Clinical Study

Molecular Analysis of TTF-1 and TTF-2 Genes in Patients with Early Onset Papillary Thyroid Carcinoma

Carla Espadinha,1 Ana Luísa Silva,1 Rafael Cabrera,2 and Maria João Bugalho1,3,4

1 Centro de Investigação de Patobiologia Molecular, Instituto Português de Oncologia de Lisboa Francisco Gentil E.P.E., 1099-023 Lisboa, Portugal
2 Serviço de Anatomia Patológica, Instituto Português de Oncologia de Lisboa Francisco Gentil E.P.E., 1099-023 Lisboa, Portugal
3 Serviço de Endocrinologia, Instituto Português de Oncologia de Lisboa Francisco Gentil E.P.E., 1099-023 Lisboa, Portugal
4 Clínica Universitária de Endocrinologia, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, 1169-056 Lisboa, Portugal

Correspondence should be addressed to Maria João Bugalho, mjbugalho@ipolisboa.min-saude.pt

Received 26 May 2011; Accepted 27 December 2011

1. Introduction

Thyroid cancer is not common among children and adolescents. Between 1975 and 2000, thyroid cancer represented approximately 7.8% of all cancers diagnosed in the 15- to 19-year age group according to data in the Surveillance Epidemiology and End Results (SEERs) database [1]. It is very rare in children younger than 15 years of age with very few cases diagnosed before 10 years of age. Differentiated thyroid cancer (DTC), that is, papillary (PTC) or follicular thyroid cancer (FTC), accounts for the vast majority of cases and medullary thyroid cancer (MTC) accounts for the most of the rest. Poorly differentiated and anaplastic cancers are exceedingly rare.

Among environmental risk factors, a well-known risk factor for PTC is exposure to ionizing radiation and for FTC is deficiency in iodine intake. Common variants on 9q22.33 and 14q13.3 were shown to predispose to thyroid cancer in European populations [2]. The gene nearest to the 9q22.33 locus is FOXE1 (TTF-2), and NKX2-1 (TTF-1) is among the genes located at the 14q13.3 locus. Both genes are important in the biology of thyroid gland, and their expression is altered in thyroid tumors [3, 4].

Based on the hypothesis that young patients are carriers of genetic variants that influence their early onset of disease, we analyzed samples from patients who developed thyroid cancer early in life. Besides screening for the classical somatic changes, known to cause neoplastic transformation, we sought for TTF-1 and TTF-2 variants.
exposition to radiation or family history of thyroid disease. Thirteen of these samples have already been included in a previous study [5]. In seven cases, it was possible to pair tumors with their normal adjacent tissue or contralateral lobe. Peripheral blood samples from patients and 32 healthy controls were also analyzed.

Tissue sample collection was carried out in accordance with protocols approved by the institutional review board, and informed consent was obtained for the study together with the consent for surgery.

2.2. DNA, RNA Extraction, and cDNA Synthesis. Total RNA was obtained from frozen tissues using Trizol reagent (Invitrogen, Paisley, UK), according to manufacturer’s instructions, and 2 µg were reverse transcribed using random primers and SuperScript II (Invitrogen). When applicable, genomic DNA was extracted from peripheral blood lymphocytes using Puragene DNA Purification System Blood Kit (Gentra, USA).

2.3. Screening for BRAFV600E Mutation and RET/PTC Rearrangements. Screening for the BRAFV600E mutation and RET/PTC rearrangements 1–3 was conducted as previously described [6].

2.4. TTF-1/NKX2-1 and TTF-2/FOXE1 Variants Screening. The mutational analysis of TTF-1/NKX2-1 and TTF-2/FOXE1 was performed in all tumor samples by RT-PCR followed by direct sequencing. Two transcript variants encoding different protein isoforms have been described for TTF-1/NKX2-1 gene. The transcript variant 1 (encoding the longer protein isoform) was amplified using 6 overlapping amplicons that encompass the 3 exons of its coding-region. Similarly, 6 overlapping amplicons (5 of which were common to both variants) were generated to span the 2 exons that comprise the coding region of variant 2 (which encodes a shorter protein isoform with a N-terminus distinct from isoform 1). The entire coding-region (a single exon) of TTF-2/FOXE1 was also amplified by producing 6 overlapping amplicons. When appropriate, germline TTF-2/FOXE1 alterations were screened in peripheral blood DNA using analogous PCR conditions. Primers were designed according to Gen-Bank sequence (GenBank accession numbers: NM_001079668 for TTF-1/NKX2-1 transcript variant 1, NM_003317 for TTF-1/NKX2-1 transcript variant 2, and NM_004473 for TTF-2/FOXE1). The primer pairs used to generate each amplicon, as well as the annealing temperature and the amplicon expected length, are described in Table 1.

