No detection of TSH or TSHR in oral lichen planus lesions in patients with or without hypothyroidism

MARI VEHVILÄINEN[1*], ABDELHAKIM SALEM[2*], MUHAMMAD YASIR ASGHAR[3], TUULA SALO[4], MARIA SIPONEN[5]

* These authors contributed equally to this work.

Author information

1 a Unit for Specialized Oral Care in the Metropolitan Area and Kirkkonummi, Department of Social Services and Health Care, City of Helsinki, Helsinki, Finland

2 b Translational Immunology Program (TRIMM), Department of Oral and Maxillofacial Diseases, Clinicum, University of Helsinki, Helsinki, Finland

3 c Cellular Physiology, Minerva Foundation Institute for Medical Research, Biomedicum Helsinki, Helsinki, Finland.

4 d Cancer and translational medicine research unit, Faculty of Medicine, University of Oulu and Oulu University Hospital; Medical Research Center, Oulu University Hospital, Oulu, Finland

Department of Oral and Maxillofacial Diseases, and Translational Immunology Program (TRIMM), University of Helsinki, Helsinki, Finland

5 e Institute of Dentistry, Faculty of Health Sciences, University of Eastern Finland, Kuopio, Finland

Oral Health Teaching Clinic and Oral and Maxillofacial Diseases Clinic, Kuopio University Hospital, Kuopio, Finland

A short title: No TSH or TSHR in OLP lesions

Corresponding author

Maria Siponen

Institute of Dentistry, Faculty of Health Sciences, University of Eastern Finland, P.O Box 1627 (Yliopistonranta 1), 70211 Kuopio, Finland

Oral Health Teaching Clinic and Oral and Maxillofacial Diseases Clinic, Kuopio University Hospital, P.O Box 100, 70029 Kuopio, Finland

maria.siponen@uef.fi, telephone number +35829445111, telefax +35817162131

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Abstract

Objective: An association between hypothyroidism (HT) and oral lichen planus (OLP) has been reported. However, the mechanisms that could explain this association remain unresolved. This study aimed to evaluate the expression of thyroid-stimulating hormone (TSH) and thyroid-stimulating hormone receptor (TSHR) in healthy oral mucosa and in OLP lesions of individuals with and without HT. ***Material and methods:*** Immunohistochemical expression of TSH and TSHR was studied in oral mucosal biopsies obtained from 14 OLP patients with HT, 14 OLP patients without HT and 10 healthy controls without oral mucosal lesions. Gene expression of *TSHR* was investigated by using three different PCR techniques in oral mucosal samples from 7 OLP patients with HT, 3 OLP patients without HT, 9 healthy controls and in cultured human oral epithelial cells. Gene expression of *TSH* was examined by employing 2 PCR techniques in oral mucosal samples from 2 OLP patients with HT, 2 OLP patients without HT and 4 healthy controls. ***Results:*** TSH and TSHR stainings were negative in the studied oral mucosal specimens. Gene quantification assays demonstrated negative gene expression of *TSH* and *TSHR* in clinical and *in vitro* samples. ***Conclusions:*** These results suggest that TSH and TSHR may not be commonly involved in the pathogenetic mechanism that could explain the association between OLP and hypothyroidism.

Keywords

Hypothyroidism; oral lichen planus; pathogenesis; TSH; TSHR

1. Introduction

Lichen planus is a chronic inflammatory condition that can affect skin and mucous membranes including oral mucosa [1]. Oral lichen planus (OLP) presents as bilateral reticular, plaque-like, papular, atrophic, erosive or bullous lesions that affect predominantly the buccal mucosa, gingiva and tongue but can also be seen anywhere on the oral mucosa [2,3]. The disease has periodic exacerbation and remission, and only symptomatic treatment exists [3].

The prevalence of OLP in general population is estimated to be 0.2-2% [2]. OLP is more common in females and the age of onset is generally between 30 and 60 years [4]. Oral lichen planus has around 1 % risk of malignant transformation [5].

The precise etiology of OLP is unknown. The pathogenesis of OLP is not completely understood, but both antigen-specific and non-specific mechanisms seem to be involved [6,7]. Oral microbiota composition, genetic factors, trauma, stress and infections have been suggested as predisposing factors for OLP [4,8].

Some systemic factors have been associated with OLP. Based on systematic reviews and meta-analyses [9 and 10], hepatitis C virus is more common in OLP patients in the Middle East (n=583, OR=6.01, 95% CI: 1.47-24.53), Asia (n=900, OR=6.87, 95% CI: 1.42-33.15), Mediterranean countries (n=1615, OR= 6.99, 95% CI: 4.92–9.94) and the USA (n=54, OR= 5.09; 95% CI: 1.33–19.41).

