CLINICAL STUDY

Genetic alterations in thyroid tumors from patients irradiated in childhood for tinea capitis treatment

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Abstract

Objective: Exposure to ionizing radiation at young age is the strongest risk factor for the occurrence of papillary thyroid carcinoma (PTC). *RET/PTC* rearrangements are the most frequent genetic alterations associated with radiation-induced PTC, whereas *BRAF* and *RAS* mutations and *PAX8–PPARG* rearrangement have been associated with sporadic PTC. We decided to search for such genetic alterations in PTCs of patients subjected in childhood to scalp irradiation.

Design: We studied 67 thyroid tumors from 49 individuals irradiated in childhood for tinea capitis scalp epilation: 36 malignant (12 cases of conventional PTC (cPTC), two cPTC metastases, 20 cases of follicular variant PTC (FVPTC), one oncocytic variant of PTC and one follicular carcinoma) and 31 follicular thyroid adenomas.

Methods: The lesions were screened for the *BRAF*^{V600E} and *NRAS* mutations and for *RET/PTC* and *PAX8–PPARG* rearrangements.

Results: *BRAF*^{V600E} mutation was detected in seven of 14 (50%) cPTC and two of 20 FVPTC (10%) ($P=0.019$). *NRAS* mutation was present in one case of FVPTC (5%). *RET/PTC1* rearrangement was found, by RT-PCR, in one of 17 cases (5.9%) and by fluorescence *in situ* hybridization in two of six cases (33%). *PAX8–PPARG* rearrangement was not detected in any carcinoma. None of the follicular adenomas presented any of the aforementioned genetic alterations.

Conclusions: The prevalence of *BRAF*^{V600E} mutation in our series is the highest reported in series of PTCs arising in radiation-exposed individuals. The prevalence of *RET/PTC1* rearrangement fits with the values recently described in a similar setting.

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Introduction

Exposure to ionizing radiation at young age is known as the strongest risk factor for thyroid carcinoma development, namely papillary thyroid carcinoma (PTC) (1), which is the most frequent histotype representing 80% of all thyroid tumors. Thyroid is very sensitive to both external and internal radiation, being the only organ with a documented major rise in cancer incidence after radiation exposure, especially in childhood (2). However, the data on the effects of low-dose radiation remain limited (3).

X-ray scalp epilation for tinea capitis treatment, applying a mean average dose to the thyroid gland of 9.3 cGy (range 4.5–49.5 cGy), has shown to increase thyroid cancer risk (4). It is not yet known whether scalp radiation-associated thyroid cancers have the same genetic alterations and clinical behaviour as the sporadic thyroid cancers (5).

Four mutation types constitute the majority of known mutations in thyroid cancer and carry the highest impact on tumor diagnosis and prognosis: *BRAF* and *RAS* point mutations and *RET/PTC* and *PAX8–PPARG* rearrangements (6). It was advanced that the dominant mutation mechanism in radiation-induced tumors is chromosomal rearrangement, whereas point mutation is the most prevalent mechanism in sporadic tumors (1). In radiation-induced tumors, namely in post-Chernobyl PTCs, *RET/PTC* rearrangements can be detected in up to 84% of PTCs (1, 7, 8) and up to 52% of thyroid adenomas (7, 8, 9, 10), but some studies showed lower values, 34–51% (11, 12), and 43% in a population exposed to external radiation (10). In sporadic, non-irradiated thyroid tumors, *RET/PTC* rearrangement frequency can be as low as 5–15% (7). *PAX8–PPARG* fusion oncogene has been identified as more prevalent in follicular carcinoma (13), but was also found in 1–38% of cases of follicular variant of PTC

(FVPTC) (14, 15, 16), and in 2–33% of cases of follicular adenoma (15, 16).

