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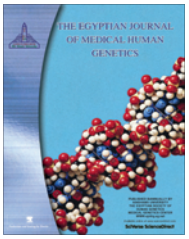

ORIGINAL ARTICLE

Molecular detection of circulating thyroid specific transcripts (TSHR/Tg-mRNAs) in thyroid cancer patients: Their diagnostic significance

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Fine-needle aspiration cytology;
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Indeterminate lesions;
Molecular marker

Abstract Thyroid cancer is the most prevalent endocrine malignancy. The preoperative diagnosis of differentiated thyroid cancer (DTC) that relies solely on fine-needle aspiration (FNAC) biopsy, sometimes possesses conflicting results. New molecular markers for thyroid cancer have been investigated with most of them based on the detection in thyroid nodules or tumor tissue specimens. Recently, it was possible to detect thyroid cancer cells in the circulation by measuring the mRNA of thyroid specific genes. Among these, thyroglobulin and more recently thyroid stimulating hormone receptor mRNAs, TSHR/Tg-mRNAs in peripheral blood might serve as cancer-specific markers. These have become promising new circulating markers for thyroid cancer.

The purpose of this study is to assess TSHR/Tg-mRNAs as diagnostic molecular markers for thyroid cancer and if they can be used preoperatively in synergy with FNAC.

This study was performed on 60 subjects; 20 healthy volunteers and 40 patients; including 16 patients with benign thyroid diseases, 24 patients with thyroid cancer; 18 patients with newly diagnosed (DTC) and 6 patients with recurrent thyroid cancer. Diagnosis of cancer was based on FNAC

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and histopathology of surgical specimens. All subjects had TSHR/Tg-mRNAs in peripheral blood measured by reverse transcriptase (RT)-PCR.

Based on cytology/pathology; 18 patients had newly diagnosed DTC and 11 had benign thyroid disease. Preoperative FNAC was performed on 29 of 40 patients; FNAC was diagnostic in 11/18 of malignant lesions (61.1%), in 8/11 of benign lesions (72.7%), while 10/29 (34.5%) were indeterminate. TSHR/Tg-mRNAs correctly diagnosed DTC in 20/24 and 19/24 (sensitivity 83.3% and 79.1%) and benign disease in 14/16 and 13/16 (specificity 87.5% and 81.3%), respectively. With indeterminate FNA, TSHR/Tg-mRNAs correctly diagnosed DTC (follicular type) in 5/7 and benign disease in 2/3 (combined sensitivity 71.4%; specificity 66.7%). There was high concordance between RT-PCR results for TSHR-mRNA and Tg-mRNA. Of the controls 19/20 (95%) and 16/20 (80%) were negative for both TSHR- and Tg-mRNAs.

With the use of a carefully selected primer pair and qualitative RT-PCR; our results indicate that TSHR/Tg-mRNAs in peripheral blood are both equally sensitive and specific markers for detection of thyroid cancer cells. Combining TSHR/Tg-mRNAs and FNAC and ultrasound enhances the preoperative detection of cancer in patients with thyroid nodules, reducing unnecessary surgeries and correctly classified most follicular cancers and could have spared surgery in patients with benign disease.

1. Introduction

Human thyroid tumors originate from epithelial follicular cells or from parafollicular C-cells. Follicular cell-derived tumors range from benign adenomas to differentiated (follicular and papillary) and undifferentiated (anaplastic) carcinomas [1].

Well-differentiated thyroid carcinoma (DTC) is the most common endocrine malignancy, with an annual incidence of 0.5–10 cases per 100,000 individuals. Its management includes thyroidectomy and I 131 ablation therapies. Differentiated thyroid carcinoma (DTC) has an overall favorable prognosis, with a 10-year survival of 90–95% [2].

Two main areas of thyroid cancer management that could benefit from improvements are the preoperative diagnosis of thyroid cancer when a biopsy suggests intermediate features, and a more reliable diagnosis of postoperative thyroid cancer recurrence.

Despite the increasing standards of imaging techniques like ultrasound, fine-needle aspiration (FNAC) remains the procedure of choice in patients presenting with thyroid enlargement. FNAC is reliable, safe and accurate method as a first line of evaluation in thyroid gland nodules before surgery. Thyroid nodules are extremely common with prevalence rates approaching 50–60% of adults under 60 years of age. Generally, most (90%) thyroid nodules are benign and can be classified as adenomas, colloid nodules, cysts, infectious nodules, lymphocytic or granulomatous nodules, hyperplastic nodules, thyroiditis, and congenital abnormalities. Because only approximately 5% of thyroid nodules are malignant, accurate preoperative characterization of thyroid nodules is critical in selecting patients appropriate for surgical thyroidectomy [2].

