REAL TIME RT-PCR ANALYSIS OF THYROGLOBULIN mRNA IN PERIPHERAL BLOOD IN PATIENTS WITH CONGENITAL ATHYREOSIS AND WITH DIFFERENTIATED THYROID CARCINOMA AFTER STIMULATION WITH RECOMBINANT HUMAN THYROTROPIN

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Objective. Thyroglobulin (Tg), measured by immunometric assay, is the most sensitive and widely used clinical marker for thyroid cancer progression and relapse. However, these Tg determinations are of limited sensitivity and susceptible to interference by Tg autoantibodies. As a possible diagnostic alternative, we tested a real time RT-PCR protocol to determine Tg mRNA levels in peripheral blood.

Methods. Tg mRNA was determined by real-time RT-PCR using total RNA from peripheral blood. Tg mRNA blood levels were calibrated to the mRNA encoding the housekeeping enzyme glyceraldehyde phosphate dehydrogenase (GAPDH); pooled blood from ten healthy subjects served as a RT-PCR positive control.

Results. Tg mRNA and serum Tg were detected in twelve patients with differentiated thyroid cancer (DTC) after thyroidectomy and radioiodine therapy, however, there was no correlation with the clinical stage. An increase in Tg mRNA and protein was observed after application of recombinant human thyrotropin (rhTSH) in four patients with DTC stimulated with rhTSH for postoperative follow up. Tg mRNA and protein were also detected in four congenital athyreotic patients. Analysis of Tg mRNA levels using a commercial multiple tissue Northern blot revealed Tg hybridization signals in several extrathyroidal tissues (salivary gland, trachea, kidney, pancreas, adrenal gland, etc.).

Conclusions. Our data suggests that RT-PCR detects Tg mRNA of extrathyroidal origin, from leukocytes or from metastasizing carcinoma cells under basal conditions or after TSH stimulation. However, considering the marked and highly variable individual Tg mRNA backgrounds, interpretation of real time PCR results requires caution. This limits the clinical use of Tg mRNA determination by real time PCR to an individual tumor progression marker in follow-up.

Key words: Tumor progression - Ectopic transcription - Molecular diagnostics - Follow-up

Differentiated thyroid carcinomas (DTC), although the most frequent endocrine neoplasm, are rare and account for about 1 % of all human cancers with a higher prevalence in women than in men (MAZZAFERRI and KLOOS 2001; SCHLUMBERGER and TORLANTANO 2000). Usually, DTC is a curable disease with a good prognosis and an overall 10-year survival rate of 70-95% (GIMM 2001). Current treatment protocols include thyroidectomy, I^{131} therapy and TSH-suppressive T4 application. However, recurrent disease is observed in 5-30 % of

the cases and metastases develop in 10-15% (Mazzaferri and Kloos 2001; Schlumberger and Torlantano 2000), with the problem of further dedifferentiation of the neoplastic tissue in 30 % (Goretzki et al. 1993). As the prognosis is better if a relapse is recognized and treated as early as possible, sensitive postoperative monitoring is crucial. In the follow up of DTC, determination of serum Tg (S-Tg) levels - complemented by ultrasonographic imaging and whole-body radioiodine scans (WBS) - is the most important and sensitive technique and its sensitivity is further increased following TSH stimulation. As Tg expression is retained also in advanced thyroid carcinomas (Pacini and Lippi 1999), any increase in S-Tg indicates metastases, recurrent or persistent disease.

