A Study on the Level of T₃, T₄, TSH and the Association of A/G Polymorphism with CTLA-4 Gene in Graves' Hyperthyroidism among South Indian Population

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Abstract Graves' disease (GD) is an organ-specific heterogeneous autoimmune disorder associated with T-lymphocyte abnormality affecting the thyroid, eyes and skin. GD is a multifactorial disease that develops as a result of complex interaction between genetic susceptibility genes and environmental factors. It has been suggested that the Cytotoxic T lymphocytes associated molecule-4 (CTLA-4) is a genetic susceptibility candidate for GD. The present study was focused on A/G polymorphism at position 49 in exon-1 of the CTLA-4 gene in 80 GD patients (GP) and 80 sex and age matched healthy individuals among South Indian (Madurai) population. Serum concentrations of thyroid hormone (T₄, T₃ and TSH) were determined by using automated analyzer. The genomic DNA was isolated from the patient and control groups and genotyping was performed using the polymerase chain reaction followed by restriction enzyme analysis using *BbvI*. Significant difference ($P < 0.001$) was observed in the level of T₃, T₄ and TSH in GD patients and healthy individuals. The results revealed the CTLA-4 gene G/G genotype to be 32 (40%) in patients and 26 (32.50%) in healthy individuals, A/G genotype to be 37 (46.25%) in patients and 25 (31.25%) in healthy individuals and A/A genotype to be 11 (13.75%) in patients and 29 (36.25%) in healthy individuals. The calculated odds ratio (OR) in individuals with mutant

genotype (GG/AG) reveal 3.6 fold risk for GD (95% confidence interval = 1.6–7.8). The mutant “G” allele frequency was observed to be 0.63 in GD patients and 0.48 in healthy individuals. Thus the present study demonstrates an association between the CTLA-4 gene polymorphism and Graves' disease.

Keywords CTLA-4 gene · Gene polymorphism · Susceptibility · Heterogenous · Autoimmune disorder · Graves' disease

Introduction

Graves' disease (GD) is an organ specific autoimmune disorder associated with T-lymphocyte abnormality [1], in which the antibodies produced by immune system stimulates thyroid gland to produce excess of thyroxine (T₄) [2]. The normal range of T₄ is suggested to be 77–155 nmol/l, T₃ to be 1.2–2.8 nmol/L and TSH to be 0.3–4 mU/l [3]. The levels of hormones above or below the normal range indicate hyperthyroidism or hypothyroidism. In Graves' hyperthyroidism, T₃ and T₄ levels have been reported to be increased and the TSH level to be decreased. Our finding also coincides with the above report.

The most common form of hyperthyroidism is GD. It is also a multifactorial disease that develops as the result of a complex interaction between genetic susceptibility genes and environmental factors. The susceptibility genes are human leucocyte antigen (HLA), GD-1, GD-2 and GD-3 [4] and cytotoxic T-lymphocyte associated molecule-4 (CTLA-4) [5–7]. It has been suggested that the CTLA-4 gene polymorphism plays an important role in the development of Graves' hyperthyroidism in various populations [8, 9].

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The CTLA-4 gene has also been implicated in several endocrine autoimmune disorders like Hashimoto's hypothyroidism [10], IDDM [5], Rheumatoid arthritis [11], Addison's disease [10] and Multiple sclerosis [12]. The CTLA-4 Ala17 is shown to be associated with IDDM and Graves' disease, whereas linkage was observed for IDDM [5].

CTLA-4 gene was discovered in a cDNA library of T-cell-specific, activation-induced genes [13]. The CTLA-4 gene is reported to be located on chromosome 2 (2q33) [14]. There is increasing evidence that CTLA-4 is an extraordinarily important molecule to down regulate the T cell expansion and cytokine production [15, 16]. The expression of CTLA-4 on T cells may affect the course of an ongoing immune process [17, 18].

Three polymorphism sites in CTLA-4 gene have been reported. They are (1) A/G polymorphism in exon 1, (2) C/T polymorphism in the promoter and (3) Microsatellite repeat in the 3'-untranslated region of exon 4 in the CTLA-4 gene. This gene has been reported to be associated with autoimmune endocrine disorders [12, 19].

