Thyroglobulin mRNA quantification in the peripheral blood is not a reliable marker for the follow-up of patients with differentiated thyroid cancer

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Abstract

Background: The detection of serum thyroglobulin (Tg) by immunoassay is widely used to detect residual, recurring or metastatic thyroid carcinoma tissue in patients with differentiated thyroid cancer (DTC) after total thyroidectomy and radioiodine therapy. However, this method requires thyroid hormone withdrawal to increase sensitivity and is limited by the interference of anti-Tg antibodies. To solve these problems, the detection of Tg mRNA from circulating thyroid cells by reverse transcription (RT)-PCR has been suggested as an alternative method. However, different previous reports show discrepant conclusions as to the clinical usefulness of Tg mRNA quantification.

Methods: We compared three methods of blood collection and RNA extraction, and quantified Tg mRNA (by real time RT-PCR) in the peripheral blood of a) probands without thyroid disease \( n = 42 \), b) thyroid autonomy \( n = 15 \), c) Graves’ disease \( n = 22 \), d) euthyroid goiter \( n = 6 \), and in DTC-patients after thyroidectomy and radioiodine therapy e) with \( n = 16 \) and f) without \( n = 37 \) metastasis. As the use of citrate blood in combination with a subsequent separation of mononuclear cells showed a significantly better RNA yield than the extraction of RNA from EDTA or citrate blood without the separation of mononuclear cells, this was the method used. Total RNA was reverse transcribed with random hexamer primers and Tg mRNA was amplified by real time RT-PCR using specific primers and hybridization probes. The Tg mRNA concentrations were normalized to \( \beta \)-actin mRNA concentrations.

Results: Mean circulating Tg mRNA for each group detailed above, expressed as the ratio of Tg to \( \beta \)-actin concentrations \( \times 1000 \), were: a) 2.3 (range 0.03 – 70.89), b) 0.25 (range 0.02 – 0.55), c) 0.31 (range 0.05 – 1.36), d) 0.18 (range 0.08 – 0.35), e) 0.57 (range 0.03 – 3.03) and f) 0.17 (range 0.02 – 0.60). Furthermore, we found no correlation between serum Tg and Tg mRNA.

Conclusions: In summary, our data do not show significant differences in Tg mRNA expression between the investigated groups. Therefore, the detection and quantification of Tg mRNA in peripheral blood is unlikely to be suitable for the follow-up of DTC.

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Introduction

Since as many as 20% of patients with differentiated thyroid cancer (DTC) may develop recurrences and metastasis even after successful initial therapy (1), effective surveillance after therapy is necessary. The follow-up of thyroid cancer patients after total thyroidectomy includes whole-body radioiodine scanning and above all the measurement of serum thyroglobulin (Tg). However, the method of the latter is characterized by some critical drawbacks, such as the requirement for thyroid hormone withdrawal or the administration of recombinant human thyroid stimulating hormone (TSH) to increase assay sensitivity (2). Furthermore, the presence of interfering anti-Tg antibodies in up to 25% of all patients with DTC may limit the measurement of serum Tg (3, 4). Additionally, different variants of Tg produced by some tumors cannot be detected by the immunoassay (5). Due to the detection of circulating cancer cells in the peripheral blood of DTC-patients (6, 7), and to circumvent the problems of serum Tg measurement, Ringel et al. (8) amplified Tg mRNA by reverse transcription (RT)-PCR from the peripheral blood of patients with thyroid ablation for DTC. Moreover, Wingo et al. (9) established a sensitive assay in which they detected Tg mRNA in the peripheral blood of healthy subjects using quantitative RT-PCR. However, the Tg mRNA concentrations determined in this
study by Wingo et al. (9) are characterized by a broad range of variation. Since the authors did not normalize their Tg mRNA expression data to a housekeeping gene like β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), it is difficult to discuss the strong variations in their assays. Both biological variations of the patients and differences in the cDNA preparation (e.g. different RNA extraction yields and differing efficiencies of RNA reverse transcription) could cause the broad range of variation seen in the Tg mRNA concentrations. Moreover, the detection of Tg mRNA in the peripheral blood of healthy persons (9) might restrict the clinical usefulness of this assay to the detection of DTC recurrence. Recently, Takano et al. (10) detected Tg mRNA in the peripheral blood of thyroidectomized patients and found no statistical difference between patients with and without metastasis. In addition, different methods of blood collection and RNA extraction have been used by different research groups and this could influence the results of mRNA quantification in the different studies, as shown for human immunodeficiency virus (HIV) type 1 RNA (11). Therefore, we investigated three methods of blood collection and established an optimized RNA extraction procedure. Furthermore, we established a sensitive and specific real time quantitative RT-PCR and compared the expression of Tg mRNA normalized to the internal control β-actin in healthy subjects, patients with Graves’ disease, patients with thyroid autonomy, patients with euthyroid goiter and in DTC-patients after thyroidectomy and radioiodine therapy with and without detectable serum Tg.

