Effect of polymorphism on CTLA-4 gene on Graves' Disease

Eman Th. Al-Fatlawy¹, Israa K. Al-Yasiri²*, Ibtihal Al-Shamarti³

1. Microbiology department, Faculty of Pharmacy, University of Kufa, Al-Najaf, Iraq.
2. Microbiology department, Faculty of Medicine, University of Jabber ibn Hyyan Medical University, Al-Najaf, Iraq.
3. Basic Science Department, Faculty of Dentistry, University of Kufa, Al-Najaf, Iraq.

* Corresponding author:
Dr. Israa K. Al-Yasiri
Microbiology department,
Faculty of Medicine,
University of Jabber ibn Hyyan Medical University,
Al-Najaf, Iraq.
E-mail: israa_alyasiri@jmu.edu.iq

Abstract

Background: Graves is one of autoimmune Thyroid Diseases. Moreover, more than 20 genes polymorphisms are associated with Graves’s diseases. Although, the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) genes play an important role in immunity system function, strategies to enhance CTL4 polymorphism gene may be impact on increase Graves’ disease. Recent research has revealed that the 49A/G polymorphism in exon 1 has a detrimental EFFECT ON this disease, results in a threonine-to-alanine conversion. Objectives: The study was carried out to investigate and affect allele polymorphism CTL4 gene in Graves’ disease in AL-Najaf province. Methods: In our study, 73(48 females and 25 males) individuals who detected Autoimmune hyperthyroidism and twenty healthy as a control groups. All Graves patients were recruited from the hormonal unite at Al-Sadder Medical City, Najaf/Iraq. The control group participating in the study did not have any history of immune diseases and chronic diseases. The study was approved by the ethical committees of Al-Sadder Medical City. Genomic DNA was extracted then polymerase chain reaction fragment length polymorphism (PCR-RFLP) technique is used, follow by Sequence data of polymorphism. Results: Results of this study found that all samples were digested with restriction enzyme Fnu4Hl in two fragments (99 and 63) bp. These results indicate that 49 A/G polymorphism GLT4 gene was detected in Graves patients and control. Conclusion: Contrary to expectations, this study did not find a significant difference between Graves patients and healthy individuals in the Najaf city population.

Keywords: CTLA-4 gene, Graves ‘disease, Gene polymorphism.


©Annals of Tropical Medicine & Public Health S276
**Introduction**

Graves’ disease (GD) is the most common and important types of Autoimmune thyroid disease (AITD). In person has GD disease, has stimulated autoantibodies to produce thyroid hormone receptors, in this situation lead to hyperthyroidism [1]. Although, the etiology of GD disease is not clear until now, genetic and environment factors have impact on causes or development Graves’ disease [2]. It has been noticed that many studies detect the concordance rates in monozygotic is the 10 times more than dizygotic twins [3,4]. Moreover, 75% of the increased AITD associated with genetic factors as mutation and recombination [3]. It has suggested that genetic factors may be increased pathogenesis of GD[4].Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is the major identified gene which influence on GD disease. However, others genes contribute to develop GD disease such as protein tyrosine phosphatase, non-receptor 22 (PTPN22), interleukin 2 and the thyrotropin receptor gene (TSHR) [5]. CTLA4 human gene locus found in chromosome 2(q33) and is expression an immunoregulatory on the surface of T cells, and this expression can be interaction with the CD 28(B7) molecule to limit T-cell activation[2,6].As results, It is suggested that CTLA4 single nucleotide polymorphisms (SNPs) such as C>T in the promoter –318 (rs5742909), A>G in exon 1 +49A/G (rs231775), microsatellite (AT)n repeat in the 3' untranslated region (UTR) may be down regulation T-cell activity [7]. A 49A/G polymorphism is the most widely investigated mutation in autoimmune diseases; this polymorphism is associated with Graves’ disease[1].In addition, The +49A/G polymorphism change an amino acid from threonine to alanine in the CTLA4 protein sequence. However, many studies mentioned that the polymorphism CTLA4 gene could be diversity according to a segregation population [7]. Thus, It hypothesized that investigation relationship between the common SNP (49A/G) within the CTLA4 gene and the susceptibility to develop GD disease in Najaf population.

Ethical Consideration: The study was approved by the a local Ethical Committee of Al-Sadder Medical City, Najaf /Iraq.

**Materials and Methods**

**Patients’ clinical characteristics**

In our study, 73(48 females and 25 males, aged 15-50 years) individuals who detected Autoimmune hyperthyroidism. Hyperthyroidism Patients were divided into two groups, Graves’ disease and Non Graves’ disease based on Elexsys Anti-TPO test (recombinant antigens and human polyclonal antibodies against thyroid peroxidase,) and Elexsys Anti -TSHR test (human monoclonal antibodies against thyrotropin receptor, TSHR-Ab) levels. The control group consisted of 20 healthy individuals with no family history of thyroid disorders and autoimmune diseases.

**DNA extraction**

Genomic DNA was extracted from 2ml blood, and transferred to EDTA tubes and mixed well, then stored at 4°C until used. The Genomic DNA Mini Kit (Geneaid-International UKAS quality management) was used, applying the buffy coat protocol. Briefly, added 3X the sample volume of RBC lysis buffer and mix, incubated for 10 minutes at room temperature. Then centrifuged for 5 minutes at 3,000 xg. 200 μl of wash buffer was added, incubated at 60 for 10 minutes. All of the mixture was transfer to the GD Column then centrifuge at 14,000-16,000 xg for 5 minutes. After
washed two times. The purified DNA was eluted in 100 µl of elution buffer. DNA quantity and purity were assessed by UV spectrophotometry.