FNAC is more specific than sensitive in detecting thyroid gland malignancy and, therefore it is used as a reliable diagnostic test. However, the instance of inadequate sampling of the lesion and overlapping cytological features of benign and malignant thyroid neoplasms are inherent limitations of this technique, often requiring multiple biopsies or unnecessary surgeries. For papillary thyroid cancer, FNAB has proven exceedingly accurate. However, 15–30% of FNAB specimens have intermediate cytology features often described as hypercellular follicular lesions of follicular neoplasm that lead to surgery for definitive diagnosis. Some of these lesions will be follicular thyroid carcinomas or follicular variants of papillary thyroid cancer, but most are benign on final pathology. The problem is that the distinction by FNAC between benign and malignant follicular neoplasms remains difficult, as the crucial criterion for FTC vs. adenoma (FA) is capsular invasion, which cannot be determined by cytology. In addition, the distinction between FA and Follicular variant of PTC (FVPTC) is also difficult, because the crucial criterion here is the aspect of the nuclei. The implication is that 70–80% of the patients with suspicious results from FNA, who undergo thyroid surgery have a benign tumor [3,4].

Based on several promising studies, molecular markers can potentially improve the diagnostic accuracy of FNAC. Molecular markers that alone or in combination with FNAC could detect cancer for preoperative evaluation is highly desirable and could save a number of unnecessary surgeries [5–7].

Thyroid-stimulating hormone (TSH; thyrotropin) and TSH receptor (TSHR) are key proteins in the control of thyroid function. The human TSHR gene is located on chromosome 14q31and is encoded by 10 exons of which the last exon encodes the entire transmembrane and intracellular region. TSHR is the receptor for thyrotropin; a member of the glycoprotein hormone family. TSH is released by the anterior pituitary gland and is the main regulator of thyroid gland growth and development. Binding of TSH to TSHR stimulates thyroid-epithelial cell proliferation, and regulates the expression of differentiation markers such as thyroglobulin, thyroperoxidase and the sodium iodide symporter (NIS), necessary for the synthesis of thyroid hormones [8]. Thyroglobulin production by both normal and neoplastic thyroid tissues depends on the presence of functional TSHR and is influenced by TSH levels. The expression of TSHR in thyroid neoplasms has been well documented as both thyroid/thyroid cancer cells express functional TSHR [9,10].

Thyroglobulin (Tg) is a 660,000-Kd glycoprotein, Tg gene mapped to human chromosome 8q24.2–8q24.3 covers at least 300 kb of genomic DNA and contains 8.5 kb coding sequence divided over 48 exons separated by introns varying in size up to 64 kb. Tg 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. However, the various sTg assays are plagued by a number of methodological problems, occasionally limiting the clinical value of routine sTg measurements [11,12].

Molecular markers have been explored as alternative method for the detection of thyroid cancer with variable success and suffer from the lack of specificity. The variable results in these studies may relate to the differences in methods used including RNA isolation, use of different primers, and the normalization process [6].

Our objective was to assess these two markers TSHR/Tg-mRNAs as diagnostic markers for thyroid cancer to determine its sensitivity and specificity and if they can be used in synergy with current diagnostic modalities; fine needle aspiration and ultrasound for preoperative diagnosis (Fig. 1).

2. Subjects and methods

2.1. Subjects

The subjects were conducted from National Cancer Institute, Cairo University and Endocrine Department of Ain-Shams University Hospitals during period from December 2009 to June 2010. Follow-up: postoperative pathological diagnosis was used to categorize the patients into benign and malignant, a total of 60 subjects including 3 groups.

2.1.1. Control group

Group A: Included 20 apparently healthy people of matched age and sex as the patient groups without history of diseases.

2.1.2. Patient groups

Group B: Comprised 16 patients with benign thyroid diseases. Among those patients; two patients had thyroiditis, 9 had thyroid nodules/multinodular goiter, and five had Grave's disease (diffuse toxic goiter).

Group C: Comprised 24 patients with differentiated thyroid cancer (DTC); 18 patients newly diagnosed cases differentiated thyroid carcinoma and 6 patients with recurrent or residual DTC on T4 replacement therapy.

