Association of CTLA-4 Gene Polymorphism in Jordanian Type 1 Diabetic Patients

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ABSTRACT

Objective: To study the association between type 1 diabetes mellitus and the allelic polymorphism of the Cytotoxic T Lymphocyte Associated-4 gene in a Jordanian population.

Methods: We studied 50 Jordanian type 1 diabetes mellitus and 50 normal subjects at King Hussein Medical Centre and Princess Haya Biotechnology Centre, during the period of September 2008 and September 2010, to determine the association between the Single Nucleotide Polymorphism A/G and – C/T and type 1 diabetes mellitus.
Chi-squared test and Fisher exact tests were used to analyze the data. P value <0.05 was considered significant.

Result: The frequency of heterozygous genotype AG= 42%, homozygous for the wild type allele genotype AA= 50%. Homozygous genotype of the mutant allele GG=8%. The allele frequency for the wild allele A=71%, and for mutant allele is G=29%, with unknown clinical findings but the frequency of phenotype for wild allele A=92% and the phenotype frequency for mutant allele G= 50%. At the same time for 50 control subjects were investigated for the Cytotoxic T Lymphocyte Association-4 +49 A/G polymorphism respectively with patients on same Single Nucleotide Polymorphism, as following : AG=40%; AA=54%; GG=6%; A=74%; G=26%; A=94% and G=46%. The distribution of Cytotoxic T Lymphocyte Association -4 + 49 A/G genotype frequency did not differ significantly between patients and controls (P=0.885). The result on the other Single Nucleotide Polymorphism -318 C/T Showing the frequency of genotype; for heterozygous (CT=16%), homozygous wild allele (CC=84%), homozygous genotype for T allele =0%. Normal allele C=92%, but for mutant allele is (T=8%) with unknown clinical finding, where the phenotype for wild allele is (C=100%), phenotype mutant allele is T=16%. On the other hand for control subject investigated for Cytotoxic T Lymphocyte Association -4 -318 C/T polymorphism were (CT=22%, CC=74%, TT=4%, C=85%, T=15%, C=96%, T=26%) respectively. The result of distribution of Cytotoxic T Lymphocyte Association -4 -318 C/T genotype frequency did not differ significantly with type 1 diabetes mellitus patients and controls (P=0.248).

Conclusion: This case-control study suggests that the +49 A/G Single Nucleotide Polymorphism of the Cytotoxic T Lymphocyte Association-4 gene is not strongly associated with type one diabetes mellitus in Jordanian population, the apparent discrepancies between the present study and other studies could be due to the genetic heterogeneity among the population studied. The Cytotoxic T Lymphocyte Association-4 49 A/G Single Nucleotide Polymorphism may not be the true disease associated variant but a marker in linkage disequilibrium in different population.

Key words: CTLA-4 gene, Polymorphism, Genotype, Homozygous, Heterozygous, Allele, Type 1 Diabetes.

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Introduction

More than three decades of genetic studies had identified several genetics disease variants and more than 20 putative loci Genetic susceptibility to Type 1 Diabetes Mellitus (T1DM).[1] In 1974, the Human Leukocyte Antigen (HLA) region was the first genetic region associated with T1DM and forms about 50% of familial basis of T1DM.[2] The HLA is located on the short arm of chromosome 6 (6p21), extends over 4 megabite (MB) and contains at least 128 genes (Sergei Nejentsev, *et al*[1]. The HLA genes encoding immune response proteins which have a central role in antigen presentation and activation of a helper T cell-mediated immune response.[3,4] Two combinations of class II HLA alleles are of a particular importance: DR4-DQ8 and DR3-DQ2 have been observed in 90% of children with T1DM,[5] while a third allele, DR15-DQ6, has a protective action against of T1DM where has been found in less than 1% of children with T1DM compared with more than 20% of control population.[6]

The Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) locus on chromosome 2q33 (IDDM1) was determined as one of the known susceptibility factor of T1DM. It encodes a vital negative of the common autoimmune disorders, like T1DM. [7]

Recently Vella *et al*.[2] identified the Interleukine-2 Receptor Alpha gene (IL2RA) on chromosome 10p15.1 as susceptibility for T1DM. Interleukin 2 (IL-2) and its receptor, (IL2RA), are expressed by T cell after activation of T cell receptors by peptide-major histocompatibility complexes. The subsequent interaction of IL-2 with its receptors leads to the stimulation of signal transduction pathway resulting in T cell proliferation and clonal expansion. [8]

Evidence for a genetic susceptibility to T1DM comes from family studies. The frequency of the disorder is higher in close relatives of T1DM patients than in general population.

