RET Expression in Papillary Thyroid Cancer from Patients Irradiated in Childhood for Benign Conditions


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Both external and internal exposure to radiation have been linked to the development of papillary thyroid cancer. Rearrangement of the gene for RET tyrosine kinase and subsequent expression of this protein has also been found to occur in many papillary thyroid cancers, and with increased frequency in radiation-related cancers following the Chernobyl accident. However, little has been reported on the frequency of RET rearrangements in cancers after exposure to external radiation. We here report on RET protein immunoreactivity in paraffin-embedded thyroid samples from 30 patients with papillary thyroid cancer who received radiation treatment during childhood for benign conditions at Michael Reese Hospital in Chicago, and in 34 patients identified from the tumor registry as having papillary thyroid cancer with no history of therapeutic radiation. The subjects were characterized by sex, age at surgery, and the following attributes of tumor pathology: size, number of lobes involved, number of foci, lymph node metastases, and soft tissue invasion. Representative tissue samples were reacted with an antibody against the RET tyrosine kinase domain whose expression has been shown to correlate highly with RET/PTC rearrangements. A greater percentage of cancers positive for RET immunoreactivity was found in the radiation-exposed group (86.7% vs. 52.9%, P = 0.006). Although the mean age at surgery of the exposed group was lower than the control group, there was no correlation of positive RET immunoreactivity with the age at surgery. No characteristics of the tumors were associated with positive RET immunoreactivity. In summary, the greater incidence of RET-immunopositives in the irradiated group indicates that the expression of RET immunoreactivity is strongly associated with radiation exposure, but the prognostic significance of this is not yet clear. (J Clin Endocrinol Metab 87: 3941–3946, 2002)

The relationship between external radiation and thyroid cancer was well established many years ago (1–3). More recently, a similar relationship was established for internal radiation from isotopes of iodine, as the accident at the Chernobyl nuclear power plant has resulted in an unprecedented number of childhood thyroid cancers (4, 5). Almost all thyroid cancers arising after external or internal radiation exposure occur after childhood exposure and are of the papillary type (2, 6–9).

Following its description in 1990 (10), the activation of the RET gene by somatic rearrangement (to form the RET/PTC oncogene) has been found to be a frequent event in papillary thyroid cancers (11, 12). As measured by immunohistochemical or RT-PCR methods and as reported from many geographical areas, the prevalence of RET expression in spontaneous papillary thyroid cancers varies widely. For example, prevalences of 3, 8, and 11% have been reported in Saudi Arabia, Germany, and France, whereas 70 and 85% have been reported in New Caledonia and Australia (12–15). Whether this wide range is due to methodological, geographical, or other variables is not fully known. The reported frequency of RET-positive papillary thyroid cases in the Chernobyl population is 51–76%, higher than that for almost all groups of spontaneous thyroid cancers (16–21).

How do the results on RET activation from Chernobyl, where radiation exposure was primarily internal through ingestion of iodine isotopes, compare to subjects receiving external ionizing radiation? Two studies have reported findings from patients receiving external ionizing radiation treatment (22, 23). However, in one of these studies, less than half were children receiving treatment for benign conditions and in the other the doses and reasons for treatment were not given. The “Chicago cohort” is a large group of patients who received radiation for benign conditions during childhood with doses comparable to those received as a result of the Chernobyl accident. Of these subjects, over 12% have developed thyroid cancer (24). In the present study, we report on the prevalence of RET protein immunoreactivity in thyroid cancers in this group, comparing it to a control group of papillary thyroid cancers in patients with no known history of radiation.

Subjects and Methods

Study subjects

At Michael Reese Hospital (Chicago, IL), 4296 children less than 16 yr of age received external beam radiation therapy for benign conditions between 1939 and 1962 (25, 26). As of January 2001, 3083 of these patients have been located. In this group, 357 have developed thyroid cancer, 121 of whom were operated on at Michael Reese Hospital. Paraffin blocks for 63 of these cases were located, of which 30 included usable papillary thyroid cancer samples. A control group of 34 cases of papillary thyroid cancer cases was compiled from 157 cases in the Tumor Registry at Michael Reese Hospital whose medical records indicated no history of
radiation treatment. An attempt was made to match the control cases to the radiation-exposed group by age and sex, but the limited availability of paraffin blocks from subjects in either group precluded a complete match. The study protocol was approved by the Michael Reese Hospital and University of Illinois at Chicago Institutional Review Boards.

