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Cytotoxic T Lymphocyte Antigen-4 Gene Variants in Type 2 Diabetic Patients with or without Neuropathy

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ABSTRACT

Many studies have shown that cytotoxic T lymphocyte antigen-4 (CTLA-4) gene variants are associated with several autoimmune diseases particularly type 1 diabetes. Due to the lack of consistent data for this association with type 2 diabetes (T2D), this study explored the possible influence of CTLA-4 gene polymorphisms at -1722 (T/C), -318 (C/T), and +49 (G/A) positions for susceptibility to T2D in relation with neuropathy.

One hundred and eleven unrelated patients with T2D [49 patients with diabetic peripheral neuropathy (DPN) and 62 patients without PDN] and 100 healthy ethnic- and gender-matched controls were included in this study. The dimorphisms at -1722 (C/T), -318 (C/T) and +49 (A/G) for CTLA-4 gene were determined using ARMS-PCR. The CTLA-4 (+49 G/G) and (+49 A/A) genotypes were found to be positively and negatively associated with T2D, respectively (p=0.03).

The -318 C/T and T/T genotypes were more frequent in patients than controls and -318 C/C genotype was shown to be protective for T2D (p=0.003). ACT and GTT Haplotypes were less and more frequent in controls and patients, respectively ($p=3.86\times10^{-7}$ and $p=2.29\times10^{-5}$). Genotypes distribution among T2D patients with and without DPN compared to healthy controls showed significantly lower frequencies for -318 C/C and +49 A/A genotypes and significantly higher frequencies for -318 C/T and T/T genotypes as well.

Our findings indicate that CTLA-4 (+49 A/G) and (-318 C/T) genotypes could be considered as genetic risk factors associated with susceptibility or protection for T2D.

Keywords: CTLA-4 antigen; Diabetes mellitus type 2; Genes

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a complex endocrine and metabolic disorder which represents a significant global health problem with high prevalence in recent years. T2DM is characterized by insulin resistance and deficient β-cell function.[1] The disease results from interaction between genetic, environmental and behavioral risk factors. In recent years, there has been a explosion of interest in the notion that chronic low-grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of type 2 diabetes. Since this hypothesis was firstly proposed in 1997 and 1998[2,3], many studies have shown that circulating markers of inflammation such as acute-phase reactants, interleukin 6 (IL-6)- (the major cytokine mediator of the acute-phase response) are strong predictors for type 2 diabetes development.[4,5] There is also increasing evidence that an ongoing T-lymphocyte activation and cytokine-induced inflammatory response are closely related to the pathogenesis of T2DM.[6]

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4/CD152), which is mainly expressed on activated T cells, is the second ligand for co stimulating molecules, B7-1 (CD80) and B7-2 (CD86), on the surface of antigen presenting cells. While the CD28-ligand interaction plays a critical role in increasing and maintaining the T-cell response, CTLA-4 ligation appears to induce negative regulation of T cell activation.[7] CTLA-4 plays a significant role in regulating peripheral T-cell tolerance and attenuating T-cell responses. The importance of CTLA-4 in regulating autoimmune diseases in humans has come from the observation that genetic variation of CTLA-4 correlates with different incidence of certain autoimmune diseases in various populations.[8,9]

Several important and functional single nucleotide polymorphisms (SNPs) have been reported in the entire region of the CTLA-4 gene located on the long arm of chromosome 2q33. Two SNPs in promoter region, -1722 T/C and -318 C/T may affect CTLA-4 at mRNA and protein levels[10] and one SNP in exon 1 (+49 G/A) promotes a Threonine (A) to Alanine (G) substitution in the protein leader sequence at amino acid position 17.[8,11] It was previously reported that the CTLA-4 +49A/A genotype is associated with impaired T cell proliferation by increasing CTLA-4 function, whereas the CTLA-4 +49G/G genotype impairs regulatory T cell function by increasing T cell proliferation and consequently causing a pro-inflammatory response.[11]

CTLA-4 gene polymorphisms have been largely studied in autoimmune diseases. High frequency of the +49*G allele has been demonstrated in Grave's disease, Hashimoto's thyroiditis, and rheumatoid arthritis, whereas +49*A allele and AA homozygosis were found more frequently in patients with celiac disease.[12-14] Nevertheless, few studies have been conducted to date to find out the association of *CTLA-4 gene* polymorphism with T2DM which revealed controversial results.[15-18]

The aim of this work was to investigate the possible influence of -1722 (T/C), -318 (C/T), and +49 (G/A) CTLA4 gene polymorphisms for susceptibility to type 2 diabetes in a population of Iranian patients.

