Direct quantification of stimulating TSH receptor autoantibodies in Graves' disease by the first *in vitro* assay suitable for routine clinical diagnosis

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Introduction

The TSH receptor (TSHR) is a seven transmembrane receptor coupling to the cAMP signaling cascade. It contains a large extracellular domain presenting epitopes for stimulating as well as blocking TSHR autoantibodies (Ab, Fig. 1). Stimulating TSHR autoantibodies (sTRAb) cause hyperthyroidism (HT) in Graves' disease (GD). Currently, assays commercially available used in GD diagnosis quantify the heterogeneous pool of all TSHR Ab indirectly by the displacement of labeled TSH from the TSHR (= competitive assay). Moreover, the TSH binding does not cover the epitopes for both stimulating and blocking TSHR Ab completely. We introduce the first *in vitro* assay for direct detection of sTRAb suitable for routine clinical diagnosis.

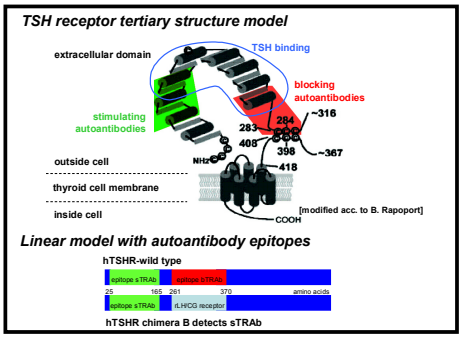

Figure 1: TSHR model illustrates epitopes for stimulating & blocking TSHR Ab and for TSH binding to the extracellular domain. The linear constructs show hTSHR & hTSHR B / rat LH/CGR chimera with autoantibody epitopes and the substituted epitope for blocking Ab. LH/CGR: luteinizing hormone / choriogonadotropin receptor

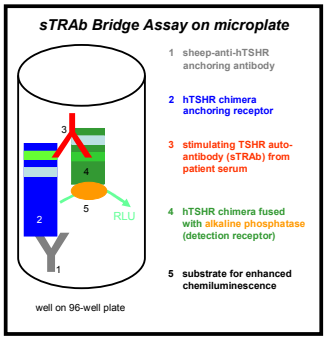

Figure 2: Direct detection of TSHR stimulating autoantibodies (sTRAb) by bridge assay. RLU = relative light units

Materials & Methods

The sTRAb bridge assay is performed on microplates (MP) using recombinant chimeric hTSHR-rLH/CG receptors (chim-hTSHR) with epitopes only for stimulating autoantibodies (Fig. 1). Chimeric TSH receptors were stably expressed in HEK293 cells as fusion proteins with alkaline phosphatase and without fusion, respectively. A C-term directed antibody anchors chim-hTSHR, to which one arm of sTRAb binds (Fig. 2). The second arm of the sTRAb bridges to chim-hTSHR fused with alkaline phosphatase. Applying chemiluminescent substrate sTRAb were quantified using a luminometer (CentroLIA LB 961). Chim-hTSHR stability allows assay performance at room temperature as well as at 37°C. Using the bridge assay WHO standard 90/672, calibrated controls, normal sera, and sera from GD patients (50µl) were tested.

Results & Discussion

The range of detection was established by using the standard for Thyroid-Stimulating Antibody WHO 90/672. High sensitivity of the assays was demonstrated: the analytical sensitivity (zero standard, mean + 3 SD) was determined below 0.3 IU / L (the low cut-off at 20% between-run CV). Polynomic correlation between relative light units (RLU) and Thyroid-Stimulating Antibody standard concentrations was shown over a broad range, at least up to 50 IU / L (Fig. 3). Using patient sera and up to five different assay material lots bridge assay was tested showing good reproducibility expressed as between-run coefficient of variation (CV) (Fig. 3). In active GD stimulating TSHR Ab levels correlated with disease activity, whereas controls were tested negative. ROC plot analysis using 182 sera (67 GD positive) revealed >0.45 IU / L as criterion for positive rating with a diagnostic sensitivity of 100.0 % with a specificity of 100.0 % (Fig. 4). Diagnostic accuracy of the sTRAb bridge assay compared to a TSH displacement assay using the human TSHR was superior: the area under ROC curve was calculated as 1.000 vs. 0.978 improving diagnosis (Tab. 1). Values obtained in GD patients by the novel sTRAb assay correlate only in part with those determined by commercial competitive TSHR Ab assays (Fig. 5). The latter measure TSH displacement from TSHR by all pathological Ab (both stimulating and blocking Ab) giving rise to higher values in these assays for patients with both types of Ab. Furthermore TSH binds only partially to the epitopes of both Ab types what might be the reason for lower values in competitive assays. Similar to the bioassay, we directly measure only stimulating Ab which cause HT in GD. However, compared to the bioassay the sTRAb bridge assay is not such time consuming and not expensive. The adaptation of direct sTRAb detection to microplate format by bridge assay design is the first step for usage in a routine laboratory. Adaptations to automated assay formats are feasible.

