



# Thyroid Stimulating Hormone Receptor Transcripts in Correlation with Clinical Parameters in Thyroid Carcinoma Patients

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## Abstract

**BACKGROUND:** Differentiated thyroid carcinomas (DTC) preserve expression of thyroid stimulating hormone receptor (TSHR).

**AIM:** The aim of our study was to evaluate the expression of mRNA-TSHR in peripheral blood of DTC patients, then to correlate the expression with clinical features: Serum thyroglobulin (sTg) value, initial staging and findings from the whole body scan (WBS), neck ultrasound (US), and total received dose of radioiodine therapy.

**MATERIALS AND METHODS:** Forty patients were divided into three groups according to the treatment response: Patients with incomplete structural response (TCs), incomplete biochemical response (TCb), and excellent responders (TCr). Total RNA was isolated from peripheral blood and used for two-step reverse transcriptase-PCR with appropriate primers. Relative quantification using the  $\Delta Ct$  and  $2^{-\Delta\Delta Ct}$  method was applied. sTg levels were evaluated with chemiluminescent assay. The statistical analysis was performed with Spearman Rank Order Correlation.

**RESULTS:** We found that TCs patients expressed mRNA-TSHR by a 5.37-fold higher level than TCr patients, TCb patients expressed TSHR by an 8.88-fold higher level than TCr patients. A significant negative correlation was detected between sTg and  $\Delta Ct$  ( $Ct_{TSHR} - Ct_{GAPDH}$ ) value ( $R = -0.475$ ;  $p < 0.05$ ), and between WBS/US findings and  $\Delta Ct$  ( $R = -0.321$ ;  $p < 0.05$ ).

**CONCLUSION:** Our data revealed higher expression of mRNA-TSHR in peripheral blood of TCs and TCb compared to TCr patients and analysis revealed a significant correlation between mRNA-TSHR and sTg and US/WBS findings. Further studies with larger number of subjects and absolute quantification are needed for understanding the real meaning of mRNA-TSHR as a biomarker in DTC.

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## Introduction

Thyroid tumors are the most common endocrine malignancies, with differentiated thyroid carcinomas (DTCs) representing more than 88% of all types of thyroid tumors [1], [2], [3], [4]. DTCs preserve expression of TSHR and the previous research found that the lower expression of TSHR was associated with a poor differentiation of thyroid carcinomas and a higher probability for distant metastases [4], [5]. Through TSH stimulation TSHR performs its own function in thyrocyte proliferation, differentiation and normal thyroid development and function. Association of higher expression of TSHR in DTC was described, with the lower expression of the two mesenchymal markers N-cadherin and vimentin, suggesting that the higher expression of TSHR inhibited cell invasion and metastasis through epithelial-mesenchymal transition [5].

Besides standard diagnostic procedures during follow-up suggested by the American and

European thyroid association guidelines [6], [7], new biomarkers in follow-up of DTC could be useful especially in patients with elevated antithyroglobulin antibodies (aTg), limiting usefulness of immunometric chemiluminescent assay in the measurement of serum thyroglobulin (sTg), as well as limiting specificity of sTg value in patients without ablative radioiodine therapy and treated only with lobectomy [8].

New molecular techniques enabled evaluation of the expression levels of specific transcripts in peripheral blood and this method was applied in other solid tumors, such as prostate, breast, and colon cancer, as one of the methods in the detection of circulating tumor cells in peripheral blood [9]. The detection of the circulating tumor cells is based on the principle of measurement expression of tumor specific transcripts in the blood sample [9]. Since Ditkoff *et al.* [10] reported a possible usefulness of RT-PCR molecular technique in the detection of thyroid circulating cells in blood samples as an indicator of metastatic TC, an increased number

of studies on this issue have been published in the past few years [11], [12], [13].

Using data from the previous investigations in relative quantification of gene expression in other malignancies and the previous experiences from evaluation of thyroid transcripts, we decided to perform a relative gene expression of mRNA-thyrotropin receptor (*TSHR*) gene in patients with DTC [14], [15]. It is considered that the presence and higher expression of tumor specific transcripts indicate the presence of circulating tumor cells.