PATIENTS AND METHODS

This case-control study was performed on 111 unrelated patients with T2DM [49 patients with diabetic peripheral neuropathy (DPN) and 62 patients without PDN] who referred to outpatient diabetes clinic of Hamadan University of Medical Sciences from March 2011 to September 2013. Diagnosis of the peripheral diabetic neuropathy was based on the recommended protocol[19] and was described in details previously.[20] The case definition criterion for confirmation of DPN was an abnormality (≥99th or ≤1st percentile) of any attribute of nerve conduction in two separate nerves, one of which must be the sural nerve. Exclusion criteria were patients with age less than 30 and more than 70 years, other causes of neuropathy, the use of any neurotoxic drugs such as chemotherapeutic agents, history of hepatic or renal dysfunction (Creatinine > 1.5 mg/dl), and alcohol abuse. The subjects completed a questionnaire including general information, smoking status, duration of diabetes, type of medication and history of foot ulcer. Additionally, one hundred healthy subjects with no clinical evidence or family history of T2DM were collected among blood donors as the control group. The healthy controls were ethnic- and gender-matched with the patients group. The mean ages of patient and control groups were 55.8±6.7 years and 34.4±9.6 years respectively. Also, the sex ratios (female/male) for patients were 46/65 versus 41/59 in controls. Informed consents were obtained from all study subjects according to the protocol approved by our institutional ethics committee (D.P.16.35.2510).

Table 1. The sequences of primers used in the study for-318, -1722 and +49 CTLA-4 dimorphisms

Position	Forward Primers	Reverse Primers	Amplicons Length
+49 A/G	5′-GGCTCAGCTGAACCTGGCCG-3′		
	5′-GGCTCAGCTGAACCTGGCCA-3′	5′-ATGCTCCAAAAGTCTCACTC-3′	102bp
-318 C/T	5′-ACTTAGTTATCCAGATCCAC-3′		
	5′-ACTTAGTTATCCAGATCCAT-3′	5′-AGGCTCTTGAATAGAAAGC-3′	185bp
-1722 T/C	5′-ATGATCATGGGTTTAGCTGT-3′		
	5′-GTGATCATGGGTTTAGCTGC-3′	5′-CCATGTTGGTGGTGATGCAC-3′	237bp

The DNA was extracted from peripheral blood samples of the patients and controls using a commercially available kit (Archive Pure DNA Blood, 5Prime, Germany). Thereafter, -1722 (C/T), -318 (C/T), and +49 (A/G) dimorphisms for CTLA-4 gene were determined by polymerase chain reaction (PCR)-amplification refractory mutation system (ARMS) using specific primers (Table 1) according to previous studies.[13,21]

Amplification was performed in 25 μL reaction volume containing 100 ng of genomic DNA, 1X PCR-buffer, 0.2 mM dNTP-Mix, 0.5U of Hot-Start Taq DNA polymerase (Promega Corporation, Mannheim, Germany), 1.5mM MgCl2 and 10 pM of each primer. The following thermal profiles were run; 5 min at 95 ºC for initial denaturation, followed by 10 cycles of 94ºC for 10s, 56ºC for 1 min and 20 cycles of 94ºC for 10s, 52ºC for 43s, 72ºC for 30s for position -318 (C/T), and 10 cycles of 94ºC for 10s, 65ºC for 55s, and 20 cycles of 94ºC for 10 s, 61ºC for 45 s, 72ºC for 35s for position +49 (G/A). Also, the PCR program for -1722 (T/C) genotyping included initial denaturation at 95°C for 5 minutes followed by 25 cycles of 94°C for 1 min, 60 °C for 28s, and 72°C for 30s and final extension at 72°C for 5 minutes. The amplified PCR products were analyzed by 2.0% agarose gel electrophoresis and ultraviolet visualization. The length of the expected PCR products were 185 and 102 bp and 237 bp for the -318 (C/T) and +49 (A/G) and -1722 (T/C) dimorphisms, respectively.