In a recent meta-analysis [11], OLP and thyroid diseases in general (n= 1846) showed a significant association (OR=2.1, 95% CI:1.47-3.01), and in two of the four studies analyzed (n=675), OLP associated with HT (OR=1.83, 95% CI:1.16-2.89). Furthermore, thyroxin medication is more common in OLP patients compared to controls [12,13,14]. However, the factors that could explain the association between OLP and HT are still unclear.

Thyroid-stimulating hormone (TSH), also known as thyrotropin, is a pituitary hormone that stimulates the thyroid gland to produce thyroxine (T4), and triiodothyronine (T3) in response to negative feedback mechanism involving free T3 (FT3) and T4 (FT4). T3 is the biologically important active hormone that stimulates metabolism [15].

Thyroid-stimulating hormone receptor (TSHR) on the thyroid follicular cells is a member of the family of G protein-coupled receptors and is the primary regulator of thyroid hormone synthesis and secretion [15]. The expression of TSHR is not restricted to the thyroid gland. Active TSHR has been detected in a variety of human and animal tissues, including osteoblasts, osteoclasts, bone marrow cells, cardiomyocytes, adipocytes, fibroblasts and skin keratinocytes. [16,17]

There is little information about the expression of thyroid proteins in the normal oral mucosa or in OLP lesions. In one study [18] TSHR expression was upregulated in OLP lesions in patients receiving thyroxine supplement medication compared to healthy oral mucosa of healthy

controls. However, thyroglobulin (Tg) and thyroid peroxidase (TPO) expression were not detected in any of the studied samples [18].

In the present study we aimed to investigate the protein and gene expression of TSH and TSHR in normal oral mucosa of healthy subjects and in OLP lesions of patients with and without HT.

2. Materials and methods

Patients and samples

The tissue samples for immunohistochemical study comprised of oral mucosal diagnostic biopsies from 14 OLP patients with HT (mean age 59.1 years, range 37–72) and 14 age- (+/- 1 year) and sex-matched OLP patients without HT (mean age 58.9 years, range 37–72) collected from the archives of Kuopio University Hospital, Department of Clinical Pathology. The diagnosis of HT was confirmed from medical records. 13 of the patients had HT due to Hashimoto´s thyroiditis and one patient had partial thyroidectomy due to goiter and subsequent hypothyroidism. In the OLP with HT group, 11 samples were from the buccal mucosa, 2 from the tongue and 1 from the gingiva. In the OLP without HT group, 11 samples were from the buccal mucosa and 3 from the tongue. Further clinical and demographic characteristics of the patients are listed in **Table I**. The diagnosis of OLP in the study was made combining the clinical and histopathological features according to the criteria presented in a AAOMP position paper [19]. The control samples consisted of 10 oral mucosal

biopsies taken from systemically healthy control donors (mean age 26 years, range 12-51). Four of the control specimens were from healthy buccal mucosa and 6 were taken during surgical removal of third molars from the mucosa next to the teeth. Seven healthy thyroid gland tissue samples from the pathology archives of Oulu University hospital were used as positive controls. All samples were fixed in 10% neutral buffered formalin and embedded in paraffin.

In addition, fresh frozen tissue samples from OLP lesions of patients with Hashimoto´s thyroiditis (n=7, mean age 68, range 60-78) and without HT (n=3, mean age 57, range 48-70) as well as control samples consisting of healthy buccal mucosa from systemically healthy donors (n=9, mean age 26, range 23-34) were collected and preserved in RNAlater (Thermo Fisher Scientific, Carlsbad, CA, USA) for later PCR based analyses. In the OLP with HT group, 5 samples were from the buccal mucosa, 1 was from the tongue and 1 from the gingiva. In the OLP without HT group, 2 samples were from the buccal mucosa and 1 from the gingiva. Further clinical and demographic characteristics of the study patients are listed in **Table II**.

This study was approved by the ethics committees of the Northern Ostrobothnian Hospital District (46/2013) and the Helsinki University Hospital. The Research Ethics Committee of the Northern Savo Hospital District in Kuopio received information of the approval. The approval for use of archival tissue material was granted by the National Supervisory Authority for Welfare and Health (VALVIRA).

Immunohistochemical staining

Tissue sections were deparaffinized in xylene and rehydrated in ethanol multi-concentration series. The retrieval of the antigens was performed as follows: the slides were placed in 700 ml of 10 mM citrate buffer, pH 6.0, and microwaved in a microwave processing labstation for Histology (HACKER Instruments & Industries Inc., Winnsboro, SC, USA) according to the manufacturer's instructions. For blocking the nonspecific background, sections were incubated in 1% H_2O_2 for 10 min and washed in 10 mM phosphate buffered 140 nM saline, pH 7.4 (PBS) and then incubated in 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA) at RT for 1 hour.

