Distinct Pattern of \textit{ret} Oncogene Rearrangements in Morphological Variants of Radiation-induced and Sporadic Thyroid Papillary Carcinomas in Children\textsuperscript{1}

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ABSTRACT

In this study, we compare the morphological and genetic characteristics of 38 post-Chernobyl thyroid papillary carcinomas from Belarussian children 5—18 years old with those of 23 sporadic papillary carcinomas from the same age children without history of radiation exposure from Los Angeles and Cincinnati. Among radiation-induced tumors, solid variant of papillary carcinoma was found in 37\%, follicular in 29\%, typical papillary in 18\%, and mixed and diffuse sclerosing variants in 8\% each. In the sporadic group, a typical papillary pattern was prevalent in 70\%, follicular in 17\%, diffuse sclerosing variant in 9\%, and solid in 4\%. In both groups, the prevalence of \textit{ret} rearrangements was high, but the frequency of specific types of rearrangement was significantly different. Among radiation-induced tumors, \textit{ret/PTC3} was found in 58\%, \textit{ret/PTC1} in 16\%, and \textit{ret/PTC2} in 3\%, whereas among sporadic tumors, \textit{ret/PTC1} was found in 47\% (P < 0.05), and \textit{ret/PTC3} was found in 18\% (P = 0.01). The morphological variants of papillary carcinoma showed different prevalence of the specific types of \textit{ret} rearrangement. Seventy-nine\% of solid variant tumors had \textit{ret/PTC3}, whereas only 7\% had \textit{ret/PTC1} (P = 0.0007). Among typical papillary tumors, \textit{ret/PTC1} was found in 38\%, \textit{ret/PTC3} in 19\%, and \textit{ret/PTC2} in 5\%. Thus, \textit{ret} rearrangements are highly prevalent in pediatric papillary carcinomas from children exposed to radiation and in those occurring sporadically. However, the types of \textit{ret/PTC} vary between these two populations, with \textit{ret/PTC3} present more commonly in post-Chernobyl tumors. Furthermore, solid variants have a high prevalence of \textit{ret/PTC3}, whereas typical papillary carcinomas do not, suggesting that the different types of \textit{ret} rearrangement confer neoplastic thyroid cells with distinct phenotypic properties.

INTRODUCTION

Thyroid cancer in children exposed to radiation as a result of the Chernobyl nuclear accident in April, 1986 is now a widely accepted paradigm of radiation-induced neoplasia. A sharp increase in the incidence of pediatric thyroid carcinoma was noted as soon as 4 years after the accident (1). Thus far, several hundred cases have been reported in children living in the contaminated areas of Belarus, Ukraine, and western regions of Russia, resulting in up to a 100-fold increased annual incidence of childhood thyroid carcinoma as compared to that prior to 1986 (2, 3).

The vast majority of these tumors are thyroid carcinomas of the papillary type. A detailed morphological analysis of 84 cases from Belarus showed that solid and follicular variants of papillary carcinoma were prevalent in these tumors (4). Spontaneous papillary thyroid carcinomas are extremely rare in children, and to our knowledge their morphological variants have not been characterized in detail.

Our understanding of the genetic events associated with radiation-induced thyroid cancer in general, and of post-Chernobyl tumors in particular, is far from complete. \textit{ras} oncogene mutations, which are found in 18—62\% of papillary carcinomas in the general population, are not observed at all in these tumors (5, 6). Mutations of the p53 gene, which are common in sporadic undifferentiated and poorly differentiated thyroid carcinomas (7, 8), have a very low prevalence in the Chernobyl population (5, 6), in spite of the fact that many papillary carcinomas in the latter population have a solid growth pattern, indicative of a less differentiated phenotype.

Recently, two studies of a small series of post-Chernobyl papillary thyroid carcinomas reported a 67\% prevalence of the \textit{ret} gene rearrangements (9, 10). \textit{ret} rearrangements are formed by the fusion of the intracellular tyrosine kinase domain of the gene with different 5' gene fragments, resulting in inappropriate constitutive expression of the truncated tyrosine kinase domain of the receptor. The authors proposed that this genetic event might reflect a radiation origin of these tumors. Indeed, the incidence of \textit{ret} alterations found in pediatric post-Chernobyl tumors is much higher than that reported previously in the general (mostly adult) population, where three types of \textit{ret} rearrangements, \textit{ret/PTC1}, \textit{ret/PTC2}, and \textit{ret/PTC3} have been detected in up to 34\% of papillary thyroid carcinomas (data summarized in Ref. 11). In addition, the induction of \textit{ret/PTC1} rearrangement has been reported after high dose X-ray irradiation in vitro (12). However, a recently published study demonstrates a similarly high incidence of \textit{ret/PTC} rearrangements in spontaneous thyroid carcinomas in Italian children but not in adults, suggesting that this might be an age-specific, rather than radiation-specific, event (13).

