Study of Cytotoxic T Lymphocyte Antigen 4 Gene Polymorphism A49G in Egyptian Children with Idiopathic Thrombocytopenic Purpura

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Abstract: Objective: The cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is transiently expressed on activated T lymphocytes to antagonize the activating signals resulting in T cell inhibition and prevention of the its clonal expansion.CTLA-4 A49G polymorphism was studied in different autoimmune disorders as it has been suggested that the presence of G allele reduce the expression and the inhibitory function of the CTLA-4 protein and this may predispose to autoimmunity. **Subjects and Methods:** In this study, we evaluated the frequency of CTLA-4 A49G polymorphism in 30 Egyptian children patients with immune thrombocytopenic purpura (ITP), and 40 healthy individuals using polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) technique. **Results:** Allele frequencies and genotype distributions were similar in both ITP patients compared to healthy individuals. **Conclusion:** Our results suggest that CTLA-4 A49G polymorphism does not contribute to the pathogenesis of immune thrombocytopenic purpura.

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1. Introduction

Idiopathic thrombocytopenic purpura (ITP) is an acquired autoimmune disease characterized by the presence of antiplatelet autoantibodies leading to premature platelet destruction and hemorrhagic manifestations **(Cines & Blanchette, 2002)**.The exact etiology of ITP disorder is still not clear, In addition to autoreactive B lymphocyte, dysfunctions of T lymphocyte and regulatory T lymphocyte may play important roles in the pathogenesis of ITP **(Zhou *et al.*, 2005)**.

In autoimmune disorders, presentation of an antigen in association with HLA (human leukocyte antigen) molecules on the surface of an antigen presenting cell to a T-cell receptor, requires a number of costimulatory signals for proper T-cell responses. On the T-cell membrane, a number of co-stimulatory molecules are present which may have stimulatory or inhibitory effects on T lymphocyte functions. The CTLA-4;cytotoxic T lymphocyte associated antigen-4, also known as CD152, is one of these costimulatory molecules (**Teft *et al.*, 2006).**

CTLA-4 appears on the surface of T cells following their activation by TCR/antigen/MHC and CD28/B7 interactions. It inhibits T cell activation and clonal expansion by reducing CD28/B7 interactions through interaction with the B-7 cell surface molecule on antigen-presenting cells. So CTLA-4 blockade leads to increase of the immune response. **(Falarino *et al.*, 1998 & Chen, 2004).** The regulation of CTLA-4 expression is important as its concentration on cell membrane determines the strength down-regulatory signals for T cells. Thus, maintenance of an optimal surface expression level of CTLA-4 is crucial for the regulation of T cell responses and peripheral tolerance, and for preventing autoimmunity **(Karabon *et al.*, 2009).**

Many single-nucleotide polymorphisms have been identified in the *CTLA-4* gene on chromosome 2q33. A/G polymorphism in exon 1 at position +49 is the only variant that results in an amino acid substitution (threonine to alanine) in the CTLA-4 protein **(Ueda *et al.*, 2003).** It has been reported that the 49G mutant allele results in decreased CTLA-4 expression due to lower mRNA efficiency and thus will result in higher T-cell proliferation and clonal expansion predisposing to autoimmunity **(Mäurer *et al.*, 2002).**

The aim of our study is to evaluate the association between the CTLA-4 gene A49G polymorphism and ITP in Egyptian children.

2. Subjects and Methods

Subjects

The study included 30 children with acute newly diagnosed ITP (14 females (46.7%) and 16 males (53.3%) who admitted to New Cairo University Children's Hospital or to Fayoum

University hospital. Their ages ranged between 1 and 16 years (median age 4 years). ITP was diagnosed in accordance with the guidelines of the American Society of Hematology [20]. Patients suffering from connective tissue diseases such as SLE were excluded. Their platelet counts ranged between 1 and 57x 10^9/L (median 20 x10^9/L, mean± SD: 23±16 x10^9/L).

Forty age and sex matched healthy children with normal blood picture were also included as control group. They were selected among cases referred to out-patient clinics in Fayoum University hospital. They were 22 males (55 %) and 18 females (45 %).Their ages ranged between 2 and 15 years and median of 8 years. Their platelet counts ranged between 152 and 586 x10^9/L (median 307 x 10^9/L, mean±SD 312±110 x 10^9/L). Consents were taken from parents of all participants before being involved in this study. This study was approved by the ethical committee of the Faculty of Medicine, Cairo University.

Methods

Extraction of Genomic DNA

Peripheral venous blood samples were collected in sterile EDTA vacutainer tubes. Genomic DNA was extracted using the GeneJET Blood Genomic DNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions.

Genotyping

Genotyping of A49G site was performed using a polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP). The primer sequences and restriction enzymes are listed in Table 1. Enzymatic amplification was performed using Dream Taq™ PCR Master Mix (2X). PCR reactions were performed with mixtures consisting genomic DNA , nuclease free water, 10 pM of each primer (1µl) and Dream Taq™ PCR Master Mix (12.5 µl) in a total volume of 25 ml. For PCR amplification, an initial denaturation at 94 °C for 4 min was followed by 30 cycles at 94 °C for 30 s, 46.8 °C for 10s, at 72 °C for 30 min, and a final extension at 72 °C for 4 min. This reaction yielded a 162 bp fragment. Cleavage was performed with Bbv 1 restriction enzyme supplied with its fast digest buffer (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's protocols. Digested fragments were separated on 3% agarose gels and restriction fragment length polymorphism (RFLP) bands were visualized by ethidium bromide staining under UV light (Table 1, Fig 1).