All subjects were subjected to history taking, clinical examination, laboratory investigations including: CBC, liver function test, kidney function test, prothrombin time, thyroid profile, thyroid antibodies and thyroid U/S, while U/S guided FNAC was done only for 29 patients who were presenting with nodular thyroid disease out of forty patients (in patients with MNG; FNAC was done on the predominant cold nodule according to isotope scan finding). The remaining 11 patients did not require FNAC as five patients were proven clinically

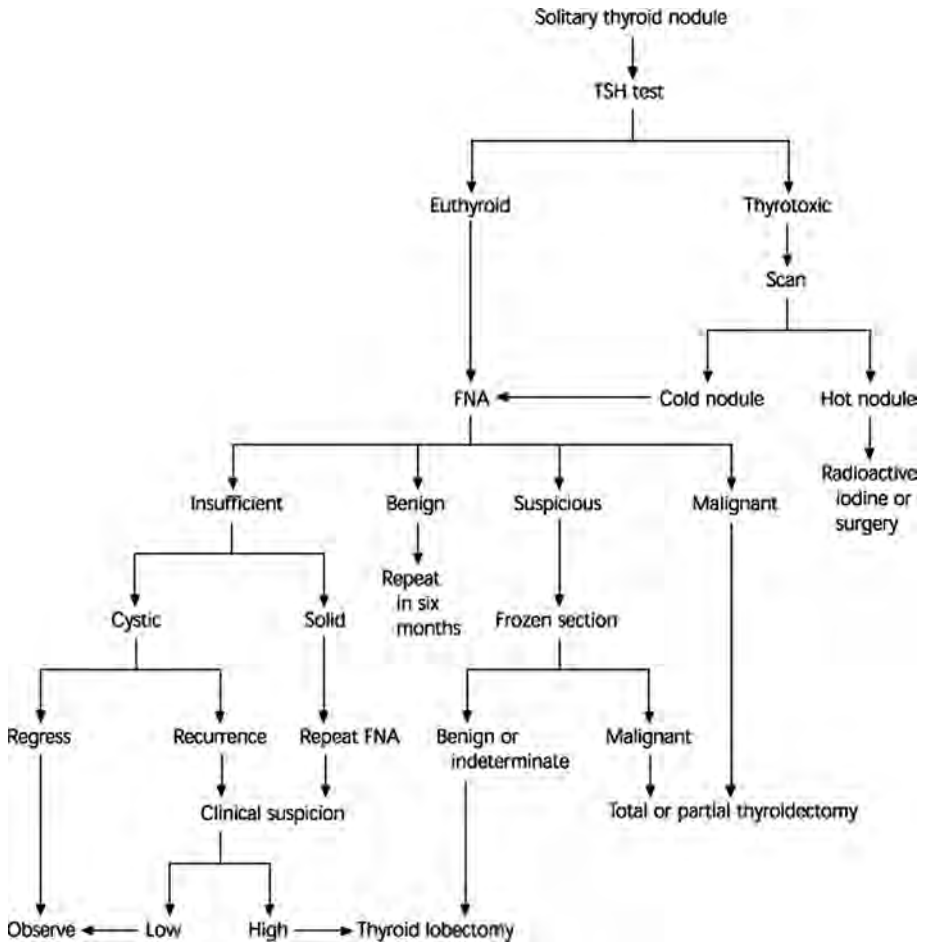

Figure 1 Algorithm for the evaluation of thyroid disorders. TSH = thyroid-stimulating hormone; FNA = fine-needle aspiration Burch HB [13].

Table 1 Setting of PCR primers 7.

		Sequence of primers	Amplified product
TSHR	F	5′-GCTTTTCAGGGACTATGCAATGAA-3’	
	R	3′-AGAGTTTGGTCAC-AGTGACGGGAA-5’	212
Tg	F	5′-AGGGAAACGGCCTTTCTGAA-3’	
	R	3′-CTTTAGCAGCAGAAGAGGTG-5’	407
GAPDH	F	5′-CGTCTTCACCACCATGGAGA 3’	
	R	5′-CGGCCATCACGCCACAGTTT 3’	300

and by lab results to have Grave's disease, while the remaining 6 patients had recurrent thyroid cancer. In selected cases thyroid isotope scans, CT scan were required and were done. Data were included in chart review. Written informed consent was obtained before enrollment into the study.