S-Tg is measured by immunometric (IRMA, ILMA) or radioimmuno (RIA) assays. Such antibody based procedures, however, present some pitfalls, such as suboptimal interassay precision, limited sensitivity, interference by Tg autoantibodies, or the so-called Hook effect. Besides, there are a limited number of tumors or tumor recurrences without any elevation of S-Tg (Spencer 2000; Westbury et al. 2000). Nevertheless the sensitivity of immunochemical Tg measurement for early cancer recognition has certain limits. The detection of circulating tumor cells, minimal residual disease and micrometastases may, therefore, have important therapeutic and prognostic implications. Several studies recently demonstrated the utility of RT-PCR based assays in the detection, staging and monitoring of minimal residual tumor burden in malignant melanoma, prostate, breast, gastrointestinal, neuroblastoma and thyroid cancer (Burchill and Selby 2000; Gossein et al. 1999; Zippelius and Pantel 2000; Fenton et al. 2001; Ringel et al. 2001; Tallini et al. 1998). PCR was shown to be superior to conventional techniques in detecting occult tumor cells, allowing the identification of one malignant cell mixed with one to ten million normal cells (Gossein et al. 1995). These findings suggest, that this might also hold true for thyroid cancer and that the measurement of Tg mRNA in peripheral blood by real time PCR might represent a more sensitive diagnostic alternative in post-operative monitoring of DTC. In the present study we established a real time PCR protocol and examined the sensitivity and specificity of real time PCR for the detection of Tg mRNA in peripheral blood in a limited number of patients. We show that relevant changes in Tg mRNA levels are detectable by RNA-based real time PCR e.g. in rhTSH stimulated patients.

Patients and Methods

Patients. S-Tg and Tg mRNA levels were measured in peripheral blood of four congenital athyreotic patients with negative I^{123} or Tc^{99m} scans. We also examined four patients treated for DTC by total thyroidectomy and I^{131} ablation, twice stimulated with 0.9 mg/d recombinant human TSH (rhTSH) on two consecutive days (Luster et al. 2000). These patients were scheduled for diagnostic whole body scanning in the follow up of thyroid carcinomas. Two of them presented with current and two without metastases as diagnosed by imaging techniques (scintigraphy, MRT, CT) or S-Tg determination; three had papillary carcinomas (PTC) and one a follicular carcinoma (FTC). Blood was obtained from three patients after the first and after the second rhTSH intramuscular injection (i.m.) and from one patient before the first and after the second rhTSH i.m. injection. In addition Tg mRNA and protein levels of twelve arbitrarily selected DTC patients were measured. Five of them had current metastases as diagnosed by imaging and S-Tg measurement and seven had not. A pool of total RNA from three resected goiters was used to generate a standard curve for real time PCR. Total RNA prepared from blood of ten healthy subjects was pooled and used as a control. All patients were treated at the Department of Nuclear Medicine of the University of Wuerzburg. Informed written consent was obtained from rhTSH and athyreotic patients and the healthy subjects.

S-Tg and thyrotropin measurements. Serum thyroglobulin levels were determined by immunoradiometric IRMA with DYNOtest® Tg-S and Tg-pluS Assay (B.R.A.H.M.S Diagnostica GmbH, Biotechnology Center, Henningsdorf/Berlin, Germany) according to the manufacturer's protocol. The lower detection limit of the DYNOtest® Tg-S was 0.3 ng/ml and of DYNOtest® Tg-pluS Assay 0.04 ng/ml. The functional assay sensitivity (FAS) with the interassay coefficient of variation < 20 % was 0.1 ng/ml (Morgenthaler et al. 2002). Thyrotropin (TSH) measurement was carried out by DYNO TSH 1 (B.R.A.H.M.S Diagnostica GmbH, Biotechnology Center, Henningsdorf/Berlin, Germany) according to the manufacturer's protocol with a detection limit of 0.03 mU/l.

RNA extraction. 2.7 ml venous blood was collected from patients in EDTA tubes (Sarstedt®, Nümbrecht, Germany) at routine appointment. From 1 ml venous EDTA blood, total RNA was extracted with QIAamp RNA blood mini kit with a DNase I treatment

(Qiagen GmbH, Hilden, Germany). Total RNAs were resuspended in 50 µl of RNAse free water and stored at –80°C. RNA integrity was controlled for representative examples by electrophoresis on denaturing formaldehyde gels.

cDNA synthesis. Quantification of isolated RNA was achieved by spectrophotometric determination at 260 nm. 2.5 µg of total RNA were reverse transcribed in a final volume of 20 µl with 2.5 µM oligo(dT)$_{12-18}$ primers; and 200 units Superscript TM II in first strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.1 M DTT, 0.5 mM each dATP, dTTP, dGTP and dCTP; 40 units RNaseOut™ according to the manufacturer's protocol [Lifetechnologies, Karlsruhe, Germany]). These samples were designated "**RT+**"**;** samples incubated without reverse transcriptase "**RT-**" were used as negative controls. Complementary DNA (cDNA) was resuspended in nuclease-free water at a final volume of 100 µl and stored at –20°C.

