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Molecular Staging Using Qualitative RT-PCR Analysis Detecting Thyreoglobulin mRNA in the Peripheral Blood of Patients with Differentiated Thyroid Cancer After Therapy

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Abstract. *Background: The prognostic significance of molecular staging using thyroglobulin (Tg)-based RT-PCR analysis in the peripheral blood of patients with differentiated thyroid cancer (DTC) was investigated. Herein, the specificity of molecular staging in DTC patients after thyroidectomy and ^{131}I ablation therapy was analysed, using different specific cDNA primers in qualitative nested and classical RT-PCR protocols. Patients and Methods: Ninty-five patients receiving TSH suppressive therapy were included, of whom 51 were disease-free and 44 presented residual/recurrent or metastatic disease as evidenced by biochemical and/or ^{131}I whole-body scan. Results: Our analysis detected RT-PCR products suggestive of the presence of Tg mRNA in all the blood samples tested, including healthy controls. A splice variant (350 bp) of Tg transcript (missing exon 3) was detected by nested RT-PCR in 34% of blood samples, however, without correlation to disease status. Conclusion: Qualitative Tg-based molecular staging in the peripheral blood of patients with DTC shows poor specificity in assessing DTC disease status.*

Well-differentiated thyroid carcinoma (DTC) is the most common endocrine malignancy, with an annual incidence of 0.5-10 cases per 100,000 individuals (1). Its management includes thyroidectomy and ^{131}I ablation therapy. However, recurrent or even metastatic disease appears to accompany almost 20% of DTC cases (2, 3). Notably, one-third of these occur one decade after the initial diagnosis. In addition, distant metastases or serious local recurrence may occur even 30 to 40 years after initial "successful" treatment (4). Finally, early detection of metastatic disease can influence dramatically the survival of patients with DTC (5).

Thyroglobulin (Tg), a 660,000-Kd glycoprotein, is considered a thyroid-epithelial-cell-specific product. Its serum levels represent a widely accepted marker for monitoring patients with DTC after thyroidectomy and ablative doses of radioiodine, as they usually show good correlation with the volume of differentiated thyroid tissue present (5, 6). Although the combination of serum Tg (sTg) measurement and whole-body scanning with ^{131}I (WBS) is highly sensitive for the diagnosis of recurrent or metastatic disease, sTg remains the principal diagnostic modality, particularly in cases of metastatic foci below the sensitivity of the conventional WBS (7, 8). However, the various sTg assays are plagued by a number of methodological problems, occasionally limiting the clinical value of routine sTg measurements. These include suboptimal sensitivity, high interassay variation, absence of international standardisation, the "hook" effect, interference of thyroglobulin autoantibodies, and the production by the tumour cells of variant Tg forms which may not be detected by some immunoassays in routine clinical practice (9-16).

The detection of tissue-specific tumour marker transcripts by RT-PCR protocols in RNA extracts derived from cells in blood has been proposed as a valuable modality for the diagnosis of early phases of metastatic disease in various solid tumours, such as prostate carcinoma, melanoma and neuroblastoma (17-19). Interestingly, the detection of Tg transcripts in fine-needle aspiration specimens from cervical lymph nodes has been suggested as a sensitive and possibly more accurate method to detect metastatic thyroid cancer than that of simultaneous measurements of sTg by immunoassay (20).

However, the clinical significance of such detections in the blood of patients with DTC receiving TSH suppressive

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therapy is still under investigation. A number of reports support the view that this method is more sensitive than sTg for the early detection of recurrent or metastatic disease (21-24). In addition, Tg-based RT-PCR detections are considered to be a useful tool mainly for papillary cancer (25) and its combined use with sTg has been advocated for the optimal follow-up of patients with DTC (26).