Patients had FNA biopsy performed and blood samples drawn before surgery. Postoperative pathological diagnosis was used to categorize the patients into benign and malignant. FNA sample adequacy included sufficient number of cells, abundant colloid, and the presence of at least six groups of benign follicular cells composed of at least 10 cells each. The results were compared with final postoperative pathological diagnosis. Indeterminate FNA included the following cytological categories: hypercellular follicular nodule (oxyphilic or non oxyphilic), atypical epithelial cells and hemorrhagic cyst contents with few follicular cells.

2.2. Methods

2.2.1. Blood samples

Ten milliliters was sampled from the peripheral veins from each patient and from controls for separation of mononuclear cells.

2.2.1.1. Isolation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation using Ficoll-hypaque 1077 (Sigma, USA) at 1200*g* for 30 min at 4 °C. The interface cells were removed, washed twice with 25 ml of sterile PBS (pH 7.3), pelleted, and resuspended in 1 ml of PBS. The cells were pelleted again at 1200*g* for 2 min. The cell pellets were kept at −80 °C until RNA extraction.

2.2.1.2. Extraction of total RNA from nuclear cells. Total RNA of nuclear cells separated from whole blood was extracted using RNA extraction kit (Qiagen). The isolated RNA was resuspended in RNAase-free water and stored at −80 °C until assay. The RNA concentration was assessed by absorbance reading at 260 nm with UV spectrophotometry (Du series 650, Beckman Inc., USA).

2.2.1.3. Reversetranscription (complementary cDNA synthesis). Reverse transcription reaction was carried out in 20 µl reaction mixture by using first strand cDNA synthesis kit (Promega; USA) according to the manufactures instructions.

2.2.1.4. PCR amplification of TSHR and Tg. PCR was performed using the selected primer pairs. Five microliters of first-strand cDNA was used as a template for the PCR reaction. Each reaction mixture consisted of 0.5 mM of each primer, 10× Taq Buffer, 2.5 mM dNTP mix, 0.5 U of Taq polymerase and nuclease-free water to a final volume of 50 µl. Thermocycling in either an MJ Research PTC 200 (MJ Research, Inc., Boston, MA) or Perkin-Elmer 9600 (Perkin-Elmer, Cambridge, UK) consisted of 38 cycles of denaturation (94 °C, 1 min; (first cycle for 2 min), annealing (62 °C, 1 min) and extension (72 °C, 1 min) (10 min for the last cycle). PCR amplification of GAPDH to assess the cDNA integrity is shown in Table 1.

2.2.1.5. Analysis of amplified thyroglobulin/TSHR cDNA. Ten microliters of PCR product was mixed with 2 µl 6× and the amplified PCR products were analyzed by agarose gel electrophoresis, stained with ethidium bromide (Sigma), and visualized with an UV transilluminator (Bio-Rad).

2.3. Statistical analysis

The results were analyzed using the Statistical Package of Social Sciences (SPSS) computer software program, version 16.0 (Chicago, IL, USA). Quantitative data were presented as mean ± SD for normally distributed data. Qualitative data were presented in the form of frequencies and percentages. For normally distributed parameters, differences among

Table 2 Patients characteristic in various diagnostic groups.

	Normal	Benign	Newly diagnosed DTC	Recurrent thyroid cancer
No. of patients	20	16	18	6 (PTC)
Age	48.8 ± 11.33	54.8 ± 10.1	60.2 ± 7.3	63.5 ± 6
Sex				
Male	6 (30%)	7 (43.8%)	6 (33.3%)	1 (16.7%)
Female	14 (70%)	9 (56.2%)	12 (66.7%)	5 (83.3%)
Surgical confirmation		11	18	
FNAC		8/11	11/18	
Cytology/pathology		nodules/MNG, 2 thyroditis and 3FA	12PTC and 6F	

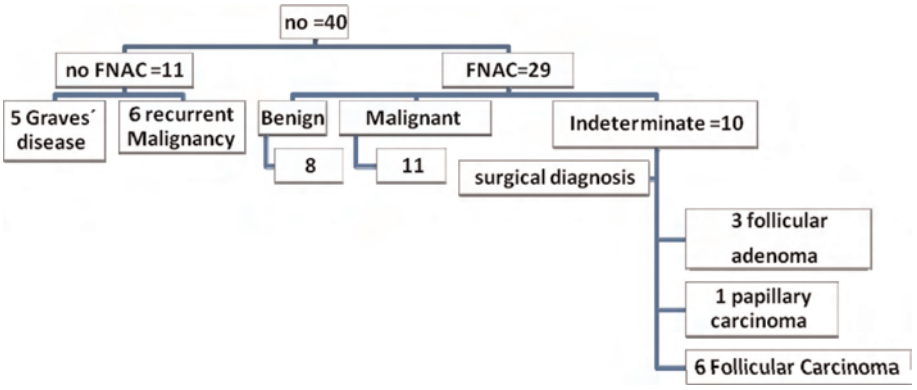

Figure 2 Categorization of patients according to FNAC and surgical results.