Polymerase chain reaction (PCR) - conventional PCR and agarose gel analysis. In conventional PCR an intron-spanning GAPDH primer set (Stratagene, Amsterdam, Netherlands) with the following nucleotide sequences was used: GAPDH$_{sense}$ (exon 3) 5'-CCACCCATGGCAAATTCCATGGCA-3' and GAPDH$_{antisense}$ (exon 7) 5'-TCTAGACGGCAGGTCAGGTCCACC-3'. These primers were used at a final concentration of 500 nM. Tg primers for conventional PCR were the same as used in the real time PCR assay (see below). PCR was performed in a final volume of 50 µl, containing 10 µl of the cDNA solution (approximately 0.125 µg cDNA), 200 µM of each dNTP (Amersham Pharmacia Biotech GmbH, Freiburg, Germany), forward and reverse primer and 1.25 units Taq DNA polymerase in a PCR buffer supplied by the manufacturer (QIAGEN GmbH, Hilden, Germany) on a thermal cycler, PTC-200 (Biozym Diagnostics, Oldendorf, Germany). One sample containing water instead of cDNA served as no template control (NTC).

After initial denaturation, 40 amplification cycles were performed for Tg and 25 for GAPDH (consisting of denaturation at 92°C/15 s, annealing at 60°C/15 s and extension at 72° C/30 s). Reaction products were analyzed on an ethidium bromide-stained agarose gel.

Real time PCR. Tg intron-spanning primers (TAKANO et al. 2000) for real time PCR were designed to amplify a 167 bp product in the cDNA sequence as follows: Tg $_{sense}$ 5'-GAGAAGAGCCTGTCGCTGAA-3' (exon 46; 7942- 7960 bp (VAN DE GRAAF et al. 2001)), Tg $_{antisense}$ 5'-CAGCTCACTGAACTCCTTGT-3' (exon 47; 8090- 8111 bp (VAN DE GRAAF et al. 2001)), Tg $_{probe}$ 5'FAM-TGAGTTCTCACGGAAAGTACCCA-3'TAMRA. Real time PCR for Tg mRNA determination was done in a final volume of 50 µl containing 10 µl cDNA solution (approximately 0.125 µg), 300 nM sense and 900 nM antisense primer and 200 nM probe in TaqMan Universal Master Mix; GAPDH mRNA was measured using the Predeveloped TaqMan Assay. All primers, probes and solutions were from P.E.-Applied Biosystems. Real time PCR reactions were performed on an ABI Prism 5700 (P.E.-Applied Biosystems) according to standard protocols of the manufacturer. "RT+" samples were measured in triplicates and "RT-" samples were assayed in duplicates. Tg and GAPDH mRNA contents of each clinical sample were simultaneously determined in a 96 well plate. The threshold cycle (C_T) represents the PCR cycle at which an increase of the reporter fluorescence (DRn) above the baseline is first detected. No increase in DRn was observed in the "RT-"or in NTC samples.

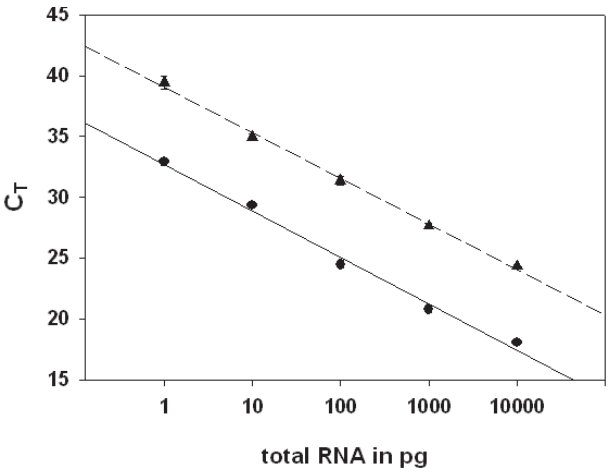

Fig. 1 Typical Standard curve of GAPDH (▲) and Tg (•) mRNA established with TaqMan PCR standard analysis software. The standard curve plots the C_T (described in "Material and methods") versus the log of input amount of total RNA (pg) of three pooled goitre samples. The slope was between –3.5 and –3.8 and the correlation was –0.99 in all experiments (n = 8).