**Study parameters**

The demographic characteristics of the study subjects included in the analyses were age at radiation, radiation dose to the thyroid, sex, and the age at thyroid cancer surgery. The characteristics of the thyroid cancers for each subject were derived from operative and surgical pathology reports. The largest dimension of the largest malignant nodule was used as a measure of size. The number of thyroid lobes involved (1 or 2), the number of tumor foci (single or multiple), lymph node metastases (present or absent), and the presence of tumor invasion into soft tissue adjacent to the thyroid (present or absent) were recorded.

**Immunohistochemistry**

Paraffin blocks had been stored for a mean of 25.7 yr for the radiation-exposed cases and 11.0 yr for the unexposed cases. Sections were cut from those blocks where the likelihood of papillary tumor samples was greatest, based on the descriptions in the surgical pathology reports. Sections of 5-μm thickness were mounted on Fisher Scientific (Pittsburgh, PA) Superfrost slides, deparaffinized with xylene, and quenched for endogenous peroxidase by reaction in a solution of 0.3% hydrogen peroxide in absolute methanol for 30 min. After washing in distilled H2O and phosphate-buffered saline, the slides were treated with diluted blocking serum for 20 min before incubating overnight at 4°C with an affinity-purified rabbit polyclonal antibody against a glutathione S-transferase fusion protein containing 116 amino acids of the RET cytoplasmic tyrosine kinase domain (27). The antibody reacts with both full-length and rearranged RET, and does not stain normal thyrocytes. Previous results obtained by immunohistochemistry have been shown to correlate highly with the molecular results obtained by RT-PCR for RET gene rearrangements in papillary thyroid cancers (18, 28–33), although recent reports indicate that the antibody may also recognize on immunoblots activated RET protein from papillary thyroid cancers in the absence of RET/PTC rearrangements (34). After incubation with the primary antibody, the slides were washed and incubated with biotinylated goat antirabbit IgG [Vectastain avidin biotin complex (ABC) kit, Vector Laboratories, Burlingame, CA] for 20 min and reacted with the ABC in the ABC kit before visualizing the reaction product with 0.05% diaminobenzidine (DAKO Corp., Carpinteria, CA) and 2% hydrogen peroxide in 0.05% phosphate-buffered saline for 5 min. The slides were washed, counterstained with hematoxylin, dehydrated through graded ethanol and xylene, and mounted in Permount (Fisher Scientific). Expression of the RET product was scored by independent observers, based on the number of cells reacting to the antibody, not the intensity of the reaction. The specimen was scored as "3+" if more than 60% of the cells stained positive, "2+" if 40–60% of the cells were positive, and "1+" if only 20–40% of the cells were positive. Visible staining of less than 20% was scored as "trace" but was considered negative for the analysis of the data.

**Statistical analysis**

Binary variables were analyzed by logistic regression and the Fisher exact test and continuous variables were analyzed by multiple regression, ANOVA, and t-tests.

**Results**

The demographic variables and the thyroid cancer characteristics in the radiation-exposed and the unexposed groups are given in Table 1. The members of the exposed group were younger and had a higher percentage of males than did the control group. The tumors in the exposed group were smaller and had a greater prevalence of lymph node metastases and multifocal lesions.

Figure 1 shows representative sections of the reacted thyroid specimens from which the data for RET immunoreactivity were drawn. A greater percentage of the radiation-exposed group had positive RET-immunoreactive cancers than did the unexposed control group: 86.7% vs. 52.9%; \( P < 0.01 \) (Table 1 and Fig. 2). When the cases were analyzed by the extent of immunoreactivity (0 to 3+) attributed to each specimen, not only were there more cases with positive RET immunoreactivity in the exposed group, but the exposed cases accounted for more extensively stained RET-immunopositive than the control group. Only one of the control cases scored above 1+ (5.5%), whereas 7 of the 26 (26.9%) cases in the exposed group were scored as 2+ or 3+.