At variance with radiation-induced tumors, the most prevalent mechanism in sporadic tumors is point mutation, typically involving the *BRAF* and *RAS* genes (1). *BRAF* mutations occur in ~45–53% of adult sporadic PTCs (17, 18), being considered as rare events in subjects exposed to radiation in children, both internal and external, with frequencies ranging from 4 to 24% (12, 19, 20). Recently, however, Dinets *et al.* (11) reported a *BRAF* mutation prevalence of 37% in a recent study of post-Chernobyl cases. These authors also detected the co-occurrence of *RET/PTC* and *BRAF* mutation (11). In atomic bomb survivors, Hamatani *et al.* (21) found that *RET/PTC* rearrangement, and not *BRAF* mutations, were closely associated with radiation-associated adult-onset PTC.

RAS mutations have been detected in 10–20% of PTCs, virtually all belonging to the follicular variant (22) and in 20–40% of follicular adenomas (15). *RAS* can present three isoforms being the *NRAS* isoform predominantly mutated in thyroid tumors (23). No major difference has been associated with the radiation in comparison with the sporadic setting (24). Recently, Leeman-Neill *et al.* (25) reported an 8% prevalence of *RAS* mutations in a post-Chernobyl cohort.

The aim of this study was to evaluate the aforementioned genetic alterations in PTCs following X-ray external radiation performed in childhood to induce scalp epilation for tinea capitis treatment, a cohort in which we have already shown a high prevalence of thyroid cancer (26). As far as we know, only *RET/PTC* rearrangements have been studied in similar cohorts (10).

Subjects and methods

Cases

We have clinically observed 1287 individuals from an original cohort of 5356 individuals who had been submitted in childhood to scalp irradiation for tinea capitis treatment (26). Briefly, the standard treatment was done according to the Kienbock–Adamson technique (27) using one X-ray epilation session (325–400R); 6% of the 5356 individuals ($n=318$) were submitted to two or three sessions (irradiation dose $\geq 630R$) (26).

Thirty-five patients with thyroid cancer and 38 patients with follicular adenoma were identified. Sixty-seven thyroid tumors from 49 participants were retrieved from several pathology departments and laboratories and were histologically re-evaluated by two pathologists (M S-S and T N). Formalin-fixed and paraffin-embedded (FFPE) blocks from 24 of the 35 patients with thyroid carcinoma (69%) and from 28 of the 38 patients with follicular adenoma (74%) were examined. Some patients presented more than one

lesion ($n=9$), leading to a total of 36 thyroid carcinomas and 31 follicular adenomas.

All the procedures were performed under strict ethical and confidentiality procedures according to the Portuguese ethical rules. The study was approved by the Ethics Committee of the Hospital Pedro Hispano and all the patients signed an informed consent.

Identification of *BRAF* and *NRAS* mutations

Genomic DNA was extracted from microdissected FFPE pathological tissue, using 10 μ m sections, by conventional overnight incubation with proteinase K. Subsequent DNA purification was performed using Invisorb Spin Tissue Mini Kit (Invitex, Berlin, Germany) according to the manufacturer's protocol.

The following specific PCR primers were used to flank the mutational hotspot region of *BRAF* activation segment (exon 15) and *NRAS* exon 2: forward primer for *BRAF*, 5'-TCATAATGCTTGCTCTGATAGGA-3' and the reverse primer, 5'-GGCCAAAAATTTAATCAGTGG-3'; for the *NRAS* exon 2 amplification, the forward primer used was 5'-GATTCCTTACAGAAAACAAGT-3' and the reverse was 5'-GAAAATAATGCTCCTAGTAC-3'. All the procedures, including automated sequencing, were performed as described previously (16).

