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Original Article

The CTLA4 -819 C/T Dimorphism Is Associated with Type 1 Diabetes in Egyptian Children

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Abstract

Background: We investigated whether the C-819T SNP of the CTLA4 gene is associated with Type 1 diabetes (T1D) for the Egyptian population, a multi-ethnic group. We determined if expression of the C-819T SNP correlated with onset of T1D for Egyptian children and the prevalence of this polymorphism with respect to gender.

Methods: The association of the C-819T SNP in intron 1 of the CTLA-4 gene with T1D was investigated in 396 Egyptian patients ≤ 14 years old and 396 control subjects >24 years old, with a similar ratio of males to females in both groups. The diagnosis of T1D was made based on ketoacidosis or ketosis with severe symptoms of acute onset at presentation and continuous dependence on insulin. Controls were negative for anti-GAD antibodies and were greater than 24 years of age. Genotyping was performed using single strand conformation polymorphism (SSCP), temperature gradient gel electrophoresis (TGGE), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and reverse dot blot (RDB).

Results: The results demonstrated an association of the C-819T SNP in the CTLA-4 gene with T1D patients (P= 0.0096). This association was more pronounced in children ≤5 years of age (P= 0.0178) than in children 6-14 years of age (P= 0.0437). Moreover, this association was stratified by gender and age to female patients with age at onset 0-5 years old (P= 0.0186) more than male patient with the age at onset 0-5 years old (P= 0.3120).

Conclusion: The results support an association of the C-819T SNP in the CTLA-4 gene with Egyptian children, specifically, females of onset age 0-5 years old.

Keywords: *CTLA-4, SNP, Mutation, Diabetes, Genotyping, Egyptian population*

Introduction

Type 1 diabetes (T1D) is a genetically complex disorder of glucose homeostasis that results from autoimmune destruction of the insulin-secreting cells of the pancreas. The development of T1D likely results from exposure to environmental factors, which interact with a number of genes that contribute to the susceptibility of the disease (1). Genetic susceptibility to T1D is conferred by more than 20 putative loci (2). Approximately 40% of the susceptibility genes are located within the HLA locus on chromosome 6p21, known as IDDM1 (3). Another significant susceptibility locus (IDDM12) maps to the CTLA-4 gene region of chromosome 2q33 (4). IDDM12 has also been implicated in systemic lupus erythematosis, autoimmune thyroid diseases, coeliac disease, and rheumatoid arthritis, underscoring the importance of this locus in autoimmune processes (5).

IDDM12 contains a cluster of T lymphocyte-regulating genes including CD28, CTLA-4, and ICOS. CD28 and CTLA4 are receptors that together with the antigen specific T-cell receptor-bind to the B7 family of receptors on the surface of antigen presenting cells. CD28 enhances whereas CTLA4 inhibits T-cell proliferation. Binding of CTLA4 to the B7 receptor limits the proliferation of T-cells and terminates the ongoing immune response (6). CTLA4 knockout mice develop a severe lympho-proliferative disorder and die within a few days after birth, highlighting the importance of this gene in the negative regulation of the immune response (7).

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Increasingly, single nucleotide polymorphisms (SNPs) of the CTLA4 gene are correlated with autoimmune diseases. Most SNPs of the CTLA4 gene associated with autoimmune diseases are located within introns, with A+49G the only SNP found within an exon (8). Overall, the data on the association of CTLA4 polymorphisms with T1D is convincing in some populations (8-11) (12-14), but not in others (15-17). The identification of new SNPs for T1D is an important ongoing task.

In this study, we investigated whether the C-819T SNP of the CTLA4 gene is associated with T1D for the Egyptian population, a multi-ethnic group. We determined if expression of the C-819T SNP correlated with onset of T1D for Egyptian children and the prevalence of this polymorphism with respect to gender.

