Evaluation of the PAX8/PPARG Translocation in Follicular Thyroid Cancer with a 4-Color Reverse-Transcription PCR Assay and Automated High-Resolution Fragment Analysis

Alicia Algeciras-Schimnich,1 Dragana Milosevic,1 Bryan McIver,2 Heather Flynn,1 Honey V. Reddi,2 Norman L. Eberhardt,2,3 and Stefan K.G. Grebe1,2*

BACKGROUND: Molecular testing of thyroid malignancies, in combination with cytologic and histologic examination, is becoming increasingly attractive as a tool for refining traditional morphologic diagnosis. The molecular changes associated with follicular thyroid carcinoma (FTC) are point mutations in RAS oncogenes or the presence of PAX8/PPARG (paired box 8/peroxisome proliferator-activated receptor gamma) rearrangement.

METHODS: We developed and validated a clinical assay for the detection of PAX8/PPARG rearrangements that uses a 4-color reverse-transcription PCR (RT-PCR) assay and high-resolution fragment analysis.

RESULTS: The RT-PCR assay is applicable for detecting the various described fusion transcripts of PAX8/PPARG in formalin-fixed, paraffin-embedded thyroid tissue and in fine-needle aspirate biopsy washes from thyroid nodules. The analytical sensitivity of the assay is 1 abnormal cell in a background of 100–10,000 translocation-negative cells. A comparison of the RT-PCR assay with dual-fusion fluorescence in situ hybridization showed an overall concordance of 95%. With this assay, we obtained a prevalence for the PAX8/PPARG rearrangement in FTC of 62% (13 of 21 cases), compared with a 5% prevalence (3 of 55) for other follicular cell–derived neoplasms.

CONCLUSIONS: The introduction of this assay into clinical practice could provide useful information for the diagnosis and possibly for the prognosis and treatment of thyroid cancer in the future.

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1 Departments of Laboratory Medicine and Pathology, 2 Medicine, and 3 Molecular Biology and Biochemistry, Mayo Clinic, Rochester, MN.
* Address correspondence to this author at: Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First St. SW, Rochester, MN 55905. Fax 507-284-9758; e-mail grebs@mayo.edu.

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1 Nonstandard abbreviations: FTC, follicular thyroid carcinoma; FNAB, fine-needle aspiration biopsy; FA, follicular adenoma; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; RT-PCR, reverse-transcription PCR; FFPE, formalin fixed, paraffin embedded; HCC, Hürthle cell carcinoma; D-FISH, dual-fusion FISH.
2 Human genes: HRAS, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), and NRAS [neuroblastoma RAS viral (v-ras) oncogene homolog] and in the PAX8/PPARG (paired box 8/peroxisome proliferator-activated receptor gamma) fusion oncogene rearrangement (3); however, RAS gene mutations (a) occur only in 10%–25% of FTC cases, (b) are also seen in papillary thyroid carcinoma, and (c) occur equally often in FA and FTC (3–5). By contrast, the PAX8/PPARG fusion oncogene (a) is detected in up to 70% of FTC cases, (b) shows a prevalence of only 5%–20% in FA, and (c) has not been reported in papillary thyroid carcinoma (6, 7). Furthermore, the presence of the PAX8/PPARG fusion oncogene has been suggested to predict clinical outcome (3, 7). Finally, the protein product of PAX8/PPARG acts, at least in part, by inhibiting wild-type PPAR signaling (8), suggesting that such tumors could be amenable to PPAR agonist therapy, similar to what has been observed in anaplastic thyroid carcinoma (9). These features make the PAX8/PPARG fusion oncogene an attractive diagnostic target.
The PAX8/PPARG rearrangement is created by a somatic genetic translocation between chromosome arms 2q and 3p, in which the entire coding region of the PPARG transcription factor gene is fused in frame with the first 8 to 10 exons of the PAX8 gene, which encodes a thyroid-specific paired box transcription factor (6). Several approaches have been used to detect this fusion gene, including fluorescence in situ hybridization (FISH), immunohistochemistry (IHC) for PPARG, and reverse-transcription PCR (RT-PCR) targeted at rearrangement-specific transcripts (6, 10–12). FISH is considered the gold standard, but it is technically challenging, slow, and labor intensive. By contrast, IHC fits well into the established work flows of cytology and histology laboratories; however, the strength of the association between PPARG staining by IHC and the presence of the PAX8/PPARG rearrangement remains a matter of debate (10, 11, 13). RT-PCR appears to offer the best balance of ease of use, speed, and good agreement with FISH results. Unfortunately, the published PAX8/PPARG RT-PCR methods struggle with reliable amplification of RNA at low concentrations or poor RNA quality, a problem frequently encountered with scanty FNAB material, FNAB needle washes, or formalin-fixed, paraffin-embedded (FFPE) tissue sections. Moreover, 4 distinct fusions between PAX8 and PPARG have been described (14). One or several of these variants may be found in any given FTC sample.