Statistical Analysis

The distributions of rs5742909 and rs231775 and rs733618 genotypes were tested by goodness-of-fit X^2 test for deviation from Hardy–Weinberg in controls. Genotypic and allelic distributions for all three SNPs between patients and controls as well as haplotype analysis were assessed by Chi Square test with Yates correction and Fisher's exact test where appropriate. Independent sample t test or Mann-Whitney U test were used for analysis of quantitative data between either cases and controls or patients with and without DPN. The Benjamini–Hochberg method was used to control false discovery rate (FDR) for multiple comparisons.[22] Statistical analyses were performed by SPSS for Windows (Version 19.0, IBM SPSS Inc., USA). Probability (p) values less than 0.05 were considered statistically significant.

RESULTS

Demographic data of the study subjects were compared between two groups of the patients and healthy controls. The mean duration of disease was 9.7±4.6 years in patients group and the mean age-at-diagnosis for T2DM was 45.8±8.5 years in patients with DPN (group a) versus 46.5±9.5 years in those patients without DPN (group b). Anti-diabetic treatment status in both groups of the patients were as follows; in group a, 17 (34.7%) cases received oral hypoglycemic agents (OHA), 20 (40.8%) cases were under insulin therapy and 12 (24.5%) patients received both OHA and insulin. The relevant percentages in group b were 43.5%, 25.8% and 30.7% respectively. The mean body mass index (BMI) was 28.8±4.6 Kg/m^2 in group a versus 27.4±4.04 in group b and 21.8±1.5 Kg/m^2 in healthy controls (p<0.001). The mean serum creatinine levels and hemoglobin A1c contents were significantly lower in the controls compared to the patients group (0.97±0.19 *vs.* 1.13±0.21 mg/dl, p<0.001 and 5.4±0.54% vs. 7.52±1.03%, p<0.001 respectively).

The frequencies of genotypes and alleles for all three SNPs are shown in Table 2. The CTLA-4 rs231775 GG genotype was found to be positively associated with T2DM whereas, rs231775 AA

genotype was negatively associated with the disease (P_{corr}=0.03, Table 2). Likewise, rs5742909 C/T and T/T genotypes were more frequently observed in patients than controls and C/C genotype was shown to be protective for T2DM (P_{corr}=0.003, Table 2). Distribution of genotypes and alleles for rs733618 among both groups of study didn't show statistical differences (Table 2). Haplotype analysis for three SNPs revealed that ACT and GTT haplotypes were significantly different between patients and controls (p=3.86×10^{-7} and p=2.29×10^{-5} respectively, Table 3).

Moreover, analysis of genotype distribution for all three SNPs among T2DM patients with and without DPN did not show any statistical differences, however,, the patients without DPN showed significantly lower frequencies for A/A genotype and A allele at +49 A/G position in the CTLA-4 gene (Table 4). In addition, both groups of patients showed significantly lower frequencies of CC genotype and C allele as well as significantly higher frequencies of C/T and T/T genotypes at -318 C/T position in comparison to healthy controls (Table 4).