Sections were then incubated overnight in 1 µg/ml mouse anti-human monoclonal thyroid stimulating hormone receptor antibody (TSHR; clone 4 C1; Bio-Rad Corp., Hercules, CA, US); 2) 10 µg/ml mouse anti-human monoclonal thyroid stimulating hormone antibody (TSH; clone 0042; DAKO, Glostrup, Denmark), at +4ºC and washed in PBS. Biotin-conjugated secondary anti-mouse antibodies (Vector Laboratories; 1:200) were applied for 1 hour at RT and washed in PBS. Later, avidin–biotin–peroxidase complex (ABC, Vector Laboratories; 1:200) were then applied for 1 hour at RT and washed in PBS. For colour development, diaminobenzidine tetrahydrochloride was applied for 10 min at RT and washed in dH_2O. Counterstaining of the slides was performed using hematoxylin and the slides were mounted in Mountex (HistoLab, Gothenburg, Sweden). The optimal working concentration for each antibody was confirmed in pilot experiments using a series of dilutions. The

specificity of each antibody was confirmed with positive control samples (thyroid tissues). Negative control sections were incubated without the primary antibody.

Cell culture and maintenance

Human oral keratinocytes HOKs isolated from normal oral mucosa (ScienCell, Uppsala, Sweden) were cultured in 75 cm^2 flask containing oral keratinocyte medium OKM (ScienCell, Uppsala, Sweden) enriched with growth factors and antibiotics. Confluent cells were detached using trypsin/EDTA solution (ScienCell, Uppsala, Sweden) and plated in 6-multiwell cell culture plates (BD Falcon, Lawrence, KS, USA). Once the wells were approximately 90% confluent, cells were lysed using RLT buffer (Qiagen, Düsseldorf, Germany) and stored at -80ºC for further use. The normal human thyroid follicular cells (N-Thyr-ori 3-1) were purchased from Health Protection Agency Culture Collection (Salisbury, UK). The cells were cultured in RPMI1640 supplemented with 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine. The human thyroid follicular cancer cells (ML-1) were generously provided by Dr. Johann Schönberger (University of Regensburg, Germany). The cells were grown in DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin. The cell cultures were maintained in the incubators with a water-saturated atmosphere of 5% CO_2 at 37°C.

Isolation of Total RNA

Total RNA was isolated from HOKs using the RNeasy Mini Kit (Qiagen, Düsseldorf, Germany), while RNA from clinical samples were isolated using miRNeasy Mini Kit and QIAcube workstation (Qiagen, Düsseldorf, Germany), where QIAzol Lysis Reagent was added and then samples were homogenized with Tissuelyser 3x2 min, 50 Hz. The assay was performed according to the manufacturer's instructions. Total RNA from N-thyr Ori 3-1 and ML-1 cells was extracted with AurumTM Total RNA Mini Kit (Bio-Rad; CA, USA) according to the manufacturer's instructions. RNA integrity was checked by gel electrophoresis and RNA concentration and purity was determined with NanoVue Plus (Healthcare Bio-Sciences AB; Uppsala, SE).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Complementary DNA strands were synthesized using iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Quantitative RT-PCR was run using 2 µl cDNA and 250 nM primers in iQ SYBR Green supermix in iQTM5 Multicolour RT-PCR Detection System (Bio-Rad Laboratories Inc.). GAPDH and RPLP0 were used as the housekeeping reference genes. Primer sequences are listed in **Table III**.

Droplet Digital Polymerase Chain Reaction (ddPCR)

The ddPCR is a highly-sensitive new technology that is able to perform an absolute nucleic acid quantification without establishment of a standard curve [20]. The ddPCR is proven to have a low detection limit and to be sensitive enough to detect only few positive molecules. The utilized final volume of 20 µl reaction mix contained 10 µl of QX200™ EvaGreen® ddPCR™ Supermix (Bio-Rad Laboratories), 2 µl of cDNA, 1 µl primers. The annealing temperature was set to 60°C. The assay was performed using QX200™ Droplet-Generator and QX200™ Droplet Digital™ PCR Systems (Bio-Rad Laboratories) according to the manufacturer's instructions.

Qualitative end point PCR

Complementary DNA was synthesized with SuperScript IV VILO kit (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA) from equal amounts of RNA (1µg). Reaction mixtures without reverse transcriptase (RNA control) or RNA (No template control-NTC) were employed as negative controls to maintain a check that the sample RNA preparations and PCR mixtures were not contaminated with any genomic DNA. The information about primer sequences and the PCR cycling settings are mentioned in **Table III**.