Standard curve. Tg mRNA was normalized to the mRNA coding for the housekeeping enzyme GAPDH. For this purpose, a standard curve comprising 1-10000 pg of total RNA (in 5 successive steps of 10-fold dilutions in water) isolated in water from three pooled goiter samples was generated (Fig. 1) to mimic a biological sample work up for DTC cells. Based on this plot the amounts of GAPDH and Tg mRNA of each test

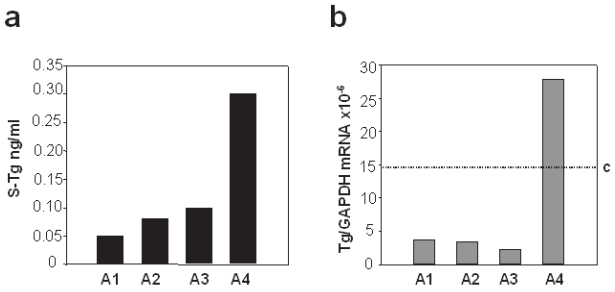

Fig. 2 Serum Tg levels and GAPDH/Tg mRNA ratios of congenital athyreotic patients (A1-4). A Serum Tg measurement with the DYNOtest Tg–pluS assay. B Tg/GAPDH mRNA ratio. TSH levels are in all subjects in the normal range (0.3-4 mU/l). c (reference line) = control of ten pooled blood samples of ten healthy subjects. In three patients Tg mRNA levels were lower than in the control, except for A 4.

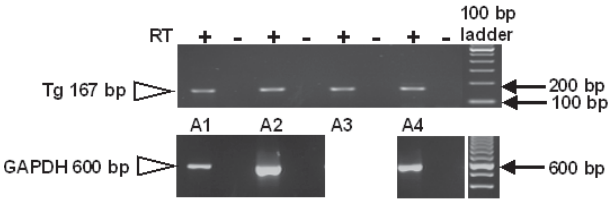

Fig. 3 Conventional RT-PCR amplification of GAPDH (A1, A2, A4) and Tg (A1- 4) mRNA from subjects with thyroid agenesis. RT +/–: cDNA synthesis reaction with/without reverse transcriptase. Expected PCR products of 660 bp for GAPDH and of 167 bp for Tg are visualized by agarose gel electrophoresis and staining with ethidiumbromide.

sample were calculated. Quantity of Tg mRNA was divided by GAPDH mRNA content, and the ratio is shown.

Northern blot. To examine the expression of Tg mRNA, the Human Multiple Tissue Expression (MTE™) Array 2 from BD Biosciences Clontech (Heidelberg, Germany) was hybridized to a [α^{32}P]dTCP- labeled 167 bp Tg PCR product (see above real time PCR). Hybridization was performed according to the manufacturer's protocol, with a final washing step using 0.3 SSPE/ 0.3% SDS at 65°C. The blot was analyzed with the Phosphoimager Cyclone™ (Packard, Meriden, USA). Signal intensity is expressed as **d**igital **l**ight **u**nits per unit area (DLU/mm^2) and is defined as total signal intensity divided by the area of the hybridizing region in mm^2 after subtraction of the background signal.

Results

Detection of Tg mRNA and S-Tg in patients with thyroid agenesis. Tg mRNA levels were determined in peripheral blood of four patients with congenital thyroid agenesis (Fig. 2). Imaging techniques, ultrasound and $I^{123/131}$- or Tc^{99m}-scintigraphy, gave no clue for ectopic or hypoplastic thyroid tissue. TSH levels in all subjects were in the normal range (0.3 - 4 mU/l) under thyroxine supplementation. As shown in Fig. 3, a 167 bp product was amplified from the cDNA of all four athyreotic patients demonstrating the presence of Tg mRNA in their peripheral blood. Similar results were obtained using two other Tg primer sets (data not shown). This was not due to contamination by genomic DNA, as on the one hand, primers were intron-spanning and on the other hand, neither GAPDH- nor Tg-specific amplicons (Fig. 3) were detected when "RT-" samples were used as templates for conventional PCR.