We have set as an objective in our study to evaluate the expression of *TSHR* gene transcripts in patients with DTC, and then to correlate the expression with clinical features: Serum thyroglobulin (sTg) value, total received dose of radioiodine therapy (RAI), initial staging and findings from the whole body scan (WBS), and neck ultrasound (US) of DTC patients.

## Materials And Methods

### Subjects

Blood sampling from 40 patients with DTC was performed. In all patients, surgery and ablation treatment with radioactive  $^{131}\text{I}$  were conducted at least 6 months before starting the research. Patients were divided into three groups according to blood levels of sTg and aTg antibodies (CRM 457, Immulite 2000; Siemens, Munich, Bavaria, Germany), US neck examination and findings from WBS, similar to the concept of risk stratification by Tuttle *et al.* [16]. The first group (TCs - 22 patients), consisted of patients with incomplete structural response to treatment and biochemical relapse of the disease; imaging confirmed metastatic spread or loco-regional relapse and elevated sTg levels > 0.2 ng/mL. The second group (TCb - 6 patients) was patients with incomplete biochemical response and indeterminate response, with only elevated sTg > 0.2 ng/mL, or elevated aTg levels, without confirmed and known morphological signs for relapse. The third group of patients (TCr - 12 patients) had complete structural and biochemical response to therapy, excellent responders. The thyroid bed and neck lymph nodes were evaluated with ultrasound (US) of the neck with linear transducer 7.5–10 MHz. WBS was performed after ablative or therapy dose of radioiodine (RAI). Planar scan and in some cases single-photon emission computed tomography (SPECT/CT) were performed on dual-head gamma camera. Scans were interpreted by two (more than ten years) experienced nuclear medicine physicians. Imaging findings from WBS and neck US were scored: (0) if there was no remnant tissue and enlarged neck lymph nodes and absence of distant metastases, (1) if remnant thyroid

tissue was detected, (3) if loco-regional relapse and local neck involved lymph nodes were detected, and (4) if distant metastases were present. Corresponding to the histopathology reports from total 40 patients, four cases were follicular thyroid carcinoma, 25 typical variants of papillary thyroid carcinoma, one papillary thyroid carcinoma in thyroglossal duct cyst, one papillary microcarcinoma, seven follicular variants of papillary thyroid carcinomas, and two Hurtle cell carcinomas. The TNM staging system for DTC developed by the American Joint Committee on Cancer (AJCC) was recorded from the histopathology report, and in 31 of 40 patients, we found detailed description of initial staging according TNM. Of them, 15 patients were classified as Stage I, two patients were Stage II, four patients were Stage III, one patient was Stage IV, five patients were Stage IVa, and four patients were Stage IVc. The study was approved by the Ethics Committee of the Medical Faculty of Ss Cyril and Methodius University, in Skopje, Republic of Macedonia, and a written consent was obtained from all subjects enrolled in the research.

### RNA extraction procedure and real-time polymerase

#### Chain reaction

The blood samples were drawn from patients in standard 3 mL EDTA blood vacutainers; total RNA was extracted using commercially available RNA isolation kit GenElute™. Total RNA purification kit (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) and isolated total RNA were used for two-step RT-PCR with ReadyScript™ cDNA Synthesis Mix kit (Sigma-Aldrich), according to the manufacturer's protocols.

#### Real-time polymerase chain reaction

The PCR step was performed using the following primer pairs: *TSHR*-F 5'-GCT TTT CAG GGA CTA TGC AAT GAA-3' and *TSHR*-R 5'-AAG GGC AGT GAC ACT GGT TTG AGA-3', targeted to amplify a segment spanning exons 6 to 9 (nucleotides 555-767 or 212 bp) [17]. The ubiquitously expressed *GAPDH* gene was used to confirm RNA extraction and RT-PCR using primers *GAPDH*-F 5'-TTC GTC ATG GGT GTG AAC C-3' and *GAPDH*-R 5'-GAT GAT GTT CTG GAG AGC CC-3', as previously reported [17], [18]. 2 µl of cDNA was added for RT quantitative PCR (qPCR), performed according manufacturer's protocol for Hot FirePol Eva Green qPCR Mix Plus (Rox) PCR master mix (Solis BioDyne, Tartu, Estonia). The reaction mixture for *TSHR* was incubated at 95°C for 15 min, followed by 38 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 20 s, and elongation at 72°C for 20 seconds, and for *GAPDH* incubated at 95°C for 15 min, followed by 38 cycles of denaturation at 95 °C for 15 seconds, annealing at 57°C for 20 s, and elongation at 72°C for 20 s. All samples were analyzed in duplicate and in some cases in triplicate.