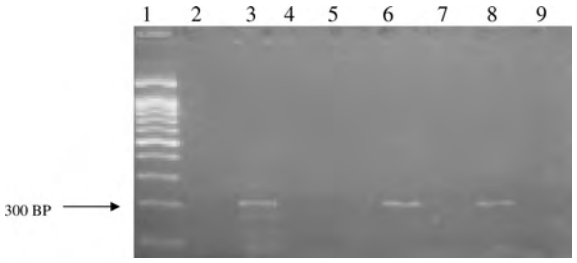

Figure 3 Electrophoretic separation of PCR amplified product. Detection of gene transcripts in blood specimens from thyroid cancer patients. Lane 1: DNA molecular size marker. Lanes 2 ,4, 5, 7: negative cases. Lanes 3, 6, 8: cases showing TSHR/Tg positive gene transcript. Lane 9: negative control.

groups were tested by one-way analysis of variance (ANOVA). For qualitative data, differences among groups were tested using Pearson's chi-square test (χ^2) and Fisher's exact test. Data were analyzed for diagnostic sensitivity and specificity of TSHR-mRNA and Tg-mRNA for detection of thyroid cancer sensitivity, specificity and accuracy was calculated. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Patients Characteristic

A total of 40 patients were enrolled in the study (27 female 67.5%; 13 male 32.5%). Demographic data represented in Table 2 in addition to 20 healthy controls.

Neither age nor sex was correlated with the presence of TSHR/Tg-mRNAs expression ($P > 0.05$). Twenty-nine patients had FNA biopsy performed before surgery. Postoperative pathological diagnosis was used to categorize the patients into benign and malignant. Diagnosis of Grave's disease as well as of healthy individuals was based on clinical assessment and appropriate ultrasound examination; thyroid profile (thyroid-stimulating hormone (TSH), total thyroxin (T4) and triiodothyronine (T3). In a hyperthyroid patient with nodular thyroid disease the risk of malignancy is low. FNAC does not need to be performed routinely in these patients unless clinical features suggestive of malignancy are present. These features include a history of neck irradiation, rapid growth, family history, hard consistency, local lymphadenopathy and recurrent laryngeal nerve palsy in the absence of previous surgery [14,15]. Tests for serum thyroid autoantibodies are done as these may indicate autoimmune thyroid disease.

Categorization of patients according to FNAC and surgical results is demonstrated in Fig. 2.

3.2. Interpretation results

After optimization of conditions for reverse transcription and polymerase chain reaction and according to the design of primer pairs we were able to detect the PCR products of 212; 407 base pairs were amplified from TSHR/Tg cDNA, respectively; while GAPDH band amplification was visualized corresponding to 300 bp. No transcripts were identified in the water control or in the RT-negative control. The sizes of PCR products were estimated by comparison with DNA molecular mass markers (Bio-Rad; EZ Load) Fig. 3.

Table 3 TSHR/Tg-mRNAs positivity in healthy individuals and in patients with benign thyroid disease and thyroid cancer.

	n	TSHR-mRNA	Tg-mRNA	P1	P2	P3
Healthy volunteers	20	1 (5%)	4 (20%)	$>0.05^a$	$<0.01^b$	$<0.01^c$
Benign thyroid disease	16	2 (12.5%)	3 (18.7%)			
Thyroid cancer patients	24	20 (83.3%)	19 (79.1%)			
χ^2		36.893	30.343			
P-value		<0.01 HS	<0.01 HS			

[a] Healthy and benign.
[b] Benign and malignant.
[c] Healthy and malignant.

Table 4 Statistical analysis of TSHR/Tg-mRNAs' results.