A/G Single Nucleotide Polymorphism (SNP) at position 49 (exon 1, codon 17) of the CTLA4 gene leads to a Thr/Ala substitution [20]. Studies conducted among different populations showed an association of CTLA-4 gene A/G polymorphism with Graves' disease. However, such studies are lacking among Indian population. Hence the present study associating this polymorphism (A/G polymorphism at position 49 in exon 1 of CTLA-4 gene) with Graves' disease was conducted among the population of South India (Madurai, Tamil Nadu).

Materials and Methods

In the preliminary study undertaken by us, collecting samples from an Endocrinology clinic in Madurai (TN) it was found that females are more affected (80%) than males (20%) and 85% of them had previous history of thyroid disease and all the patients were on antithyroid drugs. Fifty-two patients were postpartum stage. Hence female patients of age group 20–60 years were selected. The results obtained coincide with the report by Kinjo et al. [3], who studied 224 patients with GD, among whom 199 were females and only 25 were males. Their study also revealed the prevalence of GD in females than males (15–75 years old).

Eighty healthy individuals and 80 clinically proven Graves' disease patients were considered for the study. The clinical parameters used were weight, height, body mass index (BMI), TRAb level, T₃, T₄ and TSH levels. Fresh peripheral blood was collected in EDTA coated tubes from

both healthy individuals and GD patients and stored at –20°C till analysis.

The study plan was reviewed and approved by our institutional biosafety committee.

Hormone Analysis

Serum concentration of T₄, T₃ and TSH were determined by using automated analyzer—Immunochemiluminometric analyzer (ICMA) (ADVIA Centaur). Graves' disease was confirmed when the serum T₃ (normal range: 0.582–1.64 ng/dl), T₄ (normal range: 0.39–5.60 ng/dl) and TSH (normal range: 82–280 ng/dl) hormone levels exceeded the normal range.

Genotyping of CTLA-4 Gene A/G Polymorphism

Genomic DNA was isolated from all the healthy individuals and GD patients from peripheral white blood cells [21]. PCR-based restriction enzyme analysis was performed in the isolated DNA. The presence of DNA was confirmed with 0.7% Agarose gel electrophoresis and the amount of DNA was quantified using UV-Spectrophotometer (Systronics).

Polymerase Chain Reaction Analysis (PCR) [3]

The DNA sequence (162 bp) of CTLA-4 gene was amplified by PCR. Genomic DNA (0.2 µg) was incubated in a total reaction volume of 50 µl containing 10 pmol of both forward (5'-GCTCTACTTCCTGAAGACCT-3') and reverse (5'-AGTCTCACTCACCTTTGCAG-3') primers (Fermentas Life Sciences) for the CTLA-4 gene A/G Single Nucleotide Polymorphism (SNP) (A49G), using 2 Units of *Taq* DNA polymerase (Bangalore Genei, India) and dNTPs (200 µM). Amplification for the CTLA-4 A49G SNP was performed with an initial denaturation of 94°C for 5 min in a thermal cycler (Eppendorf India Limited). The PCR amplification conditions were as follows: 30 cycles consisting of 30 s denaturation at 94°C, 45 s annealing at 57°C, 30 s extension at 72°C. The final cycle included a 7 min extension step at 72°C. The PCR amplified product (162bp) was confirmed by 2% agarose gel electrophoresis under UV-transilluminator.

Restriction Enzyme Analysis [3, 9]

The CTLA-4 A49G SNP creates a *BbvI* (Fermentas Life Sciences, Germany) restriction enzyme recognition sequence site. The SNP was detected by digestion of PCR amplified product (10 µl) with *BbvI* (0.5 U) for 3 h at 37°C. Restriction fragment size analysis was performed by

visualization of digested PCR product by 2% agarose gel electrophoresis.

Statistical Analysis

Mean and Standard deviation was calculated for hormone profile for both the healthy individuals and GD patients. Student *t* test was performed to find out whether there was significant difference in hormone profile between the healthy individuals and GD patients. The significance level of genotype and allele frequency was tested by chi-square test (χ^2). Odds ratio at 95% confidence intervals were calculated.

Results and Discussion

The level of serum T_3 and T_4 were significantly increased ($P < 0.001$) in GD patients when compared to the healthy individuals and the level of TSH was significantly decreased in GD patients ($P < 0.001$) when compared to the healthy individuals (Table 1). Similar results were observed by several other studies [3, 20, 22].

