only	0	0	0	0	0	0	
Ret/PTC 1 and 2	2	4.1	2	5.4	0	0	NS
Thyroid adenoma patients[b]							
Total	13		7		6		
Ret/PTC 1 only (Southern blots)	6	46.2	3	42.9	3	50.0	NS
Agarose gel	4	30.8	3	42.9	1	16.7	NS

[a] The results of Ret/PTC 2 only, Ret/PTC 3 only, and Ret/PTC 1 and 2 were identical using the RT-PCR and the Southern blot assays.
[b] Adenoma patients showed only ret/PTC 1 rearrangements.

latter assay detected five additional positive ret/PTC rearrangements (Table 3). The associations between ret/PTC rearrangements and gender, age at surgery and at exposure to irradiation, and latency period remained similar using either genetic technique. Whereas no significant differences in rates of thyroiditis between ret/PTC-positive and -negative specimens were found using the Southern blot, using the RT-PCR, thyroiditis was more prevalent in ret/PTC 1-positive thyroid cancer specimens compared with ret/PTC 1-negative specimens with a borderline significance (31.3 *vs.* 9.4%, respectively; $P = 0.097$).

Discussion

This paper presents the molecular analysis of a series of 49 cases of thyroid carcinoma and 13 cases of benign adenomas, of which 44 are radiation induced and 18 are sporadic tumors, all derived from a homogenous population of Middle Eastern and North African, mainly Moroccan, Jewish origin.

Our results demonstrated an overall ret/PTC activation rate of 38.6% in papillary thyroid cancer specimens, of which ret/PTC 1 was the predominant rearrangement. Surprisingly, ret/PTC 1 activation was also demonstrated in follicular carcinoma specimens. In our study group, no association was found between ret/PTC protooncogene activation and exposure to low-dose external-beam ionizing radiation in childhood.

The prevalence of ret expression in spontaneous papillary thyroid cancer varies widely between countries and studies. Whereas a very low rate of 2.5% was reported in Saudi Arabia (25), and medium-range rates of 29.3 and 45–52.9% were reported in Italy and North America, respectively (21, 26, 27), the activation rate of 60% seen among the nonirradiated papillary thyroid cancer patients in our study is in accordance with 60–85% observed in Ireland, Tasmania, New Caledonia (Oceania), Australia, and Hong Kong (24, 28–30). This wide range may be attributed to methodological differences of the various studies or to ethnic/geographic variability (21, 24, 27, 28).

However, the ret/PTC activation rate of 35.1% seen in our series of patients exposed in childhood to low-dose external-beam irradiation is surprising. Most other studies demonstrated higher rates of ret/PTC activation in irradiated carcinoma patients. Collins *et al.* (27) reported ret/PTC activation rates of 86.7% in papillary thyroid cancer patients who received external-beam irradiation in childhood compared with 52.9% among nonexposed patients ($P < 0.003$). In a study by Bounacer *et al.* (4), 84% (16 of 19) of papillary thyroid cancer patients who received external irradiation were positive for ret/PTC compared with 15% (3 of 20) among those who were not irradiated. Elisei *et al.* (5) found ret/PTC activation rates of 76% in children exposed to the Chernobyl accident, compared with 40% in nonirradiated Italian children ($P = 0.02$). In Italian adults who were exposed to external irradiation, 52.9% had ret/PTC activation; however, in this latter study, a similar rate (50.0%) was also seen in nonirradiated adult controls.

Our understanding of genetic rearrangements in radiation-associated papillary thyroid cancer derives primarily from studies examining individuals who were exposed to radiation after the Chernobyl accident in 1986. Such studies have reported a high prevalence of ret/PTC rearrangement that ranges between 49 and 87% (31–34). Whereas it might be useful to make predictions about the Israeli cases based on what has been observed in the childhood cases at Chernobyl, there are important differences between the two patient populations that might affect the rates and types of molecular changes we can expect to see in both groups. These include differences in doses and methods of irradiation, as well as age at diagnosis and latency period.