To address these issues, we developed and validated a PAX8/PPARG RT-PCR method and compared its performance with PAX8/PPARG FISH and PPARG IHC methods. The method performs reliably with frozen tissues, FFPE tissues, and FNAB needle-wash samples. It has a streamlined work flow, is suitable for clinical laboratories, and allows detection of all the described PAX8/PPARG fusion transcripts.

Materials and Methods

SAMPLES
This study was approved by the Mayo Clinic Institutional Review Board.

We examined 56 FFPE tissue samples from follicular neoplasms for which clinical data and sufficient tissue were available [32 FA, 19 Hürthle cell carcinomas (HCC), and 10 FTC cases]. The characteristics and diagnostic classification of these tissue samples have previously been described (11). For all samples, the final diagnosis was blinded until the analyses were completed. Additionally, RNA from fresh-frozen tissue was available for 12 FTC, 8 FA, and 10 nonpathologic thyroid samples.

Six FNAB needle washes were obtained after routine ultrasound-guided FNAB procedures. After collection of the cytology samples, each FNAB needle was washed with 200 μL of RNAlater® (Applied Biosystems) and stored overnight at 4 °C.

POSITIVE CONTROLS
RNA from N-thy-ori 3-1 cells that had been stably transfected with a plasmid containing the PAX8/PPARG fusion gene (PAX8 exons 1–8 and 10 and PPARG exons 1–6) was used as a positive control. Additional positive controls consisted of 2 plasmids containing the following fusions: (a) PAX8 exon 8 and PPARG exon 1; (b) PAX8 exon 9 and PPARG exon 1. Plasmids were synthesized by Integrated DNA Technologies. The plasmids were transcribed in vitro with a MEGAscript® High Yield Transcription Kit (Applied Biosystems). Ten nanograms of in vitro–transcribed RNA was spiked into 1 μg of cell line total RNA to generate a single positive control containing all of the described fusion transcripts of PAX8/PPARG.

RNA ISOLATION AND RT-PCR
For FFPE samples, RNA was extracted from FFPE tissues with the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Applied Biosystems) according to the manufacturer’s instructions. The choice of this kit was based on an in-house study of 20 different FFPE samples. Use of the RecoverAll kit produced RNA of higher quality (according to the A260/A280 ratio) and more reproducible RT-PCR amplification of a housekeeping gene (PGK1, phosphoglycerate kinase 1) than guanidinium thiocyanate–phenol–chloroform extraction. In brief, we deparaffinized two 10-μm paraffin sections per sample at 50 °C with Histo-Clear (Fisher Scientific) and washed the pellet with 100% ethanol. After air drying, pellets were digested with protease at 50 °C for 3 h. RNA was purified by capture on a glass-fiber filter, treated with DNase, and eluted in 60 μL nuclease-free water. The protease, glass-fiber filters, and DNase are all components of the RecoverAll kit. RNA from FNAB needle washes was isolated with the mirVana™ miRNA Isolation Kit (Applied Biosystems), because these samples were also used in an ongoing microRNA study.

