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IDENTIFICATION OF SNPS IN GRAVES' DISEASE AMONG SOUTH INDIAN (MADURAI) POPULATION

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ABSTRACT

Graves's hyperthyroidism is an organ-specific autoimmune disorder. The genetic susceptibility to GD is conferred by genes in the human leucocytes antigen (HLA) and cytotoxic T cell lymphocytes associated molecule –4 (CTLA-4). The present study focused on A/G polymorphism at position 49 in exon - 1 of the CTLA-4 gene in 80 GD patients (GP) and 80 sex, age matched normal case (NC) among Madurai population. The levels of of T.Chol., TGL, HDL, LDL, VLD and minerals like Ca with includes Mg, Mn, Na, and K and Fe were investigated by Autoanalyzer and AAS. The results showed in GD patients vs control group TGL (223.12±54.61) vs (140.44±92.22), T.Chol. (153± 40.13) vs (165.27± 31.97), HDL (30.61±8.02) vs (57.45±12.84), LDL (77.84±36.53) vs (79.73±39.80) and the levels of K (34.93 ±19.11) vs (75.36 ±15.68) Na (2824.22±150.34) vs (2520.67 ±67.71), Ca (15.93±3.66 vs (9.36 ±1.13) Fe (0.027±0.005) vs (0.092±0.037). Mean serum concentrations of T3, T4 and TSH in GD patient vs control group were 511.57±849.03 ng/dl vs 130.72±43.26 ng/dl, 25.95±40.10 ng/dl vs 0.96±0.45 ng/dl and 0.25±0.77 ng/dl vs 2.51±1.58 ng/dl respectively. Secondary investigation was the association of CTLA-4 gene genotype between GP and NC. DNA was isolated and DNA was confirmed using AGE (0.7%) and the genomic DNA was subjected to PCR and 162 bp

fragments were obtained. The amplified PCR product is digested with *Bbv1*. The restriction enzyme acts on the G variation, but not on the A variation. If a G allele was at position 49, 88bp and 74bp two fragments were obtained. The PCR and RFLP products were detected by 2%. The significant difference ($p < 0.05$) of Genotype and Allele frequency was found between the control group and GD patients The study suggested that the to determine a clear association of the CTLA-4 gene polymorphism with the remission of GD.

Keywords: Polymorphism, mutation, hypo and hyper thyroidism, variation, genetic marker

INTRODUCTION

The most common form of hyperthyroidism is Graves' disease (GD), an autoimmune disorder in which antibodies produced by immune system stimulates thyroid gland to produce excess of thyroxine (T_4). The normal range T_4 is suggested to be 77-155 nmol/L, T_3 to be 1.2-2.8nmol/L and TSH to be 0.3-4U/L The level of hormones increase or decrease than the normal range indicate hyperthyroidism or hypothyroidism. In GD, T3 and T4 levels have been reported to be increased and the TSH level to be decreased (Kinjo *et al.*, 2002).

GD also found to be an organ-specific heterogeneous autoimmune disorder associated with T-lymphocytes abnormality affecting the thyroid, eyes, and skin. GD is a interaction between genetic susceptibility genes and environmental factors (Bednarczuk *et al.*, 2003). The genetic susceptibility candidates are human leucocyte antigen (HLA), GD-1, GD-2, and GD-3 (Tomer *et al.*, 1999) and Cytotoxic T-lymphocyte associated molecule -4 (CTLA-4) (Donner *et al.*, 1997, Yanagawa *et al.*, 1997, Park *et al.*, 2000).

CTLA-4 gene was discovered in a cDNA library of T cell – specific activation- induced genes. The CTLA-4 gene is reported to be located on chromosome 2 (2q33) (Heward *et al.*, 1999). There is increasing evidence that CTLA-4 is an extraordinarily important molecule to down regulate the T cell expansion and cytokine production. This CTLA-4 gene product is a T- cell surface molecule that binds to the B7 molecule on the antigen presenting cells (APCs) (Vaidya *et al.*, 1999). The expression of CTLA-4 on T cells may affect the course of an ongoing immune process (Vaidya *et*

al., 1999). It has been suggested that the CTLA-4 gene polymorphism plays an important role in the development of Graves' hyperthyroidism in various populations (Yanagawa *et al.,* 1995, Bednarczuk *et al.,* 2003).