2.5. QRT-PCR. The TPO and TG expression levels were quantified by QRT-PCR, as previously described [5], on an ABI Prism 7900HT Sequence Detection System using specific primers and TaqMan probes from the Assay-on-Demand products (Hs00174927_ml (TPO), Hs00794359_ml (TG); Applied Biosystems). Amplification reactions were performed in triplicate for each sample, and the results were normalized to endogenous GAPDH gene expression level (Pre-Developed TaqMan Assay Reagents; P/N 4326317E; Applied Biosystems).

2.6. Statistical Analysis. Statistical analysis was carried out using GraphPad Prism statistical software (San Diego, CA). When appropriate, values are expressed as mean ± SD. Statistical comparisons were made using the unpaired Student’s t-test (two-tailed). Statistical significance was accepted at P < 0.05.

3. Results

3.1. Screening for RET/PTC Rearrangements, BRAFV600E Mutation, and TTF-1/NKX2-1 and TTF-2/FOXE1 Variants. RET/PTC rearrangements and the BRAFV600E mutation were identified in 5/18 and 2/18 PTCs, respectively (Table 2).

No TTF-1 variants were identified in tumors or in controls. Two novel TTF-2 variants, c.200C>G (p.A67G) and c.510C>A (p.A170A), were identified in heterozygosity (Table 2). In both cases, the variants were also present in constitutional DNA. In order to ascertain whether these variants were polymorphisms, we analyzed samples from 32 controls. The p.A170A variant was detected, in heterozygosity, in 1 control; none presented the p.A67G variant. In summary, the p.A170A variant was detected in 2/100 alleles and the p.A67G variant was detected in 1/100 alleles (18 patients + 32 control samples). The patient harboring the p.A67G variant inherited it from the father who presented a normal thyroid gland and normal thyroid function.

In addition, the allelic frequency (patients versus controls) for two TTF-2 variants already described was as follows: c.387T>C (p.L129L)—0.83/0.74; c.825C>T (p.S275S)—0.67/0.66.

3.2. TPO and TG Expression Levels. Paired analysis of tumor and corresponding normal thyroid samples (possible in 7 cases) revealed a significant decrease (P < 0.0001) in TPO and TG expression among the tumor group (Figure 1), reinforcing previous results [5].

Analysis of TPO and TG expression levels, among tumors, according to the subtype of molecular alteration identified, conducted to the observation that the tumor sample presenting both the RET/PTC3 rearrangement and the p.A67G showed the lowest TPO expression level amongst all tumor samples (Figure 2).

4. Discussion

Thyroid cancer has a very low incidence in the pediatric population. The yearly incidence of DTC (PTC and FTC), in the United States, in patients from 5 to 20 years of age ranged from 0.7 to 14 cases per million, according to data in the SEER database 1975–2000 [1]. Under the age of 5, it is extremely rare [7, 8]. Exposition to ionizing radiation is a significant risk factor particularly for papillary
Table 1: TTF-1/NKX2-1 and TTF-2/FOXE1: primers and PCR conditions used.

