Analysis of BRAF Point Mutation and RET/PTC Rearrangement Refines the Fine-Needle Aspiration Diagnosis of Papillary Thyroid Carcinoma

GIULIANA SALVATORE, RICCARDO GIANNINI, PINUCCIA FAVIANA, ALESSIA CALEO, ILENIA MIGLIACCIO, JAMES A. FAGIN, YURI E. NIKIFOROV, GIANCARLO TRONCONE, LUCIO PALOMBINI, FULVIO BASOLO, AND MASSIMO SANTORO

Istituto di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche and Dipartimento di Biologia e Patologia Cellulare e Molecolare (G.S., M.S.), and Dipartimento di Scienze Biomorfologie e Funzionali (A.C., I.M., G.T., L.P.), University ‘Federico II,’ 80131 Napoli, Italy; Dipartimento di Oncologia (R.G., P.F., F.B.), University of Pisa, 56126 Pisa, Italy; and Department of Pathology and Laboratory Medicine and Division of Endocrinology (J.A.F., Y.E.N.), University of Cincinnati, Cincinnati, Ohio 45267

Point mutations in BRAF are genetic hallmarks of papillary thyroid carcinoma (PTC). In this retrospective study, we examined thyroid aspirates and corresponding paraffin-embedded surgical samples for the presence of BRAF mutations. Altogether, we examined 96 cases, including 69 PTCs, 19 follicular adenomas, and eight nontoxic nodular goiters for BRAF; 60 of these samples were also examined for RET/PTC rearrangements. The results were correlated with the cytological diagnosis and the final histopathology. The BRAF mutation (V599E) was detected in 38% of the samples that were PTC on histopathology; RET/PTC was found in 18% of the PTC cases. In all the cases, the presence of the genetic alteration was confirmed in the surgically resected tumor. The identification of BRAF mutation and RET/PTC refined the diagnosis of PTC in five of 15 samples that were considered either indeterminate or insufficient at cytology. No mutation was found in aspirates of follicular adenomas and nontoxic nodular goiters. These results indicate that BRAF mutation and RET/PTC rearrangements are molecular markers of PTC that can be applied to FNA in adjunct to traditional cytology. (J Clin Endocrinol Metab 99: 5175–5180, 2004)

THYROID CANCER is the most prevalent endocrine malignancy. In most of the cases, its initial presentation is a thyroid nodule. From 5−20% of the general population has a palpable thyroid nodule, and this figure becomes even larger with ultrasonography (1, 2). However, only a small fraction of thyroid nodules harbor a malignant disease (3). Preoperative diagnosis of thyroid nodules is based on fine-needle aspiration (FNA) cytology. Cytological examination of FNA by an expert pathologist provides the most reliable information. However, in some situations, the cytological examination cannot be conclusive either because of insufficient material or overlapping/undefined (indeterminate) morphological criteria (4). In a recent series, 23% of indeterminate FNAs were revealed to be papillary thyroid carcinoma (PTC) (5). In our institution, 6% of thyroid nodules were scored as either indeterminate or insufficient; among these, 88% were follicular neoplasms, 7.2% were papillary carcinomas, and 4.5% belonged to other histotypes (6). Thus, a fraction (from 7−23%) of PTC FNA may benefit from novel molecular determination methods. Further complicating this area is our lack of a complete biological understanding of thyroid neoplastic transformation, with lesions appearing morphologically benign but which could be the initial stage of a malignancy.

PTC is the most common malignancy of the thyroid gland (together with follicular carcinoma, it accounts for up to 90% of the cases) (7). Therefore, the evaluation of a thyroid nodule is often a search for PTC. Uncertain diagnosis may result in delays, repeated aspirations, and unnecessary surgical intervention. Cases scored indeterminate preoperatively usually undergo hemithyroidectomy. A postoperative diagnosis of malignancy (which occurs in approximately one quarter of the cases) leads, in most institutions, to a second-stage completion thyroidectomy. This two-stage surgery has higher morbidity than initial total thyroidectomy (8). Thus, there is a clear need for the development of molecular assays to reduce ambiguous diagnoses.