Patients and methods

Patients

Citrate-blood samples were obtained from 42 normal subjects with no clinical evidence of thyroid disease, from 22 patients with Graves’ disease, 15 patients with thyroid autonomy, 6 patients with euthyroid goiter and 69 patients with well differentiated thyroid carcinoma. The DTC-patients had been treated with total thyroidectomy. 53 of them received radioiodine ablation and 16 of them were investigated before radioablation. The group of DTC-patients comprised 42 patients with papillary carcinoma and 27 patients with follicular carcinoma. They were classified according to WHO criteria (12). 16 DTC-patients had metastases detected by computed tomography or 131Iodine scintigraphy. No patient had antibodies against Tg. The study was approved by the ethics committee of the University of Leipzig and informed consent was obtained from each patient.

Blood samples and RNA extraction

To optimize the RNA preparation, 12 ml EDTA blood and 24 ml citrate blood were obtained from four volunteers with no clinical, laboratory (free triiodothyronin, free thyroxine and TSH within normal limits and negative for Tg antibodies, thyroperoxidase antibodies and TSH-receptor antibodies) or ultrasound evidence of thyroid disease. Total RNA was extracted from 4 ml EDTA blood and 4 ml citrate blood using the QIAamp RNA blood kit (Qiagen, Hilden, Germany; used according to the manufacturer’s instructions) immediately, 24 and 48 h after blood collection. All blood samples were stored at 4°C. Furthermore, mononuclear cells were separated using Vacutainer CPT (Becton Dickinson, Franklin Lakes, NJ, USA; used according to the manufacturer’s instructions) from 4 ml citrate blood immediately, 24 and 48 h after blood collection. Total RNA was extracted from these mononuclear cells using the QIAamp RNA blood kit (Qiagen) immediately after separation.

Total RNA was quantified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using an RNA 6.000 LabChip kit (Agilent Technologies) according to the manufacturer’s instructions. Before extracting the total RNA from the citrate blood of the different patient groups, mononuclear cells were separated using Vacutainer CPT.

Reverse transcription

11.5 µl of total RNA were reverse transcribed in a 20 µl reaction. The reaction mixture consisted of 5 × First Strand buffer (250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2; GibcoBRL, Karlsruhe, Germany), 0.5 mM dNTPs, 5 mM DTT (GibcoBRL), 15 U prime RNase inhibitor (Promega, Madison, WI, USA) and 200 U Moloney murine leukemia virus reverse transcriptase (GibcoBRL). Reverse transcription was performed at 37°C for 60 min and 94°C for 5 min.

Real time RT-PCR

The quantification of Tg mRNA and the housekeeping genes β-actin and GAPDH by real time PCR was performed using a LightCycler (Roche, Mannheim, Germany). Oligonucleotide primers were designed to be intron spanning and were purchased from MWG Biotech AG (Ebersberg, Germany). LightCycler hybridization probes were designed and supplied by TIB Molbiol (Berlin, Germany). Sequences were obtained from the GenBank database. Both the nucleotide sequences of the two primers and of the two hybridization probes of each target are shown in Table 1. First, an optimal PCR for all investigated genes was established using the LightCycler DNA Master SYBR Green I kit (Roche) according to the manufacturer’s instructions;
Annealing temperatures and MgCl₂ concentrations were optimized to create a one-peak melting curve. Additionally, the amplicons were checked by agarose gel electrophoresis for a single band of the expected size. The β-actin PCR was processed through 40 cycles of 3-step PCR, including 0 s denaturation at 95°C, 7 s annealing at 55°C and 9 s elongation at 72°C. The cycling conditions used to amplify GAPDH were 0 s at 95°C, 7 s at 57°C and 22 s at 72°C. The Tg PCR used 40 cycles including 0 s at 95°C, 7 s at 64°C and 16 s at 72°C. Furthermore, the Tg PCR was performed as a hot-start PCR using TaqStart antibodies (Clontech, Palo Alto, CA, USA).

