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Original Research Article

Cytotoxic T-Lymphocyte Associated Antigen-4 (+49A/G) Gene Polymorphism as a Protective Factor against Toxoplasmosis

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Abstract

Cytotoxic T-Lymphocyte Associated Antigen-4 (CTLA-4) is a key factor in immune regulation. Polymorphisms in CTLA-4 gene may influence the status of this factor and eventually the immune response of host against the infectious agents. This response becomes of particular importance in cases when the pathogen is an opportunistic such as *Toxoplasma gondii.* This study aimed to explore the effect of CTLA-4+49A/G single nucleotide polymorphism (SNP) on the susceptibility to toxoplasmosis in women. Genomic DNA was isolated from 59 women with toxoplasmosis and aged matched 60 *Toxoplasma*-free women as controls. Tetra-Primer Amplification Refractory System-Polymerase Chain Reaction (ARMS-PCR) was used for amplification and genotyping of CTLA-4 gene using specific primers. The heterozygous genotype (AG) and G allele of the polymorphism CTLA-4+49A/G were less frequent among cases (17.12% and 16.95% respectively) than controls (40% and 35% respectively) with significant differences (OR=0.379, 95%CI=0.206-0.697, P=0.002). These data strongly suggested the protective role of CTLA-4+49G against toxoplasmosis among Iraqi women.

Key Words: Toxoplasmosis, CTLA-4+49A/G, single nucleotide polymorphism

التغاير الوراثي احادي النيوكليوتيد (+49A/G) في جين مستضد-٤ المقترن مع الخلايا اللمفاوية التائية السامة كعامل حماية ضد الاصابة بداء المقوسات

الخلاصة

يعد مستضد-٤ المقترن مع الخلايا اللمفاوية التائية السامة أحد العوامل الاساسية في تنظيم الاستجابة المناعية ، ويؤثر التغاير الوراثي احادي النيوكليوتيد (SNP) في جين هذا المستضد على حالة هذا العامل وبالتالي على الاستجابة المناعية للمضيف ضد العوامل الخمجية . ويكون لهذه الاستجابة أهمية خاصة في حالة كون العامل المرضي من النوع الانتهازي كما هو الحال في طفيلي المقوسات القندية . هدفت الدراسة الحالية الى استقصاء تأثير التغاير الوراثي احادي النيوكليوتيد CTLA-4+49A/G على مدى الاهبة للاصابة بداء المقوسات بين النساء . عزل الحامض النووي DNA من ٥٩ إمرأة مصابة بداء المقوسات و ٦٠ أمرأة خالية من المرض لتمثل مجموعة السيطرة، واستخدم نظام الممانعة للتضخيم رباعي البادئات (ARMS-PCR) في المضاعفة والتنميط الجيني . أظهرت النتائج ان النمط الجيني متغاير الزيجة (AG) واليل G أقل تكرارا في النساء المصابات (١٧.١٢% و ١٦.٢٥% على التوالي) مقارنة بالنساء غير المصابات بداء المقوسات (٤٠% و ٣٥% على التوالي) وبفروق معنوية . تشير هذه البيانات الى الدور الوقائي للنمط CTLA-4+49G ضد داء المقوسات بين النساء العراقيات .

الكلمات المفتاحية : داء المقوسات ، CTLA-4+49A/G ، التغاير الوراثي احادي النيوكليوتيد.

Introduction

Despite the worldwide prevalence of infection with *Toxoplasma gondii*, the disease (toxoplasmosis) rarely becomes symptomatic unless the host suffers from immunodeficiency [1]. This fact reflects the crucial importance of immune system against toxoplasmosis. Immune system does has a significant role against all infectious agents, but some of these, which are called opportunistic microorganisms such as *T. gondii*, usually exhibit a clinical significant only in immune-compromised hosts [2]. In many cases, immune deficiency statuses are easily recognized such as agamma-globulinemia and acquired immune-deficiency syndrome (AIDS). In other cases, there is no clear distinction, and even the term "deficiency" cannot be applied. Cases such as those associated with single nucleotide polymorphisms (SNPs) affecting the immune response could be placed within this category. For instance, SNPs in vitamin D receptor gene was reported to be significantly associated with toxoplasmosis among Iraqi women [3].

