Growing thyroid nodules with benign histology and RET rearrangement

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Abstract. Some benign thyroid nodules are stationary in size over time while others grow progressively, indicating that there is a broad individual variability within benign nodules. To date, it is very difficult to predict if a benign thyroid nodule will grow in size and which will be its trend over time. While BRAFV600E is a highly specific marker of thyroid cancer, RET rearrangements have been disclosed also in non malignant thyroid lesions and their biological significance is debated. We compared the clinical history of three histologically benign thyroid nodules harboring RET rearrangements with that of 6 benign nodules bearing wild type RET. The nodules negative for RET rearrangements were followed for 10 years by ultrasonographic evaluation, showing a slow, constant enlargement. Three patients with benign nodules diagnosed at FNAC, were followed for 11, 9 and 7 years by annual ultrasonographic evaluation. After several years of latency, the nodules had an unexpected and gradual increase in their dimensions, reaching a large final size. A second FNAC confirmed the previous cytologic diagnosis of benign lesion. Because of the increasing size of the nodules, the patients were advised to surgery. Before undergoing thyroidectomy, we performed molecular diagnostic tests that revealed the absence of BRAFV600E and the presence of RET/PTC-1 in one nodule and RET/PTC-3 in the two others. Despite the presence of this oncogene, the samples were histologically classified as benign hyperplastic nodules. These findings lead us to speculate that histologically benign hyperplastic thyroid nodules containing RET rearrangements might represent a subgroup of nodules with a rapid size increase.

Key words: Nodular goiter, RET/PTC, FNAC

THE CURRENT therapeutic approach for thyroid nodules requires surgery for those malignant, as well as for benign nodules in the presence of local compression symptoms, such as dysphagia or dysphonia and/or cosmetic complaints. The management of non-complicated benign nodules includes follow-up without treatment or medical treatment with suppression of TSH secretion by levo-thyroxine [1, 2]. However, surgery can be considered also for benign nodules showing a high growth rate also in the absence of local complications. Thus, it would be important to select benign nodules with a higher risk of growth advising the patients for a timely treatment.

The differential diagnosis between benign and malignant nodules is a recurring problem in routine clinical practice. Since physical examination and thyroid ultrasonography are not able to distinguish between benign and malignant lesions, the differential diagnosis of thyroid nodules is based on fine-needle aspiration cytology (FNAC). Although FNAC is the most reliable tool to select patients requiring surgery, it yields uncertain results in about 15-25% of cases [3, 4]. The management of thyroid nodules with inconclusive cytology can benefit by testing for molecular alterations known

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to occur in thyroid tumors. \textit{BRAF}^{V600E} and \textit{RET} rearrangements are oncogenes with a demonstrated pathogenetic role in the transformation of thyroid cell and could therefore be used as markers of thyroid cancer [5-8]. \textit{BRAF}^{V600E}, the result of a somatic point mutation in the \textit{BRAF} gene is the most common genetic event in papillary thyroid carcinoma (PTC) [9, 10]. \textit{RET}/PTC are chimeric oncogenes generated by the fusion of the catalytic domain of the tyrosine kinase receptor \textit{RET} to the 5’ terminal region of heterologous genes. Within the different types of rearrangements identified so far, the most frequent are \textit{RET}/PTC-1 and \textit{RET}/PTC-3, generated by the fusion of the \textit{RET} gene with \textit{H4} and \textit{ELE1} respectively. The specificity of \textit{RET}/PTC as a PTC marker is still controversial as highly sensitive detection techniques disclosed the presence of this oncogene also in non-malignant thyroidal diseases such as Hashimoto’s thyroiditis, adenomas and benign colloidal nodules [11-13]. In this report we present three cases of patients with a thyroid nodule that revealed benign at cytological and histological examinations, while harboring \textit{RET}/PTC at the molecular analysis.

\textbf{Subjects and Methods}

Patients entered in the study after giving their consent and with approval from the institutional review boards.

\textit{Ultrasonography, FNAC and histology}

All thyroid ultrasonography analysis were performed by the same examiner (M.V.) using a 7.5–10 MHz linear transducer (Esaote, Genoa, Italy). The nodule volume was calculated according to the formula of the ellipsoid model: width \times length \times thickness \times 0.52. Cytology samples of thyroid nodules were obtained using a 25-gauge needle passed three to four times. Smears were classified according to the cytological classification from the British Thyroid Association and UK Royal College of Physicians and the Italian Society of Pathological Anatomy and Diagnostic Cytology (SIAPEC) Consensus [14]. One sample was used for cytological examination and the other was dispersed into TRI-reagent buffer (Sigma, St. Louis, MO) and stored at -20°C until RNA extraction. The final histopathological findings were blindly reviewed by 2 independent pathologists.