The Israeli children were exposed to external-beam irradiation, whereas the individuals at Chernobyl were exposed to radioactive iodine via internal ingestion of contaminated milk and water. In addition, the Israeli children received a significantly lower dose of radiation than the children at Chernobyl (slightly <9 rad on average in Israel, as opposed to anywhere from 10–500 rad in Chernobyl). The hypothesis that radiation dose is associated with rate of ret activation is supported by the mentioned high activation rates of 86.7% reported by Collins *et al.* (27) among carcinoma patients who were externally irradiated in childhood for benign conditions with thyroid doses of 72.1 + 11.1 cGy, which are 7- to 8-fold higher than the mean dose of the Israeli cohort. Furthermore, Rabes *et al.* (15) demonstrated higher ret/PTC activation

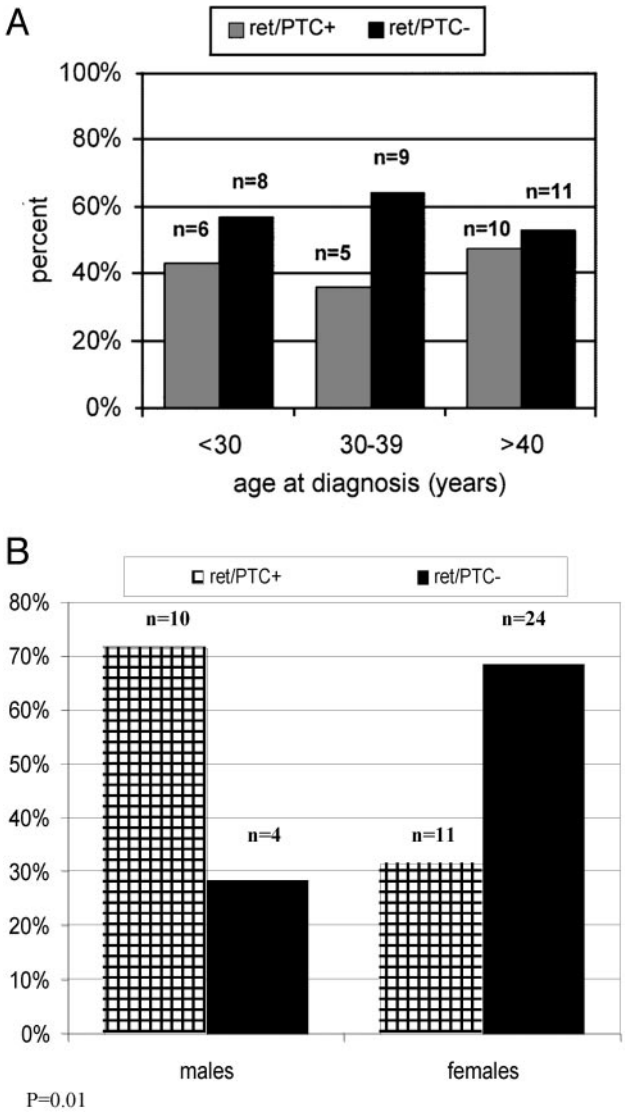

FIG. 3. Prevalence of ret/PTC (mutations 1 and/or 2) rearrangements in thyroid carcinoma patients by age at diagnosis (A) and by gender (B).

rates in thyroid cancers arising from the most heavily contaminated areas in Belarus.

Whereas most other studies, including most of the Chernobyl surveys, investigated papillary thyroid cancer patients who were irradiated and diagnosed during childhood (21, 31, 33), our series is composed of adult patients (median age at diagnosis, 38 yr) who were exposed in childhood. Indeed, Sugg *et al.* (35) investigated radiation-exposed adult patients (mean age at diagnosis, 45.2 yr) and found ret/PTC activation rates of only 5%.

