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Prevalence and clinical relevance of thyroid stimulating hormone receptor-blocking antibodies in autoimmune thyroid disease

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Summary

The prevalence and clinical relevance of thyroid stimulating hormone (TSH) receptor (TSHR) blocking antibodies (TBAb) in patients with autoimmune thyroid disease (AITD) was investigated. Serum TBAb were measured with a reporter gene bioassay using Chinese hamster ovary cells. Blocking activity was defined as percentage inhibition of luciferase expression relative to induction with bovine TSH alone (cut-off 40% inhibition). All samples were measured for TSHR stimulatory antibody (TSAb) and TSHR binding inhibiting immunoglobulins (TBII). A total of 1079 unselected, consecutive patients with AITD and 302 healthy controls were included. All unselected controls were negative for TBAb and TSAb. In contrast, the prevalence of TBAb-positive patients with Hashimoto's thyroiditis and Graves' disease was 67 of 722 (9·3%) and 15 of 357 (4·2%). Of the 82 TBAb-positive patients, thirty-nine (48%), 33 (40%) and 10 (12%) were hypothyroid, euthyroid and hyperthyroid, respectively. Ten patients were both TBAb- and TSAb-positive (four hypothyroid, two euthyroid and four hyperthyroid). Thyroid-associated orbitopathy was present in four of 82 (4·9%) TBAb-positive patients, with dual TSHR antibody positivity being observed in three. TBAb correlated positively with TBII ($r = 0{\cdot}67$, $P < 0{\cdot}001$) and negatively with TSAb ($r = -0{\cdot}86$, $P < 0{\cdot}05$). The percentage of TBII-positive patients was higher the higher the level of inhibition in the TBAb assay. Of the TBAb-positive samples with > 70% inhibition, 87% were TBII-positive. Functional TSHR antibodies impact thyroid status. TBAb determination is helpful in the evaluation and management of patients with AITD. The TBAb assay is a relevant and important tool to identify potentially reversible hypothyroidism.

Keywords: bioassay, Hashimoto's thyroiditis, Graves' disease, TSH-receptor blocking antibodies, TSH-receptor stimulating antibodies

Introduction

Thyroid stimulating hormone (TSH)-receptor antibodies (TSHR-Ab) are involved directly in the pathogenesis of autoimmune thyroid diseases (AITD) [1]. Certain TSHR-Ab with stimulating function can mimic TSH activity and promote the autoimmune hyperthyroidism of Graves' disease (GD). Less commonly, however, TSHR-Ab do not activate the TSHR, but rather block the physiological activity of TSH. This blocking function is associated often, but not uniformly, with hypothyroidism [2]. The isolation of human monoclonal TSHR-Ab has provided great insight into the molecular events associated with the interaction of autoantibodies with TSHR [3,4]. In addition, the assumption that patients have only one antibody type was negated by the demonstration that TSHR stimulating antibody (TSAb), TBAb and TSHR binding inhibiting immunoglobulins (TBII) can occur in the same patient [5]. This dual positivity (TBAb and TSAb) may, in fact, explain the variable and sometimes unpredictable clinical presentation seen in AITD patients [6]. In addition, TBII are understood to be a sum of TBAb and TSAb and, therefore, TBII levels mask the functional importance of TSHR-Ab. A number of

reports in the literature have highlighted the relevance of functional assessment of TSHR-Ab in pregnancy in which neonatal hypothyroidism can be associated with maternal AITD [7,8].

Although bioassays for measuring TSHR-blocking and stimulating antibodies were described decades ago [9], the establishment of their clinical validity and utility were hampered by their complexity and unreliability. More recently, cell-based bioassay technology has been improved significantly, which has enabled TSHR-Ab bioassays to be brought into the clinical arena [10]. While binding assays are able to detect the presence of the TSHR-Ab, they are not able to distinguish their functionality [11–13]. Recently, the highly variable sensitivity of various binding assays *versus* functional bioassays for TSHR-Ab was noted [14]. Towards the goal of establishing further the clinical relevance of assessing the functional activity of TSHR-Ab, in the present work we investigated the prevalence and utility of TBAb in AITD.