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 (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 several endocrine autoimmune disorders (Kouki *et al.,* 2000) like Hoshimotos hypothyroidism (Kotsa *et al.,* 1997), IDDM (Donner *et al.,* 1997), Rheumatoid arthritis (Vaidya *et al.,* 2002), Addision's diseae (Kotsa *et al.,* 1997, and Multiple sclerosis (Kouki *et al.,* 2000). Hence, the CTLA-4 gene is found to very important role in cause of many autoimmune disorders in all races. Therefore, the present study is focused on this CTLA-4 gene polymorphism among South Indian population.

The thyroid dysfunction is said to be associated with the metabolism of low density lipoprotein (LDL) and high density lipoprotein (HDL). HDL has been reported to be increased, normal or even decreased (Tan *et al.,* 1998). Dieckman *et al.,* (2000) have also reported that thyroid dysfunction leads to changes in lipoprotein metabolism and have been shown that the plasma low –density lipoprotein cholesterol (LDL- C) and high density lipoprotein (HDL-C) levels were also increases in hypothyroidism and decrease in hyperthyroidism. The Triglycerides (TGL) are the considered as risk factor for ischemic heart disease and commonly seen in cases of diabetes, pancreastitis, hyperthyroidism and hypoproteinemias. Under normal circumstances, triglycerides within the chylomicrons are stripped of fatty acids as they pass through various tissues especially adipose and skeletal muscles (Murray *et al.,* 2003).

Potassium (K) Sodium (Na), Calcium (Ca), Magnesium (Mg), Copper (Cu) and Zinc (Zn) are the minerals crucial for thyroid health and functions. All these minerals have been shown to be involved in the production and degradation of thyroid hormone. Edmonds and Smith (1999) have shown that potassium levels are low in patients with hyperthyroidism. The total body changes are shown to be closely related to total plasma T_3 concentration but unrelated to the thyroxine (T_4) level. So, the

determination of the plasma levels of potassium and thyroid hormone help in diagnosing the condition and establish ATD treatment and to avoid further episodes weakness (Edmonds and Smith, 1999).

A/G single nucleotide polymorphism (SNP) at position 49 (exon1, codon 17) of the CTLA-4 gene leads to a Thr/Ala substitution (Wang *et al.,* 2004). Studies conducted among different populations showed that there is an association of CTLA-4 gene A/G polymorphism with Graves' disease (Kinjo *et al.,* 2002; Yanagawa *et al.,* 1995 and Bednarczuk *et al.,* 2003). However, such studies are lacking among Indian population. Hence, the present study associating this polymorphism (A/G at 49 in exon1 of CTLA-4 gene) and variation in the level of lipid profile and elements like calcium, sodium, potassium and iron with Graves' disease conducted among the population of South India (Madurai, Tamil Nadu).

MATERIALS AND METHODS

Study subjects:

Preliminary study undertaken and samples were collected from an Endocrinology clinic in Madurai (TN). It was found that females were more affected than males. Eighty five percentages of them had previous history of thyroid disease and all the patients were on antithyroid drugs (Kinjo *et al.,* 2002). . Fifty two patients were postpartum stage. Hence female patients of age group 20-60 years were selected. Clinically proven eighty Graves' disease subjects and eighty healthy individuals were considered for the study. Peripheral blood was collected in EDTA coated tubes from both healthy individuals and GD subjects and serum was separated from the blood. Serum and the cells were stored at -20^{o}C till analysis.

The study plan was reviewed and approved by Institutional Biosafety Committee (IBSC), Department of Zoology, Lady Doak College, Madurai.

Hormones analysis:

Serum concentration of T_3, T_4 and TSH were determined by using automated analyzer-Immuno chemiluminometric analyzer (ICMA), ADVIA Centaur.

Trace elemental Analysis:

The level of trace elements like calcium, potassium, sodium and iron were estimated by atomic absorption spectroscopy (AAS), (ELICo, SL 173) and Flame photometry (ELICo CL 22).

