Quantitative Reverse Transcription-PCR Measurement of Thyroglobulin mRNA in Peripheral Blood of Healthy Subjects

Susan T. Wingo, Matthew D. Ringel, Jeffrey S. Anderson, Aneeta D. Patel, Yvonne D. Lukes, Yin-Ying Djuh, Barbara Solomon, Diamaud Nicholson, Pina L. Balducci-Silano, Michael A. Levine, Gary L. Francis, and R. Michael Tuttle*

Background: Thyroglobulin mRNA can be detected qualitatively in the peripheral blood of patients with metastatic thyroid cancer, thyroid cancer patients with residual thyroid bed uptake, and individuals with no known thyroid disease with intact thyroid glands by use of a lengthy, highly sensitive extraction technique. To improve and broaden the clinical usefulness of this assay, we developed a quantitative reverse transcription (RT)-PCR assay for thyroglobulin mRNA, using RNA recovered from whole blood with a simplified extraction technique.

Methods: Whole blood was drawn from 32 healthy subjects in standard EDTA blood collection tubes. Total RNA was extracted from whole blood, using the PUREscript RNA Isolation Kit. RT-PCR using intron-spanning primers was used to quantitatively amplify thyroglobulin mRNA, using the ABI PRISM 7700 Sequence Detection System with a fluorescent-labeled, thyroglobulin-specific oligonucleotide probe. Thyroid RNA calibration curves were created using total RNA recovered from a single nondiseased thyroid gland.

Results: Qualitative RT-PCR demonstrated the presence of thyroglobulin mRNA in the whole blood sample of each healthy subject. The mean concentration of thyroglobulin mRNA detected in these subjects was 433 ± 69 ng of total thyroid RNA per liter of whole blood (range, 26–1502 ng/L). Overall assay imprecision (CV) was 24% for five samples analyzed 10 times each in separate analytical runs on different days.

Conclusions: Thyroglobulin mRNA can be accurately detected and quantified in peripheral blood from healthy subjects. This new quantitative technique may improve the clinical utility of circulating thyroglobulin mRNA detection in patients with thyroid disease.

Current recommendations for monitoring thyroid cancer patients include periodic radioiodine scanning and measurement of serum concentrations of thyroglobulin (Tg) protein by immunoassay (1). Thyroid hormone withdrawal or administration of recombinant human thyroid-stimulating hormone (TSH) is required to produce the TSH stimulation needed to achieve maximal specificity and sensitivity for these monitoring techniques (2). Both the morbidity associated with thyroid hormone withdrawal and the presence of interfering anti-Tg antibodies in 10–25% of patients have stimulated investigations into alternative methods for the detection of recurrent or metastatic thyroid cancer.

Recent reports have demonstrated that reverse transcription (RT)-PCR can be used to detect circulating cancer cells in the peripheral blood of patients with malignancies such as differentiated thyroid cancer (3–5), melanoma (6), and adenocarcinomas of the prostate (7) and breast (8).

More recently, we have reported the detection of circulating thyroid cells in whole blood samples of indi-
viduals with no known thyroid disease, using a qualitative RT-PCR assay (3). Furthermore, we have recovered cells expressing TSH receptor and Tg protein from the peripheral blood of these same healthy subjects.

This surprising finding of circulating thyroid cells in healthy subjects may limit the usefulness of the qualitative RT-PCR assay technique in the diagnosis and follow-up of thyroid cancer patients and in the management of patients with benign thyroid diseases. Therefore, the primary objective of this study was to develop and optimize a quantitative RT-PCR assay for Tg mRNA that would circumvent the limitations of the qualitative assay. Moreover, quantification of the circulating Tg mRNA would allow for monitoring response to therapy in patients with metastases.

To facilitate the collection of samples in a busy thyroid clinic, our second objective was to optimize a simplified sample collection and processing technique that would require minimal support from the research laboratory. We believe the combination of a simplified sample collection and extraction technique with a reliable quantitative RT-PCR assay will improve the clinical usefulness of the Tg mRNA assay.