cDNA was prepared with 6 μL of DNase-treated RNA (concentration, 60–500 ng/μL) with the SuperScript® III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer’s instructions. PAX8/PPARG PCR was carried out with primers specific for PAX8 and PPARG exons (Table 1). Primer sets were designed to detect all 4 reported PAX8/PPARG fusion transcripts and 2 PCR control fragments. PCR products were detected by labeling the sense primer in each pair with a fluorescent dye. Amplifications were performed in a total volume of 25 μL containing the following: 4 μL RT-PCR product as template, 1× PCR buffer [20 nmol/L Tris-
HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L of each deoxynucleoside triphosphate, 100 mmol/L each of the sense and antisense primers, and 2.5 U Platinum Taq DNA Polymerase (Invitrogen). We used the following thermal profile: 94 °C for 10 min; 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s; a final 7-min extension at 72 °C; and cooling to 4 °C. To verify the presence of intact RNA, we amplified a final 7-min extension at 72 °C; and cooling to 4 °C. To verify the presence of intact RNA, we amplified a final 7-min extension at 72 °C; and cooling to 4 °C. To verify the presence of intact RNA, we amplified

Table 1. Amplification primers for PAX8/PPARG and PCR controls.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Fluorescent label</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAX8 Ex08S</td>
<td>CCT CTC GAC TCA CCA GAC CT</td>
<td>PET</td>
<td>NM_003466</td>
</tr>
<tr>
<td>PAX8 Ex09S</td>
<td>GCC CTT CAA TGC CTT TCC CCA TG</td>
<td>NED</td>
<td>NM_003466</td>
</tr>
<tr>
<td>PAX8 Ex10S</td>
<td>AGC GGA CAG GGC AGC TAT GC</td>
<td>6FAM</td>
<td>NM_003466</td>
</tr>
<tr>
<td>PPARG Ex01AS</td>
<td>CCA AAG TTT GTG GGC CAG AAT</td>
<td>None</td>
<td>NM_138712</td>
</tr>
<tr>
<td>PAX8 Ex05S</td>
<td>TCA ACC TCC CTA TGG ACA GC</td>
<td>VIC</td>
<td>NM_003466</td>
</tr>
<tr>
<td>PAX8 Ex06AS</td>
<td>GGA GTA GGT GGA GCC CAG G</td>
<td>None</td>
<td>NM_003466</td>
</tr>
<tr>
<td>PGK1 Ex09S</td>
<td>CAG TTT GGA GCT CTT GGA AG</td>
<td>VIC</td>
<td>NM_000291</td>
</tr>
<tr>
<td>PGK1 Ex11AS</td>
<td>GAG CTG AGA TGC TGT GCA AC</td>
<td>None</td>
<td>NM_000291</td>
</tr>
</tbody>
</table>

*a*, sense; *AS*, antisense.

*b* 6FAM, 6-carboxyfluorescein. PET, NED, and VIC are proprietary dyes (Applied Biosystems).

The limit of detection was determined with RNA from 4 tissue samples positive for a PAX8/PPARG translocation. The concentrations of translocation-positive RNA ranged from 5 pg to 500 ng diluted into a constant amount (500 ng) of translocation-negative RNA. We evaluated the amplification of the PAX8/PPARG fusion and internal controls to determine the minimum RNA concentration necessary to detect the presence of the translocation.

INTERPHASE DUAL-FUSION FISH

The PAX8/PPARG rearrangement was analyzed with dual-fusion interphase FISH (D-FISH). The probe mix consisted of the following bacterial artificial chromosome clones (Children’s Hospital Oakland Research Institute, BACPAC Resource Center): RP11-25O17, RP11-126L4, RP11-335I9, RP11-167M22, RP11-30G23, and RP11-275I11 for PPARG; and RP11-339F22, RP11-97J14, RP11-65I12, and RP11-141B14 for PAX8. The clones were labeled by nick translation with Spectrum Green (PPARG) and Spectrum Red (PAX8) (Abbott Molecular). In control experiments, the accuracy and specificity of all bacterial artificial chromosome clones were confirmed by hybridization to nonpathologic tissue.