Table 2. Distribution of alleles and genotypes of three SNPs for CTLA-4 gene among T2DM patients and healthy controls

CTLA-4 gene variants		Patients n=111 (%)	Control n=100 (%)	Odds Ratio (95%CI)	*p* values	*P_{corr}
+49 A/G (rs231775)	**AA**	9 (8.10)	20 (20.0)	1.66 (1.12-2.46)	0.02	0.03
	AG	42 (37.83)	39 (39.0)			
	GG	60 (54.05)	41 (41.0)			
	Alleles					
	A	60 (27.0)	79 (39.5)	0.57 (0.37-0.87)	0.007	0.01
	G	162 (73.0)	104 (60.5)			
-318 C/T (rs5742909)	**CC**	75 (67.6)	88 (88.0)	2.73 (1.51-4.93)	0.001	0.003
	CT	26 (23.4)	10 (10.0)			
	TT	10 (9.0)	2 (2.0)			
	Alleles					
	C	176 (79.3)	186 (93.0)	0.29 (0.15-0.56)	0.0001	0.0003
	T	46 (20.7)	14 (7.0)			
-1722T/C (rs733618)	**TT**	93 (83.8)	86 (86.0)	1.28 (0.72-2.29)	0.42	0.42
	TC	12 (10.8)	12 (12.0)			
	CC	6 (5.4)	2 (2.0)			
	Alleles					
	T	198 (89.2)	184 (92.0)	0.72 (0.35-1.46)	0.41	0.41
	C	24 (10.8)	16 (8.0)			

*: FDR-corrected probability values for multiple testing using the Benjamini-Hochberg method.

Table 3. Distribution of CTLA-4 gene haplotypes among T2DM patients and healthy controls

Haplotypes	Patients 2n=222 (%)	Controls 2n=200(%)	Pearson's P	Odds Ratio [95%CI]
ACC	4.90(0.022)	9.44(0.048)	0.15	0.46 [0.15~1.38]
ACT	40.46(0.182)	81.30(0.415)	**3.86×10^{-7}**	0.32 [0.20~0.50]
ATT	8.16(0.037)	4.32(0.022)	0.35	1.73 [0.53~5.65]
GCC	12.60(0.057)	6.49(0.033)	0.22	1.80 [0.68~4.74]
GCT	117.03(0.527)	87.76(0.448)	0.06	1.45 [0.98~2.14]
GTT	32.35(0.146)	5.61(0.029)	**2.29×10^{-5}**	5.96 [2.37~14.94]

Note: All those frequency<0.03 have been dropped for analysis.

Table 4. Distribution of alleles and genotypes for three SNPs in CTLA-4 gene according to the presence of diabetic peripheral neuropathy (DPN) among T2DM patients

CTLA-4 gene variants	Genotypes/ alleles	Patients with DPN (Group a) n=49 (%)	Patients without DPN (Group b) n=62(%)	Controls (Group c) n=100 (%)	*p Values*	
					Group a *vs.* Group c	Group b *vs.* Group c
	AA	6 (12.3)	3 (4.8)	20 (20.0)	0.36	**0.006**
	AG	21 (42.8)	23 (37.1)	39 (39.0)		1.98 (1.21-3.22)
+49A/G (rs231775)	**GG**	22 (44.9)	36 (58.1)	41 (41.0)		
	Alleles					
	A	33 (33.7)	29 (23.4)	79 (39.5)	0.33	**0.003**
	G	65 (66.3)	95 (76.6)	121 (60.5)		0.46 (0.28-0.77)
	CC	34 (69.4)	44 (71.0)	88 (88.0)	**0.006**	**0.008**
	CT	10 (20.4)	13 (21.0)	10 (10.0)	2.56 (1.31-4.98)	2.40 (1.25-4.59)
-318 C/T (rs5742909)	**TT**	5 (10.2)	5 (8.0)	2 (2.0)		
	Alleles					
	C	78 (79.6)	101 (81.5)	186 (93.0)	**0.001**	**0.002**
	T	20 (20.4)	23 (18.5)	14 (7.0)	0.29 (0.14-0.61)	0.33 (0.16-0.67)
	TT	39 (79.6)	52 (83.9)	86 (86.0)	0.20	0.63
	TC	7 (14.3)	8 (12.9)	12 (12.0)		
-1722T/C (rs733618)	**CC**	3 (6.1)	2 (3.2)	2 (2.0)		
	Alleles					
	T	85 (86.7)	112 (90.3)	184 (92.0)	0.21	0.74
	C	13 (13.3)	12 (9.7)	16 (8.0)		

Note: Differences of the allelic and genotypic distribution between group a and b were not statistically significant.