The expression levels of Tg and GAPDH mRNA were also measured by real time PCR. Tg/GAPDH mRNA ratios in athyreotic patients ranged from 3.66 x 10^{-6} to 27.8 x 10^{-6}. They were lower in three of the athyreotic patients than in the pooled normal control (14.9 x 10^{-6}) except for patient A4 (Fig. 2B), who also had the highest S-Tg level. No amplification was observed in "RT-" or in "NTC" control samples. S-Tg was not detectable in the four athyreotic subjects with DYNOtest® Tg-S assay by IRMA. When the more sensitive DYNOtest® Tg plus assay was used, S-Tg levels between 0.05 (A1) and 0.33 (A4) ng/ml were determined (Fig. 2A).

Northern blot. To find a potential source for the observed Tg mRNA in the athyreotic patients, we carried out Northern blot analysis of mRNA using a commercial multiple tissue mRNA array. A 167 bp Tg-specific, radioactively labeled PCR product was hybridized to a human Multiple Tissue Expression Array, and hybridization signals were analyzed by phosphoimaging (Fig. 4). As expected, the most intense signal (800 DLU/mm^2) was obtained in the thyroid gland. However, the salivary gland and the trachea also show prominent hybridization (around 20 DLU/mm^2), and a couple of other extrathyroidal tissues - kidney, pancreas, adrenal gland, liver and heart - still gave significant signals between 7 and 10 DLU/mm^2. Peripheral blood leukocytes and bone marrow demonstrate signals between 3.5 and 4.5 DLU/mm^2 . These data suggest illegitimate and/or ectopic Tg gene transcription in extrathyroidal tissues, which may contribute to the observed Tg mRNA in the serum of athyreotic patients.

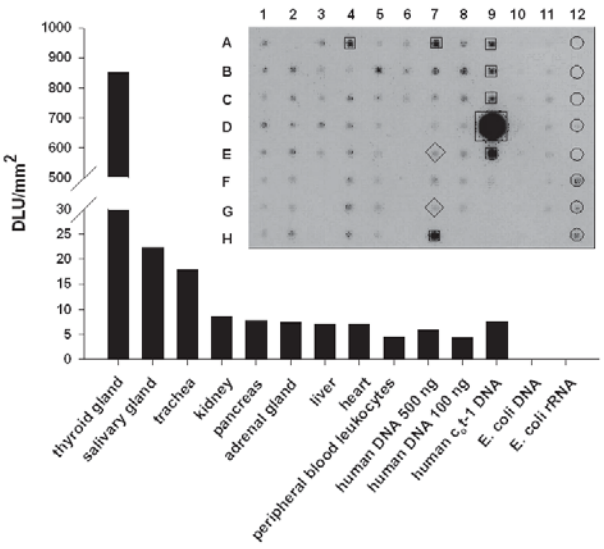

Fig. 4 Densitometric evaluation of the Multiple Tissue Expression array. The bars represent the intensity of the hybridization signals. All signals with intensities of > 7 DLU/mm² are shown. DLU: Digital Light Units. Insert: Multiple Tissue Expression array hybridized with a Tg cDNA probe (167 bp). Signals with an intensity higher than 7 DLU/mm² are marked by squares (□): A4 = heart, A7 = kidney, A9 = liver, B9 = pancreas, C9 = adrenal gland, D9 = thyroid gland, E9 = salivary gland, H7 = trachea. Peripheral blood leukocytes (E7) and bone marrow (G7) are marked by rhombes (◊). The controls are marked by circles (○): A - H 12: yeast total RNA, yeast tRNA, E.coli rRNA, E.coli DNA, Poly r(A), human C_0 t-DNA, human DNA 100 ng, human DNA 500 ng.