Kinjo et al. [3] have also reported that there is increase in the level of TRA b (TSH Receptor Autoantibody) and this leads to hyperthyroidism and increased level of T_3 and T_4 . In GD condition, binding of TRAb to the TSH receptor on the acinar cells of thyroid gland leads to continuous synthesis of thyroid hormones (T_3 and T_4) without involving the feedback mechanism. Thus TRAb prevents binding of TSH to its receptor [2]. Therefore, elevated T_3 , T_4 hormones suppressed the release of TSH and undetectable TSH hormone was noted in Graves' hyperthyroidism.

In the present study, the amplified PCR product (162 bp) was digested with enzyme *BbvI*. The restriction enzyme acts on the "G" variation, but not on the "A" variation. The presence of "G" allele at position 49 creates restriction sites for *BbvI*, there by resulting in either 3 fragments (162, 88 and 74 bp—Heterozygous mutant) or 2 fragments (88 and 74 bp—Homozygous mutant). If there is no substitution of "G" allele at position then the restriction site for *BbvI* is abolished, as a result of which there is no cleavage of the PCR product (162 bp—Wild type—Homozygous).

Table 1 Level of serum triiodo-thyroxine (T_3), tetraiodothyroxine (T_4) and thyroid stimulating hormone (TSH) in healthy individuals and GD patients

Study subjects	T_3 (ng/dl) Mean $\pm$ SD	T_4 (ng/dl) Mean $\pm$ SD	TSH (ng/dl) Mean $\pm$ SD
GD patients ($n = 80$)	511.57 $\pm$ 94.92	25.95 $\pm$ 4.48	0.25 $\pm$ 0.08
Healthy individuals ($n = 80$)	130.72 $\pm$ 4.83	0.96 $\pm$ 0.05	2.51 $\pm$ 0.17

Table 2 Prevalence of the genotype and allele of A/G polymorphism at position 49 in exon 1 of CTLA-4 gene in GD patients and controls among South Indian population

Genotype	GD patients ($n = 80$)	Healthy individuals ($n = 80$)
G/G (homozygous mutant)	32 (40%)	26 (32.50%)
A/G (heterozygous mutant)	37 (46.25%)	25 (31.25%)
A/A (homozygous normal)	11 (13.75%)	29 (36.25%)
Allele frequency		
G (mutant)	0.63	0.48
A (normal)	0.37	0.52

There was significant difference ($P < 0.05$) between the control group and GD patients both in genotype and allelic frequency.

"G" allele frequency (0.63) was found to be significantly ($P < 0.005$) higher in GD patients than healthy individuals (0.37) (Table 2). The calculated odds ratio (OR) showed that individuals with mutant genotype (GG/AG) have 3.6 fold risk for Graves' disease (95% confidence interval = 1.630–7.802).

The distribution of genotypes (GG, AG, and AA) for CTLA-4 gene polymorphism varies among different populations (Caucasians, Japanese, Koreans, Tunisians, Hong Kong Chinese children and Taiwanese) [6–8, 20, 23–26]. Till date, there is no study available for the prevalence of CTLA-4 gene polymorphism among GD patients in Indian population. Hence the present study associating CTLA-4 gene A49G polymorphism and Graves' hyperthyroidism was conducted among South Indian (Madurai) population.

The highest "G" allele frequency (0.76) was noted in Chinese GD population [20]. The lowest "G" allele frequency (0.48) was noted in Caucasian GD population and among healthy individuals in Iranian (0.37) and Caucasian population (0.38) [9, 12, 22]. The present study shows that the "G" allele frequency is 0.63 among South Indian population (Madurai), which is comparable to that of the Taiwanese "G" allele frequency (0.67) [23].

Susceptibility to GD has significant genetic components. CTLA-4 gene polymorphism has been reported to be associated with GD. CTLA-4 molecule is a member of the family of cell surface molecule as CD28, which binds to B7. The CTLA-4/B7 complex competes with the CD28/B7 complex and delivers negative signals to the T-cells, which affects T-cell expansion, cytokine production, and immune responses as evidenced in different populations [6–8, 23]. To conclude (i) The TSH was found to be decreased among GD patients whereas T_3 and T_4 were found to be increased. (ii) Prevalence of mutant genotype (GG) for CTLA-4 gene was found to be higher in GD patients than in healthy individuals and (iii) there is association between mutant

genotype and GD among South Indian (Madurai) population.

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