Materials and methods

A total of 2910 serum samples from 1079 unselected consecutive AITD patients [Hashimoto's thyroiditis (HT) and Graves' disease (GD)] and 302 unselected healthy controls (one sample each) were enrolled into the study. All controls were devoid of thyroid disorders and autoimmune diseases, had normal baseline serum thyroid-related hormones and were negative for thyroperoxidase (TPO), thyroglobulin (Tg) and TSH receptor antibodies. HT was defined as a serum level of TPO-Ab above the reference range with or without increased serum concentrations of Tg-Ab, a hypoechoic appearance at thyroid ultrasound and eu- or hypothyroidism. GD was defined as positive TSHR-Ab, suppressed baseline TSH, elevated free thyroid hormones [free triiodothyronine (fT3) and/or free thyroxine (fT4)], enhanced vascularization at thyroid ultrasound ('thyroid inferno'), with or without clinical manifestations of orbital disease. Informed consent was obtained from all participants. The study protocol was approved by the Ethics Committee of the JGU Medical Center, and was carried out in accordance with the ethical guidelines of the Helsinki Declaration.

Bioassays for TSH-receptor antibodies

Both bioassays use Chinese hamster ovary (CHO) cells expressing a chimeric TSHR (Mc4) and cyclic adenosine-5'-monophosphate (cAMP) response element (CRE)-dependent luciferase. In the Mc4 construct, the amino acid (AA) residues 262–368 of human TSHR are replaced with AA residues 262–335 from rat luteinizing and chorionic gonadotrophin (LH-CG) hormone receptor [15]. In the plasmid construct used to make the stable CHO cell line, the *TSHR* cDNA is driven by the SV40 promoter/enhancer (SV40 pro) and a preceding beta globin intron; the firefly luciferase reporter gene is driven by a glycoprotein hormone alpha subunit promoter, which contains two CREB (cAMP response element-binding protein) binding sites.

TSH-receptor blocking antibody bioassay. Serum TBAb activity was measured as described previously [12,13]. Briefly, a frozen vial of CHO-Mc4 cells ($6{\cdot}7 \times 10^4$ cells/well) was seeded and grown to a confluent cell monolayer in 96-well plates for 15–18 h at 37°C, 5% CO_2. The cells were plated in the 48 inner wells of a 96-well plate with black-walled, clear-bottomed wells. For the sample preparation, 180 µl reaction buffer, 220 µl bovine (b)TSH (Sigma Aldrich, St Louis, MO, USA) and 40 µl of the sample were mixed. The final concentration of bTSH in the well was 100 mIU/l. The final sample dilution was 1 : 11 (40 µl/440 µl) and 100 µl of each sample was added to each well. Serum samples and three controls, consisting of a reference standard bTSH, normal serum and positive TBAb control, were tested in duplicate. After 3 h induction time, the luciferase expression levels of cell lysates were measured as relative light units (RLU) directly in the wells following the addition of substrate and lysis reagent using the luminometer (Tecan Infinite M200; Tecan GmbH, Crailsheim, Germany). Blocking activity was defined as percentage inhibition of luciferase expression relative to induction with bTSH alone [100 × (1 sample + bTSH/bTSH alone)]. Prior to calculating the percentage inhibition, the RLU of the background wells (normal serum diluted 1 : 11 in reaction buffer) were subtracted from the RLU of the test wells and the bTSH-alone wells. The cut-off for the TBAb assay is at 40% inhibition.

TSH-receptor stimulating antibody bioassay. Serum TSAb activity was measured with a chimeric TSHR bioassay according to the manufacturer's instructions [15,16]. Briefly, a frozen vial of CHO-Mc4 cells ($6{\cdot}7 \times 10^4$ cells/well) was seeded and grown to a confluent cell monolayer in 96-well plates for 15–18 h at 37°C, 5% CO_2. Serum samples, positive, reference and normal controls were diluted 1 : 11 in reaction buffer, added to the cell monolayers, and each plate was incubated for 3 h at 37°C, 5% CO_2. Subsequently, the CHO-Mc4 cells were lysed and the RLU were quantified in the luminometer (Tecan Infinite M200; Tecan). The cut-off for the TSAb assay is specimen-to-reference ratio (SRR) at 140%.