For hybridization, we baked 5-μm tissue sections for 10 min at 90 °C. Slides were soaked at room temperature in CitriSolv (Fisher) for 15 min and in 100% ethanol for 5 min. Air-dried tissue sections were placed in a Coplin jar containing 1 mol/L Tris and 0.5 mol/L EDTA, pH 7.3, and microwaved in a 1000-W microwave oven for 15 min at 50% power. The sections were digested for 40 min with a pepsin solution (4 mg/L pepsin, 15 mmol/L NaCl, pH 1.8) prewarmed at 37 °C. The slides were dehydrated in graded ethanol baths of 70%, 85%, and 100% ethanol for 2 min each and air-dried. We then applied the probe hybridization mixture, cover-slipped the slides, and sealed them with rubber cement. The tissue sections and probes were denatured at 80 °C for 5 min. Hybridization was performed overnight at 37 °C in a humidified chamber. The posthybridization wash consisted of incubating the slides at 73 °C for 2 min with 2× saline–sodium citrate buffer (0.30 mol/L NaCl, 0.030 mol/L sodium citrate) with 1 g/L NP-40. We then applied counterstain solution (VECTASHIELD® Mounting Medium; Vector Laboratories) to the target areas and analyzed the slides with a fluorescence microscope equipped with a dual-bandpass filter set, as well as with single-bandpass filter sets. Two reviewers counted 100 nuclei with FISH signals per sample. Nuclei in which the 2 probes were fused, were touching, or were close to each other (distance, ≤1 probe signal) were scored as positive for gene fusions. Samples were considered
translocation positive if ≥10 cells showed the presence of the fusion.

PPARG IHC

PPARG is produced only at low concentrations in the nonpathologic adult thyroid gland and therefore serves as a marker for the expression of the PAX8/PPARG fusion gene. The PPARG IHC results for these tissue samples have previously been reported (11). In brief, tissue sections were stained with a mouse monoclonal antibody raised against the C terminus of PPARG (clone E8) (Santa Cruz Biotechnology). Antibody was detected with the EnVision nonbiotin detection system (Dako). Two independent reviewers scored the slides qualitatively. Only strong and specific nuclear staining was considered positive.

DATA ANALYSIS

Sensitivity and specificity of the RT-PCR analysis in classifying samples as nonpathologic, FA, FTC, or HCC were calculated, with histologic diagnosis used as the gold standard. Agreement between methods was assessed by means of \( \kappa \) statistics.

Results

DETECTION OF PAX8/PPARG EXPRESSION BY RT-PCR

We initially developed and validated the RT-PCR assay with RNA from the positive-control cell line N-thy-ori 3-1 that had been stably transfected with a plasmid containing the PAX8/PPARG fusion gene. As shown in Fig. 1A, the assay detects all 4 PAX8/PPARG mRNA transcripts and the 2 RNA QC products in the positive control. As part of the validation, we also conducted the RT-PCR assay with FFPE tissue samples, frozen tissue samples, and FNAB needle washes. Fig. 1, B and C, presents representative results of a translocation-positive tissue sample and a translocation-negative FNAB wash. No translocations were detected in the FNAB washes. The PAX8 control amplifications were all positive, however, and the final benign diagnoses of these samples were consistent with the absence of PAX8/PPARG rearrangements.

Sixty-one FFPE tissue samples collected between 1974 and 1991 as part of the Mayo Clinic thyroid cancer database (11) were available for method-comparison and
clinical-validation studies. Fifty-six of these samples showed amplification of at least one of the RNA positive controls and were therefore included in the analysis. This result represents an amplification success rate of 92%. Fig. 1B shows a representative sample with positive amplification of the PAX8/PPARG rearrangement. None of the tissue samples that contained the fusion oncogene showed all 4 PAX8/PPARG mRNA transcripts. The most frequent transcript combination was that of PAX8 exons 1–8 juxtaposed to PPARG exon 1 and PAX8 exons 1–9 juxtaposed to PPARG exon 1 (n = 10). In 3 FTC samples, only the expression of PAX8 exons 1–10 juxtaposed to PPARG exon 1 was observed; in the remaining 2 positive cases (1 FTC and 1 HCC), only the expression of PAX8 exons 1–9 juxtaposed to PPARG exon 1 was observed.

The assay was able to detect 5 ng of translocation-positive RNA in a background of 500 ng of translocation-negative RNA in all cases tested. In 1 case, the translocation was detected with only 50 pg of translocation-positive RNA. Assuming that 1 eukaryotic cell contains approximately 25 pg of RNA per cell (15), these results indicate a limit of detection of 1 translocation-positive cell in a background of 100–10 000 translocation-negative cells.