Recent studies have shown that activating point mutations in BRAF are present in approximately 45% of PTCs (range, 29−69%) (9−17). BRAF belongs to the RAF family of serine/threonine kinases, which includes two other isoforms, ARAF and CRAF (RAF-1). BRAF is located downstream of MAPK kinase in the classic MAPK cascade (18). Virtually all mutations identified so far in PTC affect nucleotide 1796 in exon 15 of BRAF, resulting in a thymine-to-adenine transversion, which translates into valine-to-glutamate substitution at residue 599 (V599E). Follicular variant (FV) PTCs were found to be rarely positive for BRAF (only two of 30 samples in Ref. 15). Trovisco et al. (17) reported that three of 32 FV PTCs did harbor the less common K600E

Abbreviations: FNA, Fine-needle aspiration; FV, follicular variant; PTC, papillary thyroid carcinoma; SSCP, single strand confirmational polymorphism.
mutation in BRAF. Remarkably, BRAF mutation is specific for PTC (9–17). Only one of 146 follicular adenomas reported so far scored positive for the K600E mutation (10). Moreover, no follicular carcinoma scored positive out of the 106 samples analyzed so far by different groups (zero of 10, zero of 18, zero of 13, zero of 11, zero of eight, zero of 32, and zero of 14) (9–11, 13–16).

Our results demonstrate that, together with RET/PTC, BRAF detection is a robust and specific test to improve the accuracy of FNA diagnosis of PTC.

Materials and Methods

Tumors

Retrospectively collected archival FNA thyroid smears from 96 patients were retrieved from the files of the Pathology Departments at the University of Naples and University of Pisa upon informed consent. Special care was taken to select patients whose corresponding histological samples were available for matched analysis. FNA smears were stained with May Grumwald-Giemsa or Papanicolau. As shown in Table 1, 54 cases showing branching tissue fragments, enlarged nuclei with fine dusty chromatin, vesicular and overlapping nuclei, nuclear grooves, single or multiple micro- or macronucleoli, and intranuclear inclusions fulfilled the six diagnostic criteria required for the identification of PTC on FNA smears (19–21). Special care was also taken to search for patients with PTC whose cytological diagnosis was not conclusive. In particular, we examined 11 samples that were considered indeterminate because they showed only some (from three to five) of the six diagnostic features and four other samples (all classic PTC) that were insufficient (i.e., not enough thyroid cells for diagnosis; less than six clusters of cells). The final histological diagnoses of indeterminate/insufficient samples were classical papillary (n = 5), microfollicular (n = 2), and sclerosing variant (n = 2). In addition, 27 FNA showing cytological features that were diagnostic of follicular neoplasia (n = 19) or hyperplastic nodular goiter (n = 8) were used as a controls. Immunocytochemical detection of thyroglobulin was performed according standard procedures (Dako Corp., Carpinteria, CA) to ensure that examined cells were mostly of follicular origin. For nucleic acid extraction, thyroid smears were immersed in xylene for 24 h to remove coverslip and then washed with ethanol. Slides were destained in a 1% (vol/vol) solution of HCl in 70% ethanol for 15 min, rinsed, and rehydrated in ethanol.

Matched paraffin-embedded histological samples of all cases were studied. Clinical-pathological data, including size of tumor, extrathyroid invasion, node metastasis, associated thyroid lesions, and metastatic deposits were obtained. After surgical resection, tissues were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Sections (4-μm thick) were stained with hematoxylin and eosin for histological examination. The nuclear and architectural features were carefully evaluated for final histopathological diagnosis (19–21). As shown in Table 1, at the histopathological diagnosis, the samples were grouped as 69 PTCs (54 cases with FNA evidence of carcinoma, 11 cases were indeterminate, and four cases were insufficient at FNA), 19 adenomas, and eight goiters. PTC variants included classic (n = 35), FV (n = 22), tall-cell (n = 9), and diffuse-sclerosing (n = 3) variants. FNA-identified adenomas proved to be Hurthle (n = 4), microfollicular (n = 5), microfollicular/macrol follicular (n = 9), and trabecular (n = 1). For nucleic acid extraction, 20-μm sections were cut, immersed in xylene for 20 min to remove paraffin, washed in ethanol, and processed.