Afterwards, the PCR fragments were cloned into the pGEM-T vector (Promega) and the plasmids were sequenced to confirm the specificity of the PCR. The quantification of the Tg mRNA was performed using the LightCycler DNA Master hybridization probes kit (Roche). The 20 μl reaction consisted of 2 μl LightCycler DNA Master hybridization probes (containing Taq DNA polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP) and 10 mM MgCl₂), additional MgCl₂ according to the optimization results, 0.5 μM of each primer, 0.15 μM of each hybridization probe (3'-fluorescein and 5'-LightCycler Red 640 labeled) and 2 μl of template. Dilutions of the plasmids were used to generate calibration curves for each template. The quantification of the Tg-plasmid dilution was performed in three triplicated PCR runs to determine the intra- and interassay imprecision. The measured threshold cycle of the triplicate determinations of each calibrator was used to determine the intra-assay coefficient of variation (CV) and the mean threshold cycle for each calibrator in the three independent runs was used to calculate the interassay. The quantification of each proband sample was performed in one duplicated PCR run. To normalize for differences in the amount of cDNA added to the reactions, quantification of β-actin was performed as an endogenous control. The determined concentration of Tg mRNA was then normalized with concentration of the housekeeping gene β-actin. Data are expressed as means ± S.E.M. Differences between the investigated groups of patients were evaluated by paired-sample t-test or Mann–Whitney U-test after testing for Gaussian distribution using a K–S test. A P value of 0.05 was characterized as statistically significant.

### Table 1 Sequences of the primers and the hybridization probes.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>GAPDH forward</td>
<td>5'-TTC GTC ATG GGT GTG AAC C-3'</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>5'-GAT GAT GTT CTG GAG AGC CC-3'</td>
</tr>
<tr>
<td>β-actin forward</td>
<td>5'-GGC CTC GCT GTC CAC CTT CC-3'</td>
</tr>
<tr>
<td>β-actin reverse</td>
<td>5'-TGT CAC CTT CAC GTG TCC AGT TTT-3'</td>
</tr>
<tr>
<td>TG forward</td>
<td>5'-CCT GCT GGC TCC ACC TTG TTT-3'</td>
</tr>
<tr>
<td>TG reverse</td>
<td>5'-CCT TGT TCT GAG CTT CCC ATC GTT-3'</td>
</tr>
<tr>
<td>LC640-TG</td>
<td>5'-LC640-CAT CCC ACA GTG CAG CAC CGA TGG-3'</td>
</tr>
<tr>
<td>Fluorescein-TG</td>
<td>5'-CAT GCT ACC CAC CCT TTC CGA CAC C- FL-3'</td>
</tr>
</tbody>
</table>

Figure 1 Total RNA yield extracted from EDTA blood, citrate blood and citrate blood after separating mononuclear cells using VACUTAINER CPT. The figure shows the mean of total RNA levels ± S.E.M. for two independent measurements of four probands without thyroid disease. RNA was extracted immediately, 24 and 48 h after blood collection respectively and its concentration was quantified using an Agilent 2100 Bioanalyzer. Differences between the investigated patient groups were evaluated by paired-sample t-test or Mann–Whitney U-test after testing for Gaussian distribution using a K–S test. A P value of 0.05 was characterized as statistically significant.
**Serum Tg measurement**

Serum Tg was measured using a commercial radioimmunoassay (DYNOtest Tg-pluS kit, BRAHMS Diagnostica, Hennigsdorf, Germany). The functional sensitivity of this assay was 0.3 μg/l according to the manufacturer’s specifications.

**Results**

Before the quantification of Tg and β-actin mRNA from the peripheral blood of the different patient groups, we tested three different methods of sample preparation to ensure an optimal RNA yield and quality. The use of citrate blood in combination with the subsequent separation of mononuclear cells, immediately, 24 and 48 h after blood collection, showed a significantly better RNA yield than the extraction of RNA from EDTA blood or citrate blood without the separation of mononuclear cells (Fig. 1). The total yield of RNA produced from 4 ml blood varied between 0.1 μg and 6.4 μg RNA depending on the method and time of RNA extraction (EDTA-blood 48 h after blood collection and citrate blood with separation of mononuclear cells immediately after blood collection respectively). The measured concentrations of the duplicate RNA determinations for each volunteer were used to determine the intra-assay CV for each RNA extraction method. The mean intra-assay CV was 12.1% for EDTA blood, 13.1% for citrate blood and 16.7% for citrate blood with mononuclear cells separated.