**COMPARISON OF PAX8/PPARG DETECTION BY RT-PCR AND D-FISH**

Fig. 2 are representative images of the PAX8/PPARG D-FISH assay in a translocation-negative tissue sample and a translocation-positive sample. Use of the dual-fusion probes reveals unaffected cells with 2 separated pairs of red and green signals (Fig. 2A). Translocation-positive cells have one or more fusion signals (yellow), which are caused by a colocalization of the red and green signals (Fig. 2B). The typical D-FISH pattern of positive cells consists of 1 red signal, 1 green signal, and 2 fusion (yellow) signals, but other patterns with fusion signals may emerge in cases of variant or unbalanced translocations. In this study, we observed large variation in the D-FISH patterns, including samples with only 1 fusion per cell and some samples with as many as 5 fusions per cell. D-FISH–positive cases had a mean of 70 fusion-positive cells per 100 analyzed cells (range, 16–93 positive cells). A comparison of the RT-PCR and D-FISH results for PAX8/PPARG expression revealed agreement for a positive result in 78% of cases (7 of 9 cases) and agreement for a negative result in 98% of cases (46 of 47). The overall agreement between the 2 methods was 95% (53 of 56 cases), with a κ value of 0.8 (95% CI, 0.6–1.0). Two cases were positive by RT-PCR but negative by D-FISH, and 1 case was negative by RT-PCR but positive by D-FISH. The reason for the discrepant cases is likely sampling error due to a gap between the tissue slices used for the RT-PCR and D-FISH analyses.

**COMPARISON OF PAX8/PPARG DETECTION BY RT-PCR AND PPARG IHC**

PPARG IHC results were available for 47 of the FFPE tissue samples. Comparison of PPARG IHC results with those for RT-PCR showed agreement for a positive result in 100% of cases (9 of 9 cases) and agreement for a negative result in 71% of cases (28 of 39). The overall concordance of the 2 methods was 77% (36 of 48 cases), with a κ value of 0.5 (95% CI, 0.2–0.7). Eleven samples were classified as translocation positive by PPARG IHC but translocation negative by the RT-PCR assay. With FISH analysis, we were able to determine that the discrepant cases showed copy gains for
To determine the utility of the PAX8/PPARG translocation in the various follicular lesions and nonpathologic thyroid tissue according to the RT-PCR assay.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Samples, n</th>
<th>Translocation positive, n</th>
<th>Positive, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTC</td>
<td>21</td>
<td>13</td>
<td>62</td>
</tr>
<tr>
<td>FA</td>
<td>39</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>HCC</td>
<td>16</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Nonpathologic thyroid</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

chromosome 3 (PPARG region), which could explain the increase in PPARG staining observed with IHC and the observed discrepancies between the assays.

**DISCRIMINATION BETWEEN FTC, FA, AND HCC WITH PAX8/PPARG RT-PCR**

To determine the utility of the PAX8/PPARG RT-PCR assay in differentiating FTC from benign FA, as well as from other malignancies with a follicular cell origin, such as HCC, we carried out a blinded study with 56 FFPE thyroid tissue samples and 30 frozen tissue samples (Table 2). Thirteen (62%) of 21 FTC lesions were positive for the PAX8/PPARG rearrangement. In contrast, 1 (6%) of 16 HCC samples and 2 (5.0%) of 39 FA samples were positive for the rearrangement. Histologic reevaluation of the FA-positive tissue samples confirmed the original diagnosis of FA. None of the tested nonpathologic thyroid RNA samples was positive for the translocation (n = 10). In our practice, the probability of a cytologically follicular lesion being FTC rather than FA is 1 in 5. With this prevalence, a PAX8/PPARG lesion that is positive by the RT-PCR assay has a 76% probability of being a FTC, compared with a pretest probability of 20%.

**Discussion**

We have shown that our PAX8/PPARG RT-PCR assay is applicable to FFPE tissue samples, as well as to FNAB needle washes. The latter application is confirmed by the fact that we could amplify intrinsic QC RNA, including the native PAX8 gene, from the needle washes. The ability to use FNAB washes is very appealing, because it allows a molecular signature of the lesion to be generated while ensuring that all cellular material can be used for cytologic examination, thereby avoiding compromising cytologic diagnosis.