Briefly, PCR amplifications were performed in a 25 μ l volume reaction containing 25–100 ng genomic DNA, 200 μ M each dNTP, 0.1 μ g of each, forward and reverse, primer, 1 $\times$ GoTaq Flexi Buffer (Promega), 2.5 mM $MgCl_2$ and 0.75 U of *Taq* DNA polymerase (Promega). Reaction mixtures were submitted to 40 amplification cycles preceded by a *Taq* activation step at 95 °C for 5 min, each cycle comprised a denaturation step at 95 °C for 30 s, annealing at 58 °C for 40 s and extension at 72 °C for 45 s followed by a final extension at 72 °C for 10 min. PCR products were then purified using enzymatic digestion with Exonuclease I (Fermentas, Vilnius, Lithuania) and Shrimp Alkaline Phosphatase (Fermentas) and sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Kit (Perkin–Elmer, Foster City, CA, USA) and an ABI Prism 377 DNA Sequencer (Perkin–Elmer). All the detected mutations were further validated by a new independent amplification and sequencing.

Identification of *RET/PTC* and *PAX8–PPARG* rearrangements

Total RNA was extracted from paraffin-embedded tissues using the RecoverAll Nucleic Acid Isolation Kit for FFPE (Ambion – Life Technologies, Foster City, CA, USA) according to the manufacturer's instructions.

The cDNA was obtained using the RevertAid Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA) at 37 °C for 60 min, followed by enzyme inactivation at 99 °C for 5 min. The PCR using cDNA was performed with *HotStart Taq* DNA polymerase kit (Qiagen Co.) and initiated by denaturation at 95 °C for 15 min followed

Figure 1 Photomicrographs of haematoxylin-stained slides showing histopathological findings. (A) cPTC presenting *RET/PTC1* rearrangement detected by RT-PCR and FISH; (B) FVPTC presenting *RET/PTC1* rearrangement detected only by FISH; (C) cPTC presenting *BRAF^{V600E}* mutation (100×).

by 30 cycles of 30 s at 95 °C, 75 s at 56–60 °C and 30 s at 72 °C, before a final elongation step at 72 °C for 6 min. The RT-PCR products were analyzed by 2% agarose gel electrophoresis. PCRs creating a visible band on the gel were sequenced directly (ABI 3730 DNA analyser; Applied Biosystems by Life Technologies). The housekeeping *GAPDH* gene was also amplified to control the cDNA integrity of the samples. Primers for cDNA amplification: *RET-PTC1* rearrangement: 5'-GTCTGGGGGGGCATTGTCATCT (*CCDC6*, exon 1), 3'-AAGTTCTTCCGAGGGAATTC (*RET*, exon 12). *RET-PTC3* rearrangement: 5'-TGGAGAAGAGAGGCTGTATC (*NCOA4*, exon 6); 3'-CTTTCAGCATCTTCACGG (*RET*, exon 12). *PAX8-PPARG* rearrangements: *PAX8-PPARG* (F7): 5'-AACCTCTCGACTCACCAGAC (*PAX8*, exon 7), 3'-TGGGAGTGGTCTTCCATTACG (*PPARG*, exon 1); *PAX8-PPARG* (F8): 5'-CCCTTCAATGCCTTTCCTCC, 3'-TGGAGTGGTCTTCCATTACG (*PPARG*, exon 1); *PAX8-PPARG* (F9): 5'-CTATGCCTCCTCTGCCATC (*PAX8*, exon 9), 3'-TGGGAGTGGTCTTCCATTACG (*PPARG*, exon 1).

In addition to RT-PCR approach, fluorescence *in situ* hybridization (FISH) was performed in tumor samples in order to confirm the *RET/PTC* rearrangements. Briefly, isolated nuclei were extracted from 50 µm sections of FFPE thyroid tumor tissues. The isolation of nuclei from FFPE tissue and FISH technique was performed

following the procedure described by Marques *et al.* (28). *RET/PTC* rearrangement detection was investigated with a DNA probe generated from three yeast artificial chromosome clones (313F4, 214H10, and 344H4) covering the *RET* locus (kindly provided by Prof. Horst Zitzelsberger); probe hybridization was performed as described (29). FISH signals were analyzed and recorded with a CytoVision System (Applied Imaging, New Castle, UK). For *RET/PTC*, at least 200 nuclei were scored for the presence of a split FISH signal (rearranged) in addition to an overlapping signal (normal). Only cells with either two overlapping signals or one split and one overlapping signal were counted to ensure that only intact nuclei had been scored. Only when 10% or more rearranged nuclei were present, we considered the case as positive for *RET/PTC* or *PAX8-PPARG* rearrangements (30).