Cytotoxic-T lymphocyte-associated antigen 4 (CTLA-4) is an inhibitory receptors expressed transiently especially on CD4+ and CD8+ T cell and constitutively on CD4+CD25+ T-regulator (T-reg) cells [4]. This receptor has a decisive function in fine regulation of T-cell response by exerting a negative signals for T-cell activation by interacting with its ligands, B7.1 and B7.2 on antigen presenting cells (APCs) [5].

The CTLA-4 gene is located on chromosome 2q33 and it consists of four exons and three introns. This gene encodes for two different protein forms: soluble CTLA-4 (sCTLA-4) and full length CTLA-4 (flCTLA-4) with the former is lacking the exon 3 [6]. Approximately 100 SNPs have been reported in this gene; however only two as well as one microsatellite of which are found to be significantly associated with certain diseases. These are CTLA-4+49A/G (rs231775), CTLA-4−318C/T (rs5742909) and microsatellite $(AT)_n$ repeat in the 3'-untranslated region (3'UTR) [7]. Previous reports have elucidated the effect of CTLA-4+49A/G with three kinds of diseases: autoimmune diseases such as graves' disease [8], Hashimoto thyroiditis [9], autoimmune hypothyroidism [10], systemic lupus erythematosus, celiac disease, Henoch-Schnlein purpora and type 1 diabetes mellitus [9,11,12]; cancers like breast, lung, esophageal, gastric, colorectal, oral, cervical, and renal cell carcinoma [13,14,15,16]; and finally with few infectious diseases particularly tuberculosis, visceral leishmaniasis and hepatitis B infection [17,18,19]. This study aimed to assess the effect of this SNP on Iraqi women susceptibility to develop active toxoplasmosis. To the best of our knowledge, there is no previous published work addressed such exploration.

Materials and Methods

Women attending the Department of Obstetrics and Gynecology at Al-Imamain Al-Kadhimain Medical City/ Baghdad from January, 2015 to October, 2016 were eligible for this study. The age range was 22-49 years, mean= 32.19 years. Those women were suffering from different gynecological diseases. From each woman, 5 mL of venous blood were obtained and divided into two parts. The first part (3ml) was in aplain tube from which serum was obtained. The second part (2ml) was placed in EDTA tube for DNA isolation.

For detection of anti-toxplasma antibodies, two laboratory method were used.. Those were rapid test cassette (CTK Biotech Inc., USA), and E for LISA IgG and IgM antibodies (Cusabio/China). According to these methods, 59 women out of 342 tested gave positive results for toxoplasmosis.

Sixty women from those gave negative result were randomly chosen to be control.

DNA Isolation and Polymerase Chain Reaction

DNA was isolated from whole blood using a ready kit (ZymoBead™ Genomic DNA Kit, USA) according to manufacturer's protocol. Tetra-Primer Amplification Refractory Mutation System (ARMS-PCR) method was used for CTLA-4 gene amplification with four primers (Table 1)].

Table 1: Sequences and resultant fragment lengths of primers used for CTLA-4 gene amplification with ARMS-PCR.

Primers	Sequence (5'→3')	Fragment length
Outer primers	Forward: GTGGGTTCAAACACATTTCAAAGCTTCAGG Reverse: TCCATCTTCATGCTCCAAAAGTCTCACTC	229 bp
Inner primers	A allele: ACAGGAGAGTGCAGGGCCAGGTCCTAGT G allele: GCACAAGGCTCAGCTGAACCTGGATG	162 bp 120 bp

The PCR conditions comprised of an initial denaturation for 10 minutes at 95º C, followed by 35 cycles each with denaturation for 30 sec at 94º C, annealing for 30 sec at 61º C and an extension for 45 sec at 72 ˚ C. The final steps was an elongation for 7 min at 72º C [20]. The products of PCR were undergone gel electrophoresis and stained with ethidium bromide. The results were read under UV transluminator with digital camera.

