Low prevalence of BRAF mutations in radiation-induced thyroid tumors in contrast to sporadic papillary carcinomas

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Abstract

Point mutations of the BRAF gene have been recently described with high prevalence in papillary thyroid carcinomas. However, this molecular alteration has not been studied in radiation-induced thyroid tumors. We analyzed the prevalence of BRAF point mutations and RET/PTC rearrangements in 55 post-Chernobyl papillary carcinomas, compared with 82 sporadic papillary carcinomas. Radiation-induced tumors demonstrated a low prevalence (4%) of BRAF point mutations and high prevalence (58%) of RET/PTC rearrangements. Sporadic papillary carcinomas revealed a clearly distinct pattern, with 37% of tumors harboring BRAF mutations and 20% RET/PTC rearrangements. These results demonstrate a significant difference in the molecular genetic profile of sporadic and radiation-induced thyroid tumors.

Keywords: Thyroid cancer; BRAF mutation; RET/PTC Rearrangement; Radiation exposure

1. Introduction

Activating point mutation of the serine/threonine kinase BRAF have been recently described in papillary thyroid carcinomas with the prevalence ranging from 29 to 69% [1–6]. All mutations identified so far affect nucleotide 1796 in exon 15, resulting in a thymine-to-adenine transversion, which translates into a valine-to-glutamate substitution at residue 599 (V599E).

Exposure to ionizing radiation is a well known risk factor for thyroid cancer, particularly for papillary carcinoma. In April 1986, an accident at the Chernobyl Nuclear Power Station in the former

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USSR led to a dramatic increase in the incidence of childhood thyroid cancer in contaminated areas of Belarus, Ukraine, and western Russia [7–9]. Because of the low prevalence of thyroid tumors in children and young adults, this tragic disaster has created one of the richest paradigms of radiation-induced thyroid neoplasms. Most post-Chernobyl tumors were papillary carcinomas and had an unusually high prevalence of RET/PTC rearrangements, which were found in 67–87% of tumors removed 5–8 years after exposure and in 49–65% of those removed 7–11 years after the accident [10–15].

The prevalence of BRAF mutations has not been studied in radiation-induced tumors. In this study, we determined the prevalence of BRAF mutations as well as RET/PTC rearrangements in a large series of post-Chernobyl papillary carcinomas, and compared those with sporadic papillary carcinomas.

2. Material and methods

2.1. Tumor samples and nucleic acid extraction

We analyzed a series of 55 post-Chernobyl papillary carcinomas, which included 34 tumors operated in 1991–1992 in Minsk, Belarus and 21 tumors removed in 1995–1998 in the Institute of Endocrinology and Metabolism in Kiev, Ukraine. A control group was composed of 82 cases of sporadic papillary carcinomas, i.e. tumors from patients without radiation history. Among radiation-induced tumors from Belarus, the age of patients at surgery ranged from 6 to 20 years. These patients were between one month and 14 years of age at the time of radiation exposure. Among tumors from Ukraine, the age of patients ranged from 12 to 31 years at surgery, and from nine months to 17 years at exposure. Tissues from 82 papillary carcinomas from the control group were collected at the University Hospital in Cincinnati and through the Cooperative Human Tissue Network. The age of patients in this group ranged from 9 to 77 years. All tumors were diagnosed as papillary thyroid carcinomas based on accepted histologic criteria, except for two radiation-associated cases from Ukraine that were diagnosed as well differentiated thyroid carcinoma.

DNA and RNA were extracted from paraffin-embedded tissue (34 cases) or from frozen tissue (103 cases). Genomic DNA was isolated using proteinase K digestion, phenol–chloroform extraction and ethanol precipitation as previously described [16]. RNA isolation from snap frozen and paraffin embedded tissue was performed using Trizol reagent (Invitrogen) as previously described [17] or using the RNA extraction midi Kit (Qiagen).

2.2. Detection of BRAF mutations

In 34 radiation-induced tumors from Belarus and in 82 sporadic cases, mutations in the BRAF gene were detected using LightCycler PCR and fluorescence melting curve analysis (FMCA) as previously described [18]. Briefly, we used a pair of primers flanking the mutation site and two internal fluorescent probes, with the sensor probe spanning the nucleotide position 1796. Amplification was performed using 100 ng of DNA in a 20 μl volume containing 2 μl of 10 × LightCycler DNA Master Hybridization Probes (Roche), 1.6 μl of 25 mM MgCl2, 40 pmol or each primer, and 2 pmol of each hybridization probe. The reaction mixture was subjected to 45 cycles of rapid PCR (94 °C for 1 s, 55 °C for 20 s, 72 °C for 10 s).