	Disease	Positive	Disease	Negative
Test (TSHR-mNA) $n = 40$				
Positive	20	TP	2	FP
Negative	4	FN	14	TN
Test (Tg-mNA) $n = 40$				
Positive	19	TP	3	FP
Negative	5	FN	13	TN

Sensitivity = TP/TP + FN × 100, specificity = TN/TN + FP × 100, PPV = TP /TP + FP × 100, NPV = TN/TN + FN × 100, accuracy = TP + TN/total no. × 100; TP = true positive, TN = true negative, FP = false positive, FN = false negative, PPV = positive predictive value, NPV = negative predictive value.

Table 5 Diagnostic performance of TSHR, Tg and FNA result in patients with benign thyroid disease and thyroid cancer.

Variables	TSHR-mRNA (%)	Tg-mRNA (%)
Sensitivity	83.3	79.1
Specificity	87.5	81.3
PPV	90.9	86.4
NPV	77.8	72.2
Accuracy	85	80

3.3. FNAC result compared with histological diagnosis

Based on cytology/pathology; 18 had surgically confirmed DTC. 11 patients had benign conditions; FNA result of malignant conditions: 11/18 (61.1%) were FNAC results of newly diagnosed DTC (papillary carcinoma), FNA result of benign conditions: 8 were FNAC results of benign condition 6 patients diagnosed nodule/MNG, and 2 patients with Hashimoto thyroiditis and 3/11 (27.3%) patients had sufficient specimen but inconclusive results; surgically confirmed follicular adenoma. FNAC of indeterminate result with 6 were surgically proven to be follicular carcinoma while 3 were surgically proven to be benign follicular adenoma and 1 papillary thyroid carcinoma.

3.4. Evaluation of reverse transcriptase-PCR (RT-PCR) results in controls and patients

Forty patients (24 DTC; 16 benign) were enrolled at initial diagnosis and 20 control patients without clinical signs or a history of thyroid disease.

3.4.1. TSHR/Tg-mRNAs in normal control and benign thyroid disease

Of the controls 19/20 (95%) and 16/20 (80%) were negative for both TSHR- and Tg-mRNAs. Of the patients with benign thyroid disease, 14/16 (87.5%) and 13/16 (81.3%) were negative for both TSHR- and Tg-mRNA. False positive for TSHR/Tg included two patients with follicular adenoma (Tables 3 and 4).

3.4.2. TSHR/Tg-mRNAs in thyroid cancer

Twenty four patients were evaluated (six patients had recurrent or residual malignant disease at the time of enrollment and 18 were recently diagnosed with differentiated thyroid cancer (12 papillary, 6 follicular cancers). There were 20/24, 19/24 patients (83.3%, 79.1%) with DTC who were positive for TSHR/Tg-mRNAs in the peripheral blood. (All six patients with recurrent disease were positive by RT-PCR.) (Tables 3 and 4).

There was high concordance between RT-PCR results for TSHR-mRNA and for Tg-mRNA, also combined model for both markers with FNAC showed improved sensitivity and specificity (Tables 5 and 6).

4. Discussion

Molecular detection of tissue-specific gene expression in peripheral blood is a new diagnostic approach and has been described as a tumor marker in different solid tumors. The RT-PCR is a highly sensitive technique for the detection of tissue-specific mRNA transcripts. This method has been tested with the amplification of cancer-specific mRNA from peripheral blood to detect micrometastases in cases of prostate cancer and neuroblastoma [6]. Many studies explored blood-borne thyroglobulin/TSHR-mRNAs as a potential tumor marker in thyroid cancer [16,17]. A reliable and satisfying method that is able to differentiate preoperative malignant potential in patients presenting with thyroid nodules has not yet been proposed.

Ditkoff et al. [18] reported, for the first time, a method for detecting Tg-mRNA in peripheral blood of patients with well-differentiated thyroid tumors. Since the authors were not able to document Tg expression either in controls or in patients with benign thyroid disease, Tg-mRNA appeared to be a specific tumor marker. In contrast, different studies revealed that Tg transcripts could be found in blood from healthy control [19–21] in a variety of human cell lines and in different human tissues and organs [22]. This could be attributed to illegitimate transcription of Tg-mRNA [23–26]; any gene is expressed in any cell at very low, but detectable levels, or could mean that

Table 6 Sensitivity and specificity of combined TSHR/Tg-mRNAs and FNA result in patients with benign thyroid disease and thyroid cancer.