No RET immunoreactivity was detected in normal tissue. There were six subjects in the exposed group and two in the unexposed group whose slides contained adenomas or adenomatous nodules. An additional three subjects in the radiation-exposed cohort and one subject in the control group also contained adenomas but were not included in the final analysis because samples of their previously described papillary thyroid cancer were not present in the available blocks. Of these 12 benign nodules, only one, from the exposed group, demonstrated any positive reactivity to the RET antibody, and this reactivity was restricted to the nuclei of the reactive cells. The accompanying sample of papillary thyroid cancer on this slide was also RET-immunopositive. In one case, an area of Hashimoto's thyroiditis, accompanying papillary cancer, was positive for RET-immunoreactivity.

The RET data for thyroid cancer, plotted by age at surgery (in deciles) in Fig. 3, indicate that at any given age the percentage of cases positive for RET immunoreactivity was greater in the radiation-exposed group. Controlling for age at surgery, the difference in the proportion of positive and negative RET-immunoreactive cases between the two groups persisted. Although the age at surgery was less in the radiation-exposed group (\( P < 0.001 \)), this did not explain the difference in positive RET immunoreactivity (Table 2).

The mean size of the largest malignant nodules (Table 1) in the radiation-exposed group was smaller than that in the unexposed group (12.9 mm vs. 24.9 mm, \( P < 0.001 \)). Also, when analyzed alone, there was a significant difference in size by RET status (16.0 mm for positive RET

**Table 1. Description of the members of the two study groups and their thyroid cancers (mean ± SEM)**

<table>
<thead>
<tr>
<th></th>
<th>Exposed</th>
<th>Unexposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td>Total number</td>
<td>3.4 ± 0.6</td>
<td>7.2 ± 11.1</td>
</tr>
<tr>
<td>Thyroid dose(^a) (cGy)</td>
<td>28.7 ± 1.5</td>
<td>46.4 ± 2.8</td>
</tr>
<tr>
<td>Male(^b)</td>
<td>53.3%</td>
<td>26.5%</td>
</tr>
<tr>
<td>Cancer characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RET positive(^b) (%)</td>
<td>86.7%</td>
<td>52.9%</td>
</tr>
<tr>
<td>Size(^b) (mm)</td>
<td>12.9 ± 1.4</td>
<td>24.9 ± 2.9</td>
</tr>
<tr>
<td>Lymph node metastases(^b) (%)</td>
<td>66.7%</td>
<td>32.4%</td>
</tr>
<tr>
<td>Soft tissue invasion (%)</td>
<td>26.7%</td>
<td>29.4%</td>
</tr>
<tr>
<td>Multifocal(^b) (%)</td>
<td>76.7%</td>
<td>52.9%</td>
</tr>
<tr>
<td>Bilateral (%)</td>
<td>36.7%</td>
<td>26.4%</td>
</tr>
</tbody>
</table>

\(^a\) Thyroid dose was available for 27 of the 30 cases.\(^b\) \( P < 0.05 \).
immunoreactivity, 26.5 mm for negative RET immunoreactivity, Table 3). However, analysis by two-way ANOVA or multiple regression indicated a significant difference in size by treatment (exposed vs. unexposed) group, but not by RET status (Table 3).

The 66.7% of the radiation-exposed cases having lymph node metastases was higher than the 32.4% of the unexposed group with metastases (Table 1). However, the exposed group was younger than the unexposed group and lymph node metastases are known to be more common in younger patients. To take this into account, regression analysis of lymph node metastases as a function of age at surgery and RET status was performed. Age at surgery was inversely related to lymph node metastases, whereas RET status was not. Neither age of surgery nor treatment group (exposed vs. unexposed), when analyzed together, was independently related to lymph node metastases.

The radiation-exposed group had more multifocal cancers than did the unexposed group (76.7% vs. 52.9%, Table 1). This was significant (P = 0.01) when adjusted for age and size. There was no difference in the prevalence of multifocal cancers between the cases with positive or negative RET immunoreactivity (70.5% vs. 50.0%, P = 0.06, Table 3). Analyzed together, neither the exposure group nor RET-status independently predicted multifocal cancers. There was no difference between the exposed and unexposed groups in the number of tumors that demonstrated invasion into the soft tissue, nor was there a relationship of soft tissue invasion with RET status.