To clarify the role of \textit{ret} oncogene alterations in radiation-induced carcinogenesis in the thyroid gland and to compare the morphology of radiation-induced and spontaneous thyroid cancers in children, we studied a large series of post-Chernobyl pediatric papillary thyroid carcinomas and compared them with a group of pediatric tumors without a history of radiation exposure from two different areas in the United States.

MATERIALS AND METHODS

Patient Population. Thirty-eight cases of thyroid papillary carcinoma from Belarusian children 5—18 years of age who lived in the areas contaminated as a result of the Chernobyl nuclear accident were studied. These cases were selected from a large consecutive series of thyroid tumors in children that underwent surgery at the Thyroid Tumor Center in Minsk in 1991—1992 based on the availability of RNA extracted from paraffin-embedded tissue of sufficient integrity to allow genetic analysis. The morphological and epidemiological features of these tumors have been described previously (4, 14). To perform a comparative analysis of radiation-induced and sporadic pediatric thyroid tumors, records of all cases of thyroid carcinoma in children up to 18 years old were obtained from the files of the Department of Pathology at Childrens Hospital Los Angeles (Los Angeles, CA) and the Department of Pathology at Childrens Hospital Medical Center (Cincinnati, OH). Fourteen primary cases of papillary carcinoma were found in Los Angeles since 1974 and 10 cases in Cincinnati since 1982. The medical records of each patient were reviewed for radiation history. One patient from Cincinnati had received X-ray therapy in a dose of 1800 rads to the brain area for acute lymphoblastic leukemia 8 years prior to removal of thyroid carcinoma. The latter case was eliminated from further analysis. Altogether, 23 spontaneous papillary thyroid carcinomas from children 5—18 years of age were studied.
Histology. Histological slides from primary thyroid tumors stained by H&E were reviewed by at least two pathologists (Y. E. N., J. M. R., and H. M.-M.; or Y. E. N. and K. E. B.) to confirm the diagnoses and subclassify the variants of papillary carcinomas. Simultaneously, the sections containing 70–100% of tumor from lymph node metastasis or primary nodules were selected for RNA extraction from the corresponding paraffin blocks.

Genetic Analysis. In all cases, RNA was extracted from paraffin-embedded tissue as reported previously (15, 16). In addition, in five cases from Cincinnati, RNA was obtained from frozen tissue as described by Chomczynski and Sacchi (17). After reverse transcription at 42°C by Superscript reverse transcriptase (BRL) using a random hexanucleotide mixture as a primer, cDNA was first tested to ensure that the integrity of RNA was sufficient for analysis. For this purpose, a 236-bp sequence of the c-N-ras cDNA was amplified with primers spanning intron 1 of the gene (Table 1). Only those samples demonstrating clearly detectable levels of the low abundance N-ras transcript were further analyzed. To screen for ret mRNA expression, we first amplified fragments corresponding to the TK and EC domains, respectively, using two sets of intron-spanning primers (Table 1). The cDNAs of the tumors demonstrating preferential expression of the TK domain were considered as suspicious for having ret rearrangements. Nevertheless, all tumor cDNAs were screened with primers bracketing chimeric sequences corresponding to the rearrangements of ret/PTC1, ret/PTC2, and ret/PTC3 types (Table 1). For each PCR, 2 μl of reverse transcribed mixture were amplified with 10 pmol of each primer, 200 μM deoxynucleotide triphosphates, 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 1% Triton X-100, 1.5 mM MgCl₂, and 1 unit of Taq DNA polymerase (Promega Corp., Madison, WI) in a final volume of 30 μl. Thirty-five cycles of denaturation (94°C for 1 min), annealing (55–63°C for 1 min), and extension (72°C for 1.5–2 min) were conducted on an automated heat block (DNA thermal cycler; Perkin-Elmer, Norwalk, CT). Positive controls for amplification included RNA samples from a medullary thyroid carcinoma with constitutive expression of wild-type c-ret (for amplification of ret TK and EC domains), from the TPC-1 cell line with a ret/PTC rearrangement (18), and cloned vectors containing ret/PTC2 and ret/PTC3 rearranged genes kindly provided by Dr. S. M. Jiang (Ohio State University, Columbus, OH).