Microscopic imaging

The immunostained sections were evaluated by one of the authors (AS). Fully-automated Leica DM6000 microscope with Leica DFC365-FX

camera (Leica Microsystems, Wetzlar, Germany) was used for imaging of the immunostained samples.

3. Results

Immunohistochemistry

The positive control tissue showed strong immunoexpression of TSH and TSHR. The healthy oral mucosal tissue and OLP lesional tissue from patients with and without HT did not show immunoreactivity for TSH and TSHR (Figure 1).

PCR-based analyses

qRT-PCR and ddPCR for *TSH* from OLP lesions from patients with HT (n=2) and without HT (n=2) as well as healthy oral mucosa from controls (n=4) indicated negative expression (data not shown). qRT-PCR, ddPCR and qualitative end point PCR for *TSHR* from OLP clinical samples (OLP-HT n=7, OLP-nonHT n=3), healthy control samples (n=9) were also negative, while thyroid cells (ML-1, N-Thyr-ori) indicated strong positive expression (Figures 2 and 3). Furthermore, we were not able to detect *TSH* nor *TSHR* genes in mRNA from cultured normal HOKs (data not shown).

4. Discussion

A positive and statistically significant correlation between OLP and thyroid disease and between OLP and HT was reported in a meta-analysis [11]. The authors suggest that thyroid disease might be involved in the pathogenesis of OLP, or that OLP is a clinical manifestation of thyroid disease [11]. However, the mechanism behind the association of OLP and thyroid disease is still unknown.

Thyroid medication as a cause of OLP and oral lichenoid lesions (OLL) has been suspected. In a study [21] where an association between hypothyroidism and OLP was found, a subanalysis involving only patients with a history of thyroid disease (most of them had HT) provided no evidence for thyroxin medication *per se* of having effect on OLL/OLP. Contrary to this, a study [13] where the medication profile of patients with OLP (n=956) was compared with dental patients with no oral mucosal lesions found that levothyroxine sodium medication was associated with OLP (multivariate OR 3.39, 95% CI: 2.09–5.46, $P < 0.0001$). The same authors later interpreted, based on the scarcity of reports of lichenoid reactions caused by levothyroxine and on the short half-life of the drug, that their findings are indicative of association of HT and OLP, rather than thyroxin medication and OLP [22].

A molecular mimicry hypothesis for both HT and lichen planus has been proposed. This hypothesis suggests that structural similarity between microbial antigens and human autoantigens can turn a defensive immune reaction into an autoimmune reaction in genetically predisposed subjects. [23]

The association between OLP and HT could also be explained by a common autoimmune process. OLP is considered to be a T-cell-mediated autoimmune disease which is characterized by auto-cytotoxic CD8 + T cells triggering apoptosis of the basal cells of the oral epithelium [24]. Hashimoto´s thyroiditis is the most common cause of HT. It is an organ specific autoimmune disease in which lymphocytic infiltration of the thyroid parenchyma is seen. [25] The pathogenesis of the disease involves the formation of anti-thyroid antibodies that attack thyroid stroma causing progressive fibrosis. The most common laboratory findings in Hashimoto's thyroiditis include an elevated TSH level and normal to low FT4. [26] Ninety per cent of patients with this disease have elevated levels of circulating anti-TPO antibodies, and high serum Tg antibody concentrations are present in 20 to 50 percent of these patients [27]. In Hashimoto´s thyroiditis only 6% of patients are seropositive for anti-TSHR [28].

Indeed, it has been suggested that circulating anti-thyroid antibodies could contribute to trigger an autoimmune response in the oral mucosa, also in patients with asymptomatic chronic autoimmune thyroiditis [29]. Two studies [30,31] found significantly higher titers of anti-TPO and anti-Tg in a group of patients with OLP who did not have any other autoimmune diseases. However, in another study [18] no association was found between elevated serum anti-Tg, anti-TPO or anti-TSHR antibodies and OLP; the authors found altered levels of TSH and FT4 more often in OLP patients without previously diagnosed thyroid disease than in the general population. In addition, the same study [18] reported positive TSHR immunostaining in the basal epithelial layer in OLP lesions in patients with levothyroxine

medication but not in controls and significantly higher expression of TSHR by qPCR analysis in OLP lesions compared to controls. It was therefore suggested that TSHR might be associated with OLP pathogenesis [18]. In contrast with this, the monoclonal antibodies applied in our study, showed a negative expression of both TSH and TSHR in healthy oral mucosa and OLP lesions compared with the strongly-stained thyroid tissue controls. Additionally, these findings were also in line with the gene quantification data, which indicated negative *TSH* and *TSHR* gene levels in clinical as well as *in vitro* cell culture samples. Such variation in results in our or in the reported cases could be attributed to many factors including e.g. the clonality of the antibodies, alterations in blocking, or optimization of the other methods [32].