Post-amplification FMCA was performed by gradual heating of samples at a rate of 0.2 °C/sec from 45 to 95 °C. Normal placental DNA was used as a negative control and DNA from a tumor sample with the known BRAF nucleotide 1796 mutation served as a positive control. All PCR products that showed deviation from the wild-type (placental DNA) melting peak were sequenced to verify the presence of mutation.

In 21 tumor samples from Ukraine, the detection was performed by direct sequencing of exons 11 and 15 of BRAF cDNA. Two microgram of RNA was reverse transcribed and used in a PCR reaction to amplify exons 11 and 15 of the BRAF gene as previously described [18]. Amplification was carried out for 40 cycles with a thermal cycler (Perkin Elmer) (94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min). PCR products were visualized by 2% agarose gel electrophoresis. PCR products were sequenced using an automated sequencer (Sequenase, USB, Cleveland, Ohio).
2.3. Detection of RET/PTC rearrangements

In 34 radiation-induced tumors from Belarus and in 82 sporadic cases, 3 μg of total RNA extracted from frozen tissue and one-forth (5 μl) of RNA extracted from paraffin-embedded tissue were reverse transcribed in a volume of 20 μl using random hexamers priming and Superscript II RT (Gibco BRL). All cDNA samples were tested to assess the adequacy of extracted RNA by amplifying a 236 bp sequence of the N-RAS gene using intron-spanning primers. cDNA samples in which N-RAS mRNA was detected were considered adequate for further analysis, and were studied by PCR for the two most common types of RET/PTC rearrangement, RET/PTC1 and RET/PTC3, as previously reported [17].

In 21 tumor samples from Ukraine, 500 ng of RNA were reverse transcribed and subjected to 40 cycles of PCR (Perkin–Elmer, Norwalk, Connecticut) (94 °C for 30 s, 55 °C for 2 min and 72 °C for 2 min) with primers specific for RET/PTC1 and RET/PTC3 rearrangement, as previously reported [19]. The products were analyzed on a 2% agarose gel and hybridized with an internal RET-specific oligonucleotide probe. The amplified products were sequenced to confirm the presence of rearrangement.

2.4. Statistical analysis

Comparison between two groups was performed using the Student’s t-test for continuous data, two-tailed Fisher exact test in cases where the numbers in the cell were less than five, and standard χ² test in all other cases. The difference between two values was considered significant when the probability of P was less than 0.05.
3. Results

3.1. Prevalence of BRAF mutations

In the group of 55 radiation-associated papillary carcinomas, two (4%) BRAF mutations were identified. Both cases were papillary carcinomas from Ukraine, and revealed T-to-A transversion at nucleotide 1796 (Fig. 1). No other mutations in the BRAF gene were found in exons 11 and 15 of BRAF in tumors from Ukraine by sequencing of cDNA, and in tumors from Belarus by LightCycler PCR and FMCA (Fig. 2). The tumors samples with BRAF mutations were from patients that were 12 and 25 years old at the time of surgery, and one month and 12 years old at the time of exposure, respectively. The younger patient had a papillary carcinoma composed of follicular and solid areas, which presented with extrathyroidal extension, regional lymph node involvement and distant metastasis. The other tumor was a papillary carcinoma with solid and papillary areas and no aggressive features.

In the group of sporadic papillary carcinomas, 30 (37%) tumors were positive for the T-to-A mutation at nucleotide 1796. The presence of BRAF mutation in the patients without radiation history correlated with older age, classical papillary or tall cell tumor histology, high rate of lymph node metastasis, and extrathyroidal extension, similar to the finding we recently observed in a large series of sporadic papillary carcinomas [18].

3.2. Prevalence of RET/PTC rearrangements

In the group of post-Chernobyl papillary carcinomas, 32 (58%) RET/PTC rearrangements were totally identified (Table 1). Twenty-six of them were RET/PTC3 and six were RET/PTC1 (Fig. 3). Tumors with RET/PTC3 rearrangements were typically solid and follicular variant of papillary carcinomas, whereas tumors with RET/PTC1 type were classic papillary or follicular variants of papillary carcinoma.

Among sporadic papillary carcinomas, 16 (19%) cases were found to harbor RET/PTC rearrangement, including 10 (12%) RET/PTC1 and 6 (7%) RET/PTC3 rearrangements.