Identification of BRAF point mutations

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK). DNA from each FNA smear was resuspended in 100 μl DNA from each tissue section was resuspended in 200 μl. Five microliters of DNA were used as a template for PCR amplification. So far, the BRAF mutation detected in PTC has been the V599E in exon 15; only rarely has the adjacent K600E mutation been found. However, mutations in exon 11 have been found in other cancer types (18). Thus, exons 11 and 15 of BRAF were separately amplified in a 25-μl mixture containing 10 mm Tris-HCl, 50 mm KCl, 1.5 mm MgCl2 (pH 8.3), 0.2 mm deoxyribonucleotide triphosphate, 8 pmol of primers, and 1 U of AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA). PCR primers were designed to amplify target exons plus approximately 50-bp flanking intron sequences in both upstream and downstream direction and were as follows: exon 11, forward, 5′-GGT TAA ATT TGC GTT GCT GGC ACA GTG AAT-3′; and exon 15, reverse, 5′-GGC ACA GTG AAT ATT TCC TTT GAT-3′. Amplification products were separated on 2% agarose gel and visualized by ethidium bromide staining. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the forward primers described earlier and the Big Dye terminator kit (Applied Biosystems, Foster City, CA) by the automated ABI PRISM DNA sequencer (Applied Biosystems).

The single strand confirmation polymorphism (SSCP) analysis, PCR products were diluted 1:1 with denaturing solution (1% [wt/vol] xylene cyanol, 1% [wt/vol] bromophenol blue, 0.1 mm EDTA, and 95%
formamide], boiled for 5 min, and immediately chilled in ice to prevent reannealing of single strand products. Electrophoresis was carried out on the GeneGel Electrophoresis Unit using GeneGel Excell 12.5/24 (12.5% T, 2% C; Amersham Biosciences, Freiberg, Germany) at 18 C, 600 V, 25 mA, and 15 W for 80 min. Gels were stained with PlusOne Silver Staining Kit (Amersham Biosciences) according to the manufacturer’s instructions. Altered migration patterns in two or three independent PCR-SSCP runs were considered as indicative of DNA mutations. PCR products showing mobility alterations were purified with the QIAquick PCR Purification Kit (Qiagen), and the forward strand was sequenced as outlined earlier. The anaplastic thyroid carcinoma cell lines BHT101 and 8505C were used as positive controls (15) for both SSCP and DNA sequencing. Cells were grown in DMEM containing 10% fetal bovine serum (GIBCO, Paisley, PA).

**Detection of RET/PTC rearrangements**

Roughly 90% of RET/PTC rearrangements found in thyroid tumors are RET/PTC1 or RET/PTC3 (22). Therefore, the search was focused on these two variants. RET/PTC rearrangements were analyzed by RT-PCR. RNA was extracted using the RNeasy Mini Kit (Qiagen). Forward primers, designed on the coiled-coil domains of the RET fusion partners (H4 for RET/PTC1 and RFG for RET/PTC3), were as follows: RET/PTC1, 5'-ATT GTC ATC TCG CCG TTC-3' and RET/PTC3, 5'-TGG AGA AGA GAG GCT GTA TC-3'. Reverse primers were 5’-TGC TTC AGG ACG TGT AAA-3' for RET/PTC1 and 5’-GCT TGC CTT GAC TTT TC-3' for RET/PTC3.

Five hundred nanograms of RNA were reverse transcribed and subjected to 40 cycles of PCR (94 C for 30 sec, 55 C for 30 sec, and 72 C for 1 min). The product was analyzed on a 2% agarose gel and hybridized with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement. Amplification with a RET probe covering the tyrosine kinase domain. The amplified products were also sequenced to confirm the rearrangement.

**Statistical analysis**

Correlation between BRAF mutation and clinicopathological parameters of the thyroid neoplasm was determined by two-tailed Fisher test (STATSOFT 6.0; StatSoft, Tulsa, OK). P < 0.05 was considered statistically significant.