### Relative quantification method

Relative quantification was applied by calculating fold change in the gene expression of *TSHR* target gene, normalized to the endogenous reference gene *GAPDH*. Cycle threshold (Ct),  $\Delta Ct$ ,  $\Delta\Delta Ct$ , and normalized relative expression ratio values were calculated according to  $2^{-\Delta\Delta Ct}$  method, by Livak and Schmittgen [14]. According to this method,  $\Delta Ct_{TSHR} = (\text{average } Ct_{TSHR} - \text{average } Ct_{GAPDH})$  and then  $\Delta\Delta Ct$  value was calculated using  $\Delta Ct$  value from healthy volunteers (HC), from data published previously by our group [19], or  $\Delta\Delta Ct = [\Delta Ct(TC) - \Delta Ct(HC)]$  and later normalized expression ratio  $2^{-\Delta\Delta Ct}$  was applied for fold change evaluation [14], [15], [19].

### Statistical analysis

Intergroup comparison between average  $\Delta Ct = (TSHR-GAPDH)$  values was performed with Mann-Whitney U-test. Spearman rank order correlation was performed to measure the direction and strength of the association between *mRNA-TSHR* and sTg, and *mRNA-TSHR* and total received dose of RAI, as well as between *mRNA-TSHR* and initial stage and *mRNA-TSHR* and WBS and neck ultrasound findings.

## Results

In 37 of 40 patients, the expression of *mRNA-TSHR* was detected. Three patients from the TCs group were with undetectable samples for *mRNA-TSHR*. Descriptive analysis of all 40 patients is presented in Tables 1-3. We detected that TCs patients expressed *mRNA-TSHR* by a 5.37-fold higher level than TCr patients, TCb patients expressed *mRNA-TSHR* by

**Table 1: Descriptive analysis of patients with incomplete structural response to treatment and biochemical relapse of the disease (TCs group)**

TCs Pts	$\Delta Ct = TSHR-GAPDH$	sTg ( $\mu g/L$ )	RAI (mCi)	Stage (TNM)
1	10.99	16.1	0	III
2	12.26	> 300	300	IVA
3	13.14	3.51	432.1	n*
4	Undet.*	7.73	130	I
5	9.43	254	804	n
6	8.57	12.9	263	III
7	9.58	0.38	100	I
8	10.41	3.3	252	I
9	12.69	0.19	100	I
10	10.61	231	260	n
11	12.38	>300	0	IVC
12	12.59	0.54	286.7	n
13	11.67	6.48	100	III
14	7.47	117	241	IV
15	5.35	>300	283.5	II
16	9.17	>300	250	n*
17	9.56	43.7	0	IVA
18	6.68	>300	229	IVC
19	4.66	84	0	IVA
20	5.53	>300	0	I
21	Undet.*	>300	0	IVC
22	Undet.*	>300	100	IVC

\*Undet: Undetectable expression levels of the *mRNA-TSHR*, \*n: Missing data about initial stage of the disease

**Table 2: Descriptive analysis of patients with incomplete biochemical response to treatment or indeterminate response (TCb group)**

TCb Pts	$\Delta Ct = TSHR-GAPDH$	sTg ( $\mu g/L$ )	RAI (mCi)	Stage (TNM)
1	10.77	<0.2	50	I
2	12.56	0.5	102	IVA
3	5.63	0.26	0	I
4	10.31	0.43	105	I
5	4.15	2.5	0	I
6	9.94	0.4	103	n*

\*n: Missing data about initial stage of the disease.