Lipid profile:

The total cholesterol (T.Chol.), triglycerides (TGL), high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) levels were measured by span diagnostic kit (2000). The absorbance was read at 505nm in Spectrocolorimeter.

A/G single nucleotide polymorphism (SNP) analysis in CTLA-4 gene:

Genomic DNA was isolated from all the healthy individuals and GD patients from peripheral while blood cells (Sambrooke and Russel, 2003). The DNA sequence (162bp) of CTLA-4 gene was amplified by Polymerase chain reaction (PCR). Genomic DNA (0.2µg) was containing 10pmol 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) amplification using 2U of *Taq* DNA polymerase (Banglaore Genei, India). The CTLA-4 A49G SNP creates a *Bbv1* (Fermentas Life Sciences, Germany) restriction enzyme recognition sequence site. The SNP was detected by digestion of PCR amplified product (10µl) with *Bbv1* (0.5U) for 3hours at 37^{o}C (Kinjo *et al.* 2000, Bednarczuk *et al*., 2003).

Statistical Analysis:

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

RESULTS AND DISCUSSION

The study group comprised of 80 GD patients taking treatment in the Endocrinology clinic in Madurai. Among whom females were observed to be more affected (80%) when compared to males (20%). The blood samples were collected from females of age group 20-60 years, among whom 34% were in age the group of 20-30 years and 33% of them were within the age group of 30-40 years. Hence it was observed that the women age of group 20-40 years were more affected by GD.

Lipid profile:

The present study found that most of the patients had lower level of cholesterol (153± 6.10 mg/dl) than the control group (165.27± 3.57 mg/dl) which might be due to the body's inability to utilize and synthesize cholesterol and fat contents. The present study showed that GD patients have high level of TGL (223.12±4.48 mg/dl) than the control group (140.44±10.31 mg/dl). A correlation is observed between TGL level and T3, T4, TSH levels. The current study proved that there is correlation between thyroid hormone and TGL level because other hormones may take over the lipolysis process. The study found that GD patients had low level of HDL (30.61±0.89 mg/dl) than control groups (57.45±1.43 mg/dl)**.** The study has revealed that there is no significant difference between the LDL levels in GD patient (77.84±4.08 mg/dl) and the control group (79.73±4.45 mg/dl) and the level VLDL increase in GD patients (44.62 ±1.22 mg/dl) than in control group (28.08 ± 2.06 mg/dl). Statistical analysis by *Student t- test* showed that there is a significant difference between the level of TGL and VLDL ($p < 0.05$) but T.Chol. and LDL showed that no significant difference, whereas HDL showed that less significant difference between the control group and GD patients **(Fig.1).**

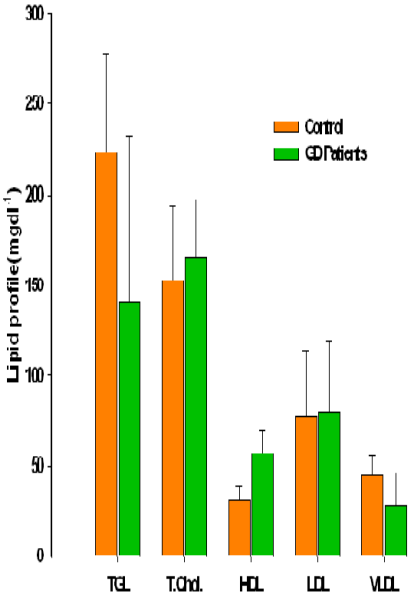

Fig 1:Analysis of Lipid Profile in controls and GD Patients

The study has revealed that the LDL cholesterol sticks to artery walls and contributes to plaque build-up. Nishitani *et al.,* (1990) accelerated catabolism and anabolism of lipid has been reported in hyperthyroidism. Transient elevation of serum lipid levels (Dieckman *et al.,* 2000) suggests a more rapid improvement in catabolism than anabolism of lipid in early stage of the medical treatment.