**Materials and Methods**

**SUBJECTS**

Whole blood was collected in 10-mL EDTA blood collection tubes from 32 healthy subjects with no current evidence or history of thyroid disease seen in the Endocrinology Clinic at the Walter Reed Army Medical Center, the Johns Hopkins University Hospital, or the Washington Hospital Center. Each subject had a normal thyroid physical examination and normal thyroid function tests [third-generation chemiluminescent TSH (Nichols) and Free thyroxine by equilibrium dialysis (Nichols)] and no evidence of anti-thyroid antibodies [anti-Tg and anti-thyroid peroxidase antibodies (Quest Diagnostics)]. This protocol was approved by the institutional review boards of the participating institutions. Informed consent was obtained from all subjects.

**RNA EXTRACTION**

Whole blood samples were separated into 0.3- to 3-mL aliquots. In 17 subjects, total RNA was extracted from each aliquot, using the PUREscript kit (Gentra Systems) according to the manufacturer’s suggested protocol for blood samples. In 15 subjects, total RNA was extracted from 3-mL aliquots of whole blood with TRIzol LS (Life Technologies) as described previously (3). All quantitative results were normalized to 1 mL of whole blood for analysis.

**RT-PCR**

Total RNA (1 μg) was reverse transcribed to cDNA, using random hexamer primers, per the manufacturer’s recommendations (ABI). Final reaction concentrations were as follows: 1× TaqMan buffer, 5.5 mmol/L MgCl₂, 500 μmol/L each dNTP, 2.5 μmol/L random hexamer, 400 kU/L (0.4 U/μL) RNase inhibitor, and 1.25 kU/L (1.250 U/μL) Multiscribe reverse transcriptase. Reverse transcription was performed at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. Twenty-five percent of the synthesized cDNA served as substrate for PCR amplification.

Quantitative RT-PCR was performed in 96-well plates using Tg-specific primers and probe with the ABI PRISM 7700 Sequence Detection System. This system identifies and quantifies amplified Tg product at 7-s intervals during PCR amplification. Tg-specific primers that spanned a 1.5-kb intron were designed to amplify an 87-bp product from bp 262 to bp 348 in the cDNA sequence as follows: sense, 5’-GTGCCAACGGCAGTGAAGT-3’; antisense 5’-TCTGCTGTTTCTGTAGCTGAAA-3’; oligoprobe, 5’-FAM-ACAGACAAGCCACAGGCCTGCTT-TAMRA-3’. To identify amplification of any contaminating genomic DNA, all PCR products were visualized by agarose gel electrophoresis.

Each sample was assayed in triplicate. Final reaction conditions were as follows: 1× TaqMan buffer; 0.05 g/L gelatin; 0.1 mL/L Tween 20; 80 mL/L glycerol; 5.5 mmol/L MgCl₂; 200 μmol/L dATP, dCTP, and dGTP; 400 μmol/L dUTP; 200 μmol/L each primer; 100 μmol/L TaqMan oligoprobe; 10 kU/L AmpErase UNG; and 25 kU/L AmpliTaq Gold. The cycling conditions included an initial phase of 2 min at 50 °C, followed by 10 min at 95 °C for AmpErase, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C.

**PCR PRODUCT CHARACTERIZATION**

In addition to quantitative analysis of PCR product amplification using the 7700 Sequence Detection System, all RT-PCR products were analyzed by electrophoresis in 3% agarose gels followed by ethidium bromide staining to ensure amplification of the appropriately sized product. Samples omitting reverse transcriptase and template were included for each sample to identify contamination.

To further confirm the identity of the product, forward and reverse strands of several of the RT-PCR products were sequenced with the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer/ABI) using a denaturing gel of 4.75% acrylamide, 8.3 mol/L urea, 1× Tris-borate-EDTA (2500 V at 30 °C for 10 h) on the Perkin-Elmer/ABI 373 DNA Sequencer.

**ASSAY QUANTIFICATION**

A calibration concentration curve was prepared from a single nondiseased thyroid immediately frozen in liquid nitrogen at the time of thyroidectomy. RNA was extracted using standard organic extraction techniques and quantified with absorbance measurements. Dilutions were made to produce a calibration curve covering a concentration range of 3.2–10 000 pg of total thyroid RNA per well. RT-PCR was then performed on these samples as described above.

RT-PCR calibration curves were created using the threshold cycle, defined as the point at which each reac-
tion number at which a reaction reaches this point in the curve is directly proportional to the amount of cDNA template in the PCR reaction. Thus, this relationship allows for the creation of linear calibration curves over a concentration range. Calibrators and subject samples were assayed in triplicate on each 96-well plate.