	Thyroid cancer		Benign conditions	
	Positive	Sensitivity (%)	Negative	Specificity (%)
FNA	11/18	61.1	8/11	72.7
TSHR/Tg-mRNA	21/24[a]	87.5	15/16[a]	93.7
FNA	20/24[b]	83.3	14/16[b]	87.5

[a] TSHR-mRNA.
[b] Tg-mRNA.

cell shedding is a physiologic rather than pathologic process, taking place even in normal thyroids.

Thyroid carcinomas are known to contain functional TSHR [27]; this target has not been exploited for detection of circulating cancer cells, perhaps due to previous reports showing the presence of TSHR-mRNA transcripts in peripheral blood mononuclear cells [28] and other extrathyroidal tissues [29]. The finding of these transcripts in extrathyroidal tissues can be explained by TSHR splice variants [30,31]. Such inconsistent results suggested that different RNA extraction methods, RT-PCR conditions, and primers could also interfere with the final results. It is possible that with certain primer pairs amplification of pseudogenes can give rise to false-positive results. Thus, selection of primers specific to thyroid cells is of paramount importance in the assay.

In the present study based on cytology/pathology, 18 patients had newly diagnosed DTC and 11 had benign thyroid disease. There was high concordance between RT-PCR results for TSHR-mRNA and for Tg-mRNA. TSHR/Tg-mRNAs correctly diagnosed DTC in 20/24, 19/24 patients (83.3%, 79.1%) and 11/18 (61.1%) of newly diagnosed cases and benign disease in 14/16, 13/16 (87.5%, 81.3%), respectively. In this study, 34.4% of FNA biopsies were called indeterminate among which 6 were surgically proved to be follicular carcinoma while 3 were surgically proved to be benign follicular adenoma and 1 papillary thyroid carcinoma. These indeterminate thyroid nodules seem to be one of the most frustrating and challenging areas for the endocrinologists and endocrine surgeons. Most studies are focused on FNAC material to identify molecular markers or a combination of markers as a means of improving the accuracy of diagnosis made by FNAC [6].

The finding of circulating thyroid cells in patients with follicular adenoma favors that some follicular adenomas may be present at premalignant stages of follicular carcinomas. The differentiation between follicular adenoma and follicular carcinoma is, at present, only possible after surgical resection and formal histological examination. To date, there are no known markers that can distinguish follicular adenomas from cancer with certainty because, like follicular cancer, a significant number of follicular adenomas harbor Ras mutations and show galectin-3 immunoassaying. Therefore, the finding of circulating thyroid cells in a patient with follicular adenoma is not unparalleled and favors the notion that some follicular adenomas may represent a premalignant stage of follicular carcinomas.

Feilchenfeldt et al. [32] observed a significant increase in galectin-3 on mRNA and protein levels in papillary thyroid cancers. No follicular thyroid cancer was positive with regard to galectin-3 mRNA levels. Hence, galectin-3 expression or immunoreactivity does not appear to be useful tool in distinguishing adenomas from follicular cancers in cytological or histological specimens. However, it remains to be seen whether galectin-3 will prove useful in detecting the follicular variant of papillary cancers or a multifocal spread on histological material.

Jakubiak-Wielganowicz et al. [33] reported that although galectin-3 was not proven to be highly specific or sensitive for given type of thyroid tumor, it seems to be a valuable tool helping in diagnosis of difficult thyroid tumors, but can not be used as a specific marker. Galectin-3 may be helpful in preoperative diagnosis of tumors diagnosed cytologically as ''follicular tumor, suspected for neoplasm''. As galectin-3 may be present in some adenomas, it does not seem to be a tool in differentiating between follicular variant of papillary carcinoma and follicular adenoma.

It is recognized that FNAC cytology has a high sensitivity for PTC. On the other hand, FNAC cannot differentiate between FC and FA [4,34]. In this study TSHR/Tg-mRNAs correctly differentiated between follicular carcinoma in 5/7 of thyroid cancer patients and follicular adenoma in 2/3 benign disease patients (sensitivity 71.4%; specificity 66.7%), similar results were found by Wagner et al. [7]. Our results suggest that circulating preoperative TSHR/Tg-mRNA acts as an adjunctive test to enhance the diagnostic accuracy of FNAC and indicate a high potential of TSHR/Tg-mRNA to differentiate FC from FA and await confirmation in future studies with a larger number of patients. This is in concordance with Wagner et al. [7] who documented in his study that preoperative TSHR/Tg classified 78% (14/18) of nodules with indeterminate FNA accurately.

