Oncogenic Rearrangements of the RET Proto-Oncogene in Papillary Thyroid Carcinomas from Children Exposed to the Chernobyl Nuclear Accident

Laura Fugazzotta, Silvana Pilotti, Aldo Pinchera, Tatiana V. Vorontsova, Piera Mondellini, Italia Bongarzone, Angela Greco, Larisa Astakhova, Marta G. Butti, Eugene P. Demidchik, Furio Pacini, and Marco A. Pierotti

ABSTRACT

Since the Chernobyl nuclear reactor accident, a striking increase of thyroid carcinoma has been reported in children exposed to radiation in Belarus. Because of its unprecedented scale and its emotional implications, this finding has raised concern and called the attention of the scientific community to this major health problem. Although epidemiologically documented, a direct correlation between thyroid cancer and radiation exposure has not been definitely proven at the molecular level. On the assumption that ionizing radiation could cause specific and common cancer-associated genetic lesions, an analysis of oncogene activation and/or tumor suppressor gene inactivation would help to define radiation-induced thyroid carcinomas.

Therefore, we have analyzed by different molecular approaches, including Southern blotting, DNA transfection assay on NIH-3T3 cells, and reverse transcription-PCR analysis, six papillary carcinomas from children living in the region of Belarus at the time of the Chernobyl nuclear accident to identify tumor-specific gene rearrangements of the proto-oncogenes RET and TRK, previously found activated in a tumor type-specific manner in papillary thyroid carcinoma. Using Southern blot analysis in four cases, we could detect specific rearranged bands indicating an oncogenic activation of RET that in three cases resulted in rearranged sequences provided by the same activating gene. Moreover, the DNA of the last three cases showed a biological activity in transforming NIH-3T3 cells after the DNA-mediated transfection assay, and the respective NIH-3T3 transfectants were found to express the oncogenic fusion transcripts.

These results support the possibility that RET oncogenic activation could represent a major genetic lesion associated with thyroid carcinoma in children exposed to the Chernobyl nuclear accident.

INTRODUCTION

The greatly increased number of thyroid carcinomas reported in children who were living in southern Belarus at the time of the Chernobyl nuclear accident has aroused a number of concerns regarding both a true increase in the incidence of thyroid cancer and its link to the disaster (1–3). Relevant answers to these questions could be provided by an analysis of oncogene activation in these thyroid tumors because of the possibility that radiation may lead with high frequency to specific genetic lesions. Potential candidates for such a role are the oncogenes derived from the activation of the tyrosine kinase receptor genes RET and TRK, which are found rearranged with significant frequency in papillary thyroid carcinomas (4). In particular, the human RET proto-oncogene was discovered following its oncogenic activation by gene rearrangement that occurred in vitro during transfection experiments (5). Subsequently, through the same technique, specific oncogenic RET rearrangements occurring at the somatic level in tumor cells have been identified and characterized in papillary thyroid carcinoma (4, 6). So far, three different oncogenic versions of RET have been characterized and designated as RET/PTC.

MATERIALS AND METHODS

Patients and Tumors. Table 1 gives a description of the characteristics of the six patients.

Microscopically, all of the tumor specimens examined on frozen sections were consistent with a diagnosis of papillary carcinoma. In two cases (patients 2 and 6), the tumor component represented <20% of the sample and in patient 3 an extensive fibrosis was present.

Southern Blot Analysis and Transfection Assay. Ten μg DNA were digested with EcoRI, blotted, and hybridized with the following 32P molecular probes: probe 1, 1-kb BglII-BamHI fragment specific for the 6.7-kb EcoRI fragment of proto-RET (7); probe 2, 1.8-kb BamHI-BamHI fragment related to the 5′ end of proto-RET (7); probe 3, 0.8-kb EcoRI cDNA fragment encompassing the ELE1/RET breakpoint (15); and probe 4, 0.8-kb fragment related to the 5′ end of the R12 intron where the two previously characterized oncogenic rearrangements of R12 occurred (9).

The transfection assay on NIH-3T3 cells was performed as previously described (14) using 16–40 μg of high molecular weight DNA.

RNA Amplification and Oligonucleotide Primers. RT-PCR analysis was performed with 2 μg RNA from each sample that was reverse transcribed and amplified as previously described (15). PCR primers were synthesized on a DNA synthesizer (Applied Biosystems, Foster, CA), and the sequences were as...
Table 1 RET rearrangements in PTCs from children exposed to the Chernobyl nuclear accident