However, several groups have raised significant concerns about its specificity, presenting data with positive detections of Tg mRNA in normal subjects, as well as even in athyreotic patients (27-29). These conflicting reports, at least in part, may be attributed to the amplification of ectopically transcribed Tg (illegitimate transcription), a phenomenon related to the specificity of the oligonucleotide primers used in various RT-PCR protocols and to the PCR conditions, such as the number of amplification cycles, which significantly alter the sensitivity of the assays (27, 30). Attempts to overcome these limitations by the use of quantitative RT-PCR methods have provided contradictory and inconclusive results (29-37). In fact, the major drawbacks to this method are the significant overlap of the Tg mRNA copies detected in the peripheral blood of patients with various stages of the disease, as well as the lack of long-term follow-up studies in a substantial number of patients, which are necessary to elucidate whether the quantitative RT-PCR detections of Tg transcripts are indeed related to disease recurrence or metastases.

Herein, the presence of Tg transcripts in the peripheral blood of a large number of patients with thyroid cancer receiving TSH suppressive therapy was analysed. Group A (n=51) patients presented disease-free status, while Group B (n=44) presented evident disease recurrence/metastasis. Of those in Group A, 16 were re-tested after T_4 withdrawal. Both nested RT-PCR and classical RT-PCR assays were used and poor correlation between positive detections in peripheral blood and disease status (disease-free and disease recurrence/metastasis) was found.

Patients and Methods

Subjects. Ninety-five patients (66 females and 29 males) with thyroid cancer (86 with papillary, 7 with follicular, 2 with anaplastic), who were followed-up at the Department of Endocrinology, Diabetes and Metabolism of Evangelismos Hospital (Athens, Greece), were studied. All of them had been initially treated with total or near total thyroidectomy and ^{131}I ablation therapy, whereas at the time of RT-PCR analysis all were on TSH suppressive treatment.

Fifty-one of the patients were disease-free, as suggested by the undetectable sTg and negative WBS performed after T_4 withdrawal (Group A; mean follow-up of 6.7 years, range 1-23; 4 patients with positive anti-thyroglobulin antibodies). Forty-four patients presented residual, recurrent or metastatic disease, as suggested by the detectable sTg and positive WBS after T_4 withdrawal [Group B; 34 with detectable sTg (5 of whom with positive anti-thyroglobulin antibodies) and 10 with undetectable sTg (3 of whom with positive anti-thyroglobulin antibodies)]. Sixteen patients of Group A were evaluated 1-24 months (mean 7 months) after molecular staging by withdrawal from TSH suppressive treatment. Healthy subjects (n=5) with no clinical evidence of thyroid disease were used as controls.

Blood samples were collected for each patient and subjected to molecular staging and analysis for measurements of sTg, TSH and anti-thyroglobulin antibodies.

Hormone assays. sTg was measured by using the hTg Immuno-radiometric Assay (DiaSorin Biomedica Saluggia, Italy) with a lower detection limit of 0.8 ng/ml. TSH was measured by the Electrochemiluminesce Immunoassay (Roche Diagnostics GmbH, Mannheim, Germany) with the lower detection limit of 0.005 μIU/ml. Serum anti-Tg antibody was detected with a Radioimmunassay (BRAHMS anti-Tgn, DYNO test, Berlin, Germany).

RNA extraction. Three mL of venous blood were collected from each subject, transferred to EDTA-coated tubes and kept refrigerated (for not more than 4 hours) to be processed immediately prior to the isolation of peripheral blood mononucleated cells. The elimination of erythrocytes from the nucleated cells was accomplished by the selective lysis of red blood cells (38). Briefly, 2.5 mL of whole blood was added to 5 mL ELB (10 mM $KHCO_3$; 155 mM NH_4Cl; 0.1 mM EDTA, pH 7.4) and gently mixed by inversion. The mixture was then placed in ice water for 30 minutes with occasional gentle mixing by inversion. Leukocytes were collected by centrifugation at 400 xg (10˚C) for 10 minutes, and the supernatant was discarded. The cell pellet was gently re-suspended in 5 mL ELB, placed in ice water for 5 minutes to remove any remaining red blood cells and was then centrifuged again at 400 xg (10˚C) for 10 minutes. The cells were homogenized by repetitive pipetting in 1 mL Tri Reagent (TR-118, Molecular Research Center Inc., Cincinati, OH, USA) and were allowed to stand at room temperature (RT) for 5 minutes. Subsequently, 200 μL of chloroform were added, mixed thoroughly and allowed to stand at RT for 12 minutes. After centrifugation at 12000 xg (4˚C) for 15 minutes, 525 μL of the supernatant were collected in a clean tube. An equivalent volume of 2-propranolol was added, mixed by inversion and allowed to stand at RT for 10 minutes. After centrifugation at 12000 xg (4˚C) for 8 minutes, the supernatant was carefully aspirated and the total RNA pellet was washed in 1.0 mL of 75% ethanol. After centrifugation at 7500 xg (4˚C) for 5 minutes, the ethanol was aspirated, and the pellet allowed to air dry for 5-10 minutes. It was then re-suspended in DEPC-treated water, and the quality of the extracted RNA was confirmed by spectrophotometric analysis (Hitach U-1500) and by amplification of the cDNA for the GAPDH house-keeping gene (Table I) (gift from Dr V. Luu The, Centre Recherche Hospital, Laval University, Quebec, Canada) (39).