Although our current results imply that TSH and TSHR, at both protein and gene level, are not at least commonly expressed in OLP lesions, it is possible that there is a subgroup of OLP patients with thyroid disease where these markers are expressed and could therefore explain, at least in part, the association between OLP and autoimmune thyroid disease. Among the limitations of the present study are a very limited number of studied samples and lack of functional assays. Therefore, based on our results, any solid conclusions cannot be made about the role of TSH and TSHR in OLP. Further investigations are needed to determine the pathogenetic basis between autoimmune thyroid disease and OLP.

This study is an extension of a previous preliminary study. [33]

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Figure legends:

Figure 1. TSH and TSHR are expressed in healthy thyroid tissue. The healthy oral mucosal tissue and OLP lesional tissue from patients without HT and with HT do not show immunoreactivity for TSH and TSHR.

Figure 2. Transcriptional levels of TSHR in healthy samples and OLP lesions. The absolute quantification by ddPCR technology revealed a negative expression of TSHR in oral mucosal biopsies obtained from healthy individuals (H1-H9) and from OLP patients (P1-P10). Human thyroid cell lines (ML-1 and N-Thyr-Ori) were used as positive controls and they revealed substantial levels of TSHR copies.

Figure 3. End point qualitative PCR of TSHR on healthy (H1-H9) and OLP (P1-10) oral mucosal samples. Normal thyroid cell line (N-Thyr-Ori 3-1) and follicular thyroid cancer ML-1 are used as positive controls. RNA C denotes negative control (without reverse transcriptase) whereas NTC denotes no template negative control (without RNA) samples. The TSHR product size is 271 bp.

Table I.

Clinical and demographic characteristics of the OLP patients (immunohistochemical study)

Characteristic	Age/Sex	Diagnosis of thyroid disease	Other comorbidities	Medications	Allergies
OLP patients with HT					
1	58/F	Hashimoto´s thyroiditis (HT)	Arterial hypertension	Candesartan, levothyroxine	-
2	61/F	HT	Sensory neural hearing loss, nasal polyposis	Mometasone furoate, levothyroxine	-
3	53/F	HT	Osteoporosis, allergic asthma, chronic brochitis, bile acid diarrhea, depression, migraine, cutaneous LP	Alendronate, escitalopram, sodium valproate, cetirizine, quetiapine, esomeprazole, montelukast, levothyroxine, fluticasone propionate/salmenterol, tiotropium, beclometasone dipropionate, buprenorphine, vaginal estrogen	Pollen, cat, dust mite, cephalosporin, penicillin
4	37/F	HT	Migraine	Levothyroxine	Pollen
5	59/F	HT	Arterial hypertension, leg and back pain, cutaneous LP	Enalapril maleate, levothyroxine, paracetamol	-
6	68/F	HT	Arterial hypertension, asthma, arthrosis, celiac disease	Estradiol, levothyroxine, bisoprolol, nifedipine	-
7	65/F	HT	Cutaneous LP	Levothyroxine	-
8	53/F	HT	Arterial hypertension, arthrosis, hypercholesterolemia	Esomeprazole, valsartan, levothyroxine	-
9	53/F	HT	-	Levothyroxine	-
10	52/F	Hypothyroidism due to goiter and partial removal of thyroid gland	DM1, arterial hypertension, hypercholesterolemia	Levothyroxine, simvastatin, insulin glargine, acetylsalicylic acid, insulin aspart	Doxycycline
11	68/F	HT	Heart failure, arterial hypertension, arrhythmia, periodical asthma, heartburn	Levothyroxine, ramipril, simvastatin, acetylsalicylic acid, amlodipine, metoprolol	Sulfonamide
12	72/F	HT	Paget´s disease	Levothyroxine, zoledronic acid	Penicillin
13	60/F	HT	Asthma, heart failure	-	-
14	68/M	HT	Arterial hypertension	Levothyroxine, amlodipine, bisoprolol	-
OLP patients without HT					
1	57/F	-	Arthrosis, arterial hypertension	Losartan, meloxicam, paracetamol, zolpidem	Penicillin
2	60/F	-	Cutaneous dermografism	-	Birch, nuts, celery, paprika
3	54/F	-	Arterial hypertension, osteoporosis, chronic cold, chronic back pain, cutaneous lichen	Bisoprolol, losartan, ebastine, lansoprazole, tramadol, gabapentin, venlafaxine, alendronate, paracetamol, temazepam	Birch
4	37/F	-	Vitamin B12 deficiency	Hydroxocobalamin	-
5	60/F	-	Lymphocytic colitis, hypercholesterolemia	Mesalazine, loperamide, acetylsalicylic acid, simvastatin	Sulfonamide
6	67/F	-	Carcinoma in corpus area, fibromyalgia	Amitriptyline	-
7	66/F	-	Arterial hypertension, DM2, bronchiectasis	Bisoprolol+hydrochlorothiazide,metformin hydrochloride, acetylsalicylic acid, glimepiride, simvastatin	-
8	53/F	-	Rheumatoid arthritis, Sjögren syndrome, psoriasis	Sulfasalazine, hydroxychloroquine	Sodium aurothiomalate, etanercept