Ten μl of PCR product were electrophoresed in a 1.5% agarose gel and blotted to a nylon membrane. Each filter was then hybridized with the corresponding specific probe (Table 1) for 16 h at 42°, washed, and exposed for autoradiography for 4–16 h.

Statistical Analysis. A χ² method was used to test homogeneity between the various groupings in the radiation-induced and sporadic sets. Because many groupings had a small number of observations, the test of two proportions with double-sided confidence limits and the Yates correction was performed to test whether specific proportions were different between two groupings. Alternatively, the two-tailed Fisher exact test was used for smaller sample sizes (19). Two values were considered significantly different when the probability of P was less than 0.05.

RESULTS

Epidemiological and Morphological Characteristics. The age of children in both groups ranged from 5 to 18 years. The mean age was 11.2 years among post-Chernobyl tumors, and 13.7 years among spontaneous thyroid carcinomas. In the former group, there were 18 girls and 20 boys, resulting in a female: male ratio of 0.9:1. In the latter group, there were 20 girls and 3 boys with a sex ratio of 6.6:1 (P < 0.01).

Morphologically, papillary carcinomas were classified according to the prevalent growth pattern in the primary tumor nodule as either typical papillary, follicular, or solid variant (Fig. 1). Typical papillary carcinomas were composed of papillae with a central fibrovascular core covered by neoplastic epithelial cells. The follicular variant tumors contained predominantly follicles, which varied in size and content of colloid, and were lined by epithelium with cytological features of papillary carcinoma. The solid variant was composed exclusively or predominantly by solid sheets of neoplastic cells surrounded by varying amounts of fibrotic stroma; the typical nuclear features of papillary carcinoma were commonly retained. Mixed variant was diagnosed in cases when the tumor nodule displayed an almost equal prevalence of any two or all three patterns of growth. In addition, tumors were classified as diffuse sclerosing variant based on a diffuse type of growth without a distinguishable solitary nodule, presence of severe fibrosis, dense lymphocytic infiltration, and areas of squamous metaplasia (20). The prevalence of morphological variants in both groups are summarized in Table 2. The distribution of histological phenotypes was found to be significantly different between radiation-induced and sporadic tumors (χ² test, P < 0.001). Typical papillary carcinoma was significantly more prevalent among sporadic tumors (test of proportions, P < 0.001), whereas solid variant tumors were more common among the exposed children (Fisher's Exact test, P = 0.006).

Molecular Genetic Analysis. Of 38 radiation-induced tumors, 33 (87%) showed preferential or exclusive expression of the TK domain as opposed to the EC domain of c-ret, indicating the possible occurrence of rearrangements with a breakpoint somewhere between these two regions (Fig. 2A). All of these positive cases were screened for the three known types of ret/PTC rearrangements. Ret/PTC1 type was identified in 6 cases (16%), ret/PTC2 in 1 case (3%), and ret/PTC3 was found in 22 cases (58%; Fig. 2B). In 4 cases with strong expression of TK domain (cases R25, R32, R33, and R35), none of the known types of ret rearrangement was identified, in spite of the fact that the experiment was replicated twice with fresh preparations of tumor RNA. The mean age at the time of radiation exposure was 4.3 years among those with ret/PTC3, 4.2 years among those who had ret/PTC1, and 5.8 years in a case with ret/PTC2 rearrangement.

In 17 of 24 sporadic tumors, the preservation of RNA was sufficient for further screening. Of these, 12 (71%) demonstrated preferential or exclusive expression of TK domain as opposed to the EC domain of the ret gene (Fig. 3A). Further screening showed the presence of ret/PTC1 in 8 (47%) cases and ret/PTC3 in 3 (18%) cases; there were no cases with the ret/PTC2 type in this group (Fig. 3B). One tumor with predominant TK expression (S14) did not show any of the known types of ret rearrangement. None of the cases from either group that lacked prefer-