Mineral profile:

Minerals (mg/dl)	Control (NC) n=80	GD patient (GP) n=80
Ca	9.36 ±1.13	15.93±3.66
K	75.36 ±15.68	34.93 ±19.11
Na	2520.67 ±67.71	2824.22±150.34
Fe	0.092±0.037	0.027±0.005

Values are Mean ± SD (mg/dl)

Table:1 Level of serum Calcium (Ca), Potassium (K), Sodium (Na) And Iron (Fe) In Graves' Patient and Control Group

In GD patient's serum showed that a low level of K (34.93 ±19.11 mg/dl) than in the control group (75.36 ± 15.68 mg/dl). Na level is found to be higher in GD patients (2824.22 ±150.34 mg/dl) than in the control group (2520.67 ± 67.71 mg/dl), Ca level is found to be more in GD patients (15.93±3.66 mg/dl) than in the control group (9.36 ±1.13 mg/dl) and the level of iron is observed to be low in GD patients (0.027±0.005 mg/dl) than in the control group (0.092±0.037 mg/dl) **(Table:1).**

The present study on the level of potassium (K), calcium (Ca), Sodium (Na) and Iron (Fe) in GD patient's serum revealed that the low level of K and Ca and higher level of Na and Ca in GD patients than in control group. The above results showed that GD patients may have problem related to muscle weakness, bone weakness and anemic condition. Fischer (1997) reported that the total K changes were shown closely related to total plasma T3 concentration but unrelated to the thyroxine (T4) level. So, the determination of the plasma levels of K and thyroid hormones helps in diagnosis and is important in establishing ATD treatment and to avoid further episodes of weakness.

Hormone profile:

Mean serum concentrations of T_3, T_4 and TSH in GD patient vs control group were 511.57±94.92ng/dl vs 130.72±4.83ng/dl, 25.95±4.48ng/dl vs 0.96±0.05ng/dl and 0.25±0.08ng/dl vs 2.51±0.17ng/dl respectively. The study shows that the mean value of T_3, T_4 and TSH are significantly different ($p < 0.05$) in GD patients compared to control group **(Fig.2,3,&4).**

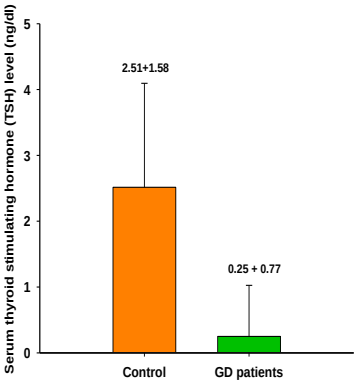

Fig 2: Level of serum thyroid stimulating hormone (TSH) in controls and GD Patients

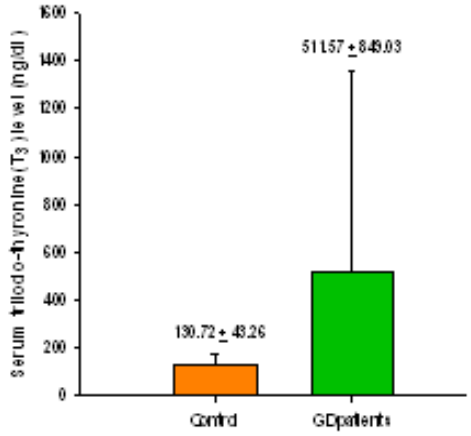

Fig 3: Level of serum triiodo-thyronine (T3) in controls and GD Patients

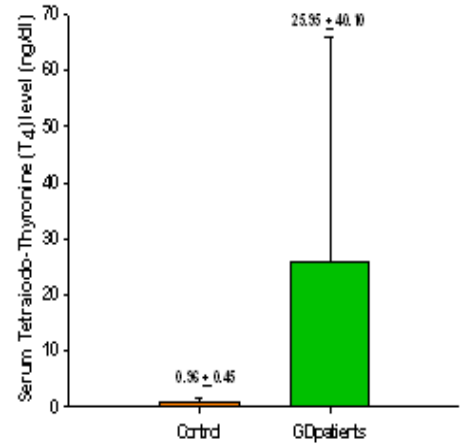

Fig 4: Level of serum tetraiodo-thyronine (T4) in control group and GD Patients

Based on the genotype and allele frequency, Kinjo *et al.,* (2002) reported that the relationship between the CTLA-4 genotype and the severity of the thyroid dysfunction at diagnosis. Free T_4 concentrations were highest in patients with GG genotype and lowest in patients with AA genotype. Graves' patients have been reported to have more G alleles than the control group (Bednarczuk *et al.,* 2003). Kinjo and his co-workers (2002) have also reported that there is increase in the level of TRAb and this leads to hyperthyroidism and increased level of T3 and TSH.

