

DETECTION OF SERUM THYROID STIMULATING IMMUNOGLOBULIN (TSI) IN GRAVES' DISEASE (GD) USING A NOVEL CELL-BASED REPORTER ASSAY

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Abstract

Graves' disease (GD) is a hyperthyroid autoimmune disorder characterized by the presence of serum antibodies directed toward the thyrotropin receptor (TR). TR antibodies (TRAbs) have been demonstrated in two major functional categories; 1) thyroid stimulating immunoglobulins (TSI or TSABs), 2) thyrotropin-binding inhibitory immunoglobulins (TBIs). The antibodies associated with GD are TSIs which function by activating thyrotropin receptor signal transduction and subsequent production of thyroid hormone. TBIs can block either thyrotropin or TSI from binding the TR, thus preventing signal transduction^[1]. TRABs are commonly measured using the thyrotropin-binding inhibitory immunoglobulin method. The objective was to establish a TSI assay cutoff along with assay sensitivity and specificity using clinical samples from subjects who had been diagnosed with GD. Sera from non-diseased subjects as well as those with other autoimmune diseases were also tested. CHO cells were stably transfected with a human TR/Luteinizing Hormone Receptor (LHR) chimera and a firefly luciferase reporter gene under the control of a tandem cyclic adenosine monophosphate (cAMP) response element (CRE). Selection of the reporter cell line was based on luciferase activity response to TSI and long term stability of the cells. Using the assay, TSI activity in patient sera was determined by the measurement of luciferase activity relative to a known bTSH (Sigma) reference. In this study we used the CHO cells to test sera from 56 GD positive subjects, 140 normal subjects, 10 pregnant subjects and 38 subjects with other diseases. ROC analysis was used to establish an optimal cut-off of 140% above the bTSH reference. The GD positive samples tested between 34% and 662% while the normal samples tested between 5% and 116%. Overall, the results demonstrate a sensitivity and specificity of 94.6% and 99.5%, respectively, for the TSI assay. The assay is novel because it utilizes a chimeric hTR which lacks the region suspected of supporting TBII^[1,ii,iii] activity. In addition, the assay provides a high degree of sensitivity (94.6%) and specificity (99.5%) for detecting GD sera while maintaining a user friendly format that requires a total assay time of less than 24 hours.

Background

Graves' disease (GD) is a hyperthyroid autoimmune disorder characterized by the presence of serum antibodies directed toward the thyrotropin receptor (TR). TR antibodies (TRABs) have been demonstrated in two major functional categories; 1) thyroid stimulating immunoglobulins (TSI or TSABs) and 2) thyrotropin-binding inhibitory immunoglobulins (TBIs). The antibodies associated with GD are TSIs which function by activating thyrotropin receptor signal transduction and subsequent production of the thyroid hormones thyroxine (T4) and triiodothyronine (T3). TBIs can block either thyrotropin or TSI from binding the TR, thus preventing the signal transduction necessary for hormone production. The presence of TSI in Graves' hyperthyroidism can cause a myriad of physical symptoms in the patient such as nervousness, irritability, heart palpitations, tremors, anxiety, sleeplessness and muscular weakness. The diagnosis of GD requires serum testing for suppressed thyrotropin levels and elevated free T4^[ii] levels. The American Thyroid Association (ATA) recommends further evaluation, including testing for thyroid autoantibodies, if GD is suspected. TRAb detection assays include (a) complex bioassays which measure cyclic adenosine monophosphate (cAMP) by RIA, (b) solid phase radioreceptor assays which measure either ¹²⁵I or chemiluminescence, (c) direct immuno-precipitation assays which measure ³⁵S and (d) saturation based ELISA assays with colorimetric outputs. The most common of these tests utilize thyrotropin-binding inhibition to detect the presence of TRABs. This assay methodology cannot distinguish TSI from TBI and as such is not a specific method for the detection of GD. The benefits of TSI specific bioassays have been clouded by researchers and clinicians who cite them as being of low practical utility because of cumbersome cell culture techniques, time-consuming sample purification steps and complicated, lengthy radioreceptor assay or radioimmunoassay (RRA or RIA) signal detection^[vi].

Methods

In order to develop an assay which could specifically detect TSI in patient serum, the human glycoprotein alpha subunit promoter was PCR amplified from chromosomal DNA that was isolated from human embryonic kidney cells. The 316 bp fragment was then subcloned into the pGL2 enhancer plasmid (Promega, Madison, WI). A second plasmid was constructed by subcloning a neomycin resistant gene fragment into the pMC4 which contained the TSHR/LH chimera receptor (from Dr. Leonard Kohn). The human glycoprotein alpha subunit promoter, with a firefly luciferase reporter gene, was then isolated and subcloned into pMC4-neo plasmid. CHO K1 cells were transfected with the new linearized plasmid using HyFect (Denville Scientific, Metuchen, NJ.) according to the manufacturer's instructions. Twenty four hours after the transfection, the cells were combined and plated into a 96 well plate and selected with 0.5mg/ml G418 in Ham's F12 Medium with 10% FBS. Final selection of the reporter cell line was based on luciferase activity response to TSI and long term stability of the cells. The system was enhanced using patented detection methods (US Patent No. 6852546) and by procedure optimization. Using the finalized Thyretain TSI Reporter Bioassay protocol, TSI activity in patient sera was determined by the measurement of luciferase activity relative to a bTSH (Sigma) reference calibrated against NIBSC standards. In this study we used the Thyretain CHO MC4 cells, which were frozen in measured aliquots, to test sera from 56 GD positive subjects, 140 normal subjects, 10 pregnant subjects and 38 subjects with other diseases. The results are reported as a ratio of the specimen signal to the reference signal (SRR%). Clinical sensitivity and specificity were calculated. Additional analyses were performed on a subset of samples which were provided to us with traditional TRAb RRA results. Of the 56 Graves' disease and 188 non-Graves' disease samples tested, 30 Graves' disease and 68 non-Graves' disease samples were further analyzed using 2x2 and 2x3 table comparisons. Clinical sensitivity and specificity were calculated.