DISCUSSION

The importance of CTLA-4 in regulating autoimmunity originates from the observations that the presence of specific CTLA-4 alleles/genotypes correlates with a higher incidence of certain autoimmune diseases.[8,9] The exact mechanism by which CTLA-4 gene polymorphisms contribute to the pathogenesis of each disease remains unclear. However, CTLA-4 (+49 A/G) dimorphism is the only variant in CTLA-4 gene that alters an amino acid in the leader peptide and therefore is known to be implicated in several immune dysregulation disorders.[23-25] In vitro study by Kouki et al. demonstrated that +49 G/G genotype is associated with reduced function of CTLA-4 protein which in turn leads to increased T cell proliferation. They speculated that the presence of GG genotype in exon 1 of CTLA-4 gene may influence the level or pattern of its expression. The other possible mechanism suggested in Kouki's study was the alteration in CTLA-4 ligation and subsequently decreased activation downstream signaling inhibitory pathway in T cells from GG expressing subjects.[11]

In the present study, we explored the possible associations between CTLA-4 gene polymorphisms at -318 C/T, +49 A/G and -1722 T/C positions and T2DM susceptibility as well as incidence of diabetic peripheral neuropathy (DPN). We observed that +49 G/G and -318 C/T genotypes were significantly more frequent in T2DM patients than healthy controls, whereas +49 A/A and -318 C/C genotypes were negatively associated with T2DM. As expected, the latter two genotypes were significantly less frequent in both groups of T2DM patients with or without DPN compared to healthy controls. However, the distribution of alleles and genotypes for three SNPs was not statistically different between both groups of patients, which is probably due to low number of samples in each group. We also found lower and higher frequencies of ACT and GTT haplotypes respectively in T2DM patients compared to healthy controls.

Our results are in line with Ma et al. study that

showed the CTLA-4 +49 AA genotype could be protective from diabetes mellitus whereas, CTLA-4 +49 GG or GA genotypes conferred an increased risk of diabetes mellitus.[15] Likewise, two studies on T1DM patients in Iranian population demonstrated a significant decrease in frequency of AA genotype and higher frequency of G allele among diabetic patients compared to healthy controls.[26,27] The differences in genotypes and alleles were greater in patients with younger age of diabetes onset (age≤15 years) compared to healthy controls.[27] In contrast to our findings, studies by Ahmadi et al. and Uzer et al. did not indicate any significant association between +49 A/G genotypes and T2DM in comparison with healthy controls.[16,26]

Recent studies suggested that the distribution of genotype and allele frequencies of *CTLA-4* (+49A/G) polymorphism were similar in T2DM patients and controls.[17,28] Rau et al. reported that this polymorphism was not associated with T2DM, although there was a significant correlation between GG genotype and younger age at disease manifestation, lower body mass index and basal C-peptide levels as well as earlier start of insulin treatment and higher portion of patients on insulin. They also reported a lower frequency of microangiopathic lesions and nephropathy in patients carrying AA genotype.[17] A similar study by Abe et al. showed a significant difference for allele frequency of CTLA-4 gene at +49 G/A position between T2DM patients who were positive for antiglutamic acid decarboxylase antibody and healthy individuals in Japanese population.[29] In contrast to above study, we found a direct correlation between +49 G/G and -318 C/T genotypes and susceptibility to T2DM. However, distribution of allels and genotypes were not statistically different between T2DM patients with and without DPN. In comparison to healthy controls, a lower frequency of +49 A/A and -318 C/C genotypes were observed among both groups of the patients. Surprisingly, patients without DPN showed a higher frequency of +49 G/G genotype compared to controls.