The sibling risk ratio (λs) is the ratio of the prevalence of disease in sibling to the prevalence in the general population and used as a quantitative measure of the genetic contribution to a disease. In T1DM, the value of λs is around 15 which mean T1DM is 15 times more common in siblings of diabetic patients than in general population. However clustering of disease in families suggests a genetic influence on a disease development, environmental factors can also cause familial clustering.[9]

Methods

Fifty patients were recruited from King Hussein Medical Center and Princess Rahma Teaching Hospital. All of the patients were diagnosed with Type 1 Diabetes Mellitus. Additionally, to make the study more effective, Selective groups were chosen from the patients who have the criteria of being below age 20 whether they have family history or not. This group was studied because the features of type 1 diabetes include multiple affected family members in several generations. Furthermore, fifty control samples were collected from healthy, unrelated and randomly selected human subjects have been referred to blood bank donation unit at King Abdullah Hospital. With keeping confidentiality, clinical and social information were obtained and recorded in a medical form (Questionnaire) especially designed for this purpose. They referred to the King Hussein Medical Center.

Sample collection

A total of three milliliters (ml) of whole blood samples were collected aseptically into EDTA-coated blood collection tubes from patients diagnosed with type 1 diabetes mellitus. To ensure proper mixing with EDTA and prevent blood clotting, every tube was mixed thoroughly, blood samples were collected, and genomic Deoxyribonucleic acid (DNA) was isolated from peripheral blood lymphocytes. Genomic DNA was visualized on 1% agarose gel to check for DNA integrity.

DNA Sequencing:

All amplified fragments of four exons and promoter region from 50 patients and 50 control subjects were purified and amplified again using the forward or reverse primers which were used in the Polymerase Chain Reaction (PCR). DNA was sequenced on 3130/3130x/ Genetic Analyzers (applied biosystems).

Statistical Analysis:

Statistical Analyses were performed with SPSS software. Chi-squared test and Fisher exact tests were used to determine the association between

Table I: Summary of the two polymorphism which have been identified in the Jordanian patients with TID. Adapted from NCBI database (http://www.ncbi.nlm.nih.gov/)

Exon no.	Variation ID	Variation Type	Allele change	Residue position	Residue change	Significance (according to NCBI database)
Promoter	Rs5742909 C ⟶ T	nearGene-5	nearGene-5 ⟶	-	nearGene-5 ⟶ nearGene-5	Unknown
Exon 1	Rs231775 A ⟶ G	Non-synonymous coding	ACC ⟶ GCC	17	T (Thr) ⟶ A Ala)	Unknown

Table II: Distribution of the +49 A/G CTLA-4 polymorphism in T1DM patients and controls.

Nucleotides at position +49	Patients (N=50)	Controls (N=50)
Genotype frequencies*		
AA	25 (50%)	27 (54%)
AG	21 (42%)	20 (40%)
GG	4 (8%)	3 (6%)
Allele frequencies		
A	71 (71%)	74 (74%)
G	29 (29%)	26 (26%)
Phenotype		
A positive	46 (92%)	47 (94%)
G positive	25 (50%)	23 (46%)

*P value=0.885

patients sample and control of CTLA-4 gene. P value of <0.05 was considered statistically significant.

Results

CTLA-4 mutational spectrum was studied in 50 patients who were diagnosed with type 1 diabetes mellitus and 50 control subjects. All of these patients have early-onset of type 1 diabetes mellitus below age 20 (years). Twenty four of them have consanguineous parents, three of them have brothers and sisters with type 1 diabetes mellitus and only one has Celiac disease associated with type 1 diabetes mellitus.

Polymerase Chain Reaction (PCR) Results:

The four exons and the promoter region of CTLA-4 gene for 50 patients with type 1 diabetes mellitus and 50 control subjects were amplified by PCR, followed by 2% gel electrophoresis in order to visualize PCR product and check the size of the amplified exons.The expected product size for promoter region was 225 base pair (bp), for exon 1 was 466 bp, for exon 2 was 489 bp, for exon 3 was 417 bp, for exon 4 was 308 bp.

The two important variations were identified in the promoter region and exon one of CTLA-4 among the 50 T1DM patients and 50 healthy control subjects who were included in this study, and those are -318C>T and +49A>G.

The first and the important Single Nucleotide polymorphism (SNP) is +49 A/G, which is located in the coding region of CTLA-4 exon one, this polymorphism changes A→G at nucleotide +49 predicting an amino acid change, Threonine (codon ACC) to Alanine (codon GCC) at residue 17 and this SNP has been reported to ensemble database (http://www.ensemble.org) and to NCBI data base (http://www.ncbi.nlm.nih.gov) under rs2317755 accession number (Table I).

Twenty one patients were observed to have heterozygous genotype AG with genotype frequency (AG: 42%) (Table II).