\textbf{Genetic analysis}

Total RNA was extracted using TRI Reagent following the suggested protocol. The pellet of RNA was resuspended in 10μL DEPC water and reverse transcribed with SuperScript III (Invitrogen, Milan, Italy) in a 20 μL reaction volume with random primers. The presence of thyroid follicular epithelial cells, the integrity of the RNA and the efficiency of the RT reaction in each sample was confirmed by PCR for thyroglobulin mRNA. Searching for the \textit{BRAF} mutation was performed by mutant allele-specific polymerase chain reaction (PCR) amplification (MASA) on cDNA as described [15]. \textit{RET}/PTC-1 and \textit{RET}/PTC-3 were analyzed by PCR using previously reported primers [16] and oligoprobes specific for the TK domain, \textit{H4} or \textit{ELE1} (Table 1). The PCR conditions were the same as those used for thyroglobulin. The annealing temperature was 56°C for \textit{RET}/PTC-1 and 54°C for \textit{RET}/PTC-3. cDNA from WRO cells was used as a negative control, while cDNA from TPC-1 cells and from a PTC sample were used as \textit{RET}/PTC-1 and \textit{RET}/PTC-3 positive controls, respectively. Ten μL of PCR product was electrophoresed in a 1.5% agarose gel and blotted onto a nylon membrane (Amersham Hybond N+, Amersham Place Little Chalfont, UK). The filter was then hybridized with two different internal probes specific for each amplified fragment, labeled with biotin.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Gene} & \textbf{Primer sequences (5’–3’)} & \textbf{Gene} & \textbf{Oligoprobes (5’–3’)} \\
\hline
\textit{RET}/PTC-1 & For: GCTGGAGACCTACAAACTGA & \textit{H4} & GGCACCTGCAAGAGAAGAGCGGAC- B \\
& Rev: GTTGCTCTGACCTTTTTC & \textit{TK} & GGAATTCCTCGGAAGAAC- B \\
\textit{RET}/PTC-3 & For: AAGCAAACCTGCACGTTGG & \textit{ELE1} & GTTCGGTGCTGGTATGTAAGGA- B \\
& Rev: CTTTCAGCATCTTCAAGG & \textit{TK} & GGAATTCCTCGGAAGAAC- B \\
\textit{BRAF} & For wt: GTGATTTTGGTCTAGCTACAG & & \\
& For mut: GTGATTTTGGTCTAGCTACAG & & \\
& Rev: GGCACAAATTTATCAGTGGA & & \\
\hline
\end{tabular}
\caption{Primers and probes used for \textit{RET}/PTC and \textit{BRAF} screening}
\end{table}

\textit{BRAF}\textsuperscript{V600E} and \textit{RET} rearrangements are oncogenes with a demonstrated pathogenetic role in the transformation of thyroid cell and could therefore be used as markers of thyroid cancer [5-8]. \textit{BRAF}\textsuperscript{V600E}, the result of a somatic point mutation in the \textit{BRAF} gene is the most common genetic event in papillary thyroid carcinoma (PTC) [9, 10]. \textit{RET}/PTC are chimeric oncogenes generated by the fusion of the catalytic domain of the tyrosine kinase receptor \textit{RET} to the 5’ terminal region of heterologous genes. Within the different types of rearrangements identified so far, the most frequent are \textit{RET}/PTC-1 and \textit{RET}/PTC-3, generated by the fusion of the \textit{RET} gene with \textit{H4} and \textit{ELE1} respectively. The specificity of \textit{RET}/PTC as a PTC marker is still controversial as highly sensitive detection techniques disclosed the presence of this oncogene also in non-malignant thyroidal diseases such as Hashimoto’s thyroiditis, adenomas and benign colloidal nodules [11-13]. In this report we present three cases of patients with a thyroid nodule that revealed benign at cytological and histological examinations, while harboring \textit{RET}/PTC at the molecular analysis.