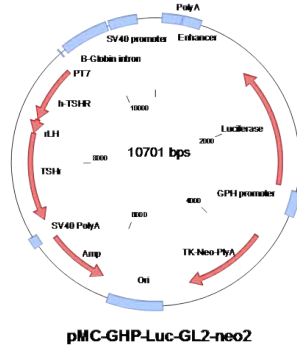


Figure 1: Schematic Diagram for the Construction of the Thyretain Cell Line

The chimeric hTSHR-containing plasmid with a luciferase reporter gene was permanently transfected into CHO-K1 cells. After G418 selection, the functional clones were identified by the signal response when the reporter gene expression was induced by TSI-containing samples.

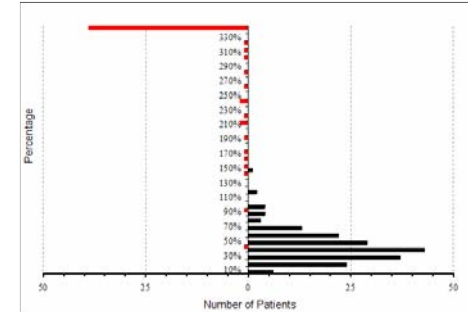
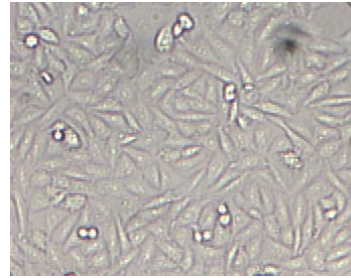


Figure 2: Frequency of Occurrence of TSI-containing Samples
The 244 samples tested on the Thyretain bioassay are represented as the frequency of signal to reference ratios for each sample tested. This graph shows that the average range of normal samples is near 40% of the bTSH reference control.

Figure 3: Clinical Sensitivity of the Thyretain TSI Reporter BioAssay

Thyretain	Diagnosis		
	Positive (Graves' disease)	Negative (Non-GD)	Total
Positive	53	1	54
Negative	3	187	190
Total	56	188	244

Sensitivity: 95%
Specificity: 99%

Figure 3: The 56 GD and 188 non-GD samples tested to confirm the 140% Thyretain bioassay cutoff result in a Clinical Sensitivity of 95% and Specificity of 99%.

Figures 4a and 4b: Clinical Sensitivity of the Thyretain TSI Reporter BioAssay versus Traditional TRAb Assay

Thyretain	Diagnosis		
	Positive (Graves' disease)	Negative (Non-GD)	Total
Positive	29	1	30
Negative	1	67	68
Total	30	68	98

Sensitivity: 97%
Specificity: 99%

Figure 4a: A subset of samples were analyzed to determine the sensitivity and specificity of the Thyretain bioassay compared to the traditional TRAb assay. This figure shows the results obtained with the Thyretain bioassay compared to diagnoses. The 68 negative samples had the following diagnoses: 11 were hyperthyroid non-Graves', 12 were Hashimoto's disease, 3 were rheumatoid arthritis, and 40 were clinically healthy normal samples.

TRAb Assay	Diagnosis		
	Positive (Graves' disease)	Negative (Non-GD)	Total
Positive	20	0	20
Negative	4	67	71
Indeterminate	6	1	7
Total	30	68	98

Sensitivity: 67%
Specificity: 99%

Figure 4b: This figure shows the results of traditional TRAb assay when the subset of samples analyzed in Figure 4a are analyzed. TSI was positively detected in 20/30 Graves' disease samples.

Summary of Results

The Graves' disease, TSI positive samples tested between 34% and 662% while the normal samples tested between 5% and 116%. Overall, the results demonstrate a clinical sensitivity and specificity of 94.6% and 99.5%, respectively, for the Thyretain TSI Reporter BioAssay.

Further analysis showed that Thyretain TSI Reporter Bioassay positively detected TSI in 29/30 Graves' disease samples while the TRAb assay positively detected TSI in 20/30 of those same Graves' disease samples.

Conclusion:

A novel Thyretain TSI Reporter Bioassay has been developed which provides a high degree of sensitivity (94.6%) and specificity (99.5%) for detecting TSI in Graves' disease positive sera while maintaining a user friendly format that requires a total assay time of less than 24 hours. The luciferase reporter technology can replace the traditional radioisotope usage with a sensitive assay utilizing a chimeric hTR which lacks the region suspected of supporting TBII activity.