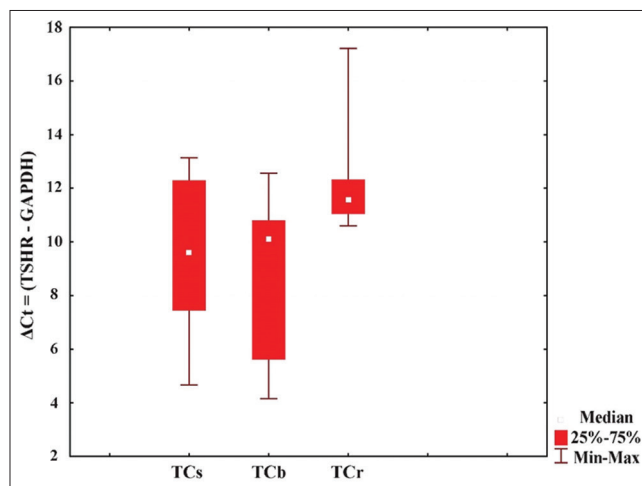
an 8.88-fold higher level than TCr patients, as was previously reported in our previous study [19].

**Table 3: Descriptive analysis of patients with excellent response to treatment (TCr)**

TCr Pts	$\Delta Ct = (TSHR-GAPDH)$	sTg ( $\mu g/L$ )	RAI (mCi)	Stage (TNM)
1	11.21	<0.2	100	IVA
2	17.21	<0.2	102	I
3	11.89	<0.2	94	*n
4	11.015	<0.2	87.3	n
5	12.87	<0.2	100	I
6	11.20	<0.2	100	I
7	10.93	<0.2	0	n
8	10.60	<0.2	243.8	III
9	12.37	<0.2	113	I
10	12.22	<0.2	100	II
11	11.11	<0.2	100	I
12	11.91	<0.2	99.5	I

\*n: Missing data about initial stage of the disease.

Further intergroup comparison between average  $\Delta Ct = (TSHR-GAPDH)$  values was performed and a significant difference was detected between TCs and TCr group (Mann-Whitney U-test:  $Z = -2.231$ ,  $p = 0.026$ ) and TCb and TCr group (Mann-Whitney U-test:  $Z = -2.341$ ,  $p = 0.019$ ) (Figure 1).



**Figure 1: Intergroup comparison between average  $\Delta Ct = (TSHR-GAPDH)$  values. Range of  $\Delta Ct = (TSHR-GAPDH)$  values in three groups, patients with incomplete structural response to treatment and biochemical relapse of the disease or imaging confirmed metastatic spread or loco-regional relapse and elevated sTg levels,  $>0.2 \text{ ng/mL}$  (TCs patients); second group was patients with incomplete biochemical response and indeterminate response, with only elevated sTg ( $>0.2 \text{ ng/mL}$ ), or elevated aTg levels, without confirmed and known morphological signs for relapse (TCb patients); third group of patients had complete structural and biochemical response to therapy, excellent responders (TCr). Significant difference in average  $\Delta Ct = (TSHR-GAPDH)$  values was detected between TCs and TCr group (Mann-Whitney U-test:  $Z = -2.231$ ,  $p = 0.026$ ) and TCb and TCr group (Mann-Whitney U-test:  $Z = -2.341$ ,  $p = 0.019$ ).**

Correlation between sTg and  $\Delta Ct = (TSHR - GAPDH)$  for all three groups of patients showed a

significant negative correlation with Spearman Rank Order Correlation ( $R = -0.475$ ,  $p < 0.05$ ) (Figure 2). This finding suggest that the increased sTg value correlated with the lower value of  $\Delta Ct = (TSHR - GAPDH)$  for *TSHR* gene, or higher expression of the evaluated transcripts.

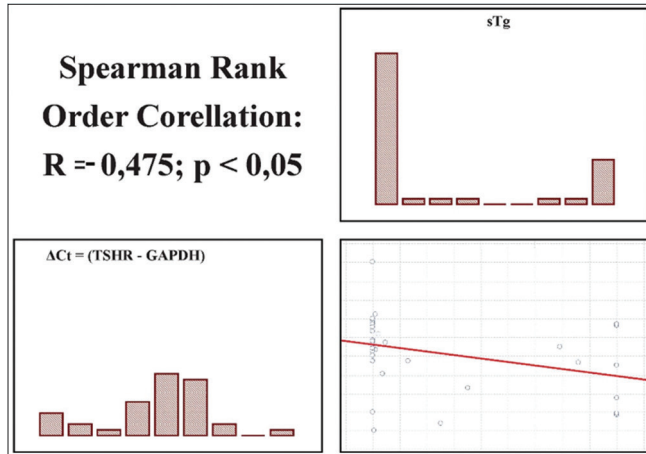


Figure 2: Correlation between sTg and  $\Delta Ct = (TSHR - GAPDH)$  for all three groups of patients. sTg values were correlated with  $\Delta Ct = (TSHR - GAPDH)$  values from all patients using Spearman Rank Order Correlation. Results showed significant negative correlation ( $R = -0.475$ ,  $p < 0.05$ ).