Reverse transcription. 2.5 μg of total RNA was reverse transcribed to complementary DNA (cDNA) in a final volume of 20 μL using 200 U of Superscript II RNAase H-Reverse Transcriptase (Life Technologies Inc., USA, Cat. No. 18064-014), 0.5 μM of the reverse specific primer for thyroglobulin and the oligo T18 primer for the GAPDH positive control, 1xRT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, Life Technologies Inc.), 500 μM of each deoxy NTP (Life Technologies Inc.) and 10 mM Diothiothreitol (DTT) (Life Technologies Inc.). Each sample was overlaid with

Table I. Overview of primer sequences

Detection of primer	Sequence	Product size	Exon
Nested RT-PCR			
Tg sense (1st PCR)	5'-GCCTCCATCTGCTGGGTGTC-3'	529	1
Tg antisense (1st PCR)	5'-CTCCCTCCGCAGAACACTGGGGT-3'		5
Tg sense (2nd PCR)	5'-TGCCCAGCCCCTTCGTCCCT-3'	447	2
Tg antisense (2nd PCR)	5'-CCCACCCCGTGCTGGAGAAGACG-3'		5
Normal sensitivity RT-PCR			
Tg sense	5'-ACCAGAACATCCACGTCTCC-3'	240	14
Tg antisense	5'-AACTCAGGAAGCCGAAGACA-3'		15
GAPDH sense	5'-AACGGATTTGGTCGTATTGGGC-3'	599	
GAPDH antisense			

Thyroglobulin gene: Gene Bank Acc. NM_003235.

mineral oil (Sigma Chemical Co., M-5904), and the reverse transcription was carried out for 50 minutes at 42˚C. The reaction was terminated by incubating the mixture for 20 minutes at 70˚C. A negative control was included in each sample, where the cDNA was replaced with an equal volume of water. RNA extracted from the thyroid tissue of a patient undergoing thyroidectomy (for reasons not associated with malignancy) served as a positive control.

PCR protocol. High sensitivity assay (nested). PCR was performed using 5 μL of the RT solution in a 50 μL reaction volume containing 1xPCR buffer (20 mM Tris-HCl pH 8.3, 50 mM KCl, Life Technologies Inc.), 200 μM of each deoxy NTP, 1.5 mM $MgCl_2$ (Life Technologies Inc.), 0.05 U/μL Taq polymerase (Life Technologies Inc.) and 0.4 mM of the thyroglobulin specific primers (Table I) (23). The cycling condition (Thermal Cycler, PTC-100, MJ Research Inc., USA) for the first round of amplification included an initial phase of 5 minutes at 94˚C, followed by 25 cycles of 45 seconds at 94˚C, 90 seconds at 60˚C, 2 minutes at 72˚C and final extension of 5 minutes at 72˚C.

Two μL of the PCR product of the first amplification were used as a template for the nested PCR in a final volume of 50 μL, using the same reagents as before and with a set of primers for thyroglobulin internal to those of the first step (23) (Table I). The cycling conditions included an initial phase of 5 minutes at 94˚C, followed by 19 cycles of 45 seconds at 94˚C, 2 minutes at 66˚C, 2 minutes at 72˚C and final extension of 5 minutes at 72˚C. For each experiment water replacing the patient's DNA was used as a negative control.