Investigation CTLA-4 polymorphism

CTLA-4 polymorphisms gene (49A/G) was assessed by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism), using the restriction enzyme Fnu4HI from New England Biolabs, USA. A PCR-RFLP experiment was set up with specific primers for detection CTLA4 49A/G (marker IDs according to dbSNP: rs231775 for CTLA4 49A/G) polymorphism, and 20 ng gDNA, the final volume 25 µl. The primers sequences were: Forward Primer: 5’- GCTCTACTTCTGAAGACCT-3’, Reverse Primer: 5’-AGTCTCACTCCTTGGACAG-3’. The PCR was carried out in a Eppendorf PCR machine and the thermal conditions were: Initial denaturation at 94°C for 4 min. 35 cycles of denaturation at 94°C for 30 sec, annealing at 50.1°C for 10 sec and extension at 72°C for 30 sec. Final extension was conducted at 72°C for 5 min. 5 µl of PCR products were run on 1% gel agarose, and comparison to the 100bp marker was used to confirm the size of the product. The rest of the PCR product was split into two aliquots, one digested with the Fnu4HI restriction enzyme, for overnight in a 37°C water bath, according to manufacturer's protocol. All digested products were run on 2.5% agarose gel 1 hour; result in fragment of 63 and 99 bp. This step is done to confirm PCR was successful. While the second aliquot was sequenced to investigate genotypes (A/A, A/G and G/G) respectively, the polymorphism occur on exon 1 in (5003-5266) site. This experiment included control for PCR blank sample was used with nuclease-free water instead of gDNA. sequencing of study was carried out in Molecular Medicine Department /Biotechnology research Center, Pasteur Institute of Iran.

Results

The sequencing of CTLA4 gene is taken from NCBI sequence website. CTLA4 gene locus in chromosome 2 from 4986-11180 bp. Partially sequence of CTLA4 gene as illustrated in (Figure 1).

![Figure 1](https://www.ncbi.nlm.nih.gov)

©Annals of Tropical Medicine & Public Health S276
Figure 2: Sequence data of polymorphism +49A>G position in the sequencing trace of CTLA4 gene shown 49 A/G polymorphism is indicated by a red arrow.

The results of analysis CLTA-4 genotype (A/A), (A/G) and (G/G) polymorphism distribution in GD patients in comparison with the healthy individuals were no statistically significant (p > 0.05), regarding to A and G allele polymorphism, our results showed that no association with Graves' disease patients in our study area (Najaf population).

Figure 3: Bioinformatic analysis of +49 A/G CLTA-4 polymorphism. DNA was extracted and sequenced. The Fnu4HI restriction site is shown in purple colored box.

Discussion

The distribution of polymorphism CTLA4 in GD disease confirmed by many studies worldwide [8-10]. However, this relationship is not clear until now. Thus, Sanger analysis of CTLA4 gene to confirm position of +49 A>G as illustrated in (Figure 2). Although, this was not statistically significant, the +49A/G Polymorphism may play an important role in develop or causes this disease because it likely to an autosomal recessive [7]. The bioinformatics analysis of CTLA4 polymorphism showed position of SNP and digestion site with restriction enzyme Fnu4HI, as results the two fragments were showed 63 and 99 bp in all study groups (Figure 3). The association of CLTA-4 gene polymorphisms with clinical
manifestations of GD was not strong to influence on development this disease. It has noticed that the genetic and environmental factors are very involving in GD increased development [5]. The present paper confirmed that CTLA4 49A/G polymorphism might impact on the susceptibility to GD development, however, the results showed no significantly. Our results confirm by Ting, ET AL. [4] mentioned that two variations +49A/G and CT60 may be susceptibility reasons for GD in adults and children. In addition, they found weakly associations between +49A/G, CT60 polymorphisms and GD diseases. In contrast, Lewandoska, ET AL. 2011 investigated the increased frequencies of A/G and G/G polymorphism could impact on the development of GD. In addition to Chinas population, some research have noted no association between the CTLA4 polymorphism and GD diseases in the UK [3, 4, 10]. It is also suggested that CTLA4 polymorphisms is not related with autoimmune thyroid disease. Although, the polymorphism +49A/G does not effect on the expression CTLA4 protein, several studies have found that variety levels of expression of CTLA4 is associated with the polymorphism +49A/G [11]. Our study had limitations results. First, we did not include children GD patients. Second, the number of sample were limited, finally although, CTLA4 polymorphism is not significant in Najaf population, it may be different in others population. Fathima ET AL. [1] found that the 49A/A genotype predominates in whites population, while the 49G/G genotype is found in the Asian population.

**Conclusion**

The CTLA4 polymorphism gene did not find a significant difference between Graves patients and healthy individuals in the Najaf city population. Although, the negative correlation between Graves’ disease and CTLA4 49A/G genotype, an association research using a larger number of sample should be performed to further study. Moreover, further studies need to be carried out in order to validate the relationship between CTLA4 polymorphisms and GD disease.

**Conflict of interest**

None of the authors have any conflicts of interest relevant to this research subject.

**References**