Thyroid-related hormones and antibodies

Serum levels of baseline TSH, fT3, fT4, Tg and TPO concentrations were measured using electrochemiluminescent immunoassays (CLIA; Abbott, Wiesbaden, Germany). TBII were measured with the Cobas e 411 (Roche Diagnostics, Mannheim, Germany). Both were utilized according to the manufacturer's instructions.

Table 1. Demographic and laboratory data

$N = 1079$	GD	HT
n (%)	357 (33)	722 (67)
Female, *n* (%)	276 (77)	598 (83)
Age (years)	47·9 (34·5–56)	35·6 (17·4–49)
Children, *n* (< 18 years)	14 (4)	189 (26)
Adults, *n* (> 18 years)	343 (96)	533 (74)
Duration of AITD (years)	2 (1–5)	2 (0·1–8)
TAO, *n* (%)	229 (64)	44 (6)
TSH (0·4–4·9 mU/l)	1·6 (0·01–4·7)	2·0 (1–4)
fT3 (1·7–3·7 pg/ml)	4·7 (3·7–5)	3·0 (2·6–3·5)
fT4 (0·7–1·5 ng/dl)	2·0 (1·2–16·2)	1·2 (1–1·4)
Tg-Ab (< 4·1 IU/ml)	8·4 (1·8–58)	58·4 (10–295)
TPO-Ab (< 6 IU/ml)	93 (7–519)	182 (38–537)

Values are represented in median and 25 and 75 quartiles [interquartile range (IQR)]. The normal range is shown for the thyroid-related hormones and autoantibodies. GD = Graves' disease; HT = Hashimoto's thyroiditis; AITD = autoimmune thyroid disease; TAO = thyroid-associated orbitopathy; TPO = thyroperoxidase; Ab = antibody; fT3 = free triiodothyronine; fT4 = free thyroxine.

Statistical analysis

The baseline serum sample values of each patient were considered for statistical analysis. Data are presented as median and quartiles (25 and 75% percentiles) and analysed with SPSS version 23 (SPSS, Inc., Chicago, IL, USA) and SAS version 9.3 (SAS Institute, Cary, NC, USA). Repeatability of the TBAb bioassay was analysed on five repeated measurements in 47 initially positive patients by fitting a linear random-effects model and report the square root of the within-patient variance as the precision parameter. This analysis was repeated with 45 patients with mean TBAb > 30% and 39 patients with mean TBAb > 40% to reduce a risk of bias due to accumulation of false-positive findings near the cut-off. A possible relationship between level and precision was investigated by Spearman's correlation coefficient between the means and standard deviations calculated for each patient. The correlation between assay findings with Spearman's correlation coefficients was quantified and tested for zero correlation with the corresponding test. All *P*-values were two-sided and a result was significant when $P \leq 0{\cdot}05$.

Results

Demographic data

A total of 1079 unselected, consecutive patients with AITD and 302 euthyroid healthy controls were included. The demographic and serological data are demonstrated in Table 1. The age of controls (median, 25 and 75% percentiles) was 25 (24–29) years, with 155 (51·3%) females. Compared with patients suffering from GD, the HT patients included in the study were younger (fivefold more children), included only 10% as many patients with thyroid-associated orbitopathy (TAO), and had higher titres of TPO-Ab and Tg-Ab.

TBAb positivity

All healthy controls were negative for TBAb and TSAb (bioassay specificity 100%). In contrast, the prevalence of TBAb-positive patients with HT and GD was 9·3 and 4·2%, respectively (Table 2). Approximately 82% of TBAb-positive patients were diagnosed with HT and 18% GD. However, 3·1% (51 of 1621) samples of GD patients and 6·4% (82 of 1289) samples of HT patients were TBAb-positive. The corresponding median (25 and 75%

Table 2. Prevalence and percentage inhibition in 82 thyroid stimulating hormone receptor-blocking antibodies (TBAb)-positive autoimmune thyroid disease (AITD) patients