Classical PCR assay. Samples from 25 patients of Group A and 19 from Group B were tested with the normal sensitivity assay. PCR was performed by using 2.5 μL of the RT solution in a 25 μL reaction volume containing 10xPCR buffer, 200 μM of each deoxy NTP, 1.5 mM $MgCl_2$, 0.05 U/μL Taq polymerase and 0.15 mM of the thyroglobulin specific primers (Table I). The cycling conditions included an initial phase of 5 minutes at 95˚C, followed by 30 cycles of 65 seconds at 95˚C, 65 seconds at 55˚C, 50 seconds at 72˚C and final extension of 2 minutes at 72˚C. For each experiment water replacing the patient's DNA was used as a negative control.

Analysis of the RT-PCR products. RT-PCR products were electrophoresed on a 2% agarose gel and visualised with ethidium bromide under ultraviolet light. The nested RT-PCR products were subjected to digestion with the restriction enzyme Bgl II (Life Technologies Inc.) according to the conditions specified by the manufacturer, analysis on a 2% agarose gel and visualisation with ethidium bromide under ultraviolet light. To confirm Tg expression in the samples, the amplicon was column-purified (Quiaquick Nucleotide Removal Kit; Cat. No. 28304; Qiagen, Hilden, Germany) and sequenced on the 373A automated DNA Sequencer (ABI Biosystems, England). Briefly, direct sequencing of the PCR product was performed in 20 μL volumes using dye-labelled dideoxy terminator chemistry (ABI Prism 373; Applied Biosystems of Perkin Elmer, Foster City, CA, USA). The reaction contained 8 μL Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitag FS (ABI Biosystems, Cat. No. 402079), 0.2-0.4 ng of the PCR product and 0.25 μM of the thyroglobulin primer. The cycling conditions were set at 24 cycles at 96˚C for 30 seconds, 50˚C for 15 seconds and 60˚C for 4 minutes. The sequencing reaction products were then purified by ethanol precipitation, re-suspended in formamide EDTA and loaded on the sequencer for analysis.

Results

Nested RT-PCR. A 447-bp product was detected in 35 patients (68.6%) of Group A, 28 (63.6%) of Group B, 3 (60%) controls, as well as in the thyroid tissue extracts tested. In addition, 2 products of 447 and 350 bp were detected in 11 patients (21.6%) of Group A, 14 patients (31.8%) of Group B (4 of them with undetectable sTg and positive WBS) and 2 (40%) of control healthy subjects. Interestingly, only one PCR product of 350 bp was detected in 5 patients (9.8%) of Group A and in 2 patients (4.5%) of