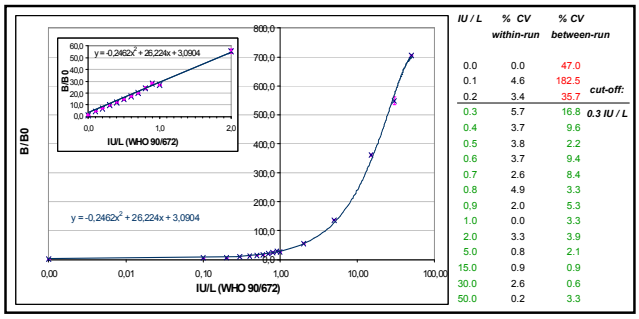

Figure 3: Standard curve with WHO-Standard for thyroid stimulating autoantibodies WHO 90/672. B/B0: RLU-mean of each standard value divided by RLU mean of zero standard value (n = 3). Inter assay precision profile created by 5 runs with triplicates. cut-off: 20 % between-run variance.

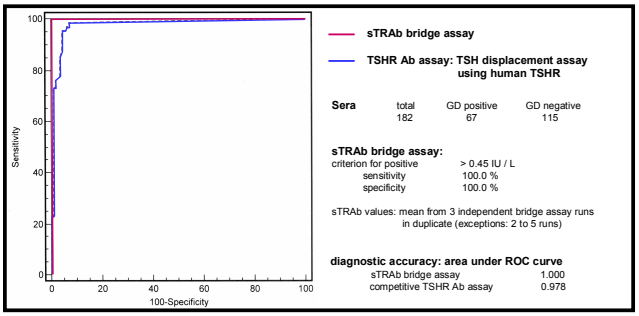

Figure 4: Comparative ROC plot analysis of patient and normal sera: the sTRAb bridge assay shows higher diagnostic accuracy compared to a commercial TSHR Ab assay using TSH displacement at the human TSHR. For the sTRAb bridge assay criterion (treshold concentration) for correctly classifying subjects into positive and negative group was determined as sTRAb > 0.45 IU / L with 100.0 % diagnostic sensitivity and 100.0 % specificity.

serum	activity of autoimmune process evaluated by clinical diagnosis (ATD treatment, tachycardia, etc.) and thyroid laboratory tests (TSH, T3, T4): 0 - negative, 1 - positive		sTRAb >0.45 positive	TRAb * 1.0 < grey zone ≤ 1.5	improvement by sTRAb
B002	1		5.50	1.60	well-defined diagnosis
B001	1		2.35	1.10	explicit diagnosis
B003	0		*0.0*	1.40	
PL04	0	euthyroid: normal values TSH, T3 & T4; no ATD	0.45	1.20	
PL05	0	normal values of thyroid parameters	0.21	1.40	
PL08	1		2.26	1.50	
B039	1		1.14	1.30	
PL02	0	thyroxin substitution essential for normal thyroid metabolic status	*0.0*	18.80	no false diagnosis based on serum Ab concentration: measured TRAb do not induce hyperthyroidism; e.g. blocking Ab; particularly PL02 with blocking Ab
PL11	0	euthyroid metabolism after ATD treatment, actual no ATD	0.37	2.20	
B028	0		0.12	2.20	
B027	0	euthyroid metabolism after ATD treatment, actual no ATD	0.43	2.80	
B013	1		1.10	<1.0	no false diagnosis based on serum Ab concentration
B052	0	Thyroiditis de Quervain	*0.0*	1.80	no interference

ATD: antithyroid drug ★: TRAb measurement by commercial TSH displacement assay using human TSHR *0.0*: sTRAb value < negative standard

Table 1: Portrayal of patient sera with discrepancies in diagnosis based on TRAb measurement compared to clinical diagnosis. Determination of sTRAb activity by sTRAb bridge assay improves diagnosis for GD based on serum parameter.

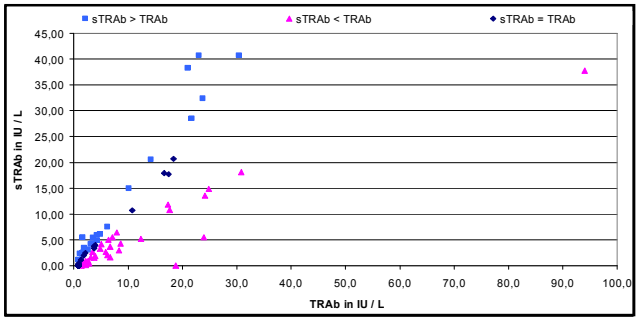

Figure 5: Comparison of direct measured stimulating TSHR Ab (sTRAb) and values obtained by competitive assay measuring all TSHR autoantibodies (TRAb) in patient n = 62 and normal n = 115 sera. Values correlate only partially, e.g. 107 normal and 10 patient sera; the remaining 52 patient and 8 normal sera do not correlate.

Literature

Kim TY, Kohn LD et al (2003). Epitope Heterogeneity of Thyroid-Stimulating Antibodies Predicts Long-Term Outcome in Graves' Patients... *JCEM* 88:117-124; Minich WB, Loos U: Detection of Functionally Different Types of Pathological Autoantibodies Against TSH-R... Exp Clin Endocrinol Diabetes 108 (2000) 110-119