The analysis of the correlation between RAI and  $\Delta Ct = (TSHR - GAPDH)$  showed a weak non-significant positive correlation with Spearman Rank Order Correlation ( $R = 0.114$ ,  $p > 0.05$ ) (Figure 3). Data showed that with increase of the total received RAI therapy, there was a non-significant increase in the  $\Delta Ct = (TSHR - GAPDH)$  for *TSHR* gene, or increased RAI dose correlated with the lower expression levels of transcripts for *TSHR* gene.

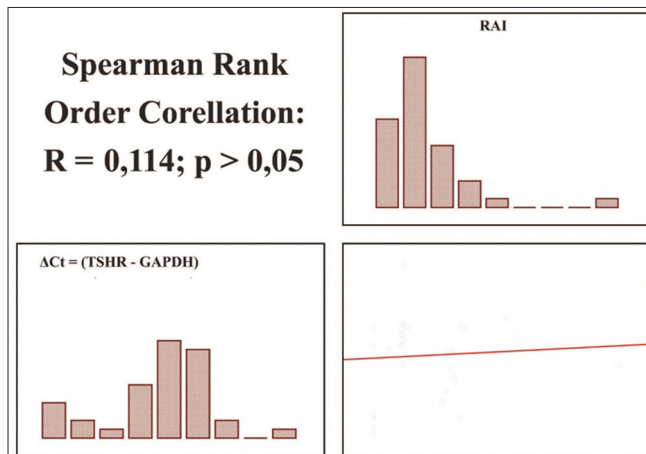


Figure 3: Correlation between received total dose of RAI (mCi) and value for  $\Delta Ct = (TSHR - GAPDH)$  for *TSHR*. Received doses of radioiodine therapy in milliCuries were correlated with  $\Delta Ct = (TSHR - GAPDH)$  values using Spearman Rank Order Correlation. Results showed weak non-significant positive correlation ( $R = 0.114$ ,  $p > 0.05$ ).

Correlation between initial stage of the disease according to TNM staging and  $\Delta Ct = (TSHR - GAPDH)$  revealed non-significant negative correlation, with Spearman Rank Order Correlation ( $R = -0.0009$ ,  $p > 0.05$ ) (Figure 4). This means that with increase of

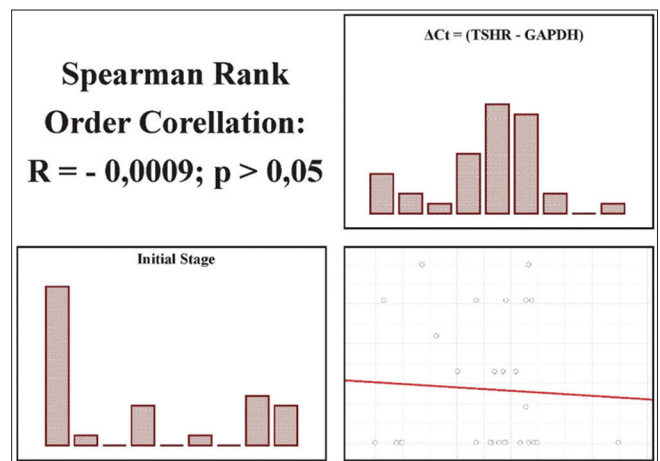


Figure 4: Correlation between initial stage and  $\Delta Ct = (TSHR - GAPDH)$ . Initial stages of the disease according TNM staging were correlated with  $\Delta Ct = (TSHR - GAPDH)$  using Spearman Rank Order Correlation. Results revealed non-significant negative correlation ( $R = -0.0009$ ,  $p > 0.05$ ).

the initial stage of the disease,  $\Delta Ct = (TSHR - GAPDH)$  value was non-significantly lowered, or increase expression of the transcripts of the *TSHR* gene.