| Amplicon | Primers (5’–3’) | Annealing temperature (°C) | Expected length (bp) |
|----------|-----------------|-----------------------------|----------------------|
| 1sv1     | agactcgcgctgcctcatttgt ctccatgccccacttctgtt | 58                          | 231                  |
| 1sv2     | gctccacactcaagccacatta ctccatgccccacttctgtt | 62                          | 273                  |
| 2        | tgtcgtagcttcaacagcac gctttcctctggtttccttct | 62                          | 320                  |
| TTF-1    | ctactgcaacggcacaactctgccctgcctccattcttgagcttgctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa
gagatcgcgcagtgcgtgctgctgccctgcctccattcttgagcttgctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 56                          | 404                  |
| 3        | ccagcatgatccacctgac gctgttcctcatggtctgctcctgcccgccggttgtagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 56                          | 222                  |
| 4        | gctgttcctcatggtctgctcctgcccgccggttgtagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 62                          | 317                  |
| 5        | gccaggtatccagcctgctgcccgccggttgtagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 60                          | 210                  |
| 6        | acgttccctgtgacgcgcggtggcgtcttgtctgctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 301                  |
| 7        | tcctgaggtgtcgcctgcctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 300                  |
| 8        | gcggacctgttgcagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 256                  |
| 9        | gctctgaggtgtcgcctgcctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 300                  |
| 10       | gcggacctgttgcagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 300                  |
| 11       | gcggacctgttgcagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 256                  |
| TTF-2    | gcggacctgttgcagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 300                  |
| 12       | gcggacctgttgcagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 256                  |
| 13       | gcggacctgttgcagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 300                  |
| 14       | gcggacctgttgcagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 256                  |
| 15       | gcggacctgttgcagctgcatctcacatgcttaagagctgagcgcgtgtagctgtaaaaa | 58                          | 300                  |

Figure 1: mRNA expression of TG and TPO: comparative analysis between tumors and corresponding normal thyroid tissue (possible in 7 cases, referred in Table 2 as 4, 5, 6, 8, 9, 16, and 17). Quantitative PCR results are expressed as arbitrary units (a.u.) representing x-fold differences relative to the reference sample.

Figure 2: mRNA expression of TG and TPO among tumors according to the molecular alteration identified. Quantitative PCR results are expressed as arbitrary units (a.u.) representing x-fold differences relative to the reference sample.
Table 2: Demographic, histological, and molecular characteristics of study subjects. M: male; F: female; −: without alteration; +: with alteration; y: yes; n: no.

| Patient | Gender | Age at diagnosis (years) | Pattern                  | Histopathology | Multifocality | Extra thyroid extension | Lymph node metastases | Ret/PTC rearrangements | BRAFV600E mutation | TTF1 | TTF2 |
|---------|--------|--------------------------|--------------------------|----------------|---------------|------------------------|------------------------|------------------------|---------------------|------|------|
| 1       | M      | 5                        | Follicular Variant       | y              | y             | y                      | y                      | PTC3                   | −                   | −    | −    |
| 2       | F      | 9                        | Follicular Variant       | y              | y             | y                      | y                      | −                      | −                   | −    | −    |
| 3       | F      | 10                       | Classic                  | y              | n             | y                      | y                      | PTC1                   | −                   | −    | −    |
| 4       | F      | 10                       | Solid-Follicular Variant | n              | n             | n                      | n                      | −                      | −                   | −    | −    |
| 5       | F      | 11                       | Follicular Variant       | y              | y             | y                      | y                      | −                      | −                   | −    | −    |
| 6       | F      | 11                       | Classic                  | y              | n             | y                      | y                      | −                      | +                   | −    | −    |
| 7       | M      | 12                       | Follicular Variant       | y              | y             | y                      | y                      | −                      | −                   | −    | −    |
| 8       | M      | 13                       | Classic                  | y              | y             | y                      | y                      | PTC1                   | −                   | −    | −    |
| 9       | F      | 13                       | Follicular Variant       | y              | y             | y                      | y                      | −                      | −                   | −    | −    |
| 10      | F      | 15                       | Multinodular Follicular Variant | y         | n             | n                      | n                      | −                      | −                   | −    | A170A|
| 11      | F      | 15                       | Classic                  | y              | y             | y                      | y                      | −                      | −                   | −    | −    |
| 12      | F      | 16                       | Classic                  | y              | y             | y                      | y                      | PTC3                   | −                   | −    | A67G |
| 13      | M      | 17                       | Follicular Variant       | y              | y             | y                      | y                      | −                      | −                   | −    | −    |
| 14      | F      | 17                       | Encapsulated Follicular Variant | n         | n             | n                      | n                      | −                      | −                   | −    | −    |
| 15      | F      | 19                       | Encapsulated Follicular Variant | n         | n             | n                      | n                      | −                      | −                   | −    | −    |
| 16      | F      | 19                       | Encapsulated Follicular Variant | n         | n             | n                      | n                      | −                      | −                   | −    | −    |
| 17      | F      | 21                       | Diffuse Sclerosing       | y              | y             | y                      | y                      | PTC1                   | −                   | −    | −    |
| 18      | F      | 21                       | Classic                  | n              | n             | n                      | n                      | −                      | +                   | −    | −    |
thyroid cancer, the most common type of DTC [9, 10]. In childhood, the thyroid presents a higher susceptibility to the carcinogenic effect of ionizing radiation than in adulthood [11]. Nonetheless, a large percentage of DTC in young patients cannot be explained by this external risk factor.