A/G single nucleotide polymorphism (SNP) analysis in CTLA-4 gene:

In the present study genomic DNA was isolated from GD patients and control groups and was subjected to Agarose gel electrophoresis (0.7%). This enables easy visualization of DNA band patterns. After confirming the presence of genomic DNA, it was subjected to PCR and 162bp fragments were obtained. The amplified PCR product was digested with enzyme *Bbv*1. The restriction enzyme acts on the "G" variation, but not on the A variation. If a "G" allele was at position 49bp, 88bp and 74bp fragments were obtained. The PCR products were detected by 2% Agarose gel electrophoresis.

The results showed that the G/G genotype is observed in 32 (40 %) patients and in 26 (32.50 %) persons of the control group, A/G genotype in 37 (46.25 %) patients and in 25 (31.25 %) persons of the control group, A/A genotype in 11 (13.75 %) patients and in 29 (36.25 %) persons of the control group and "G" allele is found in 50

(62.5%) GD patients and in 38 (47.5%) persons of the control group and **"**A" allele is found in 30 (37.5 %) GD patients and 42 (52.5%) in control group. There was significant difference ($p<0.05$) between the control group and GD patients both in genotype and allelic frequency. The study also demonstrated that an association between the CTLA-4 gene polymorphism in Graves' disease and with the remission rate of Graves' hyperthyroidism. Among the GD cases studied, only 2% had remission and the frequencies of GG genotype (40 %) and "G" allele (62.5%) were higher when compared to A/A genotype (13.75%) and "A" allele (37.5 %) **(Fig.5& 6).**

Bednarczuk *et al.,* (2003) and Yanagawa *et al.,* (1997) analyzed the association of CTLA-4 A49G polymorphism with Graves' disease in Caucasian and Japanese population. There are several reports described (Park *et.al.,* 2000 (Koreans); Yanagawa *et al.,* 1997 (Japanese) and Yanagawa *et al.,* 1995 (Caucasian).) the association of GD with CTLA-4 gene among different populations.

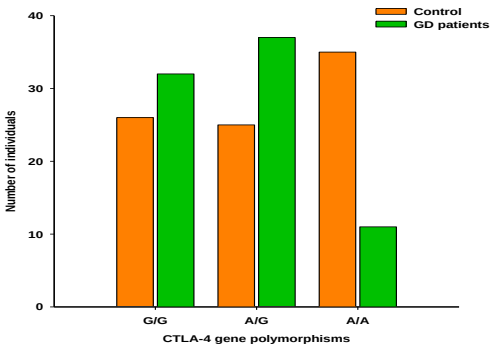

Fig.5: Prevalence of CTLA-4 gene genotype

in control group and GD patients among Madurai population

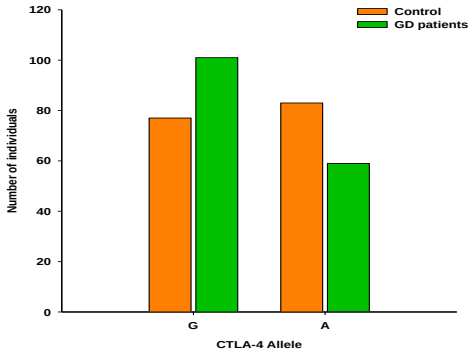

Fig.6: Prevalence of CTLA-4 gene allele frequency in control group and GD patients among Madurai

CONCLUSION

Therefore, in accordance with previously published results, the present study also demonstrates an association between the CTLA-4 gene polymorphism in Graves' disease and with the remission rate of Graves' hyperthyroidism among South Indian (Madurai) population. Hence, the study

suggested that GD patients with G allele in exon 1 of the CTLA-4 gene were required to continue ATD treatment for longer periods to achieve remission. Further studies will be required to determine a clear association of the CTLA-4 gene polymorphism with the remission of GD.

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