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at the moment of the accident (4-26-1986)</th>
<th>Region of Belarus</th>
<th>Age at surgery (yr)</th>
<th>TNM$^a$</th>
<th>RET rearrangement</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Female</td>
<td>14 mo</td>
<td>Gomel</td>
<td>6</td>
<td>T$_2$N$_1$BM$_0$</td>
<td>RET/PTC3$^b$</td>
</tr>
<tr>
<td>C2</td>
<td>Female$^c$</td>
<td>7 yr</td>
<td>Brest</td>
<td>13</td>
<td>T$_1$N$_1$AM$_0$</td>
<td>None</td>
</tr>
<tr>
<td>C3</td>
<td>Female$^d$</td>
<td>8 yr</td>
<td>Mogilev</td>
<td>14</td>
<td>T$_1$N$_1$BM$_0$</td>
<td>None</td>
</tr>
<tr>
<td>C4</td>
<td>Male</td>
<td>5 yr</td>
<td>Gomel</td>
<td>11</td>
<td>T$_1$N$_1$BM$_0$</td>
<td>RET/PTC2.</td>
</tr>
<tr>
<td>C5</td>
<td>Female</td>
<td>8 yr</td>
<td>Minsk</td>
<td>14</td>
<td>n.a.$^e$</td>
<td>RET/PTC3$^a$</td>
</tr>
<tr>
<td>C6</td>
<td>Female$^c$</td>
<td>7 yr</td>
<td>Brest</td>
<td>13</td>
<td>T$_1$N$_0$M$_0$</td>
<td>RET/PTC3$^a$</td>
</tr>
</tbody>
</table>

$^a$ Microscopically, all of the thyroid tumor specimens examined on frozen section were consistent with a diagnosis of papillary carcinoma.
$^b$ Transforming activity confirmed by DNA transfection on NIH-3T3 cells.
$^c$ Tumor component <20%.
$^d$ Stromal component >50%.
$^e$ n.a., not available.

follows: primer 1, 5'-CTTTCAGCATCTTCACGG-3' and primer 2, 5'-TGGAAGAGGAGCTGTATC-3'.

Fig. 2B shows the approximate localization of the two primers based on the published sequence of RET/PTC3 cDNA (15).

RESULTS

Southern Blot Analysis to Detect RET and TRK Gene Rearrangement The presence of oncogenic RET and/or TRK rearrangements was initially investigated by Southern blot analysis in tumor DNA from six children living in the region of Belarus at the moment of the Chernobyl nuclear accident (see Table 1 for a description of patient characteristics). No rearranged bands were identified in the blot probed with TRK-related sequences (data not shown). Fig. 1A shows the restriction pattern obtained by using EcoRI-digested DNAs, and as a RET-related probe a 1-kb BgIII-BamHI fragment of proto-RET that is able to detect the region within the RET gene where all of the oncogenic rearrangements have been found to occur (15). Besides the RET germ line band of about 6.7-kb, four cases (C1, C4, C5, and C6) showed an additional band of different size, indicating a RET rearrangement. In keeping with our previous results, these abnormal bands correspond to activated alleles of proto-RET (14). By analyzing in Southern blot a number of human papillary thyroid tumor DNAs, we found that in most cases the probe 2 in Fig. 1E specific for the 5' region of the RET locus was able to detect tumor-specific RET rearranged bands, corresponding to the reciprocal event of the RET oncogenic recombination. Therefore, to better characterize the present cases, the presence of the predicted products of the reciprocal rearrangements was also investigated. As shown in Fig. 1B in tumor DNA

Fig. 1. Southern blot analysis of RET rearrangements in six PTCs of children living in the region of Belarus (A-D) and schematic representation of the molecular probes used (E). Germ-line (−) and rearranged (−) bands are indicated. The additional bands seen in B and C with 5' end proto-RET and ELE1-related probes, respectively, are due to still uncharacterized cross-hybridizing sequences also present in normal control DNA (15). Lanes C1–C6, tumor DNAs; lane NC, human normal control DNA.
of Cl, C4, C5, and C6, probe 2 detected, besides the germ line 6.7-kb band and a high molecular weight band due to cross-hybridizing sequences (also present in normal control DNA, lane NC), additional rearranged bands that were not present in normal human control. This finding indicates that the amino terminal part of the RET proto-oncogene was rearranged and not deleted in these tumors.

To determine the identity of the gene(s) rearranged with RET in Chernobyl tumors, the same blot was sequentially hybridized with probes related to the different RET-activated genes, so far identified. In particular, using an ELE1-specific probe (probe 3, Fig. 1E) we identified in both tumors and control DNAs several bands due to sequences representing the germline configuration of ELE1 (16-kb band) and to still uncharacterized cross-hybridizing sequences (Ref. 15 and Fig. 1C). The same probe detected in tumors Cl, C5, and C6 additional bands (9.0 kb in Cl; 13 kb in C5; 8.5 kb in C6) are identical in size to those identified by probe 1 in the same tumors (Fig. 1A). The RET/PTC3 oncogene was therefore identified in these three Chernobyl tumors. Similarly, we demonstrated that the RET rearranged band of tumor C4 was due to a rearrangement with RIA sequences, thus indicating the occurrence of a RET/PTC2 oncogene (Fig. 1D and Ref. 16).

These molecular data are in keeping with the previously reported findings that a chromosomal inversion and a balanced reciprocal translocation generated RET/PTC3 and RET/PTC2 oncogenes, respectively (8, 9).