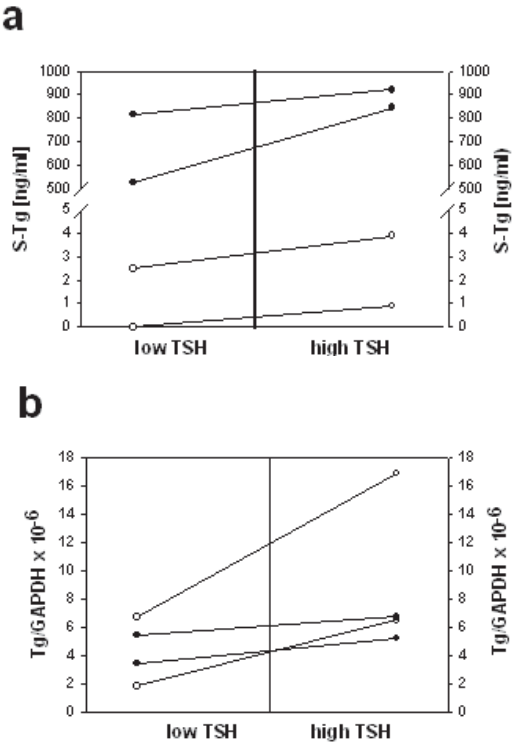

Fig. 5 A S-Tg levels (ng/ml) and B Tg/GAPDH mRNA ratios (x 10^{-6}) at low and high serum TSH levels after rhTSH application in 4 DTC patients.

Detection of S-Tg and thyroglobulin mRNA in rhTSH treated patients. Tg mRNA expression was also examined in four patients with DTC (Table 1) who were treated with rhTSH before diagnostic radioiodine scintigraphy during postoperative follow up (LUSTER et al. 2000). Peripheral blood samples were obtained on two consecutive days after they (R1-R4) had received 0.9 mg/d i.m. rhTSH. Blood was obtained from R1, R2 and R3 before and after the second rhTSH application; from R4 before and after the first rhTSH stimulation. S-Tg was detectable in patient R1, R2 and R4 at low or high serum TSH levels caused by exogenous rhTSH stimulation. In patient R3 S-Tg was measurable only after rhTSH stimulation. In all patients, rhTSH stimulation caused a rise in S-Tg. In the two patients with metastases (R1, R4), S-Tg levels were higher than in the metastasis-free subjects (R2, R3) (Fig. 5a). Tg mRNA was detectable in all of the individuals and was increased after rhTSH stimulation in all 4 patients (Fig. 5b); Tg/GAPDH mRNA ratios were between 1.8 x 10^{-6} and 16.9 x 10^{-6}, i. e. below or in the range of the value observed in the healthy control.

Detection of thyroglobulin mRNA by RT – PCR in DTC patients during the follow up. Finally, we examined a heterogeneous set of 12 randomly selected patients with DTC after thyroidectomy and ablative radioiodine therapy in the follow up (Table 2). Five (M1 – M5) of them had known metastases diagnosed by imaging and /or by increased S-Tg levels, seven (N1 – N7) showed no evidence of metastatic disease. The median of the Tg/GAPDH mRNA ratios was 2.9 in the first group (M1 – M5) and 5.5 in the second group (N1 – N7). No obvious correlation was found between S-Tg, clinical stage (TNM, MACIS) and mRNA ratios. Interestingly, three patients of the second group developed local recurrence (N3, N7) or increased S-Tg (N4) half a year after Tg mRNA measurement

Discussion

We developed a RNA based real time PCR assay to measure Tg mRNA in peripheral blood. This assay was

Table 1
Patients treated with rhTSH during follow-up

Patient	DYNOtest Tg–plus (ng/ml)		TSH (mU/l)		Tg/GAPDH x 10^{-6}		TNM	Histology	Imaging WBS[1]
rhTSH	low	high	low	high	low	high			
R 1	815	921	125	136	5.44	6.67	$pT_3N_0M_0$	FTC[2]	distant metastases[4]
R 4	525	845	0.03	138	3.40	5.18	$pT_3N_0M_1$	PTC[3]	distant metastases[4]
R 2	2.5	3.9	17.4	113.7	1.84	6.49	$pT_2N_0M_0$	PTC[3]	NED[5]
R 3	0	0.9	87.3	93.5	6.69	16.9	$pT_2N_0M_0$	PTC[3]	NED[5]

[1] WBS: whole-body radioiodine scan; [2] FTC: Follicular Thyroid Carcinoma; [3] PTC: Papillary Thyroid Carcinoma; [4] pulmonal, osseous metastastes; [5] NED: No Evidence of Disease

Table 2
DTC patients after thyroidectomy and ablative radioiodine therapy in the follow up