9	52/F	-	Asthma	-	Hydrocortisone, nickel, tixocortol pivalate, mercury
10	51/F	-	Arterial hypertension	Medication for hypertension	-
11	68/F	-	Arthrosis	Glucosamine, estradiol	-
12	72/F	-	Arterial hypertension, restless legs syndrome	Amiloride hydrochloride dihydrate, pramipexole, tafluprost	pollen
13	60/F	-	Otitis media serosa, hypercholesterolemia	Simvastatin	-
14	67/M	-	Coronary artery disease	Nitroglycerin, metoprolol	-

Table II.

Clinical and demographic characteristics of the OLP patients (gene expression study)

Characteristic	Age/ sex	Diagnosis of thyroid disease	Other comorbities	Medications	Allergies
P1	68/F	Hashimoto´ s thyroiditis (HT)	Heart failure, arterial hypertension, arrhythmia, periodical asthma, heartburn	Levothyroxine, ramipril, simvastatin, acetylsalicylic acid, amlodipine, metoprolol	Sulfonamide
P2	76/F	HT	Essential thrombocythemia	Levothyroxine, acetylsalicylic acid, pantoprazole, hydroxyurea, estradiol	Penicillin, acetylsalicylic acid
P3	48/M	-	Cerebral infarction, sleep apnea	Dipyridamole + acetylsalicylic acid, simvastatin	-
P4	70/M	-	Arterial hypertension	Bisoprolol, felodipine, losartan, cetirizine	-
P5	67/F	HT	Arterial hypertension, glaucoma, depression	Levothyroxine, enalapril+hydrochlorothiazide, citalopram	-
P6	78/F	HT	Asthma, arterial hypertension, celiac disease, arthrosis, restless legs syndrome, leaky heart valve, pulmonal hypertension, previous breast cancer	Levothyroxine, bisoprolol, candesartan+hydrochlorothiazide, levomepromazine, melatonin, salbutamol, fluticasone propionate	Pollen, odors
P7	60/F	HT	Arterial hypertension	Levothyroxine, candesartan	-
P8	66/F	HT	Arterial hypertension, Sjögren syndrome, musculoskeletal disorder	Levothyroxine, candesartan, hydroxychloroquine	-
P9	61/F	HT	Arterial hypertension, arthrosis, osteoporosis, hypercholesterolemia, transient ischemic attack, celiac disease, mental issues	Levothyroxine, alendronic acid, losartan, escitalopram, acetylsalicylic acid, rosuvastatin	-
P10	53/M	-	Arthrosis	-	-

Table III.