Table (1): Primers used and fragment sizes after digestion with restriction enzyme

SNP	Primer sequence	Restriction enzyme	Alleles	Fragment size (pb)
A49G	Forward 5′-GCT CTA CTT CCT GAA GAC CT-3′ Reverse 5′- AGT CTC ACT CAC CTT TGC AG-3′	Bbv 1	A G	162 88,74

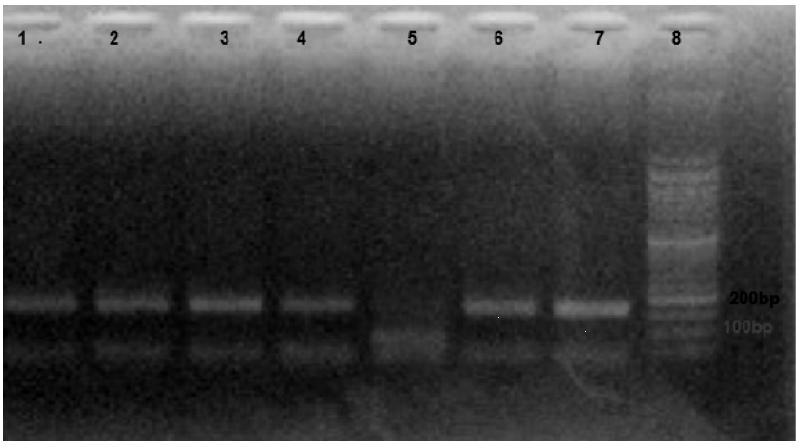

Figure (1):PCR-RFLP results of CTLA4 A49G polymorphism.
Lane (1,2,3,4,6,and7): AA genotype (wild type) showing bands at 162 bp.
Lane (5):GG genotype (mutant type).
Lane(8): marker ladder which shows bands at 50,100,150,200,250,300,400,500,600, 700,800,900 and1000 bp.

Statistical Analysis

Data was analyzed using SPSS win statistical package version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and

percentage. For quantitative data that not normally distributed, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Chi-square test was used to examine the relation between qualitative variables. Odds ratio (OR) with it 95% confidence interval (CI) were used for risk estimation. A *p*-value < 0.05 was considered significant.

3. Results

No statistically significant difference in genotype frequencies of CTLA-4 A49G between ITP patients and healthy control (*P*-value 0.834, Odds ratio 0.902, 95% Confidence interval: 0.345-2.362). As in the control group, we found that twenty three children had AA genotype (57.5%), twelve children had AG genotype (30%) and five children (12.5%) had GG genotype, while in ITP children, eighteen patients had AA genotype (60 %), twelve patients had AG genotype (40%) and no one had GG genotype.

The distribution of the CTLA-4 alleles among the studied groups is shown in Table (2). Statistical analysis reveals no significant difference in allele frequency of CTLA-4 A49G between ITP patients and healthy control (*P*-value >0.05, Odds ratio 0.659, Confidence interval: 0.341-1.273).

There was no statistically significant difference in genotype (*P*-value 0.796) or allele frequencies (*P*-value 0.796) between males and females in the ITP group (Table 3). There was no significant difference in total leucocytic count, platelet count and absolute lymphocytic count between ITP patient with AA genotype and those with AG genotype (Table 4).

Table (2): Statistical analysis of allele frequencies of CTLA-4 A49G polymorphism in both ITP and the control groups.

Allele	ITP group (No :30)	Control group (No :40)	*P*-value	Odds ratio	Confidence interval
A (wild allele) No (%)	48/60 (80%)	58/80 (72.5%)			
G (mutant allele) No (%)	12/60 (20%)	22/80 (27.5%)	>0.05	0.659	0.341-1.273

Table (3): Comparison of genotype/allele frequencies in relation to gender in ITP group:(No: 30).

Genotype/Allele frequency	Male (No :16)	Female (No :14)	*P* value
AA (wild type): N (%)	10/16 (62.5%)	8/14 (57.1%)	0.765
AG (mutant): N (%)	6/16 (37.5%)	6/14 (42.9%)	
A (wild): N (%)	26/32 (81.3%)	22 /28 (80%)	0.796
G (mutant): N (%)	6 /32 (18.2%)	6 /28 (20%)	

P-value more than 0.05 is of no significant difference.