Statistical Analysis

Graphpad prism (version 6) was used for data analysis. Chi-square and independent t-tests were used for comparing categorical and continuous data respectively between patients and controls. Adjusted binary logistic regression was used to find out any significant difference in genotypes and allele frequency between cases and control. Through this test, odds ratio (OR) with 95% confidence interval (CI) were got. The significant level was determined at P-value less or equals to 0.05.

Results

Characteristics of the Study Population

Table 2 shows the demographic characteristics of the study population. According to results of this table, there are no significant differences in term of age, parities, body mass index (BMI) or dwelling between toxoplasma-infected women and toxoplasma-free women.

Table 2: demographic data of the study population.

Characteristic	Cases (59)	Controls (60)	*P*-value
Age (years)	34.41±8.12	35.83±10.22	0.802
Parities (No)	1.92±0.74	2.21±0.91	0.532
BMI kg/M^2	26.18±4.32	25.85±4.8	0.452
Dwelling			
Rural	21(35.59%)	32(53.33%)	0.066
Urban	38(64.41%)	28(48.67%)	

BMI: body mass index

Figure 1 shows the result of ARMS-PCR, and genotype patterns of CTLA-4+49A/G in cases and controls. All samples gave positive results for the outer primers with 229 bp, which indicates the integrity of DNA and the successful amplification of the gene.

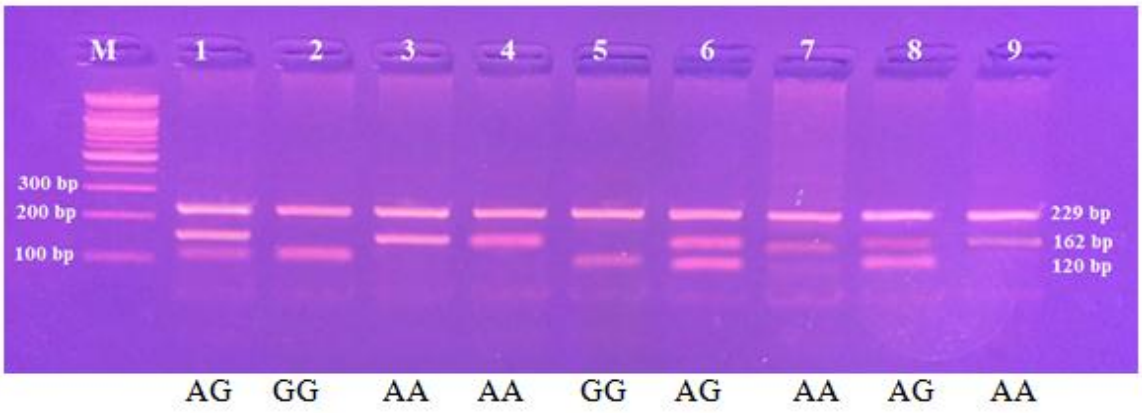

Figure 1: Genotype patterns of cytotoxic T-lymphocyte associated antigen-4 +49A/G polymorphism using ARMS-PCR visualized under UV transluminator. M: DNA marker, lanes 1,6 and 8: AG genotype, lanes 2 and 5: GG genotype, lanes 3,4 and 9: AA genotype.

The SNP appeared in the three genotypes both in cases and controls. These are AA, AG and GG. The genotype AA was more frequent among cases than controls (69.49% vs. 45%) with significant difference. In contrast, AG genotype was more frequent among controls (40%) than cases (27.12%) with significant difference (OR=0.146, 95%CI=0.029-0.730, P= 0.019). Also, there was higher percentage of GG genotype (15%) among controls than cases (2.39%); however the difference was insignificant (OR=0.333, 95% CI=0.064-1.749, P=0.194) as shown in table 3.

Table 3: Frequency of genotypes and alleles of CTLA-4+49A/G polymorphism in women with toxoplasmosis and toxoplasma-free women.