**Transfection Assay and RT-PCR Analysis of NIH-3T3 Transfectants.** The tumor of the cases Cl, C5, and C6 yielded adequate high molecular weight DNA to confirm the already reported relationship between RET rearrangements and transforming activity of tumor DNA (6, 14). Following the standard procedures, 16–40 μg of high molecular weight DNA were transfected onto NIH-3T3 cells. After 2 weeks, transformation foci were scored (on average, 0.033 foci/μg DNA), and three foci, positive for Alu repetitive sequences for each tested DNA were collected and expanded as cell lines.

Fig. 2 shows the results of a RT-PCR experiment where the presence of the chimeric transcripts ELE1/RET, relative to the RET/PTC3 oncogene, in a representative NIH-3T3 transfectant from cases Cl and C6 was demonstrated by the detection of an amplified specific band of 329 bp representing the fusion region between ELE1 and RET sequences (Ref. 15 and Fig. 2). In case C5 the amplified band resulted in about 400 bp, thus indicating a different breakpoint presently under investigation (data not shown).

**DISCUSSION**

Since 1990 a great frequency of thyroid cancer in children from Belarus has been reported and related to the accident at Chernobyl (1). Moreover, a direct relationship between the radiation exposure from the nuclear disaster and the increased incidence of thyroid carcinoma has been also supported on clinical and histopathological ground (2). Although obtained in a small number of cases (six), our results definitely show that a biologically assessed RET oncogenic activation could be associated with the papillary thyroid carcinomas in children from the region of Belarus (four of six). In addition, we have recently examined an additional group of six thyroid tumors from Chernobyl children and found the presence of RET rearranged bands in three of them as assessed by Southern blot (data not shown). The DNA status of the latter samples has not allowed their use in the transfection assay; the nature of the rearranging partner gene(s) is not therefore yet identified. Moreover, Ito et al. (17) reported essentially the same results by showing, following RT-PCR of RNA extracted from formalin-fixed, paraffin-embedded blocks of seven PTC from children living in the Chernobyl contaminated area, the presence of RET amplification bands in four cases (17). Altogether, the present results and the results of Ito et al. (17) indicate that 11 of the 19 Chernobyl tumors examined showed a RET activation, apparently in keeping with the results obtained by in vitro irradiation of human undifferentiated thyroid carcinoma cells (11). However, a definitive evidence that exposure to radiation has directly resulted in the rearrangement of the RET gene and consequently in its oncogenic activation is still lacking. Clearly, more cases need to be investigated to support a direct cause-effect relationship between nuclear irradiation and RET activation as suggested by these preliminary observations.

In this context it is worth noting that following a review of our cases of PTCs, previously characterized for RET and TRK activation (4), we have identified five under 16-year-old patients. Two cases of RET (one RET/PTC1 and the other RET/PTC3) and one of TRK activation were scored, indicating a similar ratio (about 2:1) of RET versus TRK activation found in adult papillary carcinomas (4). Therefore, the question of whether, and to what extent the age per se rather than radiation, or the combination of both, is responsible for a RET activation remains to be answered. To this purpose, a more extensive analysis of thyroid tumors from Chernobyl exposed children and of a sufficient number of sporadic tumors, preferably from the same area and arising in the same genetic background, with no obvious radiation associated etiology is needed. The extreme rarity of sporadic childhood thyroid carcinomas will however make difficult this comparison. Our past experience in molecular genetics of PTC outlines several peculiarities in the present group of tumors. First, the percentage of carcinomas with RET activation significantly varies in cases collected in different countries from 2.5 to 6% in Saudi Arabia and Japan patients (18, 19) to about 35% in our tumor collection, 18 RET-
positive tumors of 52 examined including the 2 positive in the group of the 5 under 16 year-old patients (4). In Chernobyl tumors, RET activation is the highest reported accounting for about 60% of the cases (our results and Ito et al.'s (17) results). Second, in all of the available studies the most frequently detected RET rearrangement involves the activating gene H4/D10S170 (RET/PTC1; Ref. 7). In three of the four positive cases, completely characterized here, the RET/PTC3 oncogene (ELE1/RET) is found. The peculiarity of RET/PTC3 holds on the fact that the two rearranging genes are located within the same chromosomal band 10q11.2 (8). Therefore, among the different available examples of RET activation, the inversion generating the fusion between the closely located ELE1 and RET genes represents the more conserved chromosomal rearrangement event. In conclusion, the present results along with those obtained by Ito et al. (17), the previous observation of RET activation in a patient with a documented radiation exposure (10) and the reported induction of RET rearrangements by \textit{in vitro} irradiation of tumor cell lines (11) seem to indicate that the RET oncogenic activation could be a relevant genetic change associated with the thyroid tumor in children exposed to the Chernobyl nuclear accident.

\textbf{ACKNOWLEDGMENTS}

We are grateful to Professor E. D. Williams for reviewing the pathology. The assistance of Mario Azzini, Giovanna Raineri, and Anna Grassi is also acknowledged.

\textbf{REFERENCES}

Oncogenic Rearrangements of the RET Proto-Oncogene in Papillary Thyroid Carcinomas from Children Exposed to the Chernobyl Nuclear Accident

Laura Fugazzola, Silvana Pilotti, Aldo Pinchera, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/55/23/5617

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.