Table 1 Primers and probes used for the screening

<table>
<thead>
<tr>
<th>Size (bp)</th>
<th>Primer sequences (5'→3', a-sense, b-antisense)</th>
<th>Nucleotide positions</th>
<th>Probes for hybridization (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-ras 236</td>
<td>a-ATGACTGGTACAACACTCTT</td>
<td>727–746</td>
<td>CAAGTCGGTATAGTGTTGA</td>
</tr>
<tr>
<td>c-ret, TK 155</td>
<td>a-AGGAACCTTGCTGCCTGCTTC</td>
<td>943–962</td>
<td>AGCCGAAATGTTGATAGTTC</td>
</tr>
<tr>
<td>c-ret, EC 184</td>
<td>a-CGCGCTAGGAAATCCAGGATTA</td>
<td>2654–2677</td>
<td>AGCCGAAATGTTGATAGTTC</td>
</tr>
<tr>
<td>ret/PTC1 165</td>
<td>a-GCTTTGGAGAACTTGCTTTT</td>
<td>1285–1306</td>
<td>GAGCCGACAGCCTAAGAGATC</td>
</tr>
<tr>
<td>ret/PTC2 202</td>
<td>a-GCTTTGGAGAACTTGCTTTT</td>
<td>1352–1374</td>
<td>GAGCCGACAGCCTAAGAGATC</td>
</tr>
<tr>
<td>ret/PTC3 242</td>
<td>a-AAGCGAcACCTGCCAGTG</td>
<td>1513–1535</td>
<td>GAGCCGACAGCCTAAGAGATC</td>
</tr>
</tbody>
</table>

a Nucleotide positions of the primers are based on the sequences deposited in GenBank: c-N-ras (accession no. X02751), c-ret (accession no. X12894), ret/PTC1 (accession no. M31213), ret/PTC2 (accession no. L03357), and ret/PTC3 (accession no. S71225).

The abbreviations used are: TK, tyrosine kinase; EC, extracellular.
ent TK over EC ret mRNA had evidence of either ret/PTC1, ret/PTC2, or ret/PTC3. These findings are summarized in Table 3. The frequency of specific types of ret rearrangement was significantly different between radiation-induced and sporadic tumors (χ² test, \( P < 0.03 \)). Specifically, ret/PTC1 was the most prevalent type in the sporadic group (test of proportions, \( P \) ret/PTC3 was the most prevalent type in radiation-induced tumors. This was particularly obvious for the solid variant, which constituted 37% of radiation-induced but only 4% of sporadic tumors. In addition, three cases of mixed variant tumors (composed of equal portions of solid and follicular pattern in two cases, and of a mixture of solid, follicular, and typical papillary patterns in one case) were found only in the radiation-induced group, making the predominance of solid growth pattern in this group even higher. The solid variant of papillary carcinoma, which is a rare finding in either children or in the adult population (21), is poorly characterized from the point of view of biological behavior and patient prognosis but is generally considered to be a more aggressive form of the disease. In contrast to radiation-induced neoplasms, tumors displaying the typical papillary pattern were characteristic of the sporadic group. In this respect, sporadic pediatric neoplasms are similar to papillary carcinomas in the adult population.

ret rearrangements were highly prevalent in both groups. However, the prevalence of specific types of rearrangement was markedly different; ret/PTC1 was the dominant form in sporadic tumors, and ret/PTC3 was the dominant form in radiation-induced neoplasms. Arguably, pediatric tumors from the United States are not the best control group for comparison with tumors found in Belarusian children. However, sporadic thyroid carcinomas were collected from children residing in two different areas in the United States. In addition, similar data on predominance of ret/PTC1 over ret/PTC3 types of rearrangement in sporadic pediatric thyroid carcinomas have been reported in Italian children and adolescents younger than 19 years of age (13). In contrast, the predominance of ret/PTC3 in post-Chernobyl pediatric carcinomas has been reported before in the series of 6 and 12 cases (9, 10).

The histological variants of papillary carcinoma exhibited strong correspondence with the type of ret rearrangement. Thus, among post-Chernobyl carcinomas, solid variant tumors exhibited a striking

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**Table 2 Prevalence of morphological variants of papillary carcinoma among radiation-induced and sporadic pediatric thyroid tumors**

<table>
<thead>
<tr>
<th>Variants of papillary carcinoma</th>
<th>No. of cases</th>
<th>Solid</th>
<th>Follicular</th>
<th>Typical papillary</th>
<th>Mixed</th>
<th>Diffuse sclerosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiation-induced</td>
<td>38 (100%)</td>
<td>14 (37%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11 (29%)</td>
<td>7 (18%)</td>
<td>3 (8%)</td>
<td>3 (8%)</td>
</tr>
<tr>
<td>Sporadic</td>
<td>23 (100%)</td>
<td>1 (4%)</td>
<td>4 (17%)</td>
<td>16 (70%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>2 (9%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> \( P = 0.006 \) in comparison with the prevalence of solid variant in sporadic tumors.