The plausible explanations for these discrepancies could be ethnic variation and implementing different methodology and designing in each study. Therefore, more replicative studies are required to find out a true association between CTLA-4 gene variants and T2DM and diabetic neuropathy as well. Collectively, because of the paucity of studies on T2DM in relation to CTLA-4 gene polymorphism, particularly in Iranians, and due to variations in the study design among these reports, our data regarding to direct association between +49 G/G genotype and T2DM should be interpreted with caution. Another limitation for better interpretation of the current results is the lack of proper evaluation of CTLA-4 at protein level. These important issues besides the more complexity of genetic aspect of T2DM compared to T1D indicate that drawing any conclusion regarding to genetic predisposition for T2DM based on CTLA-4 gene is still hard to reach. More importantly, discussing the link between CTLA-4 genetic variation and peripheral neuropathy in T2DM is even more difficult. Because, the pathogenesis of diabetic neuropathy with regard to immune responses in these patients is not yet well understood and the exact role of immunological mediators in this disease, converse to T1DM, remained to be elusidated.

Based on the literature, CTLA4 gene variants at -318 C/T and -1722 T/C positions have not yet been investigated in T2DM. While, CTLA-4 (-318C/T) dimorphism has been evaluated in a number of diseases and various results have been reported, from significant association of this SNP with systemic sclerosis and COPD,[30,31] to observation of no correlation with rheumatoid arthritis, Graves' disease, Hashimoto's thyroiditis and inflammatory bowel disease.[12,13,32] To our knowledge, this is the first report on negative association between -318 C/C genotype and T2DM and positive association of -318 C/T genotype with T2DM in Iranian population. Definitely, our results need to be verified by replicative studies in our population particularly and other ethnic groups as well. It has been documented that -318 C/T genotype may regulate the expression level of CTLA-4 either as soluble (s) or as membranous (m) CTLA-4 molecule. It was also, shown that the presence of -318 T allele is associated with increased expression of CTLA-4 molecule in both forms and is associated with inhibition of T cells.[33,34] In this context, the inhibitory effect of sCTLA-4 through competing with mCTLA-4 for binding to CD80/CD86 ligand on antigen presenting cells may lead to over-activation of T cells.[33,34] Elucidation of higher frequency of -318 C/T genotype along with high levels of sCTLA-4 in patients with COPD indicates the possible role of this genetic risk factor in favor of disease progression.[31] With bearing this in mind, we observed that T2DM patients carried higher frequency of -318 C/T genotype than healthy controls which could be indicative for contribution of this SNP in

susceptibility or pathogenesis of T2DM. Likewise, significantly higher frequencies of -318 CT and TT genotypes were observed among patients either with or without DPN than healthy controls. Supporting these results, higher significant frequency of GTT haplotype was found in T2DM patients compared to control group.

Taken together, elucidation of true association and more importantly the exact mechanism of contribution of CTLA-4 gene variants in T2DM pathogenesis and diabetic neuropathy are particularly difficult due to the presence of limited number of related but not similar investigations, ethnic variations and various study designs which leads to divergent results. Hence, more replicative studies preferentially genome wide association studies (GWAS) are needed to find out the exact role of CTLA-4 gene and probably its related genes in T2DM as well as in post-diabetes microvasular complications like neuropathy.

Considering the -1722 T/C genotypes, positive and negative correlations between this SNP in the CTLA-4 gene and several autoimmune diseases such as systemic lupus erythematus, bechet's disease and multiple sclerosis have been reported.[21,33,35,36] Whereas, the distribution of alleles and genotypes for -1722 T/C were not statistically different between T2DM and healthy controls in the present study. Due to the lack of previous reports on this CTLA-4 SNP in relation to T2DM, further studies warranted to confirm our findings. In conclusion, this is a new and quite different report postulating the potential role of CTLA-4 gene variants in susceptibility for T2DM which is in agreement with some previous studies and in converse to other studies considering only +49 A/G genotypes, but not for -318 C/T and -1722 T/C genotypes. Further studies on larger groups of T2DM patients and different ethnic populations are required to clarify a true association of these SNPs in the CTLA-4 gene with T2DM and secondary lesions particularly peripheral diabetic neuropathy in these patients. Furthermore, CTLA-4 protein and function analysis besides the other related and closed gene map would be useful to define the exact role of these molecules in predisposition or pathogenesis of T2DM and even post diabetes complications such as diabetic neuropathy.

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