3.3. Statistical analysis

The prevalence of genetic alterations in the two groups were summarized in Table 2. Radiation-induced and sporadic thyroid tumors had dramatic difference in the incidence of BRAF mutations and RET/PTC rearrangements, which showed strong statistical significance ($P < 0.0001$). The difference in RET/PTC was exclusively due to the RET/PTC3 type of rearrangement, since the prevalence of RET/PTC1 was comparable in both groups. In addition to radiation history, the two groups differed in the average age of patients, which was $14.1 \pm 8.2$ years in the radiation-associated group and $39.5 \pm 16.4$ years in the sporadic group ($P < 0.0001$).

4. Discussion

Previous studies of thyroid carcinomas in children and young adults exposed to radiation after the Chernobyl accident have established that these radiation-associated tumors have a significantly higher prevalence of RET/PTC rearrangement, especially of the RET/PTC3 type [10–15]. In this study, we confirm this association, and demonstrate

<table>
<thead>
<tr>
<th>Tumors from Belarus $n = 34$</th>
<th>Tumors from Ukraine $n = 21$</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET/PTC1</td>
<td>4 (12%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>RET/PTC3</td>
<td>20 (59%)</td>
<td>6 (29%)</td>
</tr>
<tr>
<td>Total</td>
<td>24 (71%)</td>
<td>8 (38%)</td>
</tr>
</tbody>
</table>

Fig. 3. PCR-based detection of RET/PTC1 and RET/PTC3 rearrangements in post-Chernobyl tumors from Ukraine. $\beta$-actin-control of RNA quality and amplification.
that by contrast, the most common genetic change associated with sporadic papillary carcinomas, \textit{BRAF} point mutations, is present infrequently in radiation-associated papillary carcinomas. These data provide additional evidence for the unique nature of the genetic changes involved in radiation carcinogenesis in the thyroid gland.

The search for \textit{BRAF} mutations in this study was restricted to the functionally active regions of the gene previously found to be affected, i.e. exon 15 (34 cases) or exons 11 and 15 (21 cases). Therefore, we cannot rule out the possibility that mutations in other, less functionally important regions of the gene, may occur in these radiation-associated tumors.

Our results provide additional clear evidence that the \textit{RET}/PTC \textit{B} type of rearrangement is responsible for the difference in \textit{RET}/PTC prevalence between the two groups, whereas \textit{RET}/PTC \textit{C} was present at a similar rate among sporadic and radiation-associated tumors. It is important to realize, however, that an additional difference between the two groups of papillary carcinomas analyzed in this study was that radiation-associated tumors were in significantly younger patients. Although the age of tumor presentation is by itself determined by the radiation history, it remains unclear whether or not younger age is an independent factor influencing the low rate of \textit{BRAF} mutations in this group. In our series of sporadic tumors, the number of pediatric cases was too small to test this possibility.

Interestingly, among 48 \textit{RET}/PTC positive tumors and 32 \textit{BRAF} positive tumors in both groups, only three (4%) cases showed the occurrence of two genetic events in the same tumor. This was observed in two sporadic and one radiation-induced tumor, all of which revealed both \textit{BRAF} point mutation and \textit{RET}/PTC \textit{C} rearrangement. The fact that 96% of tumors harbored either one or another alteration, provide additional evidence that these molecular alterations are likely to activate effectors along the same signaling pathway, leading to the activation of mitogen-activated protein kinase (MAPK) cascade [1]. The results of this study suggest that radiation exposure and etiologic factors involved in sporadic thyroid carcinogenesis act by affecting in a distinct manner intermediates along the MAPK signaling pathway, all resulting in the promotion of papillary thyroid carcinoma.

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\section*{References}


\begin{table}
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\begin{tabular}{|l|c|c|c|}
\hline
 & Radiation-associated & Sporadic & \textit{P} value \\
\hline
\textit{BRAF} point mutations & 2 (4\%) & 30 (37\%) & <0.0001 \\
\textit{RET}/PTC rearrangements (total) & 32 (58\%) & 16 (19\%) & <0.0001 \\
\textit{RET}/PTC \textit{C} & 6 (11\%) & 10 (12\%) & 1.0 \\
\textit{RET}/PTC \textit{C} & 26 (47\%) & 6 (7\%) & <0.0001 \\
\hline
\end{tabular}
\caption{Correlation between the prevalence of \textit{BRAF} mutation and \textit{RET}/PTC rearrangements in sporadic and radiation-associated thyroid cancer}
\end{table}