<sup>b</sup> \( P < 0.001 \) in comparison with the prevalence of typical papillary variant in radiation-induced tumors.
preponderance of ret/PTC3, which was detected in 79% of cases. In addition, all three mixed variant tumors, consisting of 30–50% of solid growth pattern also had ret/PTC3 rearrangements. In contrast, ret/PTC1 was associated more commonly with typical papillary carcinomas both in radiation-induced and sporadic groups. Almost one-third of carcinomas with typical papillary growth had no detectable rearrangements of ret.

These data indicate that different types of ret rearrangement are associated with distinct phenotypes of papillary carcinoma. This observation was not expected, because all types of ret rearrangement lead to the inappropriate expression of the intracellular domain of the ret receptor. This is thought to be a major mechanism of thyroid cell transformation (22, 23). The three types are formed by the fusion of the TK domain of ret to different 5' partners. ret/PTC1 is formed by a paracentric inversion of the long arm of chromosome 10, leading to fusion with a gene named H4/DJOSJ7O (24). ret/PTC3 is also a result of an intrachromosomal rearrangement and is formed by the fusion with the RFG/ELE1 gene also located on chromosome 10 (25, 26). ret/PTC2 is formed by a reciprocal translocation between chromosomes 10 and 17, resulting in the fusion of the TK domain of c-ret with 5' terminal sequence derived from the regulatory subunit of Ria cAMP-dependent protein kinase A (27). In all cases, fusion points are located within the intron bracketed by the transmembrane domain and the TK region of ret. The truncated fragment of ret lacks the transmembrane domain and has been shown to be located within the cytosol (28). The expression of this chimeric product is driven by the respective promoters of the genes upstream of the rearrangement. The contrasting phenotypes reported here between tumors harboring ret/PTC1 and ret/PTC3 translocation may be due to unique effects of the sequences upstream of the breakpoint or to the levels of activity of the ret/PTC1 and ret/PTC3 promoters, which may result in important differences in the absolute amounts of the truncated ret mRNA and protein. These data, therefore, point to important structural differences between the various rearranged forms of ret, which are worthy of further study.

### Table 3 Prevalence of ret rearrangements in radiation-induced and sporadic pediatric thyroid tumors

<table>
<thead>
<tr>
<th>TK positive</th>
<th>ret/PTC1</th>
<th>ret/PTC2</th>
<th>ret/PTC3</th>
<th>Novel ret/PTC ?</th>
<th>TK negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiation-induced</td>
<td>6 (16%)</td>
<td>1 (3%)</td>
<td>22 (58%)</td>
<td>4 (10%)</td>
<td>5 (13%)</td>
</tr>
<tr>
<td>Sporadic</td>
<td>8 (47%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>3 (18%)</td>
<td>1 (6%)</td>
<td>5 (29%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> P = 0.01 in comparison with the prevalence of ret/PTC3 in sporadic tumors.

<sup>b</sup> P < 0.05 in comparison with the prevalence of ret/PTC1 in radiation-induced tumors.
Five of the cases reported here had clear evidence of preferential expression of the TK fragment of ret, which was not due to either ret/PTC1, ret/PTC2, or ret/PTC3, suggesting that it may have resulted from novel forms of ret rearrangement. While this report was in preparation, a subtype of ret/PTC3 containing an additional 93 bp was reported and termed ret/PTC4 (29). However, it appears that at best only one of the five tumors may be explained by ret/PTC4 (data not shown), indicating that additional rearrangements of ret are present in tumors from this pediatric population.

The prevalence of ret rearrangements found in both pediatric groups is much higher than in adults. This invites the speculation that almost all thyroid carcinomas in children may be radiation induced. If this were the case, the differences observed in this study may be due to the fact that in the documented radiation-induced cases, the high-dose exposure led primarily to ret/PTC3 types of rearrangement and a solid growth pattern. The so-called sporadic tumors may have also been induced by radiation but by inadvertent exposure to lower doses that led to tumor development in children with a higher susceptibility to radiation-induced DNA damage, and are characterized primarily by ret/PTC1 types of rearrangement and a typical papillary growth pattern. Alternatively, only post-Chernobyl tumors may be truly radiation induced, and the high prevalence of ret rearrangements in the sporadic cases are due to alternative mechanisms of tumor initiation. Regardless of the manner in which these mutations take place, this study shows that the different types of ret rearrangements present in tumors from this pediatric population.

ACKNOWLEDGMENTS

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