INTRA- AND INTERASSAY IMPRECISION

The threshold cycle was determined in triplicate for each calibrator in six independent analytical runs over 6 different days. The measured threshold cycle of the triplicate determinations of each calibrator was used to determine the intraassay CV. An interassay CV was calculated for each of the calibrators in each of the six independent analytical runs. The mean threshold cycle measured for each calibrator in six independent analytical runs on 6 separate days was used to determine the interassay CV.

VALIDATION OF ACCURACY

Known amounts of normal human thyroid RNA (0–4.3 ng) were added to the total RNA recovered from whole blood samples obtained from a single athyreotic individual. This subject had been treated previously with total thyroidectomy and radioactive iodine, leaving him with no functional thyroid tissue visible by standard nuclear medicine radioactive iodine imaging and no detectable Tg mRNA in our previously reported qualitative RT-PCR assay (3). Each sample was assayed in triplicate in three separate assays for Tg mRNA.

QUALITY CONTROL

Quality-control (QC) samples were prepared using known amounts of normal total thyroid RNA (3.2 and 80 pg). QC samples were chosen to represent the measurement region of the assay thought to have the most clinical importance in the evaluation of thyroid cancer patients. Each QC sample was assayed in triplicate in six separate assays on different days. The overall CV was determined using the triplicate assay values (picograms of total thyroid RNA) determined in six separate assays on 6 separate days for each of the QC samples.

OVERALL ASSAY IMPRECISION

Whole blood samples from each of five healthy subjects were divided into 10 aliquots. These 10 aliquots underwent RNA recovery, cDNA synthesis, and quantitative RT-PCR analysis as described above. Aliquots from each patient were processed and analyzed in separate assays on different days. An overall CV for each of these five healthy subjects was determined from the Tg mRNA values (picograms of total thyroid RNA) obtained from the 10 independent measurements. The mean of these five individual CVs represented the overall CV for the entire assay.

Results

The appropriately sized 87-bp Tg cDNA RT-PCR product was visible on agarose gel electrophoresis for each calibrator. Sequencing of the PCR product confirmed the expected Tg cDNA sequence. Visual inspection of the Tg PCR products by gel electrophoresis after 40 amplification cycles revealed only subtle differences in band intensity despite the wide range of concentrations used. Negative controls did not demonstrate amplified product.

The calibration curve of the triplicate assay values demonstrated a strong linear relationship, with $r = 0.991–0.996$ (Fig. 1). The mean interassay CV for the threshold cycle of the calibrators in six separate amplification runs was 1.6%. The intraassay CV for the threshold cycle of the calibrators assayed in triplicate was consistently <1% for amounts >3.2 pg of total thyroid RNA. Although amplification was seen with known samples that contained only 0.5 pg, the intraassay CV was 4.3%. Negative controls consistently failed to demonstrate detectable PCR product at 40 cycles.

The results of 18 assays in six separate analytical runs of two known amounts of thyroid RNA (3.2 and 80 pg) were used to establish day-to-day QC for the assay. Quantification of the 3.2-pg QC sample demonstrated $2.6 \pm 0.6$ pg of total thyroid RNA ($CV = 23\%$). Similarly, the 80-pg QC sample demonstrated $67 \pm 12$ pg of total thyroid RNA ($CV = 17\%$).

Whole blood samples from five healthy subjects were divided into 10 separate aliquots and analyzed in separate analytical runs on different days. These five samples contained 17.9–20.9 pg of total thyroid RNA (mean, $19.3 \pm 4$ pg of total thyroid RNA). The overall assay imprecision for repetitive analysis of the same samples in these five healthy subjects was 24% (range, 19–32% in individual subjects).

The addition of increasing amounts of total thyroid RNA to samples obtained from the whole blood of an
athyreotic patient revealed a strong linear relationship, with $r = 0.996$ – 0.998 (Fig. 2). The amount of Tg mRNA detected in the whole blood of this athyreotic patient without added exogenous RNA was at the limits of detection of our assay in three separate analytical runs (mean, $1.5\pm 0.2$ pg).

The use of 0.3 mL or more of whole blood per aliquot consistently provided RNA of sufficient quality and quantity for RT-PCR amplification [PUREscript kit (Gentra Systems)]. In general, 0.3 mL of whole blood yielded $\sim 1\mu g$ of total RNA.