Patient	TSH (mU/l)	DYNOtest Tg–S (ng/ml)	Tg/GAPDH x 10^{-6}	TNM	MACIS	Histology	Imaging			
							Ultra-sound	WBS[7]	CT	PET
M1[1]	0.04	352	1.0	$pT_xN_0M_0$	10.9	FTC[4]	local	distant uptake		∅[8]
M2	>80	39	2.6	$pT_3N_0M_0$	7.06	FOTC[5]	NED[3]			
M3	>80	108	2.9	$pT_{4a}N_{1b}M_0$	7.1	PTC[6]	NED[3]			
M4	0.4	32	5.1	$pT_3N_0M_1$	12.09	PTC[6]	local	distant uptake		∅[8]
M5	< 0.3	63358	5.6	$pT_{4a}N_0M_1$	8.75	FOTC[5]	local	distant uptake		∅[8]
N1[2]	< 0.03	**NED**[3]	0.04	$pT_{2a}N_0M_0$	3.7	FOTC[5]	**NED**[3]			
N2	< 0.03		0.1	$pT_{1b}N_0M_0$	4.3	PTC[6]				
N3	< 0.03		4.4	$pT_4N_0M_0$	6.08	FTC[4]				
N4	< 0.03		5.5	$pT_2N_{1a}M_0$	4.52	PTC[6]				
N5	0.2		8.7	$pT_{4b}N_1M_0$	4.49	FTC[4]				
N6	< 0.03		9.4	$pT_4N_0M_0$	6.6	PTC[6]				
N7	< 0.03		44.0	$pT_{4a}N_{1b}M_1$	7.85	PTC[6]				

[1]M1 – M5: with current metastases or local recurrency; [2]N1 –N7: No metastases; [3]NED: no evidence of disease; [4]FTC: Follicular Thyroid Carcinoma; [5]FOTC: Follicular- Oncocytic Thyroid Carcinoma; [6]PTC: Papillary Thyroid Carcinoma; [7]WBS: Whole- Body radioiodine Scan; [8] ∅: PET: Positron-Emission Tomography not performed

applied to healthy individuals, congenital athyreotic patients and patients with treated DTC. The real time PCR protocol was sensitive enough to quantify Tg mRNA in peripheral bood of healthy subjects. By examining congenital athyreotic patients we had intended to create truly negative controls, especially by avoiding any interference with thyroid remnant tissue after surgical therapy. Surprisingly, we detected Tg mRNA in the peripheral blood of these congenital athyreotic patients. Imaging techniques gave no indication for hypoplastic or ectopic thyroid tissue in these athyreotic patients, and contamination by genomic DNA in our PCR assays was also excluded by appropriate controls. These results suggested that there must be an extrathyroidal source for Tg mRNA in the sense of illegitimate (Chelly et al. 1989) or ectopic transcription (Sarkar and Sommer 1989), where expression levels of one transcript in 500 to 1000 cells (Chelly et al. 1989; Cooper et al. 1994) may be expected.

Indeed, using a multiple tissue expression array screened with high stringency, we detected Tg signals also in non-thyroid tissue, e.g. salivary gland, trachea, kidney, pancreas, adrenal gland, liver and heart. These

results are in line with similar data obtained by other experimental approaches: SPITZWEG et al. (1999) reported that, according to RT-PCR data, normal thymus tissue expresses thyroid-related genes and proteins like sodium iodide symporter, thyrotropin receptor, thyroid peroxidase and thyroglobulin. SELLITTI et al. (2000) amplified by RT-PCR thyroglobulin mRNA from mesangium cells associated with glomerular basal membrane of the kidney. BOJUNGA et al. (2000) found Tg mRNA in thymus, suprarenal gland, hypophysis, lung, testis and vermiform appendix after 40 cycles of PCR. KIMOTO et al. (1998) published that lymphocytes express virtually all human mRNAs. CHELLY et al. (1989) postulated illegitimate transcription in any cell type and anticipated that all promoters could be minimally active when ubiquitous transcriptional factors reach their DNA binding site, leading to very low, but not null, gene transcription. Recently, BUGALHO et al. (2001) published expression of Tg mRNA in lymphocytes and granulocytes. Thus, Tg mRNA observed in athyreotic patients most likely results from an ectopic or illegitimate transcription of the Tg gene in extrathyroidal tissues including blood cells. Basal levels of Tg mRNA strongly vary from patient to patient. With respect to a diagnostic application of real time PCR, this means that Tg mRNA levels cannot be used as absolute markers for the status of thyroid cancers. However, this problem also arises with the new and more sensitive immunological Tg–pluS assay, which also detected the low levels of Tg protein in the peripheral blood of athyreotic patients given that the ectopically expressed mRNA is faithfully spliced an translated. In conclusion, both assays might be interference-prone and given the high sensitivity of the two methods, there seems to be no advantage in any of them regarding the point of specificity.