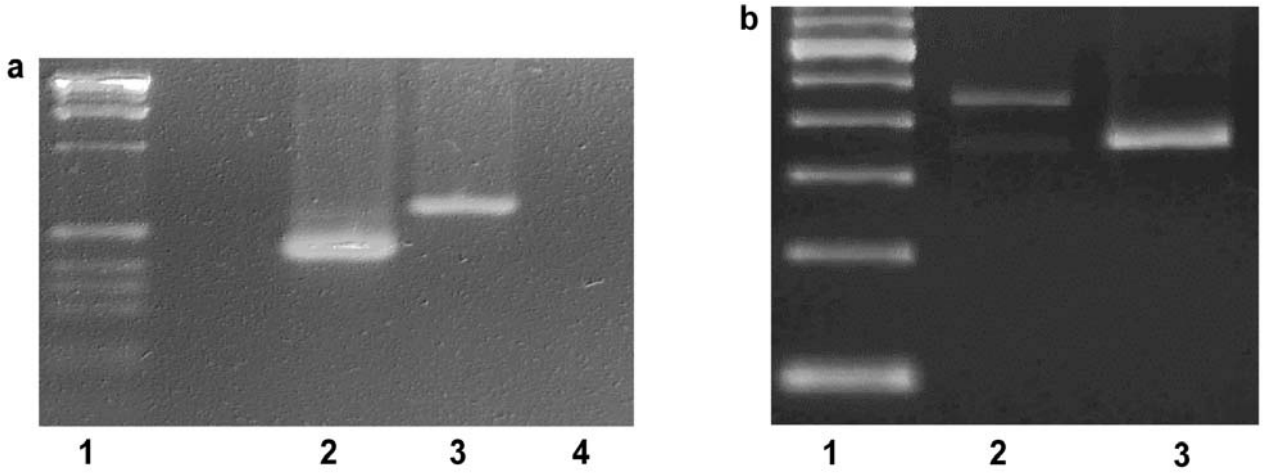

Figure 1. *An example of the Tg-based nested RT-PCR product depicted in the peripheral blood of patients with DTC. Note that in several analyses only the 447-bp PCR product was detected (a): Lane 1: molecular markers, lane 2: RT-PCR product from the peripheral blood of patient (447 bp), lane 3: GAPDH-positive control (599 bp), lane 4: negative control. Other analyses revealed both 447- and 350-bp PCR products and in other samples only the 350-bp PCR product was detected. (b): Lane 1: molecular markers, lane 2 : RT-PCR product from the peripheral blood of patient (447 and 350 bp), lane 3: RT-PCR product from the peripheral blood of patient (350 bp).*

Group B (1 with undetectable sTg and positive WBS). Considering the total number of patients, the product of 447 bp was found in 66.3%, both products (447 and 350 bp) were detected in 26.3% of the patients, while only the 350-bp product was detected in 7.3% of all patients (Figure 1).

Restriction digestion and direct nucleotide sequencing analysis indicated that the 447-bp product was derived from Tg mRNA, whereas the smallest band of 350 bp did in fact represented a splice variant of Tg (missing exon 3) (Figure 2).

Classical PCR ssay. The expected PCR product (240 bp) was detected in all the samples of DTC patients, including those of control healthy subjects (Figure 3). Direct sequence analysis indicated that this product was derived from Tg mRNA.

None of the 16 patients of Group A (follow-up of 1-24 months) showed any evidence of recurrent or metastatic disease upon re-evaluation after T_4 withdrawal.

Discussion

Based on data from other solid tumours, indicating that the detection of tissue-specific gene transcripts, normally absent in peripheral blood, may be valuable for the detection of metastatic spread (40, 41), several investigators have presumed that Tg-based RT-PCR protocols may be useful for the early diagnosis of metastatic disease in DTC. Dikoff *et al.* (21) were the first to amplify Tg transcripts from the peripheral blood of patients with DTC, with quite promising results. Subsequent studies have supported the notion that Tg mRNA detections are possibly more sensitive than sTg measurements for the diagnosis of recurrent or metastatic disease (22-24). However, among these reports there were differences in the sample handling and RNA isolation methods, in the RT-PCR protocols as well as in the sets of primers (22-24). It should be noted that there were substantial concerns about the specificity of our method, since positive detections have been documented in normal healthy volunteers, as well as in a number of athyreotic patients (22-24, 27, 42).

Although Ringel *et al.* (22) have detected TSH-receptor and Tg-positive cells by magnetic cell sorting in the peripheral blood of 2 control subjects (but not of one athyreotic patient), and identified them as thyrocytes, this method was mainly focused on the amplification of illegitimate Tg transcripts derived from the peripheral blood cells (43).

In order to overcome the limitations of the qualitative assays, investigators have proposed quantitative RT-PCR detections as an alternative method. Nevertheless, while a few studies have suggested that these assays are useful for monitoring patients, particularly in patients with positive anti-Tg antibodies (26, 31, 33), most of them have provided rather disappointing results (29, 34-37).

The results of our study clearly indicate that qualitative Tg-based RT-PCR protocols in the peripheral blood (high and normal sensitivity PCR assays) have no specificity for detecting the disease status of post surgery and radioiodine ablation therapy DTC patients who were receiving TSH suppressive therapy. Furthermore, none of the 16 patients who underwent T_4 withdrawal presented evidence of