Common variants on 9q22.33 and 14q13.3 were shown to predispose to thyroid cancer in European populations [2]. The gene nearest to the 9q22.33 locus is FOXE1 (TTF-2), and NKX2-1 (TTF-1) is among the genes located at the 14q13.3 locus. Almost at the same time, Landa et al. [12] showed that the variant rs1867277 within the TTF-2 5′UTR confers thyroid cancer susceptibility.

We hypothesized that children and adolescents, who develop thyroid cancer, without a prior exposition to radiation and without a family history of thyroid disease, might represent a group with a higher susceptibility. Therefore, we decided to assess the potential contribution of TTF-1 and TTF-2 genes as sources of genetic susceptibility to thyroid cancer in this particular group.

Eighteen patients were included in the present study. Multifocal carcinoma was found in 13/18 (72,2%), extrathyroidal extension in 10/18 (55,6%), and neck lymph node metastases in 12/18 (66,7%). Such a high frequency of multifocality, as reported in other studies [13], reinforced the hypothesis of a genetic background.

Screening for TTF-1 and TTF-2 variants disclosed two novel variants in the TTF-2 gene. One variant is a silent one, the other predicts an amino acid substitution, c.200C>G (p.A67G), located within the forkhead domain and conserved between species. The former variant was observed in 1/32 healthy individual, whereas the latter variant was not observed in the control group. However, the c.200C>G variant was also present in the patient's father who had a normal thyroid and a normal thyroid function. Moreover, the patient presenting this alteration had also a RET/PTC3 rearrangement sufficient to justify the PTC phenotype. Thus, we considered both variants as polymorphisms. A possible interplay between the RET/PTC3 and the c.200C>G TTF-2 variant cannot be excluded and awaits further clarification. The allelic frequencies of the other TTF-2 variants were not significantly different between patients and controls.

We assessed the functional consequences of the variants described herein by comparing the thyroglobulin (TG) and thyroid peroxidase (TPO) expression among tumors. Noteworthy, the patient with the c.200C>G variant presented the lowest TPO expression suggesting that this variant might alter the TPO transcription normally regulated by the TTF-2 transcription factor [14].

Despite the small number of cases, based on present results, TTF-1 and TTF-2 variants do not seem to contribute greatly to an earlier age of onset of thyroid cancer.

5. Conclusions

The aim of the study was to investigate whether TTF-1 and TTF-2 variants might contribute to thyroid cancer risk in children and adolescents. This hypothesis derived from the fact that two common variants, close from these genes, known to play an important role for thyroid organogenesis and differentiation, have been reported to be associated with a 5.7-fold increase in the risk of thyroid cancer in homozygous individuals.

No TTF-1 variant was identified. For TTF-2, 2 variants already identified had the same allelic frequency in patients and controls. Two new TTF-2 variants (c.200C>G and c.510C>A) were identified in 2 adolescents. The variant c.510C>A (p.A170A) was also identified in a control subject. The patient harboring the c.200G variant (p.A67G) inherited it from the father who presented a normal thyroid morphology and normal thyroid function. In addition, the latter variant was associated, in the patient, with a RET/PTC3 rearrangement sufficient to respond for the neoplastic phenotype. Altogether, results suggest that the two new TTF-2 variants are likely to be polymorphisms.

Noteworthy, the c.200C>G variant was associated with the lowest TPO expression suggesting that this variant might alter the TPO transcription, normally, regulated by the TTF-2 transcription factor. Whether this occurred by chance or influenced by the RET/PTC3 cannot be excluded.

The frequency of RET/PTC rearrangements and BRAF mutations observed was in accordance with previous reports, thus showing that RET/PTC is the most common genetic alteration in papillary carcinomas from young patients.

Although limited by the number of patients studied, our results suggest that TTF-1 and TTF-2 variants do not make a major contribution to an earlier onset of thyroid cancer.

Conflict of Interests

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

Disclosure

This research did not receive any specific grant from any funding agency in the public, commercial or non-for-profit sector.