**Results**

We retrospectively studied 96 patients from whom we had available preoperative FNA smears and surgical follow-up. Clinicopathological characteristics of the patients are summarized in Table 1. The average age was 42 ± 16 yr, and the female to male ratio was 2:1. We initially extracted blindly genomic DNA from FNAs and studied the presence of BRAF mutations in exons 11 and 15 by both SSCP and DNA sequencing. Then, we extracted DNA from corresponding paraffin-embedded surgical samples and performed the same analysis. Finally, the data from FNA and surgical samples were matched and compared with cytological and histological diagnoses. Two thyroid cell lines, the 8505C and BHT101, showing homozygous/hemizygous or heterozygous V599E BRAF mutation, respectively, were used as positive controls. Representative examples of direct sequencing of FNA-extracted DNA from one PTC that did not harbor the mutation and one PTC carrying the heterozygous V599E mutation are reported in Fig. 1. Representative examples of SSCP analysis of FNA-extracted samples are reported in Fig. 2. Results are summarized in Table 1. Because SSCP and direct sequencing gave completely overlapping results, the results are referred to both techniques. Detection of BRAF mutation on FNA demonstrated 100% sensitivity compared with the subsequent analysis of paraffin-embedded samples. The only BRAF mutation that we found was the V599E substitution in exon 15. BRAF mutations were restricted to PTC (38% of positivity); we did not find any mutation in adenomas and goiters (zero of 27 samples). Among PTC subtypes, classic, tall-cell, and diffuse-sclerosing variants showed the highest prevalence of BRAF mutations, whereas PTC-FV had a lower prevalence (14%). However, due to the small sample size, the clinicohistology analysis could not achieve statistical conclusions.

Eleven PTC cases were classified as indeterminate at FNA based on the presence of only some atypical features on the
cytological evaluation. Four additional smears (classic PTC) were insufficient because they did not contain enough cells. The molecular analysis proved most useful in four of these cases (one insufficient and three indeterminate), which showed the presence of the BRAF mutation (Table 1).

The second more frequent genetic alteration found in PTC is RET rearrangement leading to the generation of RET/PTC oncogenes. RET/PTC1 (H4-RET) and RET/PTC3 (RFG-RET) are the most prevalent variants (22). Cheung et al. (21) reported that RET/PTC detection in FNA may help refine preoperative diagnosis of PTC. BRAF mutation and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC and do not overlap in the same patients (9, 10). Thus, to further increase the sensitivity of our method we analyzed 60 of the samples for RET/PTC1 and RET/PTC3 rearrangements by RT-PCR on RNA extracted from smears. The assay was performed with primers designed to flank the fusion point between RET and its partner gene (H4 or RFG) as illustrated in Fig. 3. Rearrangements were found in six (18%) of 33 PTC (Table 1). Four cases were positive for RET/PTC1, and two were positive for RET/PTC3. The presence of the rearrangements was confirmed in the corresponding surgically resected tumor. RET/PTC rearrangement was not detected in follicular adenomas or nodular goiters. Thus, taken together, BRAF and RET/PTC detection helped to correctly identify 56% (38% + 18%) of PTC FNA samples. One of the insufficient FNA smears scored RET/PTC1 positive. Thus, the search for BRAF mutations and RET/PTC rearrangements allowed us to correctly identify 34% (27% + 7%) of the patients whose cytological diagnosis would otherwise have been not considered conclusive for PTC.

Discussion

The increasing detection of subclinical thyroid lesions places high demands on preoperative evaluation, especially because surgical procedures for benign and malignant lesions differ substantially. The best diagnostic tool currently available for the differential diagnosis of thyroid nodules is FNA biopsy. The procedure, however, could have limitations because of nondiagnostic yield and indeterminate results. Several genes have been proposed as markers for preoperative diagnosis in FNA such p27 (KIP1) (23), DAP4 (24), thyroid peroxidase (TPO) (25), and galectins (26). Unfortunately, data from multicenter studies showed limitation of these markers because of lack of specificity or sensitivity or both.