Analytically, the assay proved itself robust, with few samples failing amplification, even when very old paraffin blocks were used. The limit of detection was also well suited for analysis of scanty material, with between 1 in 100 and 1 in 10 000 cells carrying PAX8/PPARG copies being detectable. This result suggests that the assay may also be applicable for detecting minimal residual disease.

The PAX8/PPARG RT-PCR results agree closely with those obtained with FISH. Furthermore, RT-PCR is less time-consuming, is easier to perform, does not require a large amount of cellular material, and has greater detection sensitivity, especially in cases when few translocation-positive cells are present. The few samples with discrepant D-FISH and RT-PCR results are most likely attributable to intra-tumor heterogeneity, because different portions of the tumor were sampled for the 2 assays. Also notable is that the RT-PCR assay provides information beyond what is offered with the D-FISH assay. One of the advantages of RT-PCR analysis is that it provides information about the specific breakpoints in the PAX8/PPARG fusion gene. Breakpoints may be located not only after exon 10 of PAX8 but also after exon 8 or 9 (6, 10). Given that the fusion gene encoding the PAX8/PPARG fusion protein appears to act as an oncogene (7), it is possible that the various splice forms have different pathogenic potentials. This assay could therefore be useful in addressing some of the questions related to the clinical significance of the various splice forms.

In terms of clinical performance, this study of PAX8/PPARG detection addresses important clinical diagnostic challenges. Cytologic discrimination between malignant and benign follicular neoplasms remains difficult. Recent advances in the molecular characterization of thyroid cancer have revealed a small number of genetic changes in these lesions (2). FTC is characterized by the presence of RAS gene mutations or PAX8/PPARG rearrangements. These 2 types of alterations have collectively been identified in approximately 80% of cases, and they seem to be mutually exclusive (3). Molecular characterization of cytologic samples has been proposed to have the potential to improve the diagnosis of thyroid nodules, especially in cases of indeterminate cytology. A recent study analyzed a number of thyroid-specific genetic alterations, including RAS gene mutations and PAX8/PPARG, to determine the feasibility of molecular testing of thyroid FNAB samples in clinical practice (16). The study showed that the introduction of molecular testing of nodules with an indeterminate cytology diagnosis had a positive predictive value of 97% for malignancy when molecular testing was used alone and a value of 100% when it was combined with cytology. This result supports the diagnostic utility of molecular testing for thyroid cancer diagnosis (16). Unfortunately, that study in-
cluded only 3 tumors that were later definitively proved to be FTC (most of the several hundred biopsies indicated benign lesions or papillary thyroid carcinoma). Only a single PAX8/PPARG rearrangement was detected, diminishing the study’s strength with regard to conclusions about follicular lesions. Our results, however, strongly suggest that the study’s conclusions can be extended to follicular lesions. Our own and other data show that 1 to 2 of 10 follicular lesions are confirmed to be FTC at the final histology analysis. Identifying PAX8/PPARG in an FNAB sample in advance would substantially alter the odds that a given lesion is malignant, increasing it to about 58% and 76% from cytologic pretest probabilities of 10% and 20%, respectively. We have previously described similar, but smaller, diagnostic improvements for PPARG IHC (11). The further improvement observed in the present study can most likely be attributed to the significant false-negative rate of PPARG IHC. In contrast to the excellent agreement between the D-FISH and RT-PCR results obtained for PAX8/PPARG, the agreement between the PPARG IHC and RT-PCR results was only 76%, with D-FISH analysis of the discordant cases demonstrating multiple copies of PPARG, a result that probably explains the increase in PPARG immunostaining in the absence of the translocation. Therefore, use of PPARG immunostaining as a surrogate for the PAX8/PPARG rearrangement will produce a larger number of false-positive results, because polysomy is often observed in FTC (17–20).

In summary, we have developed a reliable RT-PCR assay for the detection of PAX8/PPARG translocations in thyroid tissue samples. The assay provides a rapid, sensitive, and reproducible detection method that can be performed with a wide range of tissue specimens. Detection of this translocation could provide clinically useful information for the diagnosis, prognosis, and treatment of thyroid cancer.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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