Table (4): Statistical analysis of different laboratory parameters in relation to the genotypic frequencies in the ITP group (No: 30):

Parameter	Genotype AA No:18		Genotype AG No:12		*P value
	Median	Range	Median	Range	
Platelets (10^9/L)	21	6-55	17	1-57	0.439
WBCs (10^9/L)	9.09	4.30-22.0	10.54	4.53-12.1	0.983
absolute lymphocytic count(10^9/L)	4.98	1.91-16.3	5.06	1.21-16.28	0.851

**P*-value more than 0.05 is of no significant difference.

4. Discussion

Immune thrombocytopenic purpura (ITP) is a heterogeneous disorder characterized by increased platelet destruction resulting in thrombocytopenia. A number of features indicates that this destruction is immune-mediated and that it may involve also the inhibition of platelet release from the megakaryocyte. The exact mechanism of the immune dysfunction, however, is generally not known. For example it is unclear whether ITP is initially caused by a B-cell abnormality, T-cell dysfunction, or abnormal mononuclear phagocytic cells function **(Aledort *et al.*, 2004).**

Polymorphisms in CTLA-4, have been widely studied relation to genetic predisposition to various autoimmune diseases, but studies have led to contradictory results in different populations.

In our study we found that in there was no statistically significant difference between cases and controls as regards to genotype and allele frequencies .Similar finding was achieved by **Marica *et al.* (2003)** who examined the prevalence of the A and G alleles of the CTLA-4 gene in 60 patients with immune thrombocytopenic purpura (ITP) and in 100 control subjects and found that no difference was detected between patients with ITP and controls (*P*>

0.05).Also **Aktürk *et al.*(2010)** evaluated the frequency of *CTLA-4* A49G polymorphism in 62 patients with ITP, and 150 healthy individuals and found that in ITP group thirty-eight had AA (61.3%), 21 had AG (33.9%), and 3 had GG (4.8%) while in the control group, seventy two had AA(48%), seventy one had AG(47.3 %)and seven had GG (4.7 %) and found that there was no statistically significant difference between the ITP group and control group for the risk allele.

This is also in agreement with different studies of CTLA-4 A49G polymorphism in other autoimmune diseases. For example, **Wang *et al.* (2002**) indicated no association between *CTLA4* gene polymorphism at position +49A/G and myasthenia gravis, **Xia *et al.* (2002)** found that A49G *CTLA4* gene polymorphisms and their haplotypes are not associated in Dutch Caucasian patients with inflammatory bowel disease (IBD) and in Chinese patients with ulcerative colitis (UC), **Tomasz *et al.* (2003**) found that *CTLA-4* A49G polymorphism is not associated with the development of grave´s ophthalmopathy (GO). **Barton *et al.* (2004)** found lack of association between *CTLA4* +49A/G and rheumatoid arthritis(RA), **Roxburgh *et al.* (2006**) found no evidence for an association or the susceptibility of *CTLA4* polymorphism in exon 1 and multiple sclerosis(MS), and **Aktürk *et al.* (2010)** found no association between the A49G polymorphism of the *CTLA-4* gene and autoimmune haemolytic anemia (AIHA).

However other studies found an association between CTLA-4 A49G polymorphism and several autoimmune diseases. For example, **Yanagawa *et al.* (2000)** found an association of G allele at position +49 of CTLA4 in rheumatoid arthritis patients, **Ahmed *et al.* (2001)** reported an allele G of *CTLA4* A49G polymorphism at the leader sequence to be associated with susceptibility to SLE, **Cosentino *et al.* (2002)** confirmed the association of CTLA4 +49A/G and susceptibility to type-I diabetes, **Takeuchi *et al.* (2002)** found that in a Japanese population , the frequency of A allele at position +49 of the leader sequence was associated with systemic sclerosis (Ss) ,and **Tomasz *et al.* (2003**) found that allele G and G/G genotype confer genetic susceptibility to Grave´s disease (GD).

The effect of CTLA-4 gene polymorphism on the pathogenesis of autoimmune disorders has been a point of controversy among several authors and this could be attributed to the suggestion that differences in CTLA-4 function are most likely to be associated with the haplotype rather than with individual single nucleotide polymorphism of the gene **(Downie-Doyle *et al.*, 2006)** so studying other common CTLA-4 gene polymorphisms such as CT60 and 318 T/C may be helpful to conclude the relationship between CTLA-4 gene and pathogenesis of ITP or other autoimmune disorders, in addition quantitative expression of CTLA-4 protein on the surface of lymphocytes in patients with ITP may help studying the effect of CTLA-4 in the pathogenesis of ITP

In our study there was no statistically significant difference in genotype or allele frequency between males and females in the study group as p-value> 0.05.This in agreement with **Mohammad *et al.* , 2005** who studied the *CTLA-4* A49G polymorphism in vitiligo patients and found no association between *CTLA-4* exon 1 (A49G) genotypes and gender. The number of investigated patients in this study might not be sufficient to draw definite conclusions on the role of CTLA-4 A49G polymorphism as genetic risk factor in the pathophysiology of ITP in relation to gender. Larger scale studies could help in eliciting the exact role of CTLA-4 in pathogenesis of ITP in males and females.

In summary, the negative results of our study indicate that neither gene polymorphism nor allele frequency of CTLA-4 A49G might play a role as genetic risk factor in the pathogenesis of ITP in Egyptian children. Further studies about other polymorphisms of the CTLA-4 gene may facilitate exploration of associations between this genetic alternation and pathogenesis of ITP

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