Yet contrary to our results Torosian et al. [20] found that Tg and TSHR-mRNA signals were detected equally in all subjects (patients with DTC with and without evidence of disease and in normal subjects).

The variability in Tg-mRNA results in various studies may be explained by the alternative splicing of Tg-mRNA in thyroid cells, as at least 16 alternative splicing sites have been reported. 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 [10]. Another explanation 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 [23,24].

Previous report demonstrated that RT-PCR has been demonstrated to be more sensitive than sTg assays, especially during T4 therapy or in the presence of anti-Tg antibodies. However, the existence of several alternative splicings of the Tg-mRNA has raised the question of the frequency of these variants in thyroid tissues in diseased and normal subjects. In fact, the transcription pattern of Tg-mRNA is heterogeneous in thyroid cancer tissues, as well as in normal thyroid tissues, and no specific pattern has been found in differentiated thyroid carcinoma.

A Bojunga et al. [25] clearly documented the fact that a change in the number of PCR cycles was sufficient to alter the percentage of positive cases obtained in different groups of individuals with benign and malignant thyroid diseases.

A major finding in the present study is that the presence of TSHR/Tg-mRNAs signals in blood is specific for patients with thyroid cancer, being undetectable in the vast majority of healthy volunteers and patients with benign thyroid diseases. The high specificity of TSHR-mRNA and Tg-mRNA for thyroid cancer because carefully selected primers are used in the assay.

A model for combined sensitivity and specificity of TSHR/Tg-mRNAs and FNAC results in patients with benign thyroid disease and thyroid cancer revealed 21/24 (87.5%), 20/24 (83.3%) in patients with thyroid cancer and 15/16 (93.7%), 14/16 (87.5%) in patients with benign thyroid disease compared to (61.1%) in thyroid cancer and (72.7%) in benign thyroid disease when using FNAC alone, this algorithm can contribute to a more accurate diagnosis prior to operation especially of indeter-

minate thyroid lesions or follicular neoplasms than other currently available strategy, nevertheless this algorithm requires prospective validation and comparison with other suggested models combining molecular markers with US features .Finally, it is worth noting that TSHR/Tg-mRNAs correctly diagnosed all recurrent thyroid cancer cases, which implies the utility of TSHR/Tg-mRNAs in long-term monitoring and detection of cancer recurrence and residual disease which is concordant with earlier reports of correlation of elevated levels with the presence of residual/ metastatic disease and that it could postoperatively predict recurrent cancer [6,10].

A standardization of cDNA preparation, gene-specific primer pairs, optimal RT-PCR conditions and the determination of possible threshold caused by cells of non-thyroid origin is mandatory to obtain reliable and comparable results. In view of our data obtained for sensitivity and specificity, we believe that a clinically useful assay will require further assay refinement, e.g. quantitative assay to permit the distinction of weakly and more strongly positive RT-PCR results. A number of questions remain to be answered and further studies are necessary.

5. Conclusion

In summary, the presence of either TSHR-mRNA or Tg-mRNA in peripheral blood is specific for the presence of circulating thyroid cells; and represent specific and sensitive markers for thyroid cancer and expression of this marker shows promise for detecting FC, which is often missed by FNAC. Overall, the management of cancer in general and thyroid cancer in particular is moving away from reliance on a single test that replaces others. The ultimate usefulness of molecular markers as TSHR/Tg-mRNAs may reside in being part of a multimodality panel of clinical tests. An envisioned new algorithm may combine the best of all management criteria clinical assessment, radiologic and histologic information, and the biologic implications of a molecular marker.

5.1. Future perspectives

The use of molecular diagnostic techniques, such as RT-PCR, which enable the specific amplification of small numbers of mRNA molecules, has led to the development of several methods for the specific amplification of small foci of malignant tissue, either primary or metastatic. PCR-based techniques may improve the preoperative diagnosis of patients with thyroid malignancies and therefore help to avoid unnecessary radical procedures. Quantitative TSHR-mRNA assay is helpful in the preoperative diagnosis of thyroid nodules, particularly in the subgroup of patients with indeterminate FNA. The immediate postsurgical levels can serve as a sensitive marker for detecting residual/metastatic disease. Furthermore, it detects recurrent cancer with high sensitivity and can be valuable as an alternative to serum Tg measurement in patients who harbor Tg antibodies. Future studies are required to establish its role in long-term monitoring.

Conflicts of interest

The authors indicate that they do not have any conflict of interest.

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