Therefore, the discrepancies between the relatively low rates of ret/PTC activation found among the irradiated patients of our series and the higher rates described in other studies may suggest that radiation-induced ret/PTC activation is correlated with early-onset papillary thyroid cancer or short latency period (15).

Collins *et al.* (27), Bounacer *et al.* (4), and Elisei *et al.* (5) tried to investigate the association of ret/PTC activation with age at diagnosis, but none of them found such a correlation. However, these studies described either limited age range (27) or small numbers of irradiated adult patients that did not enable the researchers to detect statistically significant changes of ret/PTC with age (4, 5).

Because all of our study population was irradiated during the 1950s and in a relatively narrow age range (median age at irradiation, 6 yr; range, 1–15 yr) and because significantly more specimens were allocated from the more recent period compared with the earlier one (71.7% in 1981–2000 *vs.* 46.8% in 1959–1980; $P = 0.005$), it is possible that we allocated more specimens of older patients. If indeed ret/PTC activation is more prevalent among early-onset radiation-induced papillary thyroid cancer, this might reduce the activation rates seen in our series of radiation-induced tumors. To examine this assumption, we compared the age distribution at thyroid cancer diagnosis of the irradiated individuals of the whole TC cohort (n = 86) with that of our group and did not find significant differences.

It is important to mention that the issue of ret/PTC activation and age at diagnosis was also investigated among nonirradiated patients, and no association of ret/PTC activation with age was demonstrated, neither in our series nor in others (4, 36). Williams *et al.* (36) studied ret activation in sporadic papillary thyroid tumors from both children and adults. Similar ret expression rates of 59 and 47% were found in adults and in children, respectively. Bounacer *et al.* (4) found an overall frequency rate of 7–8% among nonirradiated papillary thyroid cancer and adenoma patients, with no correlation with age. Contrasting results were found by Bongarzone *et al.* (26), who studied 92 consecutive nonirradiated papillary thyroid cancer patients and found significantly higher rates of ret and neurotrophic tyrosine kinase receptor activation in patients younger than 30 yr of age compared with older patients (57 *vs.* 30–35% in older patients; $P = 0.019$).

There is conflicting evidence in the published literature as to which type of ret/PTC mutation is radiation induced. In the Chernobyl experience, the prevalence of specific types of ret/PTC mutations has changed over the years. In the earliest cases of radiation-induced thyroid cancer in Belorussian children, the predominant rearrangements reported were ret/PTC 3 (5, 15, 31, 37). Molecular analysis of cases developing after a longer latency period, 10 yr or more after the accident, demonstrated a lower prevalence of ret/PTC 3 mutations and a higher prevalence of ret/PTC 1 mutations (15). Similarly, Smida *et al.* (32) found almost equal frequencies of ret/PTC 1 and ret/PTC 3 (23.5 and 25.5%, respectively) among post-Chernobyl children, whereas post-Chernobyl adult patients exhibited only ret/PTC 1, as did nonirradiated German adults. Whereas the highest rates of ret/PTC 3 mutations in the post-Chernobyl thyroid cancers were seen in those cases with the shortest latency period between radiation exposure and development of thyroid cancer (5–10 yr latency), in the Israeli carcinoma patients, there was only one patient who had a latency period of less than 10 yr, and the mean latency period for the total group was 29.4 yr (range, 9–45 yr). Thus, it may be possible that ret/PTC 3 plays a role in the development of early cancer, whereas it may not play a role in the development of cancers arising in adults, many

years after radiation exposure. Externally irradiated adults were also studied by Bounacer *et al.* (4) and Elisei *et al.* (5). Whereas the former found the ret/PTC 1 rates to be higher than those of ret/PTC 3 in French patients (83.3 *vs.* 22.2%, respectively), the latter found higher rates of ret/PTC 3 that were similar to those found in nonirradiated adult patients. Irradiation of both cell lines *in vitro* and human thyroid tissue that was grafted into mice *in vivo* produced mainly ret/PTC 1 rearrangements (38).