However twenty five patients are homozygous for the wild type allele with genotype frequency (AA: 50%). In addition, 4 patients were had homozygous genotype of the polymorphism allele with genotype frequency (GG: 8%). The allele frequency for the wild type allele is (A: 71%) and for the mutant allele is (G: 29%) with unknown clinical finding, where the phenotype frequency for wild type allele is (G: 50%) (Table II).

Table III: Distribution of the -318 C/T CTLA-4 polymorphism in T1DM patients and controls.

Nucleotides at position -318	Patients (N=50)	Controls (N=50)
Genotype frequencies*		
CC	42 (84%)	37 (74%)
CT	8 (16%)	11 (22%)
TT	0 (0%)	2 (4%)
Allele frequencies		
C	92 (92%)	85 (85%)
T	8 (8%)	15 (15%)
Phenotype frequencies		
C positive	50 (100%)	48 (96%)
T positive	8 (16%)	13 (26%)

*P value 0.248

At the same time, the 50 control subjects were investigated for the CTLA-4 +49 A/G polymorphism, where twenty control subjects observed to have a heterozygous genotype AG with genotype frequency (AG: 40%) (Table II). While twenty seven control subjects have homozygous genotype of wild type allele with genotype frequency (AA: 54%) (Table II).

However three controls have homozygous genotype of the mutant allele with genotype frequency (GG: 6%).

The allele frequency for the wild type allele is (A: 74%) and for the mutant allele is (G: 26%) with unknown clinical findings, where the phenotype frequency for the wild type allele is (A: 94%) and the phenotype frequency for mutant allele is (G: 46%) (Table II).

The distribution of CTLA4 +49 A/G genotype frequency did not differ significantly between patients with type 1 diabetes and control subjects (P=0.885).

The second important SNP is -318 C/T, was identified in the promoter region of CTLA4, this polymorphism changes C→T and this SNP has been reported to ensemble database (http://www.ensemble.org) and to NCBI database (http://;www.ncbi.nlm.nih.gov) under rs5742909 accession number (Table I).

About eight patients observed to have a heterozygous genotype with genotype frequency (CT: 16%) (Table III). But forty two patients had the homozygous state for the wild type allele with genotype frequency (CC: 84%) (Table III). And there is no any patient has a homozygous genotype for T allele. The allele frequency for the normal allele is (C: 92%) and for the mutant allele is (T: 8%) with unknown clinical findings, where the phenotype frequency for wild type allele is (C: 100%) and the phenotype frequency for mutant allele is (T: 16%) (Table III).

On the other hand, the 50 control subjects were investigated for CTLA-4 -318 C/T polymorphism. About eleven control subjects observed to have a heterozygous genotype with genotype frequency (CT: 22%) (Table III). But thirty seven control subjects had the homozygous state for the wild type allele with genotype frequency (CC: 74%) (Table III). Interestingly, two control subjects were homozygous state for the rare mutant allele with genotype frequency (TT: 4%) (Table III).

The allele frequency for the wild type allele is (C: 85%) and for the mutant allele is (T: 15%) with unknown clinical finding, where the phenotype frequency is (C: 96%) and the phenotype frequency for mutant allele is (T: 26%) (Table III).

The distribution of CTLA4 -318 C/T genotype frequency did not differ significantly between patients with type 1 diabetes mellitus and control (P=0.248).

Interestingly, the diabetic two siblings have been shown homozygous state for the wild type alleles with AA genotype at +49 A/G and CC genotype at -318 C/T.

Discussion

The incidence of diabetes is increasing in most countries of the world, making efforts of finding genes and environmental factors contributing to this disease particularly important. The identification of genes involved in the development of type 1 diabetes mellitus is a major challenge, because each gene may account for only a small percentage of susceptibility, or

certain mutations may not exist in all ethnic groups or geographical populations.

An additional complicating factor is genetic heterogeneity in different populations. One of the main non-HLA genes linked to the disease, CTLA-4 was investigated as a third susceptibility gene for T1DM.[4] Since the CTLA-4 plays a critical role for regulating peripheral self-tolerance and prevention of autoimmunity, abnormality of this gene may result in activated T cells attacking self antigens,[10] and may be involved in the pathogenesis of multiple T cell-mediated autoimmune disorders.[7] Therefore, CTLA-4 gene is the main interesting focus for our research in the current study. This is the first study of a possible associating between CTLA-4 polymorphism and predisposition to type 1 diabetes in the Jordanian population.

Linkage of CTLA-4 to type 1 diabetes has been continually demonstrated as has the association of its two unknown polymorphisms (the +49 A/G transition resulting in a Threonine / Alanine substitution in the leader peptide, and the -318 C/T in the promoter region) with the disease in different population. [11-17] neither linkage nor association was consistently observed in all populations.