The data for the radiation group alone were analyzed for
FIG. 3. The number of cases of RET immunopositive and immunonegative tumors by age at surgery (in deciles) in the exposed and unexposed groups. Each bar represents the number of cases whose surgery occurred in the age decile bracketed by the age at either side of the bar. The RET-positive cases are represented by the shaded portion of the bar and the RET-negative cases by the open bar. The percentage of the positive cases in each decile is indicated at the top of the bar.

TABLE 2. Description of the study groups by RET status

<table>
<thead>
<tr>
<th>Group</th>
<th>RET +</th>
<th>RET -</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>44</td>
<td>20</td>
</tr>
<tr>
<td>Exposed</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Age at radiation</td>
<td>3.65 ± 0.62</td>
<td>1.39 ± 0.48</td>
</tr>
<tr>
<td>Thyroid dose (cGy)</td>
<td>69.1 ± 12.2</td>
<td>89.3 ± 29.0</td>
</tr>
<tr>
<td>Age at surgery</td>
<td>36.9 ± 2.3</td>
<td>40.8 ± 3.7</td>
</tr>
<tr>
<td>Exposed</td>
<td>29.2 ± 1.7</td>
<td>25.4 ± 3.6</td>
</tr>
<tr>
<td>Unexposed</td>
<td>48.0 ± 3.9</td>
<td>44.7 ± 4.0</td>
</tr>
<tr>
<td>Male</td>
<td>19 of 44 (43.2%)</td>
<td>6 of 20 (30.0%)</td>
</tr>
<tr>
<td>Exposed</td>
<td>15 of 26 (57.7%)</td>
<td>1 of 4 (25.0%)</td>
</tr>
<tr>
<td>Unexposed</td>
<td>4 of 18 (22.2%)</td>
<td>5 of 16 (31.3%)</td>
</tr>
</tbody>
</table>

* P < 0.05.

possible correlations between RET status and the dose of radiation, age at treatment or latency (time between radiation and surgery). No associations were found for any of the variables (Table 2). Also, none of these factors was related to the characteristics of the thyroid cancers in the exposed group.

Discussion

Most of the data related to the expression of the RET proto-oncogene in papillary thyroid cancers have been either from sporadic cases or from cases related to radiation exposure after the Chernobyl accident. In this paper, we report results of RET protein expression in thyroid cancers in a population exposed to external radiation during childhood for the treatment of benign conditions, a group that has been subject to much less analysis of RET expression. Our data show that there is an increased expression of positive RET immunoreactivity in the thyroid cancers of the radiation-exposed group, with 86.7% of the radiation group positive for RET immunoreactivity vs. 52.9% of the control group. The frequency of RET protein expression in our control group is higher than the frequency of RET expression reported in many, but not all studies of spontaneous papillary thyroid cancers (15, 35) and is similar to what has been observed in the Chernobyl population (56–76%) (16, 19, 20, 23, 36). The frequency of RET expression we observed by immunohistochemistry in the radiation group is comparable to that reported by Bounacer et al. (22) for RT-PCR data, who found 84% RET positives in 19 externally irradiated subjects. However, they included irradiated adults and patients who received high dose radiation therapy in their population. It is also comparable to the RT-PCR data recently reported by Elisei et al. (23) who observed 76% (19 of 25) RET-positive thyroid cancers removed from adults who had been exposed to external irradiation in childhood. Because they did not report the dose of treatment and whether it was administered for benign or malignant conditions, the comparison to our study and to Chernobyl cases is uncertain.

As the exposed and unexposed groups were not balanced in demographic characteristics, we performed multivariate analyses. The subjects in our radiation-exposed group were significantly younger than the control group (Table 1). However, we found no association of positive RET immunoreactivity with age at surgery. Similarly, no relationship between the frequency of RET rearrangements and age was found for nonradiation related cases in Japan, England, and Italy (23, 37, 38). Thus, the age difference in the two groups does not explain the higher frequency of positive RET immunoreactivity in irradiated cases.

The fact that the radiation-exposed group contained more lymph node-positive cases might be attributed to the lower age of this group and the well-known association of young age with lymph node metastases. In fact, an age difference between lymph node positivity and negativity existed only in the unexposed group, suggesting that the association of
young age and lymph node metastases may be stronger in the absence of radiation treatment.