Final analysis in our study was correlation between WBS/US findings and  $\Delta Ct = (TSHR - GAPDH)$ , resulting with significant a moderate negative correlation using Spearman Rank Order Correlation ( $R = -0.321$ ,  $p < 0.05$ ) (Figure 5). Increase of the score from the findings from WBS/US correlated with significant lowering of the  $\Delta Ct = (TSHR - GAPDH)$ , or increase of the score was associated with a significant increase of the expression level of *TSHR* gene transcripts.

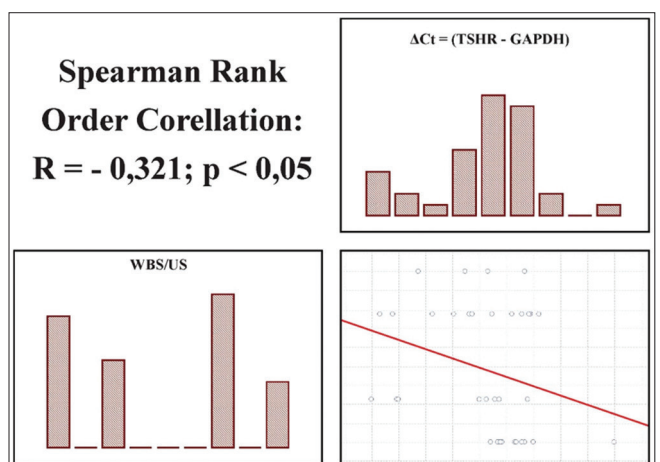


Figure 5: Correlation between WBS/US findings and  $\Delta Ct = (TSHR - GAPDH)$ . Radioiodine WBS were analyzed and neck ultrasound examinations were performed and findings were scored with: (0) if there was no remnant tissue and enlarged neck lymph nodes and absence of distant metastases, (1) if remnant thyroid tissue was detected, (3) if loco-regional relapse and local neck involved lymph nodes were detected, and (4) if distant metastases were present and those scores were correlated with  $\Delta Ct = (TSHR - GAPDH)$  using Spearman Rank Order Correlation. Results revealed significant moderate negative correlation ( $R = -0.321$ ,  $p < 0.05$ ).

## Discussion

Data from our analysis revealed a higher expression level of *mRNA-TSHR* in peripheral blood of TCs and TCb compared to TCr group of patients. In only three samples from all TCs patients, expression levels were undetectable. This finding may be due to dedifferentiation of tumor in the TCs group of patients and low expression of the evaluated transcripts for the target genes. Correlation of  $\Delta\text{Ct}$  value for *TSHR* gene with sTg level indicated a significant negative correlation, or in other words, elevation of sTg value correlated with an increase in the expression of *mRNA-TSHR*. Increase in expression of *mRNA-TSHR* results in lowering  $\Delta\text{Ct}$  value representing the difference between threshold cycles for the amplicons for target gene and reference gene. In DTC patients besides sTg level, neck US and WBS are an important and standard part of the follow-up routine. Evaluated association between US/WBS findings and  $\Delta\text{Ct}$  value for *TSHR* gene also revealed a significant negative correlation, which means extensiveness of the loco-regional status or distant metastatic state confirmed with imaging modalities correlated with higher expression of *mRNA-TSHR*. Ringel *et al.* [20] evaluated the expression of *mRNA-Tg* and they found that quantitative detection of circulating *mRNA-Tg* corresponded with the finding from last radioiodine WBS. They analyzed and found a positive connection of TSH value and expression of *mRNA-Tg* in patients with DTC, and although the exact mechanism was not known, they suggested that this relation was due to an increased Tg gene transcription under stimulation of TSH [20]. The need of new molecular biomarkers in diagnosis and follow-up of DTC is important in patients with elevated aTg antibodies and in patients with lobectomy, without ablative radioiodine therapy, which is recommended in low-risk group of patients according to the new ATA 2015 guidelines [8], [16]. The detection of mRNA of Tg gene was first introduced by Ditkoff *et al.* and the results were encouraging [9], [10], initiating many researchers to work in this field, but methodology varied significantly among research groups in respect of used primers, number of cycles, selection of reference gene, and quantification. In 2002, Gupta *et al.* [17] concluded that *mRNA-TSHR* could be tumor marker for DTC patients [13]. In 2004, Chinnappa *et al.* [18] found that DTC patients had a higher rate of *mRNA-TSHR* expression in the peripheral blood than healthy individuals [14]. Wagner *et al.* [21] using RT-PCR detected a higher level of *mRNA-TSHR* in patients with benign thyroid nodules. We applied the semi-quantitative analysis using  $2^{-\Delta\Delta\text{Ct}}$  method by Livak [14] in the previous research including healthy individuals and DTC patients and detected significantly higher expression in TCs and TCb group of patients compared to healthy control group [19]. Our further analysis was aimed at correlation of expression