	GD (*n* = 357)	HT (*n* = 722)
TBAb-pos. *n* (%)	15 (4·2)	67 (9·3)
TBAb (% inhibition)	76 (51–86)	55 (45–83)
Age (years)		
Adults, *n*	14	43
Adults, % inhibition	76·5 (54–86·3)	56 (46–79)
Children, *n*	1	24
Children, % inhibition	44	51·5 (42–92)
Gender		
Female, *n*	13	55
Female, % inhibition	77 (48–86·5)	55 (44–78)
Male, *n*	2	12
Male, % inhibition	70·5 (65–70·5)	60 (47–99)
Thyroid function, *n* (%)		
Hypothyroid	5 (33)	36 (54)
Euthyroid	4 (27)	27 (40)
Hyperthyroid	6 (40)	4 (6)
Percentage inhibition		
Hypothyroid	77 (76–77)	60 (45–92)
Euthyroid	83·5 (61·5–93)	54 (43–78)
Hyperthyroid	48 (43–66)	66·5 (47–82)
TAO		
n (%)	2 (13·33)	2 (3)
% inhibition	42·5 (41–43)	74·5 (43–82)
Specific treatment		
• L-T4, *n*	3	23
• L-T4, % inhibition	86 (84–86)	47 (41–84)
• Methimazole, *n*	3	0
• Methimazole, % inhibition	76 (44–76)	–
TBAb% inhibition (10 TSAb-positive patients)	44 (41–44)	60 (50·5–68)

Values are represented in median and 25 and 75 quartiles (interquartile range). GD = Graves' disease; HT = Hashimoto's thyroiditis; TAO = thyroid-associated orbitopathy; TBAb = thyroid stimulating hormone receptor-blocking antibodies; TSAb = thyroid stimulating hormone receptor-stimulating antibodies.

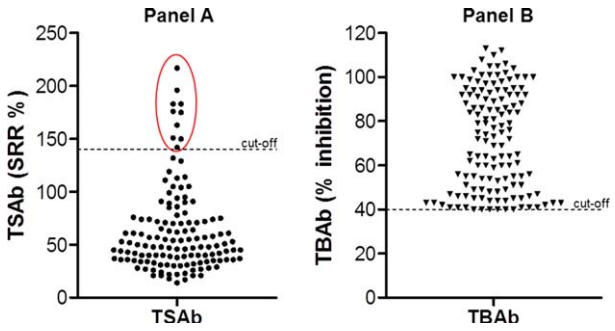

Fig. 1. Distribution of thyroid stimulating hormone receptor-stimulating antibodies (TSAb) (a) and TSHR-blocking antibodies (TBAb) (b) in 133 TBAb-positive serum samples with autoimmune thyroid disease (AITD). (a) The dots demonstrate TSAb measured in the TSAb bioassay (cut-off at 140 specimen-to-reference ratio (SRR %) as a dotted line. Included in the red circle are the 10 TBAb- and TSAb-positive samples. (b) The triangles TBAb measured in the TBAb bioassay (cut-off at 40% inhibition as a dotted line). [Colour figure can be viewed at wileyonlinelibrary.com].

percentiles) TBAb percentage inhibition levels were −107 (–158·5; −60·5) for GD and −1 (–23·8; 16) for HT, respectively. The distribution of percentage inhibition of TBAb-positive samples, according to thyroid function, reveals GD patients to be more hyperthyroid; HT patients, however, were more hypothyroid or euthyroid (Table 2). The prevalence and serum levels of TSAb and TBII have been investigated in all 1079 AITD patients (Table 3). TBAb percentage inhibition shows a clear trend to thyroid function (Table 4). The higher the percentage inhibition, the more hypothyroid was the patient. Of 82 TBAb-positive patients, 39 were hypothyroid, 33 euthyroid and 10 hyperthyroid, with four (10·3%), two (6%) and four (40%) additionally testing TSAb-positive. Four TBAb-positive patients displayed TAO, two of whom had HT and two GD. Three of these four were additionally TSAb-positive.

Both TSAb (Fig. 1a) and TBAb (Fig. 1b) bioassays were utilized simultaneously along with the binding (TBII) test. Ten patients (six with HT and four with GD) were positive in all assays (Fig. 1a, red circle). The thyroid function of these patients was as follows: four hypothyroid, two euthyroid and four hyperthyroid. Among all 133 TBAb-positive samples, 59% were TBII-positive and TSAb-negative, 7·5% were positive for both TBII and TSAb and 33·5% were negative for both TSAb and TBII (Table 5). Not surprisingly, TBAb correlated positively with TBII ($r = 0{\cdot}67$, $P < 0{\cdot}001$) and negatively with TSAb ($r = -0{\cdot}86$, $P < 0{\cdot}05$). Samples with high TBAb positivity (> 70% inhibition) were more likely to be positive in the binding assay. When comparing the TBAb percentage inhibition to TBII positivity (Table 4) an ascending relation was apparent, such that of the samples with 40–50% inhibition only 17% were TBII-positive, whereas of those samples with > 70% inhibition, 87% were TBII-positive.