As there were differences in the presenting features of the cancers in the exposed and unexposed groups, we analyzed the data to see if RET status was related to any pathological characteristic. The smaller size of the cancers in the radiation group is due, in large part, to the effect of surveillance of this population, which has been screened on a regular basis since 1975 for the presence of thyroid nodules. The control group, on the other hand, represents spontaneous cancers that likely had gone undetected until they reached a larger size. Although treatment (exposed vs. unexposed) and RET status were independently related to size, when combined, only radiation exposure status was significant.

Attributes of the thyroid cancers were analyzed for a relationship to RET status. None were found for lymph node metastases, multifocality, and local invasion. However, the number of cases was too small to determine whether RET status has prognostic significance in general or in radiation-related cases. A recent study (32) similarly found no prognostic impact of RET protein expression on the long-term outcome of papillary thyroid cancers.

The specificity of RET/PTC rearrangements to papillary carcinomas has been questioned by findings in “benign” thyroid neoplasms in patients who were exposed to radiation, either for therapeutic purposes or after the Chernobyl accident. Bounacer et al. (22) found RET rearrangements in 45% of radiation-related follicular adenomas. Elisei et al. (23) found rearrangements in 52.4% “benign thyroid neoplasms” following exposure from Chernobyl, in 37.5% (3 of 8) cases following external irradiation, and in 13.9% of sporadic cases. In contrast, we observed positive RET immunoreactivity in only one of nine (11%) external radiation-related benign nodules. The reason for this discrepancy, possibly due to the limited number of cases studied or the difference in methodologies, and the potential role of RET activation in radiation-related benign nodules requires further investigation.

It has recently been shown that, contrary to previous assumptions, the RET gene may not be completely silent in normal thyroid follicular cells and that the expression of wild-type RET may be increased above low basal levels in many papillary thyroid cancers (34, 39). Bunone et al. (39) found RET/ELE1 transcripts, the reciprocal form of the oncogenic ELE1/RET translocation, in thyroid carcinomas, confirming the activity of the RET promoter in thyroid cells. Fluge et al. (34) confirmed the presence of low levels of wild-type c-ret mRNA in normal thyroid and overexpression of these wild-type transcripts in papillary thyroid carcinomas by RT-PCR, although the latter also harbored translocations. They also demonstrated RET immunoreactivity by Western blotting of a large amount (250 μg) of protein, using the same antibody against RET which we have used in this study and two other antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

These findings show that wild-type RET mRNA is expressed at a low level in normal thyroid tissue, but the protein from normal cells was not detectable on Western blots (34) and therefore is not likely to be detectable on tissue sections. In our study and in other studies using this antibody, positive RET immunoreactivity has not been detected in normal thyroid. However, the correlation reported between histologically detectable RET immunoreactivity using the antibody employed here and the finding of a somatic RET rearrangement is very high (18, 28–33), although some discordance between immunohistochemical and RT-PCR data has been observed (18, 28). Absence of RET protein expression in the presence of a RET/PTC translocation may arise because RT-PCR is a more sensitive technique than immunohistochemistry. Conversely, expression of the RET protein in the absence of a detectable translocation may have been due to the presence of a translocation that was missed by the RT-PCR primers used, or in accordance with Fluge’s work, may represent a splice variant or some alternative mechanism of activation. We cannot, therefore, be sure that every positive RET immunoreactive case in our study represents a corresponding gene rearrangement. However, the immunoreactivity recognized by the antibody in this study is cytoplasmic, indicating overproduction of RET protein that has lost its transmembrane localization due to transposition of the tyrosine kinase domain with the N terminus of a cytoplasmic protein. The wild-type and splice variants observed by Fluge et al. (34) do not have altered transmembrane and tyrosine kinase domains, indicating that the protein product should still be targeted to the plasma membrane. Further work is necessary to confirm the cellular localization and detectability of the reported splice variants and wild-type RET under different fixation and detection protocols.

In conclusion, positive RET immunoreactivity in radiation-related thyroid cancers is more common than in cancers from patients without a history of radiation exposure. The frequency of activation reported in cases arising from internal, Chernobyl-related cases is similar to the frequency reported here. The cases reported here were exposed to radiation at about the same ages and with similar doses. The findings predict that as cases continue to occur, as a result of either external or internal radiation, they will be associated with a high frequency of RET activation. How this will affect their clinical behavior, if at all, remains to be seen. This is important because of the well-recognized poorer prognosis of thyroid cancer with advancing age.

Acknowledgments

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