Moreover, the quantification of the housekeeping gene GAPDH (Fig. 2) confirms the greater RNA amount extracted from citrate blood with mononuclear cells separated in comparison with the RNA extracted from EDTA or citrate blood without a subsequent separation of mononuclear cells. In addition, the quantification of Tg mRNA in probes which were prepared using citrate-blood with mononuclear cells separated showed an average fivefold increase in Tg mRNA level over probes in which the RNA was extracted from citrate blood without the separation of mononuclear cells (data not shown). Subsequently therefore, for the different patient groups (i.e. normal subjects, patients with Graves disease, thyroid autonomy, euthyroid goiter or DTC), total RNA was extracted after the separation of mononuclear cells from citrate blood.

Using the calibration curves generated for each template by the dilution of the appropriate plasmid, Tg mRNA was quantified in all investigated samples. The detection limit of the Tg PCR was 0.42 fg pGEM-T vector containing the 352 bp thyroglobulin insert, as determined by using the plasmid calibration curves.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>TG mRNA*1,000/β-actin mRNA ratios for the different patient groups.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No thyroid disease</td>
</tr>
<tr>
<td>n</td>
<td>42</td>
</tr>
<tr>
<td>Mean</td>
<td>2.3</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>1.7</td>
</tr>
<tr>
<td>Range</td>
<td>0.03–70.89</td>
</tr>
<tr>
<td>Significance (P) in comparison to subjects without thyroid disease</td>
<td>0.214</td>
</tr>
</tbody>
</table>
The intra-assay CV for the threshold cycle of the calibrators assayed in triplicate was <1% and the intra-assay CV in three independent runs was 2.3%. The comparison of 42 patients with papillary carcinoma (with or without metastasis or residual thyroid tissue) with the subjects without thyroid disease revealed a significantly decreased expression of Tg mRNA in the peripheral blood of the DTC-patients ($P < 0.05$) (Table 2, Fig. 3). Furthermore, the comparison of the Tg mRNA expression of the 27 patients with follicular carcinoma (with or without metastasis or residual thyroid tissue) with that of healthy subjects also showed a significantly decreased expression of Tg mRNA ($P < 0.05$) (Table 2, Fig. 3) in the patients with DTC. There were no statistical differences between the group without thyroid dysfunction and the patients with thyroid autonomy ($P = 0.14$), Graves’ disease ($P = 0.21$) or euthyroid goiter ($P = 0.06$) (Table 2, Fig. 3). Moreover, we found no statistical difference in the Tg mRNA expression between the DTC-patients (including both patients with papillary carcinoma and patients with follicular carcinoma) with and those without metastasis ($P = 0.14$) or between the DTC-patients with thyroid ablation and without metastasis and those DTC-patients with residual thyroid tissue before radioablation ($P = 0.09$; Table 3, Fig. 4).

Serum Tg was measured in 69 patients with DTC. While serum Tg could be detected in DTC-patients with metastasis and in 13 of the 16 DTC-patients with residual thyroid tissue, it was undetectable in the DTC-patients without metastasis or residual thyroid tissue. In contrast, Tg mRNA was detectable in all investigated samples. The comparison of serum Tg

**Table 3** TG mRNA*1,000/β-actin mRNA ratios of DTC-patients without metastasis, with metastasis and with residual thyroid tissue.

<table>
<thead>
<tr>
<th></th>
<th>DTC without metastasis/residual thyroid tissue</th>
<th>DTC with metastasis</th>
<th>DTC with residual thyroid tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>37</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Mean</td>
<td>0.17</td>
<td>0.57</td>
<td>0.39</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0.02</td>
<td>0.23</td>
<td>0.13</td>
</tr>
<tr>
<td>Range</td>
<td>0.02–0.60</td>
<td>0.03–3.03</td>
<td>0.01–2.18</td>
</tr>
<tr>
<td>Significance ($P$)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3 Tg mRNA levels of probands without thyroid disease, patients with Graves’ disease, with thyroid autonomy, with euthyroid goiter, with papillary carcinoma or with follicular carcinoma, respectively. Tg mRNA amounts are shown normalized to the housekeeping gene β-actin. Differences between the investigated patient groups were evaluated by Mann–Whitney U-test. *$P < 0.05$. 

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with Tg mRNA showed no correlation between these two parameters ($r = 0.01, P = 0.91$).