Table 3. Prevalence and serum levels of TSAb and TBII in all 1079 AITD patients

	GD (n = 357)	HT (n = 722)
TSAb-positive, n (%)	309 (87)	68 (9)
TSAb (SRR %)	422 (280–516)	47 (36–71)
TBII positive, n (%)	269 (75)	68 (9)
TBII (< 1·75 IU/l)	6·65 (2.53–19·8)	0.5 (0·5–0·5)

Values are represented in median and 25 and 75 quartiles (IQR). TSAb = thyroid stimulating hormone receptor (TSHR) stimulating antibody; TBII = TSHR binding inhibiting immunoglobulins; AITD = autoimmune thyroid disease; SRR = specimen-to-reference ratio.

Repeatability

For the TBAb assay, reproducibility data were generated from 622 sera of 47 AITD patients, initially TBAb-positive. The root mean squared error was 9·1% for patients with mean TBAb from 40 to 50% inhibition and of 8·8% when restricting data to patients with mean TBAb > 30 or > 40%. A significant trend towards increasing precision with higher TBAb levels was observed (Spearman's $r = -0{\cdot}33$, $P = 0{\cdot}02$, between individual means and standard deviations).

Discussion

This work entailed the largest study of TSHR-blocking antibodies from consecutive and unselected patients with AITD from a single institution. It shows clearly the high prevalence and clinical relevance of TBAb such that, overall, approximately 10% of patients with AITD are TBAb-positive. Most TBAb-positive patients have HT, but patients with GD may also be TBAb-positive, particularly during or after antithyroid drug treatment. Consistent with the functional relevance of TBAb, the majority of TBAb-positive patients were hypothyroid. TBAb-positivity, however, was not predominant in patients with TAO. TBAb was correlated highly with TBII. Gender, age and treatment had no impact upon either TBAb prevalence or degree of inhibition.

The high number of unselected subjects emphasizes the importance of this field of research. As well as the high number of patients, the novelty of this paper encompasses diligent testing involving not only the functional blocking

Table 4. Thyroid function in 82 TBAb-positive patients with AITD according to TBAb percentage inhibition

% Inhibition	40–50	51–60	61–70	71–100	Σ (%)
Hypothyroid	14	7	1	17	39 (48)
Euthyroid	12	7	2	12	33 (40)
Hyperthyroid	4	2	2	2	10 (12)
Patients (n)	30	16	5	31	82
TBII	5	6	3	27	41
	(16·6%)	(37·5%)	(60%)	(87·1%)	(50%)

TBII = thyroid stimulating hormone receptor (TSHR)-binding inhibitory immunoglobulins; AITD = autoimmune thyroid disease; TBAb = TSHR-blocking antibodies.

Table 5. Classification of TSHR-antibody positivity in bioassays and a binding assay in 133 TBAb-positive samples

TBAb	TSAb	TBII	*n* (%)
+	–	+	78 (59)
+	–	–	45 (33·5)
+	+	+	10 (7·5)

TBAb = thyroid stimulating hormone receptor (TSHR)-blocking antibodies; TSAb = TSHR stimulating antibodies; TBII = TSHR-binding inhibitory immunoglobulins.

and stimulating cell-based bioassays, but also the conventional binding assay. This allowed us to detect the seldom prevalence of dual TSH receptor antibody positivity. In the meantime, we were able to detect a further 'double-positive' patient (with two samples) who was not included in the following data. In addition, our serological data enabled us to identify the thyroid state responsible for symptoms and relevant for clinical presentation. Thus far, the investigation of the results obtained with thyroid function is unique to bioassay testing.