In the last year, our understanding of the molecular biol-
ology of PTC has made a step forward with the discovery that roughly 45% of PTC harbor one specific activating point mutation in the BRAF gene (9–17). The high prevalence combined with the PTC specificity render BRAF an attractive molecular marker for PTC diagnosis. Furthermore, the specificity (one selective codon) and the nature of the mutation (a single nucleotide change) render PTC-associated BRAF mutations easily detectable on a technical point of view. Here, we demonstrate that BRAF mutations can be easily detected in genomic DNA extracted from FNA. SSCP and direct sequencing were equally good and gave overlapping results. There were no false-negative results for BRAF detection in FNA compared with the analysis of follow-up surgical samples. There were no false-positive results because neither adenomas nor goiters scored BRAF positive. 
BRAF analysis of FNA correctly identified 38% of the PTC. Another genetic lesion that is often present in PTC is the recombination of the RET kinase to heterologous genes, leading to the generation of chimeric RET/PTC oncogenes (22). Although the prevalence of RET/PTC rearrangements varies greatly according to different reports, on average, it is smaller than that of BRAF mutations. In contrast to BRAF mutations, which appear to be restricted to PTC, RET/PTC rearrangements can also be present in benign conditions, including trabecular adenomas and, in some studies, Hashimoto thyroiditis (27–30). This may complicate the use of RET/PTC detection in the molecular diagnosis of PTC. Nonetheless, we also analyzed 60 of the samples for the presence of either RET/PTC1 or RET/PTC3 and found that 18% of PTC samples were RET/PTC positive. Samples harboring RET/PTC were negative for BRAF. Thus, the molecular diagnosis of both BRAF and RET/PTC could increase the fraction of PTC identified on FNA. It is noteworthy that molecular detection of BRAF mutation and RET/PTC rearrangements in FNA refined the diagnosis of five (four BRAF and one RET/PTC) of the 15 indeterminate/insufficient PTC FNAS. It should be pointed out, however, that we sorted out from our institutional series those indeterminate samples that revealed to be PTC at the final histopathological diagnosis. In clinical practice, only a fraction of indeterminate FNA samples do correspond to PTC. For instance, in recent series, 7–23% of indeterminate FNAs were finally revealed to be PTC (5, 6). Only in this group of samples, BRAF and RET/PTC detection may prove to be useful.

Thus, we envisage the possibility that the BRAF mutation analysis could be combined with RET/PTC detection to increase the fraction of identifiable PTC. A larger and prospective study will be necessary to calculate the diagnostic utility of FNA molecular analysis. Of course, the absence of BRAF mutations will not exclude a malignant condition. Nonetheless, a positive finding can support decision making about the extent of surgery, indicating the need of performing total thyroidectomy rather than lobectomy. Also, it should be recognized that, with the exclusion of insufficient samples, many indeterminate FNA smears are represented by PTC-FV (six of 11 in our PTC series). The FV of PTC was originally described as an invasive tumor with follicular architecture and nuclear features of PTC (31). The cytological diagnosis of PTC-FV can be extremely challenging due to overlapping morphological features with nonneoplastic or benign follicular lesions. PTC-FV are frequently RET/PTC (32) and BRAF negative, although a variant BRAF mutation (K600E) was recently reported in three of 32 PTC FV samples (17). In our series, only one PTC-FV sample showed RET/PTC, and three PTC-FV samples showed a V599E BRAF mutation. This means that a large fraction of PTC-FV would remain unrecognized at the molecular analysis of FNA smears if only RET/PTC and BRAF detection were applied. RAS mutations are frequent in PTC-FV (43% of the samples in Ref. 32), but they are also prevalent in follicular adenomas (33). Therefore, there is an urgent need for novel molecular markers for this specific PTC subtype that can be used in adjunct to RET/PTC and BRAF detection. In this frame, it is interesting to observe that deletion of a putative tumor suppressor on chromosome 22 has been reported in PTC-FV (34).

Finally, small-molecule BRAF inhibitors are available and are now tested in clinical trials (35). Should these compounds prove useful in treating BRAF-positive thyroid cancer, preoperative diagnosis of BRAF mutation on FNA will have an important therapeutic value.

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Address all correspondence and requests for reprints to: Massimo Santoro, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia, University ‘Federico II’ via’ Sergio Pansini 5, 80131 Naples, Italy. E-mail: masantor@unina.it.

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