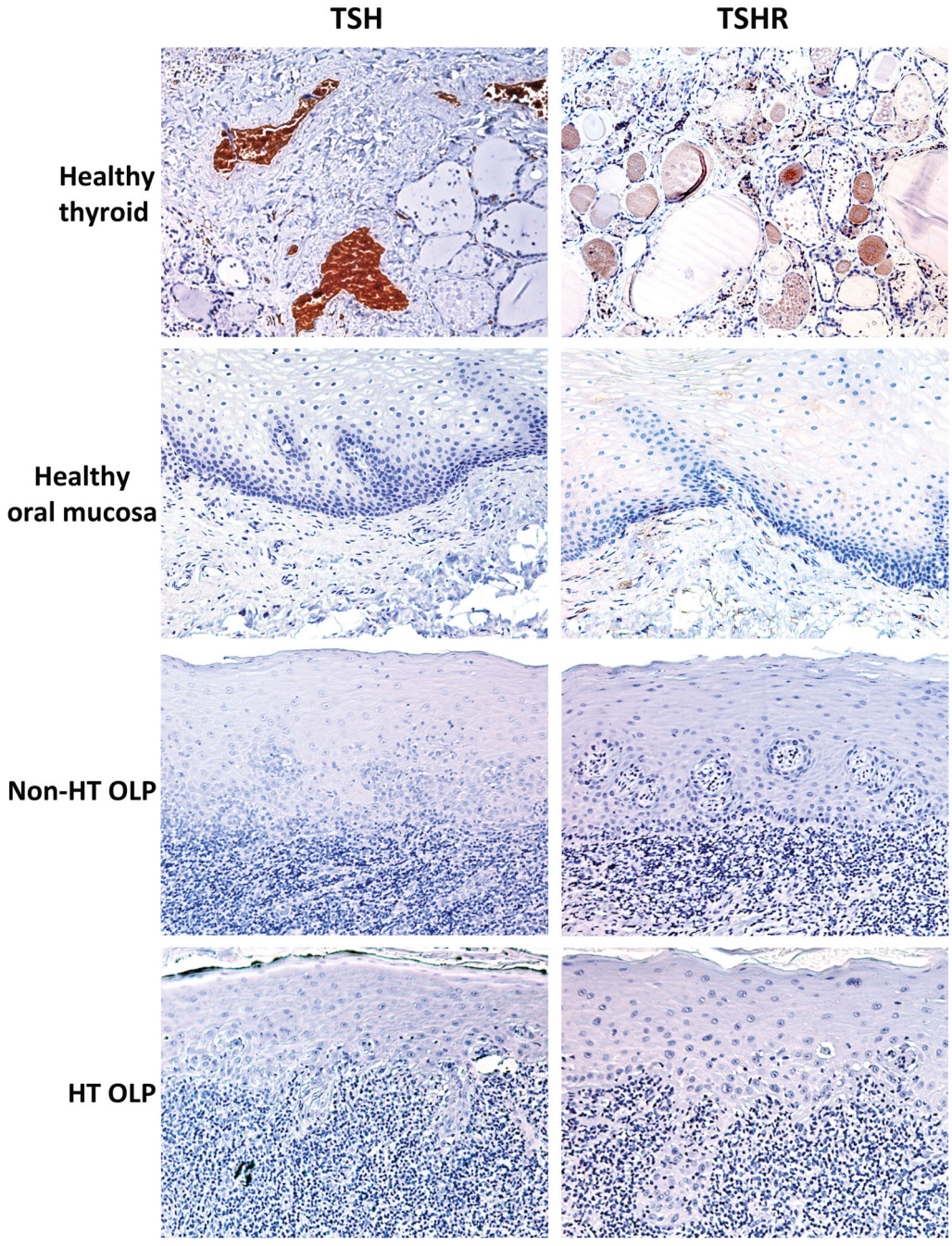
A. Sequences of the used human primer sequences.

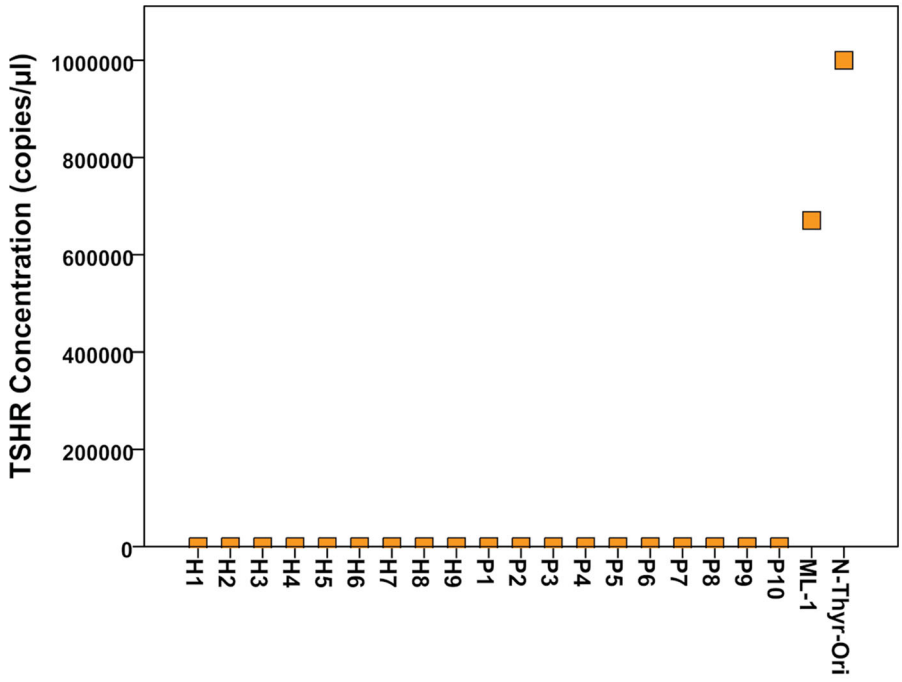
Gene	Forward	Reverse
TSH	5′-ATGGATTACTACAGAAAATATGC-3′	5′-AGATTTTGTATAATAACAAGTACT-3′
TSHR	5′-GCTTTTCAGGGACTATGCAATGAA-3′	3′-AGAGTTTGGTCACAGTGACGGGAA-5′
GAPDH	5′-AAGGTCATCCCTGAGCTG-3′	5′-TGCTGTAGCCAAATTCGTTG-3′
RPLP0	5′-GGCGACCTGGAAGTCCAACT-3′	5′-CCATCAGCACCACAGCCTTC-3′