Subsequent to development [12], analytical performance and validation of this TBAb bioassay was performed recently [13]. The goal of the present study was to illuminate the prevalence and clinical importance of TBAb, an under-studied biomarker despite its increasing relevance in AITD. Due possibly to its ephemeral nature, measurement of TBAb presents far more difficulty than measurement of TSAb. A mass screening of more than 1000 AITD patients detected 82 TBAb-positive patients, thus allowing closer examination of this uncommon biomarker. Results that compare the binding assay and bioassay emphasize the sensitivity of the cell-based bioassay and highlights that the binding assay is unable to distinguish the functionality of TSHR antibody.

This is the only study, to our knowledge, where an unselected, consecutively followed collection of AITD patients were compared with a large unselected control group devoid of thyroid or autoimmune disease. In contrast, several previous publications have encompassed smaller numbers of patients and did not compare the results of various testing methods, including established commercial assays [2,17,18]. To the best of our knowledge, only one study looked at a relatively large paediatric collective in which TBAb percentage inhibition was analysed after neonatal screening of potentially hypothyroid newborns. A prevalence of TBAb-positivity of 1·4% (11 of 790) was shown to be associated mainly with mothers with AITD [7].

The large number of patients is the relevant pool, and the novel functional bioassays are important tools. TBAb determination primarily provides an important tool to identify AITD, mainly in the form of reversible hypothyroidism. Independently from its detection, the progress, development or remission of the disease can be measured by the percentage of antibody inhibition. The blood withdrawals took part at different stages of the respective AITD patients: some were undergoing therapy, others were at the beginning of disease progress and some had previously unknown antibody titres. The TBAb testing, however, showed a tendency of according thyroid functions. The higher the percentage of TBAb inhibition, the more clinically apparent (hypothyroidism). Therefore, antibody monitoring corresponds to the severity of the disease.

TBAb percentage inhibition shows a clear trend towards thyroid function. The higher the percentage inhibition, the more hypothyroid was the patient. Of those patients with TBAb positivity in GD, a higher percentage inhibition was detectable in patients during or post-anti-thyroid drug treatment. However, 33 TBAb-positive patients were euthyroid at the time of sampling. The presence of concomitant TSAb positivity in the serum may explain biochemical euthyroidism. Another possibility is the magnitude of TBAb level in the serum, with hypothyroidism occurring above a certain antibody concentration.

As understanding of the pathogenesis of GD has evolved, it has been suspected that certain patients might produce not only TSAb but also TBAb, and that this might account for their alternating clinical presentation [19,20]. The isolation of separate monoclonal antibodies (MAbs) with stimulatory and blocking activity from the same patient's lymphocytes confirmed this suspicion [5]. Structure–function analyses using these MAbs have provided insight into the mechanism of their functional activity [3].The co-presence of TBAb and TSAb in the serum of a patient has been speculated to have a counteracting effect, similar to a tug-of-war [6]. In TSAb bioassays, the co-presence of TBAb and TSAb created a net sum, possibly evening out and remaining non-detectable. TSAb detects net TSAb, but cannot detect TBAb. TBAb detects either net TBAb (positive inhibition) or net TSAb (negative inhibition). Neither assay detects them 'independently' [12].

Despite the large collection of patients in the present study, because of the low prevalence of TBAb, the number of TBAb-positive patients was relatively small. The cross-sectional nature of this study limits the ability to obtain information about TBAb development. New trials are planned to utilize prospective longitudinal studies as well as long-term follow-up. The depicted patients were determined via cross-section and do not display the fluctuation of TSHR-Ab in a singular patient's lifetime – in particular, in extreme situations such as puberty and pregnancy [21]. Antibody-switching between TBAb and TSAb has been reported in recent literature [22,23]. These switches have not only become apparent during the therapy of HT, with L-T4 resulting in hyperthyroidism, but also during antithyroid drug therapy in GD leading to hypothyroidism [6]. Therefore, in AITD ongoing monitoring of both is essential, independently of what could be a misleading clinical appearance. Integrating the measurement of functional TSHR-Ab into the management of AITD can assist

physicians with difficult cases, and simultaneous measurement of TBAb and TSAb can help in the interpretation of thyroid dysfunction.

Disclosure

T. D., J. K., J. Kö. and M. K. have nothing to disclose. P. D. O. and G. J. K. consult for Quidel, USA. This paper entails parts of the PhD thesis of J.K.

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