Agarose gel electrophoresis of the RT-PCR product demonstrated the appropriately sized product in each healthy subject. The measured threshold cycle for each healthy subject is overlaid on the calibration curve in Fig. 1. The measured value for each healthy subject was well within the limits of detection of our calibration curve.

By correcting for the amount of whole blood used to extract RNA and the amount of cDNA loaded into each reaction tube, the Tg mRNA concentration can be expressed as the amount of thyroid RNA per milliliter of whole blood. For these 32 healthy subjects, the amount of Tg mRNA detected in 1 mL of whole blood was equivalent to that found in 26–1502 pg of total thyroid RNA (mean, $433\pm 69$ ng/L; Fig. 3).

**Discussion**

In the present study, we developed a sensitive, specific, and reproducible RT-PCR assay for the measurement of Tg mRNA from whole blood. These results confirm our previous qualitative finding of circulating Tg mRNA and Tg and TSH-receptor expressing cells in peripheral blood of healthy subjects (3).

The quantity of circulating Tg mRNA detected is similar when expressed per milliliter of whole blood from each of these healthy subjects. Assuming that each thyroid cell contains $\sim 20$ pg of total RNA (9), the amount of Tg mRNA detected in this assay would roughly approximate 20–25 thyroid cells per milliliter of whole blood in healthy subjects, a surprisingly large number. It is possible that we are detecting ectopically transcribed Tg; however, the majority of athyreotic thyroid cancer subjects had no detectable circulating Tg mRNA in our qualitative assay (3). Moreover, the current system utilizes only a single PCR reaction without a second “nested” PCR using internal primers. In general, identification of ectopic transcription has required a second nested PCR reaction.

Studies of quantitative assays in athyreotic thyroid cancer patients are underway by at least two groups of investigators (10, 11). Our preliminary data suggest that the amount of circulating Tg mRNA in the majority of these subjects is either undetectable or so low that it cannot be reliably quantified (11).

If we assume that the number of circulating cells is dependent on the volume of thyroid tissue, thyroidal blood flow, and the type of thyroid tissue, the reasons for the broad range of values in healthy subjects are unclear. Careful clinical re-evaluation of the subjects with the highest values revealed no evidence of thyroid disease. It is unclear whether these subjects represent the highest points in the reference range, have a subtle enlarged thyroid gland, or have some as yet unidentified condition associated with increased Tg mRNA.

One limitation of this assay is the finite amount of total RNA available from the thyroid gland used to create the calibration curve. Because of the sensitivity of this quantitative RT-PCR assay, it would not be surprising if small differences in Tg mRNA expression between healthy...
individuals could be detected. Although this will not affect the relative concentrations calculated with a new calibration curve, it may make comparisons between different calibration curves questionable. We have seen little difference between the amount of Tg mRNA detected in the normal thyroid RNA between three distinct thyroid specimens (data not shown). To create reproducible calibration curves that will be unaffected by variations in the abundance of Tg transcript in thyroid cells, in vitro transcription can be performed using a plasmid containing the appropriate Tg cDNA insert as template for the RNA polymerase.

A second limitation of the assay is that the RT-PCR assay will only detect Tg mRNA species that contain the region of interest bounded by our primers. The clinical significance or prevalence of Tg splice variants that would not be detected by this assay is uncertain.

Although our previously developed RNA extraction procedure is highly sensitive and does allow long-term freezing at $-70 \, ^\circ C$ between cell lysis and RNA extraction (3), it is also quite time-consuming. The RNA extraction kit from Gentra offers several advantages. No hazardous chemicals are needed in patient care environments, and organic waste quantities are substantially reduced. From specimen collection to RNA pellet resuspension, this procedure takes $<2 \, h$, compared with 4–5 h for the older procedure.

In summary, we believe the use of this simplified blood collection and RNA extraction procedure in combination with the newly described quantitative RT-PCR assay will allow for accurate quantification of circulating Tg mRNA in peripheral blood of patients with thyroid disease. The assay is simple, rapid, and reproducible and may provide a new method for monitoring the response of patients with thyroid cancer and benign thyroid diseases to therapy, particularly in the presence of circulating anti-Tg antibodies that interfere with the currently available immunoassays. Further clinical studies are needed to better assess the clinical utility of quantitative measurement of circulating Tg mRNA.

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References