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incubated with streptavidin-conjugated peroxidase and visualized using a chemiluminescent method (ECL Direct Nucleic Acid Labeling and Detection Systems Detection reagent, Amersham). Labeling, hybridization, and detection were performed according to manufacturer’s protocol. The PCR products of samples positive for RET rearrangements were sequenced with the ET-terminators kit (Amersham) and run on the MegaBACE 500 (Amersham) capillary sequencer.

**Results**

**Patient 1**

A 69 years-old woman presented to our attention in 1998 since she noticed a slight increase in volume in the anterior region of the neck. The patient reported a previous subtotal thyroidectomy at the age of 40 years with a histological post-operative diagnosis of benign nodular goiter. After surgery, the patient practiced replacement therapy with levo-thyroxine for an indefinite period of time and was followed by biochemical and instrumental examinations for many years. The physical examination of the neck revealed a mobile thyroidal left mass with increased hardness of about 2 cm of diameter, and absence of palpable cervical lymphadenopathy. The patient did not receive neck irradiation and did not complain cervical compressive symptoms.

Thyroid function tests were in the normal range and thyroid auto-antibodies were absent. Thyroid ultrasonography revealed a recidivism of multinodular goiter. The dominant nodule was localized in the left lobe as an hyso-hypoechoic solid mass of 20x15x15 mm size and a volume of 2.34 mL. FNAC was performed and revealed negativity for malignant cells (Thy 2) [14]. Taking into account the benign cytology, the absence of compressive symptoms and the age of the patient, we decided to discontinue the medication with levo-thyroxine and to follow-up the patient by annual ultrasonographic evaluation. Until 2004, the size of the nodule remained unchanged. From 2005 to 2009 the nodule had an unexpected and gradual increase in its dimensions, reaching the final size of 37x25x18 mm with a volume of 7.48 mL (Fig. 1). During the follow-up, thyroid function tests and thyroid auto-antibodies were always in the normal ranges. Given the increasing size of the nodule, a second FNAC was performed which confirmed the previous cytologic diagnosis of benign lesion.

Since the patient reported the onset of mild dysphagia, a new x-ray of the neck was performed which revealed the presence of right tracheal deviation. Despite the fact that cytology yielded repeated negative results for malignancy, we advised the patient to surgery because of the increasing size of the nodule and the onset of compressive symptoms. Before undergoing thyroidectomy we performed a new biopsy by a 25 gauge needle to search for BRAFV600E, RET/PTC-1 and
RET/PTC-3 oncogenes.

BRAF<sup>V600E</sup> and RET/PTC-3 were not detected, while Southern blot clearly showed the presence of RET/PTC-1 in the nodule (Fig. 2). Direct sequencing of the PCR product was performed, demonstrating the presence of a chimeric RNA containing the exon 1 of H4 and the exon 12 of RET.

The patient underwent near-total thyroidectomy with an uneventful post-operative course. The thyroid tissue was examined by 2 independent pathologists that agreed to classify the sample as benign hyperplastic nodule (Fig. 3). In the light of the presence of RET/PTC-1, a careful histological review was performed, and neither microscopic foci of malignancy nor features of lymphocytic thyroiditis were disclosed. The presence of RET/PTC-1 in the patient nodule was definitely confirmed by Southern-blot in the histological sample.

**Patients 2 and 3**

A 56 years-old woman (patient 2) presented to our attention in 2003 with an about 3 years pre-existing multi nodular goiter. Thyroid ultrasonography revealed a dominant nodule in the left lobe, hypoechoic, sized 21x15x12 mm with a volume of 1.87 mL, which resulted Thy 2 at FNAC. The size of the nodule increased slowly but progressively until 2008 when it reached a volume of 3.2 mL. In the following two years, the nodule dimension increased greatly, reaching a final size of 35x20x28 mm with a volume of 10.2 mL (Fig. 1, patient 2).

A 49 years-old women (patient 3) came to our attention in 2001 for a thyroidal right mass of about 2 cm diameter. Thyroid ultrasonography revealed a solid, hypoechoic nodule with a volume of 1.98 mL. The nodule was Thy 2 at FNAC. The nodule size was stable until 2006. Then it increased progressively in dimension, reaching the final volume of 7.1 mL (Fig 1, patient 3). In both patients the neck never received external radiations, thyroid function tests were in the normal range and thyroid auto-antibodies were absent. A second FNAC was performed that confirmed the diagnosis of benign lesions, and molecular testing was also performed for BRAF<sup>V600E</sup>, RET/PTC-1, and RET/PTC-3. In both nodules, only the presence of RET/PTC-3 was demonstrated (Fig. 2). Direct sequencing of the PCR product demonstrated the chimeric cDNA containing the exon 11 of ELE-1 and the exon 12 of RET.