In the present case-control study, we investigated if the CTLA-4 +49A/G and -318 C/T polymorphism are associated with development of early-onset type 1 diabetes in Jordanian population, where all of them were reported previously to ensemble and NCBI database. Whereby, the exonic polymorphism at +49 A/G (rs231775) represents the sole polymorphism, that can change amino acid sequences, among the polymorphisms in the encoding regions of three genes; CD28, CTLA-4 and Inducible-T Cell co-stimulator gene (ICOS) which co-localize in 300-kilobase Kilo base pair (1000bp) (Kb) on chromosome 2q33.[7] CTLA-4 exerted less intense inhibitory effect on T-cell proliferation in patients bearing the G/G genotype than in patients bearing the A/A genotype, so the presence of G allele showed an increased mRNA and protein expression of the primary T-cell growth factor IL-2 and increased T cell proliferation.

Our results showed that a half of T1DM patients were bearing normal A/A genotype with frequency (50% *vs*. 54%) when compared with the control group, however, patients whose bearing the heterozygous A/G genotype (42% *vs*. 40%), when compared with the control group, although a small number of patients and controls were bearing the mutant G/G genotype with frequency (8% *vs*. 6%) respectively. So no significant differences were observed in +49 G allele frequencies (29% *vs*. 26%) in the group T1DM patients when compared with the control group. The genotype, phenotype and allele frequencies did not differ significantly between type 1 diabetes patients and non-diabetic controls (P=0.885), our results were consistent with several previous reports in different populations. Lack of association for the +49G allele has been reported in a large US Caucasian data set and in small Chinese data sets,[13] as well as in addition study in UK by McComack *et al*.[18] Additionally, in Czech children,[19] and in Japanese subjects.[20] Recently reports confirmed lack association of +49 G in Korean diabetic children and adolescents,[16] as well as in Portuguese populations.[21] In contrast with another reports in another populations confirmed CTLA-4 G allele has been associated with genetic susceptibility to T1DM in Spanish and Italian families by Nistico *et al*.,[11] Japanese diabetic patients,[22] Russian population (Chistiakov *et al*.,[23] Filipinos,[24] and in Lebanese population.[25]

The reason for this discrepancy is currently unknown. One possibility is that there may be differences between populations in disequilibrium between the G allele and the allele at the site of pathogenetic mutation.[19] Another possibility is the heterogeneity of the disease; different combinations of gene loci or environmental factors may produce a similar phenotype with features of IDDM.[19] Therefore, our result does not support the involvement of the CTLA-4 gene in the pathogenesis of T1DM in Jordanian population.

The unknown CTLA4 promoter polymorphism at -318 C/T (rs5742909) and its effect on protein expression was investigated.[17,26,27] The -318 T allele may contribute to increased expression of CTLA-4 and consequently to the inhibition of excessive immune activity, thus reducing the risk of autoimmune disorders.[17,27]

Recent studies examined the associating of this polymorphisms and susceptibility to T1DM in

various populations as Chilean and Korean diabetic patients.[15, 16]

Our finding indicated that, frequency distribution of alleles and genotype of -318 promoter polymorphisms did not differ significantly between T1DM cases and controls (P=0.248). Where the frequency of C/C genotype for T1DM was (84% vs 74%) compared with the control group. By contrast, Steck *et al.*,[28] observed that the frequency of the C/C genotype was significantly lower in patients with T1DM compared to controls, and they concluded that this polymorphism was associated with T1DM.

In the current study, a lower frequency of the -318 C/T genotype (16% *vs*. 22%) was observed in the group of T1DM patients when compared with the control group. The more interestingly, the T/T genotype was absent in T1DM patients, compared with (4%) for controls. Thus, our results confirmed that, -318 T/T homozygotes are rare among populations,[27] and the -318T mutation could be considered as protective against increased T cell stimulation, as well as protective for autoimmune disease.

Conclusions

This case-control study suggests that the +49 A/G SNP of the CTLA4 gene is not strongly associated with type 1 diabetes mellitus in Jordanian populations. The apparent discrepancies between the present study and other studies could be due to the genetic heterogeneity among the populations studied, to different interactions with environmental factors involved in the pathogenesis of type 1 diabetes. Furthermore, the CTLA4 49 A/G SNP may not be the true disease-associated variant, but rather a marker in linkage disequilibrium with the casual variant and the discrepant findings may reflect variable strengths of linkage disequilibrium in different populations. The -318 C/T SNP was not associated with T1DM in Jordanian population but it is still possible that an unknown SNP in TID with -318 C/T might be responsible for the independent genetic effect. From our own data it appears that the rare -318T allele at the promoter offers a dominant protective effect against increased T cell stimulation, as well as protective for autoimmune disease.

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