Samples of thyroid cancer, positive for *RET/PTC1* or *RET/PTC3* rearrangements by RT-PCR and cDNA sequencing, were used as positive controls for FISH.

Results

Histopathology

All the lesions were observed and reclassified independently by two pathologists (M S-S and T N), being 36 malignant and 31 follicular adenomas. The malignant lesions included 12 conventional PTC (cPTC), 20 FVPTC, one oncocytic variant of PTC, two lymph nodes metastases of cPTC and one follicular carcinoma.

BRAF and NRAS mutations

The *BRAF^{V600E}* mutation was detected in nine of 36 malignant lesions (25%), two cases of FVPTC (10%) (Fig. 1A) and seven of cPTC (50%) (Fig. 1B), this difference being significant (Table 1) (*P*=0.019). In one case of cPTC, there was insufficient material from the tumor for further analysis.

There were no differences in the *BRAF^{V600E}* mutation prevalence according to gender, age at irradiation, age at diagnosis, latency period (time between irradiation and surgery) nor any clinicopathological parameter, either considering tumors or individual patients. Only

Table 1 Frequency of *BRAF^{V600E}* and *RAS* mutations in 67 thyroid tumors from childhood scalp-irradiated individuals.

Histological type or subtype	<i>BRAF^{V600E}</i> positive	<i>RAS</i> positive
Conventional PTC (12 primary tumor + two metastasized lymph node) (<i>n</i> =14)	7/14 (50%)	None
Follicular variant of PTC (<i>n</i> =20)	2/20 (10%)	1/20 (5%)
Oncocytic variant of PTC (<i>n</i> =1)	None	None
Follicular carcinoma (<i>n</i> =1)	None	None
Follicular adenoma (<i>n</i> =31)	None	None

Figure 2 Sequencing profile/electropherogram of the sample with the *BRAF*^{VK600E-1E} mutation. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-13-0543>.

two thyroid cancer patients had been submitted to a higher irradiation dose, so it was not possible to evaluate the dose effect in the *BRAF*^{V600E} mutation prevalence. One of the 20 cases of FVPTC showed the rare *BRAF*^{VK600E-1E} mutation, described by our group (31) (Fig. 2). None of the benign lesions – follicular adenomas – presented *BRAF* mutation. *NRAS* mutation was detected in only one case of FVPTC.

RET/PTC and PAX8–PPARG rearrangements

RNA was successfully recovered in 32 thyroid tumors (15 benign and 17 malignant). By RT-PCR, *RET/PTC1* rearrangement was detected in only one of 17 cases of thyroid cancer (5.9%), a case of cPTC (Figs 1C and 3). The *RET/PTC1* rearrangement was not identified in any of the follicular adenomas ($n=15$).

All the carcinomas for which there was enough material for FISH study ($n=6$) were evaluated by this assay. In two of six cases (33.3%), the *RET/PTC1* rearrangement was detected, confirming the case already observed by RT-PCR (Fig. 4). The other case detected by FISH was a case of a FVPTC. No *PAX8–PPARG* rearrangements were detected in any case. All the mutations/rearrangements were mutually exclusive.

Discussion

Almost all the carcinomas found in this series of childhood-irradiated individuals were PTCs (35/36, 97.2%). Radiation exposure has been described as significantly increasing the risk for thyroid malignancies, particularly PTC (32), in accordance with the results of the present series. We observed the FVPTC in

55.6% of the PTCs (20/36). In other series of PTCs, the prevalence of the FVPTC varied from 7 to 53% (33, 34, 35, 36, 37, 38), our frequency being similar to the higher value of this range, but no reports were found referring in particular the prevalence of FVPTC in the irradiation context. The high variation in the frequency of FVPTC observed can be due to different evaluation criteria adopted in the diverse studies published to date.