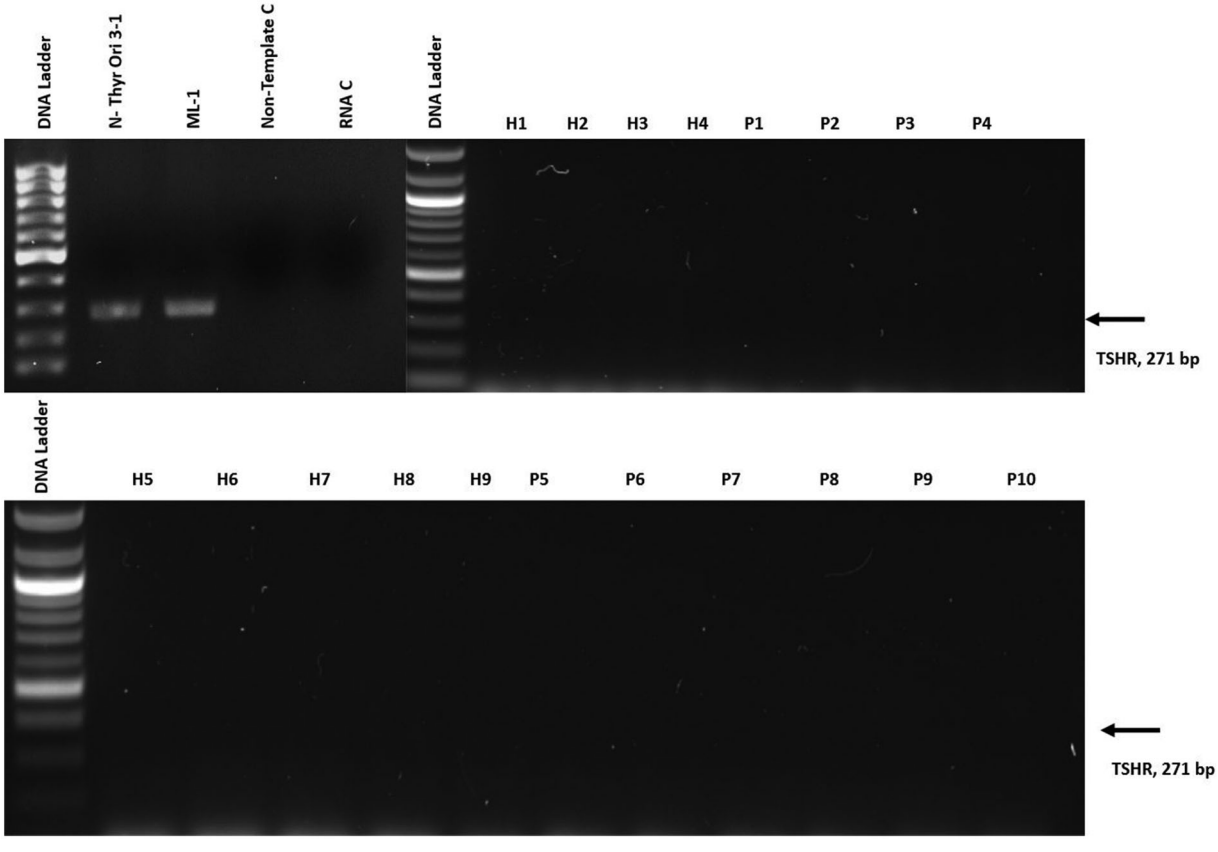
TSH: Thyroid stimulating hormone; TSHR: Thyroid stimulating hormone receptor; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; RPLP0: Ribosomal protein, large, P0.

B. PCR primer and cycling conditions for qualitative PCR for TSHR.

Gene	Primer Sequence	Amplicon	$MgCl_2$, mM	Annealing T °C	Melting T °C
hTSHR	for GTGAATGCTTTTCAGGGACTATG Rev GTCCAGGTGTTTCTTGCTATCAG	271	2	60	95


TSH
TSHR
Healthy thyroid
Healthy oral mucosa
Non-HT OLP
HT OLP


TSHR Concentration (copies/µl)
1000000
800000
600000
400000
200000
0
H1
H2
H3
H4
H5
H6
H7
H8
H9
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
ML-1
N-Thyr-Ori


DNA Ladder
N- Thyr Ori 3-1
ML-1
Non-Template C
RNA C
DNA Ladder
H1
H2
H3
H4
P1
P2
P3
P4
TSHR, 271 bp
DNA Ladder
H5
H6
H7
H8
H9
P5
P6
P7
P8
P9
P10
TSHR, 271 bp