We found three specimens (6.1%) with ret/PTC 2 rearrangements, all derived from irradiated patients. In most of the studies in which ret/PTC 2 appeared, it was at very low rates of 3–17% (26, 31, 33); in conjunction with other types of ret/PTC, it reached 35% (21, 24). One study that compared post-Chernobyl children with papillary thyroid cancer with American children with spontaneous papillary thyroid cancer found ret/PTC 2 only in the radiation-induced tumors (31). However, there are studies that demonstrated this rearrangement in nonirradiated patients as well (21, 24, 26). In sporadic cases, our results demonstrated only ret/PTC 1 rearrangements. Whereas some studies agreed that ret/PTC 1 is the most common rearrangement among sporadic cases (United States, 21 cases; Italy, 39 cases; Germany, 32 cases), others reported similar rates of ret/PTC 1 and ret/PTC 3 (New Caledonia and Australia, 27 cases; Italy, 25 cases). Moreover, in Hong Kong, the only and highly prevalent rearrangement among spontaneous papillary thyroid cancer cases was ret/PTC 3 (28). Studies conducted in Italian nonirradiated papillary thyroid cancer patients demonstrated inconsistent results, with either similar prevalence of both ret/PTC 1 and ret/PTC 3 or with each of them as the predominant rearrangement (5, 26, 39).

Although it was postulated at first that ret/PTC rearrangements occur specifically in papillary thyroid cancer (40), ret/PTC activation has been recently described in other conditions, such as Hashimoto's thyroiditis (2) and benign adenomas (4, 5, 7). In our series, ret/PTC 1 was found in six adenomas, three of which were post irradiation with a prevalence rate of 42.9%, and three of which were spontaneous with a prevalence rate of 50%. Similar ret/PTC rates of 37.5–45.0% in benign tumors among irradiated patients and 13.9–0% among spontaneous tumors were found by Elisei *et al.* (5) and Bounacer *et al.* (4), respectively.

The activation of ret/PTC in follicular carcinoma specimens deserves some attention. As far as we know, this is the first time that ret/PTC 1 rearrangements are found in specimens of follicular carcinoma. However, Bunone *et al.* (41) demonstrated ret protooncogene expression in follicular thyroid carcinoma cells derived from four neoplasms and two lymph-node metastases. Because our analysis was done using total RNA recovered from thyroid surgical specimens, we cannot be certain of the cell of origin of the ret/PTC mutation. It is possible that microscopic papillary thyroid cancer cells, which are not readily apparent on microscopy, could have been present in our follicular carcinoma samples and could have been the source of the ret/PTC transcripts. Thus, the cell of origin of these ret/PTC transcripts remains unclear.

In our series, thyroiditis in the tissue surrounding the neoplastic nodules was evident in 27.8% of the ret/PTC 1-positive and only 10.3% of the ret/PTC 1-negative specimens (nonsignificant). A possible underestimation of the true number of patients with thyroiditis might be due to underreporting of thyroiditis in pathological reports. Wirtschafter *et al.* (2) tested specimens of 21 Hashimoto's thyroiditis patients for ret/PTC 1 and/or 3. Although six of the patients had microscopic evidence of papillary carcinoma, the patients without evidence of carcinoma had ret/PTC rearrangements 10% of the time. An association between Hashimoto's thyroiditis and papillary carcinoma was suggested by other studies, in which variable frequency of Hashimoto's thyroiditis in papillary thyroid cancer patients ranged from 0 to nearly 60% (42–47). The histological features of Hashimoto's thyroiditis-associated papillary thyroid cancer were described and included fibrosis and atypical nodules that could represent precursor lesions of papillary thyroid cancer in patients with Hashimoto's thyroiditis (48).