The patients underwent near-total thyroidectomy and the thyroid tissues were classified by the pathologists as benign hyperplastic nodule without foci of...
malignancy nor features of lymphocytic thyroiditis (Fig. 3).

Patients 4 - 9
Six subjects (mean age 48 +/-8 years old, 5 female and 1 male) with a Thy 2 benign thyroid nodule were followed by ultrasonography for 10 years (Fig. 1). The mean initial nodule volume of 1.8 mL increased slowly and constantly, reaching a final volume of 2.9 mL. In all patients, the thyroid function tests were in the normal range and thyroid auto-antibodies were absent. Neither BRAlv600E, nor RET rearrangements were demonstrated by molecular testing.

Discussion

Although the three described nodules were classified as benign at FNAC, their unusual behavior prompted us to investigate for the presence of genetic alterations specific for thyroid cancer. While BRAlv600E is a specific PTC hallmark as it has been demonstrated to be present in about 45% of PTC, and absent in follicular cancer and in benign nodules, the cancer specificity of RET rearrangements has been questioned. Different studies reported a variable prevalence of RET rearrangements in PTC, in part due to the sensitivity of the method used. By means of interphase fluorescence in situ hybridization (FISH), RET rearrangements appear to be a very frequent genetic alteration in PTC, being found in 53% of micocarcinomas and 72% of post-Chernobyl papillary thyroid tumors [17, 18]. Comparative analysis of different techniques demonstrated that the sensitivity of the detection method is a crucial factor affecting the prevalence of RET/PTC in thyroid tumors [19]. By means of immunohistochemistry and RT-PCR, RET/PTC has been detected in a large proportion of occult microscopic papillary thyroid carcinomas, its prevalence is higher in radiation-induced PTC, and it is thought therefore to represent an early event in the malignant transformation of the follicular cell [20, 21]. Although sporadic, RET rearrangements have been found also in non malignant thyroid lesions such as Hashimoto’s thyroiditis and thyroid adenomas [11-13, 22]. PTC and Hashimoto’s thyroiditis partly overlap in nuclear morphological features and immunohistochemical patterns, and patients with Hashimoto’s thyroiditis have a higher incidence of PTC [23, 24]. RET rearrangements were sporadically detected also in benign nodules [11, 13]. Noteworthy, in a study that considered patients with a history of external radiations, the frequency of RET rearrangements in follicular adenomas was 45% (9/20) [22]. In both radiation-associated and non associated adenomas, the authors hypothesized the presence in the same sample of morphologically indistinguishable heterogeneous cellular populations, either positive or negative for RET/PTC. Later studies on post-Chernobyl PTC demonstrated that irradiated PTC are composed of a mixture of cells with and without RET rearrangements [18]. These data suggest that RET rearrangement alone may represent an event in thyroid tumorigenesis not sufficient to induce the typical PTC morphological features. In the clinical history of our patients, a change from indolent to rapid growth of an histologically proven benign nodule has been documented. Although a direct correlation between the rapid size increase of the nodule and the appearance of RET/PTC cannot be demonstrated, it is possible that the cell growth is empowered by the presence of this oncogene. As in PTC, the recombination could be present only in a fraction of cells. However, its effect can be extended to the oncogene-negative cells by a paracrine action. RET/PTC induces the expression of the chemokines CXCL1 and CXCL10 and their corresponding receptors, by which it modulates cell proliferation by an autocrine/paracrine mechanism [25]. Although to date there is no evidence that the presence of RET rearrangements worsen the prognosis of nodules with benign cytology, it is conceivable that it could determine a more rapid growth or a fully transformation into papillary cancer. This hypothesis could be verified by a sufficiently long follow-up study of a cohort of RET/PTC positive and negative benign nodules. A preliminary study confirms this hypothesis (manuscript submitted).

In summary, we report the case of three patients with benign thyroid nodules, with a very long ultrasonographic follow-up, suggesting that benign hyperplastic thyroid nodules containing or developing RET rearrangements might represent a subgroup of nodules with propensity to a more rapid enlargement.

Acknowledgments

We thank Dr. Juan Rosai for the review of histology samples. This work has been supported in part by Ministero dell’Istruzione, dell’Università e della Ricerca (to M.V., and G.R.).
References