Characteristic	Cases (59)	Controls (60)	*P*-value	OR(95%CI)
Genotypes				
AA	41(69.49%)	27(45%)	0.017	Reference
AG	16(27.12%)	24(40%)	0.019	0.146(0.029-0.730)
GG	2 (3.39%)	9(15%)	0.194	0.333(0.064-1.749)
Alleles			0.002	
Allele A	98(83.05%)	78 (65%)		0.379(0.206-0.697)
Allele G	20(16.95%)	42 (35%)		
Dominant model				
AA+AG	57(96.61%)	51(85%)	0.045	
GG	2 (3.39%)	9(15%)		0.199(0.041-0.963)
Recessive model				
AA	41(69.49%)	27(45%)	0.008	
AG+GG	18(30.81%)	33(55%)		0.359(0.169-0.762)

OR: odds ratio, CI: confidence interval

Analysis of allele frequencies revealed a significantly higher frequency of allele G in controls compared to cases (35% vs. 16.95%, OR= 0.379, 95%CI=0.206-0.697P=0.002). The effective role of G allele was significant in both dominant and recessive models, although its more prominent in recessive models.

Discussion

So far, this is the first published study exploring the effect of CTLA-4+49A/G polymorphism on the susceptibility to toxoplasmosis in Iraq. The study revealed significant protective role of AG genotype (OR=0.146, 95% CI=0.029-0.730, P= 0.019) and G allele (OR= 0.379, 95% CI=0.206-0.697P=0.002) against the disease. These results are in line with that obtained by Hajilooi *et al.* [18] in term of leishmaniasis, Paad *et al.* [17] regarding tuberculosis, and Xu *et al.* [19] in hepatitis B infection. All these microorganisms share *Toxoplasma* with their intracellular residency. However, Lazano *et al.* [21]did not find any significant effect of this polymorphism on the incidence of the fungal disease, paracoccidioidomycosis, among Brazilian population. These disparities may be due to the apparent heterogeneity between different populations, and to the influence of environmental factors affecting different diseases.

The CTLA-4+49A/G polymorphism involves the substitution at the site 49 in CTLA-4 gene of adenine with guanine. Accordingly, the codon 17 (ACC) which encodes threonine is substituted by GCC which encodes alanine. The CTLA-4 receptor achieves essential regulatory function during immune response by controlling the overall strength of T-cell activation [22]. In fact, two mechanisms have been postulated for this regulatory effect. The first one is interacting of CTLA-4 with its ligands B7.1 and B7.2 depriving the homologue receptor CD28 from their ligands, while the second mechanism is the inhibition of T-cell activation through signal transduction pathway which down-regulates the T-cell receptor dependent signaling [23]. Substitution of threonine by alanine results in many phenotypic changes affecting one or both of these two mechanisms. It was postulated that alanine-containing CTLA-4 protein suffers from an altered spatial configuration which causes a fault in handling of this protein in the endoplasmic

reticulum with less efficient N-glycosylation [24]. This glycosylation is very important in the dimerization and then the triggering of inhibitory function of CTLA-4 [25]. Furthermore, some evidence suggested a significant decrease in mRNA for CTLA-4 protein associated with GG genotype in patients with autoimmune diseases [26]. Sun *et al*. [27] suggested that G allele do not only cause down-regulation in the production of CTLA-4 but also results in a protein with lower affinity to B7.

Regardless of the mechanism by which G allele-bearing CTLA-4 affecting the immune response, there is almost a general agreement that this variant has less ability to control the activation of T-cells compared to A allele-bearing CTLA-4. But this is a double-edge sword. From one edge, the +49G variant carriers have the merit of robust immune response with long lasting T-cell activation, and this hypothetically protects them from different infectious agents and may be some malignancies. However, from the other edge this variant predispose its carriers to wide range of autoimmune diseases such as Graves' disease and type 1 diabetes mellitus.

In conclusion, G allele of the SNP CTLA-4+49A/G appears to have a protective role against toxoplasmosis in Iraqi women. Further studies with a larger sample and different ethnic population are required for more solid conclusion.

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