The finding of ret/PTC activation in specimens of benign adenomas, undifferentiated carcinoma, and follicular carcinoma may suggest that ret/PTC mutations are not specific for papillary thyroid cancer. It is possible that papillary carcinomas as well as other thyroid neoplasms have common genetic lineage, as been suggested for neoplastic lesions such as Hurthle cell tumors (49, 50) and trabecular adenomas (51), although we cannot rule out the possibility of microscopic papillary thyroid cancer samples that are inadvertently analyzed with the predominantly benign lesion. Additional studies that can precisely pinpoint the cell of origin of the ret/PTC transcript within a clinical thyroid sample will be required to definitively address this issue. Another possibility is a common genetic lineage for papillary carcinoma and other thyroid diseases, as was suggested for Hashimoto's thyroiditis and Hurthle cell tumors.

Our study has certain limitations. First is the relatively low percentage (47%) of specimens that were retrieved out of the original target of 161 tumors. As mentioned above, six hospitals did not agree to participate in the study, and not all paraffin-embedded thyroid tissue samples stored in pathology archives for up to 30 yr could be located. We believe that the nonparticipating hospitals and the lost specimens were not of distinctive characteristics and therefore did not create a selection bias. Nevertheless, the higher success in retrieving specimens from the more recent years raises the question of selection bias of overrepresentation of older patients compared with younger ones; however, no significant differences were noted in the distribution of diagnosis by age between the located thyroid cancer cases and the total TC cohort, and therefore this bias could not influence the results significantly. In addition, this study did not confront the issues of correlation of ret activation with either papillary thyroid cancer subtypes or clinical outcomes (34, 52, 53).

Technical factors in the selection of tumor samples for analysis, recovery of the RNA, amplification of mRNA, detection of the amplified RT-PCR product and the specific immunohistochemical antibody, and technique also have significant impact on published prevalence rates. RT-PCR-based amplification techniques coupled with Southern blot mutation detection have the highest sensitivity for detection of ret/PTC transcripts but cannot accurately identify the specific cell that contained the mutation. Immunohistochem-

ical detection of the ret/PTC protein is less sensitive, and probably less specific, but does allow for localization of the abnormal transcript to a specific cell (27). Many authors are now using laser capture microdissection to carefully select a small number of specific cells for RNA extraction and analysis (30). Because the methods for RNA extraction and amplification can vary widely between laboratories, a wide range of specificity and sensitivity is seen between assays. These technical factors make comparison of data between published reports quite difficult.

In the present study, the Southern blot assay confirmed and validated all of the results of the ethidium bromide visualization of the RT-PCR products on agarose gel, and, as expected, added five new positive results (supporting the higher sensitivity of the former assay). This establishes the specificity of the RT-PCR data and confirms the well-known increase in sensitivity for detection of low-quantity RT-PCR products with Southern blotting. Low-level RT-PCR amplification of ret/PTC products could be caused by low copy number of transcripts in the original tumor specimens, by RNA degradation in processing, and by storage of the clinical thyroid samples. The possible disadvantage of using the Southern blot method, which has a higher sensitivity, is that it might detect very low levels of ret/PTC expression with limited or unknown clinical significance. Nevertheless, no major discrepancies in the trend of our results were seen when comparing the results of both assays. Therefore, we presented most data for both assays and detailed the data of the more-sensitive Southern blot.

In conclusion, this study presents for the first time the molecular analysis of a subset of the Israeli TC cohort, adding to the data on frequency of ret rearrangements in cancers after exposure to external radiation. As opposed to most previously published cohorts of radiation-associated thyroid cancer, the subjects examined in this study were exposed to low-dose external-beam irradiation during childhood and had a very prolonged latency period before the development of clinically apparent thyroid nodules. One hypothesis that explains the present results could be that ret/PTC activation is dose dependent, and, once activated, these rearrangements are associated with a short latency period between exposure and development of clinically apparent thyroid neoplasia. If this hypothesis is correct, one would not expect to find a large prevalence of ret/PTC mutations in thyroid cancers that develop many years after exposure to low-dose external-beam irradiation. These data suggest that genetic alterations other than ret/PTC are important in the development of thyroid cancer that arises many years after low-dose radiation exposure.

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