Several groups (HAUGEN et al. 1999; LADENSON 2000; MCDOUGALL and WEIGEL 2001) reported that rhTSH stimulates the radioiodine uptake and S-Tg production as measured by immunological methods in cancer patients undergoing DTC monitoring for thyroid remnant and/or tumor recurrence. We here report on an increase in S-Tg in four rhTSH-treated DTC patients. Furthermore, according to our real time PCR assay, Tg/GAPDH mRNA ratios rose in all four patients after rhTSH stimulation. This sensitivity to alterations in Tg mRNA levels due to rhTSH stimulation shows that the assay should also be able to measure increases in Tg mRNA due to tumor progression, relapse or metastases independent of any high background levels caused by ectopic Tg gene transcription. If Tg mRNA levels are regarded as an individual tumor progression marker, i.e. if they are compared with one or more reference S-Tg values obtained from the same patient at earlier time points, then real time PCR determinations of Tg mRNA may serve as a useful tool for monitoring DTC patients during follow up.

Tg mRNA was also observed by real time PCR in all DTC patients. However Tg/GAPDH ratios and S-Tg levels did not correlate in this limited number of patients. This is not unexpected, because on the one hand the exact amount of neoplastic tissue required to increase serum Tg levels in DTC patients is unknown and probably varies with the biological characteristics of the tumor (VAN HERLE and VAN HERLE 1997), on the other hand JOHNSON et al. (1995) reported that every tumor cell synthesizes different amounts of mRNA. Also, we did not observe a correlation of Tg mRNA levels with the TNM staging in 16 patients. TANAKA et al. (2000) reported that Tg mRNA levels positively correlate with the prognostic MACIS score for papillary thyroid cancer. FENTON et al. (2001) published a correlation between Tg mRNA levels with total body $^{(131)}$I uptake and S-Tg in children with previously treated papillary thyroid cancer. In 12 DTC patients, we did not find such correlations either. Several studies have been performed trying to establish the clinical value of Tg mRNA measurement for the follow-up of thyroid cancer patients. The results are controversial, as some of them demonstrate a correlation between Tg mRNA levels in peripheral blood and the progress of thyroid malignancies, some of them do not (RINGEL at al. 1999; BISCOLLA et al. 2000; GRAMMATOPOULOS et al. 2003; TAKANO et al. 2001; ESZLINGER et al. 2002; SPAN et al. 2003). One study confines such a correlation to a subtype of thyroid carcinomas, i. e. PTC (BELLANTONE et al. 2001), one recommends the combined evaluation of circulating Tg mRNA and serum Tg by means of an immunoassay (FUGAZZOLA 2002), and one allowed the definition of a positive cutoff point at 1 pg/μg total RNA which produced fewer false negative results than those obtained with S-Tg assays (SAVAGNER et al. 2002). Altogether, this data leave the diagnostic value of Tg mRNA quantification in peripheral blood still open.

In conclusion, our assay demonstrated no direct correlation between Tg mRNA levels and S-Tg as well as between Tg mRNA and protein levels. However increased Tg mRNA levels after exogenous stimulation with rhTSH were observed. It may be assumed that the additional amount of Tg mRNA derives from residual thyroid tumor or from their metastases and not from non-thyroidal sources, as the extrathyroidal tissues are not supposed

to react to stimulation by TSH. This indicates, that real time PCR should also be able to show rising Tg mRNA levels accompanying thyroid tumor progression, relapse or metastasis, underscoring the diagnostic potential of real time PCR assays of Tg mRNA as an individual tumor marker. Further studies are needed to confirm whether stimulation with rhTSH will amplify circulating Tg mRNA signals originating from TSH receptor-positve (residual) thyroid tissue and metastases.

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