Our results show that the prevalence of *BRAF*^{V600E} mutation in our series is in the high range of that reported in PTCs of radiation-exposed individuals (internal or external) (4–24%) and lower than the values referred for sporadic PTCs (45–53%) (39). We found that the *BRAF*^{V600E} mutation was significantly more prevalent in the cPTC (50%) than in FVPTC (10%), in accordance with the 35–70 vs 5–20% reported by us and others for sporadic PTC (39, 40). We did not find any significant difference regarding the prevalence of histological subtypes or presence of *BRAF* mutation according to age, gender, latency and clinicopathological parameters.

RET/PTC1 rearrangement was detected in 6% of the thyroid carcinomas using RT-PCR ($n=17$), but this value increased to 33% in the six cases in which it was possible to use FISH. FISH has been considered as the assay of choice for rearrangement detection, although its use in FFPE tissue is hampered by the quality of the material and size of the lesions. In fact, the technique is not feasible in small PTCs that represented part of the cases of our series. A prevalence of 33% may still be assumed as a low figure for this rearrangement in the context of thyroid irradiation, although the values reported in the literature have been decreasing recently. Dinets *et al.* (11) found a 29% prevalence for *RET/PTC1* rearrangement and a 6% prevalence for *RET/PTC3* rearrangement in a recent study of adult post-Chernobyl

Figure 3 Sequencing profile/electropherogram of one sample with the *RET/PTC1* rearrangement. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-13-0543>.

Figure 4 *RET/PTC1* rearrangement (arrow) (this case was also positive by RT-PCR). Magnification: $\times 1000$. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-13-0543>.

PTC cases. The variation in the prevalence and specificity of *RET/PTC* rearrangements reported in different series can be due to differences in the prevalence of this alteration in specific age groups (15).

Nevertheless, Elisei *et al.* (9) reported that the presence of *RET/PTC* rearrangements was not higher in radiation-induced tumors compared with those naturally occurring, nor different after exposure to radioiodine or external radiation; it was also not dependent on age at irradiation. At variance with Elisei *et al.*, other authors advanced that the prevalence of the different genetic alterations can vary with the time of latency of the tumors. In Chernobyl context, it was verified that the prevalence of *RET/PTC* in short latency period tumors ('first wave') was higher than in tumors that arise later ('second wave') (41). The tumors we have analysed in the present series have a long latency period (≥ 30 years).

By RT-PCR, the *PAX8-PPARG* rearrangement was not observed in any of the FVPTC of the present series. In a previous study, we have detected, by FISH, *PAX8-PPARG* rearrangement in 37.5% of sporadic cases of FVPTC (16). In another study of sporadic FVPTC, this rearrangement was undetectable by RT-PCR in all the seven cases studied (42). One limitation of this study was the fact that we had insufficient tumor material, due to small tumor size, to apply the FISH technique to most of our cases. The rearrangement was not detected in any follicular adenoma, in accordance with Dwight *et al.* (43) and differently from the 33% prevalence in sporadic follicular adenomas (16).

In conclusion, we report a thorough histological and genetic characterization of thyroid tumors arising, after a long latency period, in the context of low-dose

external irradiation (scalp). We also report for the first time data on the prevalence of FVPTC in tinea capitis irradiation context, the results of the present series being slightly above the highest values reported in sporadic PTCs. Moreover, with the exception of *RET/PTC* rearrangements, the other common genetic alterations in thyroid cancer (*BRAF* and *NRAS* mutations and *PAX8-PPARG* rearrangements) had never been studied in this low-dose external radiation context. Further studies are needed, with larger series, in order to clarify, using FISH technique, the actual importance of these rearrangements when the thyroid is submitted to low-dose external radiation. This is important if we take into account that radiotherapy for head and neck cancers, thorax cancers, lymphomas, breast cancers and even all body irradiation may expose the thyroid to radiation due to its anatomical position (44).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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