References

[1] S. Waguespack, S. Wells, J. Ross, and A. Bleyer, “Thyroid cancer,” in Cancer Epidemiology in Older Adolescents and Young Adults 15 to 29 Years of Age, Including SEER Incidence and Survival: 1975–2000, A. Bleyer, M. O’Leary, R. Barr, and L. A. G Ries, Eds., pp. 143–154, National Cancer Institute, Bethesda, Md, USA, 2006.
[2] J. Gudmundsson, P. Sulem, D. F. Gudbjartsson et al., “Common variants on 9q22.33 and 14q13.3 predispose to thyroid cancer in European populations,” Nature Genetics, vol. 41, no. 4, pp. 460–464, 2009.
[3] M. J. Sequeira, J. M. Morgan, D. Fuhrer, M. H. Wheeler, B. Jasani, and M. Ludgate, “Thyroid transcription factor–2 gene expression in benign and malignant thyroid lesions,” Thyroid, vol. 11, no. 11, pp. 995–1001, 2001.
[4] P. Zhang, H. Zuo, Y. Nakamura, M. Nakamura, T. Wakasa, and K. Kakudo, "Immunohistochemical analysis of thyroid-specific transcription factors in thyroid tumors," *Pathology International*, vol. 56, no. 5, pp. 240–245, 2006.

[5] C. Espadinha, J. R. Santos, L. G. Sobrinho, and M. J. Bugalho, "Expression of iodine metabolism genes in human thyroid tissues: evidence for age and BRAFV600E mutation dependency," *Clinical Endocrinology*, vol. 70, no. 4, pp. 629–635, 2009.

[6] R. Domingues, E. Mendonça, L. Sobrinho, and M. J. Bugalho, "Searching for RET/PTC rearrangements and BRAF V599E mutation in thyroid aspirates might contribute to establish a preoperative diagnosis of papillary thyroid carcinoma," *Cytopathology*, vol. 16, no. 1, pp. 27–31, 2005.

[7] J. Estevão-Costa, M. J. Gil-Da-Costa, A. M. Medina, and M. Sobrinho-Simões, "Thyroid carcinoma in a newborn: clinical challenges in managing the first recorded case," *Medical and Pediatric Oncology*, vol. 34, no. 4, pp. 290–292, 2000.

[8] L. Khara, A. Silverman, C. Bethel, C. D’Cruz, and X. Sun, "Thyroid papillary carcinoma in a 3-year-old american boy with a family history of thyroid cancer: a case report and literature review," *Journal of Pediatric Hematology/Oncology*, vol. 32, no. 3, pp. e118–e121, 2010.

[9] C. J. García, A. Daneman, P. Thorner, and D. Daneman, "Sonography of multinodular thyroid gland in children and adolescents," *American Journal of Diseases of Children*, vol. 146, no. 7, pp. 811–816, 1992.

[10] G. A. Thomas, H. Bunnell, H. A. Cook et al., "High prevalence of RET/PTC rearrangements in Ukrainian and Belarusian post-chernobyl thyroid papillary carcinomas: a strong correlation between RET/PTC3 and the solid-follicular variant," *Journal of Clinical Endocrinology and Metabolism*, vol. 84, no. 11, pp. 4232–4238, 1999.

[11] D. B. Richardson, "Exposure to ionizing radiation in adulthood and thyroid cancer incidence," *Epidemiology*, vol. 20, no. 2, pp. 181–187, 2009.

[12] I. Landa, S. Ruiz-Llorente, C. Montero-Conde et al., “The variant rs1867277 in FOXE1 gene confers thyroid cancer susceptibility through the recruitment of USF1/USF2 transcription factors,” *PLoS Genetics*, vol. 5, no. 9, Article ID e1000637, 2009.

[13] K. Pazaitou-Panayiotou, A. Kaprara, M. Boudina et al., "Thyroid carcinoma in children and adolescents: presentation, clinical course, and outcome of therapy in 23 children and adolescents in Northern Greece," *Hormones*, vol. 4, no. 4, pp. 213–220, 2005.

[14] I. Cuesta, K. S. Zaret, and P. Santisteban, "The Forkhead factor FoxE1 binds to the thyroperoxidase promoter during thyroid cell differentiation and modifies compacted chromatin structure," *Molecular and Cellular Biology*, vol. 27, no. 20, pp. 7302–7314, 2007.