of *mRNA-TSHR* with sTg values, initial staging, US/WBS findings and total received RAI therapy. Our findings revealed that expression of the transcripts corresponded with clinical and laboratory findings in the evaluated patients. On the other hand, we found non-significant correlation with the initial stage of the disease and expression levels. This might be due to a great difference in the follow-up period of patients which varied from only 1 month to 405 months from diagnosis till including in the study. Furthermore, there is a possibility of the lower expression of the transcripts for *TSHR* gene in more aggressive variants, or development of dedifferentiation of the tumor in the evolution of the disease. In light of this explanation is the non-significant positive correlation of  $\Delta\text{Ct}$  with the total dose of RAI therapy, or increased dose correlated non-significantly with the lower expression of *mRNA-TSHR*.

Mira Milas *et al.* have published several studies comprising the largest number of patients with expression of *mRNA-TSHR* in DTC cases and found that expression levels were lower or undetectable in more aggressive forms of thyroid carcinomas and dedifferentiation, or mutation of the evaluated gene was pointed as a possible reason [22]. Qiu *et al.* [23] investigated the possible role of *mRNA-TSHR* measurement in peripheral blood preoperative detection of microcarcinomas in suspicious nodules smaller than 1 cm. They evaluated *mRNA-TSHR* in peripheral blood in healthy volunteers and in benign and malignant papillary microcarcinomas and *mRNA-TSHR* correctly classified 77.8% of patients preoperatively (sensitivity = 76.5%; specificity = 79.4%). Combining *mRNA-TSHR* and US examination correctly detected 97.1% of PTMC and could spare surgery in 86.8% of patients with benign thyroid nodules [23]. Riming Liu *et al.* [24] correlated expression levels of *mRNA-TSHR*, and besides significantly higher expression of *mRNA-TSHR* preoperatively in patients with papillary thyroid carcinoma compared to patients with benign nodules, they found that PTC patients' pre-operative *mRNA-TSHR* expression level was associated with age, histological type, capsular invasion status, lymph node metastasis status, and BRAFV600E mutation status, but not with gender, tumor size, number of cancer foci, or presence of autoimmune thyroiditis [24].

## Conclusion

Data from our research revealed a higher expression level of *mRNA-TSHR* in peripheral blood of TCs and TCb compared to TCr group patients. Our findings revealed a significant correlation between

*mRNA-TSHR* and sTg and US/WBS, or expression of the transcripts corresponded with clinical and laboratory findings in the evaluated patients. On the other hand, we found no significant correlation with an initial stage of the disease and expression levels. Further studies with larger number of subjects included, preoperatively and postoperatively, as well as absolute quantification of the transcripts are needed for complete understanding of the possible usefulness of *mRNA-TSHR* as a potential biomarker in DTCs.

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## Statement of Ethics

The study was approved by the Ethics Committee of the Medical Faculty of Ss Cyril and Methodius University, in Skopje, Republic of Macedonia, and a written consent was obtained from all subjects enrolled in the research.

## Author Contributions

Makazlieva T, Jakovski Z, and Eftimov A constructed the study design, Vaskova O has been involved in revising the manuscript for important intellectual outcome; Tripunoski T, Jovanovic H, and Risteski S have given substantial contributions to conception and design; Makazlieva T and Eftimov A have given substantial contribution to acquisition, analysis, and interpretation of data; Makazlieva T, Stojanoski S, and Manevska N have been involved in drafting the manuscript and looking for the most suitable references. All authors read and approved the final manuscript.

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