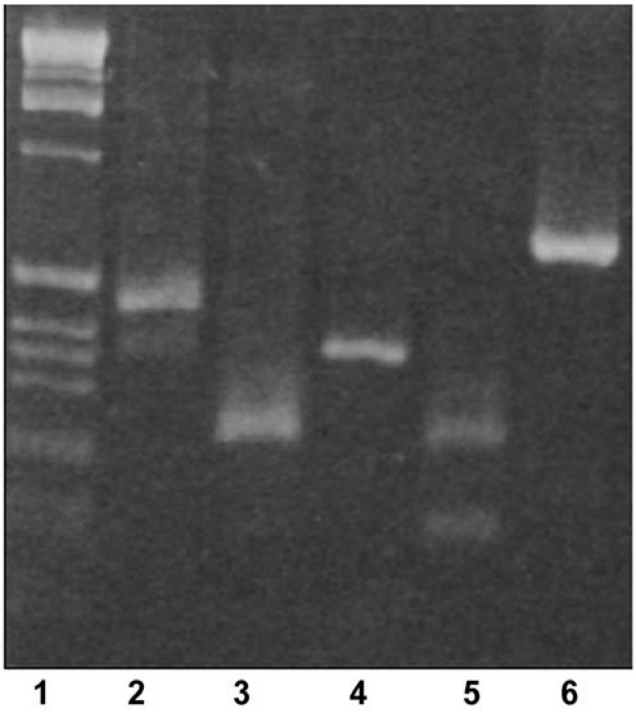

Figure 2. *An example of the fragments derived by enzymatic digestion of the 447-bp and 350-bp products Lane 1: molecular markers, lane 2: RT-PCR product from the peripheral blood of patient (447 bp), lane 3: products of digestion (222 bp and 225 bp), lane 4: RT-PCR product from the peripheral blood of patient with the alternative spliced form (350 bp), lane 5: products of digestion (222 bp and 128 bp), lane 6: GAPDH positive control (599 bp).*

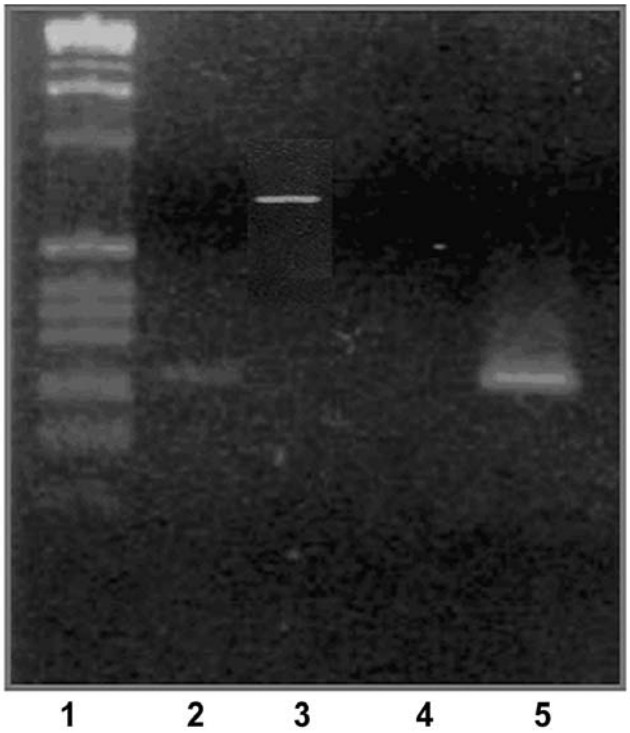

Figure 3. *An example of the Tg-based classic PCR product depicted in the peripheral blood of patients with DTC. Lane 1: molecular markers, lane 2: RT-PCR product from the peripheral blood of patient (240 bp), lane 3: GAPDH-positive control (599 bp), lane 4: negative control, lane 5: RT-PCR product from thyroid tissue (240 bp).*

recurrent/residual or metastatic disease, as assessed by sTg measurements and WBS performed 1 to 24 months (mean 7 months) after molecular staging. Moreover, the Tg splice variant (350 bp), depicted by the specific Tg primers of nested RT-PCR (primers annealing between exons 1 and 5) was not associated with disease status. These data are in concert with previous reports (29, 36). In our study these findings were confirmed in a higher number of patients using assays of high and classic sensitivity with different sets of primers. Consequently, we can conclude that extensive illegitimate Tg transcription, as noted by blood leukocytes, compromises the clinical use of the qualitative PCR protocol in evaluating the disease status of DTC patients receiving TSH suppressive therapy.