**Discussion**

The detection of tissue-specific gene expression in the peripheral blood of tumor patients by RT-PCR has been reported for prostate cancer (13), neuroblastoma (14) and breast cancer (15). In a subsequent study, Ditkoff et al. (6) reported that the detection of Tg mRNA in the peripheral blood of DTC-patients correlates with the presence of extrathyroidal disease. Moreover, Tg mRNA extracted from the peripheral blood was explored as a potential tumor marker in thyroid cancer in two further studies (7, 8). These reports suggested the use of Tg mRNA detection as an early indicator of thyroid tumor metastasis. However, the detection of Tg mRNA in the peripheral blood of healthy subjects by quantitative RT-PCR (9) and findings of Bojunga et al. (16) showing that the detection of Tg mRNA is not specific for thyroid tissue cast doubt on the specificity of Tg mRNA as a tumor marker. Moreover, Takano et al. (10) found no statistical difference between the Tg mRNA levels measured in the peripheral blood of DTC-patients with and those without metastasis.

However, one possible explanation for these differing results are reports that different methods of blood collection and RNA extraction influence the quantification of RNA from blood by RT-PCR. In order to determine the effects of blood collection and handling procedures on the quantification of plasma HIV-1 RNA, Dickover et al. (11) compared anticoagulants and sample processing. The HIV-1 RNA levels in plasma collected in Vacutainer tubes with EDTA as the anticoagulant were, on average, 14% higher than those measured in samples from heparinized tubes (11). Furthermore, they could show that, in comparison to baseline EDTA plasma, acid-citrate-dextrose and heparinized plasma samples measured at 24 h post-collection had 39% and 60% less HIV-1 RNA respectively (11). Therefore, we investigated the influence of three different methods of blood collection on RNA yield and the level of the housekeeping gene GAPDH before we quantified the Tg mRNA. We restricted our investigations to EDTA blood and citrate blood since heparin has been shown to inhibit PCR (17) and because of this heparinized blood has to be pretreated with heparinase. Moreover, the study of Dickover et al. (11) showed significantly lower RNA levels in heparinized blood samples even after treatment with heparinase.

The finding that RNA concentrations and GAPDH levels decreased over time in the stored blood of all investigated blood collection methods, but were most stable in citrate blood with mononuclear cells separated (Figs 1 and 2), is of importance in clinical situations involving the shipment of whole blood overnight at ambient temperatures. Our data show that it is necessary to extract RNA as fast as possible from whole blood to detect low copy transcripts in a reliable manner. Moreover, this study shows that the anticoagulant influences the quantification of target transcripts distinctly. Therefore, the occurrence of negative peripheral blood samples for Tg mRNA in previous studies (6–8) is likely to be caused by both less optimal blood collection (e.g. using RNA STAT-60 or heparinized tubes for blood collection) and RNA extraction procedures, and also differences in the sensitivity of the PCR-primers. To ensure a high RNA quality with minimal loss of RNA quantity, we therefore performed RNA extraction on mononuclear cells within two hours of citrate blood collection.

By real time RT-PCR we found a high variability in the Tg mRNA levels of subjects without thyroid

![Figure 4 Tg mRNA levels of DTC-patients without metastasis or residual thyroid tissue, of DTC-patients with residual thyroid tissue (before radioablation) and DTC-patients with metastasis respectively. Tg mRNA amounts are shown normalized to the housekeeping gene β-actin. Differences between the investigated patient groups were evaluated by Mann–Whitney U-test.](image-url)
Thyroglobulin mRNA in peripheral blood

Acknowledgements

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References

7. Tallini G, Ghosee S, Emanuel J, Gill J, Kinder B, Dimich AB et al. Detection of thyroglobulin mRNA in the early detection of recurrent thyroid carcinomas since the detection of Tg mRNA derived from recurrent thyroid carcinomas would be affected by the expression of Tg mRNA in blood cells and/or other tissues.
8. Our Tg mRNA expression data did not allow us to distinguish between DTC-patients with and without metastasis whereas the measurement of serum Tg clearly detected DTC-patients with metastasis. Moreover, we found no correlation between Tg mRNA expression and serum Tg level. Despite different blood collection and RNA extraction methods, our data are in line with the findings of Takano et al. (10), which support the hypothesis that Tg mRNA and serum Tg come from different sources and Tg mRNA derives not only from thyroid epithelial cells.
9. In conclusion, our findings show that by using Tg mRNA quantification it is not possible to distinguish between the investigated groups: persons without thyroid disease, patients with Graves’ disease, thyroid autonomy, euthyroid goiter or DTC-patients after thyroidectomy with and without residual thyroid tissue or thyroid carcinoma metastasis. Therefore, the detection and quantification of Tg mRNA in the peripheral blood is unlikely to be a suitable alternative for the follow-up of patients with DTC.