Indeed, it has been recently reported that Tg transcripts are found in normal blood cells (28, 44) and other tissues, such as thymus, suprarenal gland, hypophysis, lung, testis, veniform appendix and kidney (27, 45). In these tissues, the detection of Tg transcripts was directly related to the number of amplification cycles of PCR protocols. Thus, Bungalho *et al.* (28) have shown ectopic transcription after 32 cycles and Bojunga *et al.* (27) after 40 cycles. In our study, the ubiquitous presence of Tg mRNA (including in the normal subjects and the 2 patients with anaplastic carcinoma) was shown even with the normal sensitivity assay using 30 cycles, whereas no message was detected in our samples with 25 cycles (data not shown).

In addition, Gupta *et al.* (30) have suggested that the specificity of RT-PCR depends, to a great extent, on the design of the oligonucleotide primers, and that the primers should be specific to the thyroid tissue and without reactivity with normal peripheral blood mononuclear cells. Indeed, they have shown that primers spanning nucleotides 112-519 fulfill these criteria even after substantially increasing the sensitivity of their assay. However, it should be pointed out that in our study, using primers spanning a similar region of nucleotides (82-529), we were not able to obtain such results. Furthermore, contrary to Bellantone *et al.* (25) who have proposed that this method may be of value only in cases of papillary carcinoma, we found no differences in the RT-PCR results between the papillary- and follicular- type of DTC.

It has recently been suggested that the detection of Tg mRNA in the peripheral blood of patients without evident metastases may reflect clinically minimal disease. However, apart from Grammatopoulos *et al.* (24) who have followed-up 5 patients for 11 months and found a significant correlation between the detection of Tg mRNA and the subsequent diagnosis of metastatic disease by classic methods, there are not, as yet, similar reports on qualitative RT-PCR assays. In our study, however, the detection of Tg transcripts in 16 patients with disease-free status did not predict recurrence after a mean follow-up of 7 months.

The detection of a spliced variant of Tg mRNA in our nested RT-PCR assays is noteworthy. At least, 16 alternative spliced forms have been described for human Tg mRNA, located mainly on 4 exonic cassettes (46-49). Differential splicing of Tg mRNA has been observed in normal, benign and malignant thyroid tissues with no particular relationship between the presence of these forms and the underlying pathology (46). Furthermore, detection of splice variants in the peripheral blood has been previously reported (22, 33). In our nested RT-PCR assays, using primers encompassing exons 1-5, cDNAs with complete absence of exon 3 were identified in 7.3% of the patients with DTC, while both the expected PCR products and the splice variant forms were detected in 26.3% of them. In addition, this Tg mRNA splice was detected in 2 out of 5 control healthy subjects. Surprisingly, Biscolla *et al.* (23), using the same primers, did not report such variants.

It has been proposed that alternative splicing may be responsible for the heterogeneity of the Tg structure (50). Moreover, thyroid carcinomas may produce Tg molecules of altered structure, a phenomenon that may be associated with difficulties in the detection of these forms by various immunoassays (9-13). Whether the alternative spliced forms detected in peripheral blood are associated with Tg heterogeneity in serum remains to be elucidated. Notably, in one of the patients with papillary carcinoma and the presence of only the 350 bp variant, Tg was and remained undetectable even after T_4 withdrawal, despite the presence of pulmonary metastases. Although de-differentiation of the tumour seems more likely, the possibility of the production of an antigenically different Tg molecule cannot be excluded. In line with the above is the report by Savagner *et al.* (33), who proposed that the presence of alternative splicing Tg mRNA variants is associated with underestimation of the quantity of Tg mRNA. In their experiments with primers encompassing exons 6 and 7, they showed that spliced variant detection may account for about 33% of total RNA.

In conclusion, Tg transcripts are ubiquitously detected by both high and classic PCR assays in the peripheral blood of patients with DTC after surgery and radioiodine treatment. Optimisation of the assay protocols, in order to obtain reliable and comparable results, seems extremely difficult, rendering qualitative RT-PCR a rather compromised tool for the follow-up of such patients. Whether quantitative Tg-based PCR assays will be proven significant in clinical practice remains to be clarified.

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