Materials and Methods

Subjects

All individuals who participated in this study gave informed written consent. The study was approved by the Egyptian Bioethics Review Committee for Bioethics (BERD) VACSERA. Three hundred and ninety six patients with T1D from different governorates of Egypt were recruited. The diagnosis of T1D was made based on ketoacidosis or ketosis with severe symptoms of acute onset at presentation and continuous dependence on insulin. All patients were non-obese and ≤14 yr of age at enrolment. Patients were separated into two groups for statistical analysis: Less than 6 yr of age (0-5 age group) and six to less than 15 yr of age (6-14 age group). Healthy individuals (n= 396) selected randomly from the Egyptian population to serve as controls were greater than 24 yr of age, as recommended by the DiaMond protocol (18). None of the controls had a family history of T1D. A standardized medical history questionnaire was completed by all patients and controls. The anti-GAD antibodies and C-peptide tests were performed to confirm the diagnosis of T1D for patients or to rule it out for controls. Individuals who were suspected to have Maturity-Onset Diabetes of the Young (MODY) or Wolfram syndrome were excluded from the study.

SNP genotyping

Blood (1 ml) was collected in a tube containing EDTA, and the DNA was extracted by a salting out method (19). The DNA was dissolved in 50 µl of Tris-EDTA (20 mM Tris, 2mM EDTA, pH 7.5) buffer and stored at -20 °C until use. The details of the single strand conformation polymorphism (SSCP), temperature gradient gel electrophoresis (TGGE), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and reverse dot blot (RDB) experiments are reported in the supplementary material.

Statistical Analysis

Differences in genotype and allele distribution in cases and controls were tested for significance by the chi-squared test with Yates' continuity correction. Statistical significance was defined as $P< 0.05$, achieved when the calculated $\chi2>3.841$ for 1 degree of freedom, and $\chi2>5.991$ for 2 degrees of freedom. Hardy-Weinberg equilibrium was tested by comparing the expected and observed genotypes in 2 x 3 $\chi2$ tables. Odds ratio (OR) was calculated for allele distributions using the statistical program EpiCalc2000, http://www. brixtonhealth. com/epicalc.htm. No adjustments to the level of significance were made for multiple analyses.

Results

The 396 T1D patients included 198 males and 198 females, a ratio of 1:1 (Table 1). The 396 healthy individuals selected randomly from the Egyptian population to serve as controls included 198 males and 198 females, also a ratio of 1:1 to facilitate the statistical calculations to avoid any bias toward specific gender in the study. All controls were negative for anti-GAD antibodies and were 25 yr of age or older, as recommended by the DiaMond protocol (18). This age restriction is placed on the controls to exclude subjects from the high-risk period of 0-14 yr of age, during which T1D is most likely to develop (11). Furthermore, no control had a family history of T1D.

The difference in the disease onset age for female T1D patients (3.9±0.2) versus male T1D patients (3.7±0.4) in the 0-5 age group was not statisti-

cally significant (Table 1). Similarly, the difference in the disease onset age for female T1D patients (9.8±0.3) versus male T1D patients (9.4±0.7) in the 6-14 age groups was not statistically significant. The disease onset ages for the non-stratified age groups (female+male) were 3.8±0.3 for the 0-5 age group, 9.6±0.5 in the 6-14 age groups and 6.7±0.4 for the 0-14 age group respectively. The genotype distributions of the patient and control groups were tested for deviations from Hardy-Weinberg equilibrium (HWE): For the control group, P= 0.05, $\chi2$=3.73; for 0-14 patients (P= 0.53, $\chi2$=0.40); for 0-5 patients, P= 0.67, $\chi2$= 0.19; for 6-14 patients, P= 0.64, $\chi2$= 0.22; for the female control group, P= 0.18, $\chi2$= 1.8; for female 0-14 patients, P= 0.58, $\chi2$= 0.31; for female 0-5 patients, P= 0.53, $\chi2$= 0.40; for female 6-14 patients, P= 0.87, $\chi2$=0.05; for the male control group, P= 0.16, $\chi2$=1.98; for male 0-14 patients, P= 0.58, $\chi2$=0.30; for male 0-5 patients, P= 0.01, $\chi2$= 0.92; and for male 6-14 patients, P=0.49, $\chi2$=0.48. Thus, no patient or control group deviated from HWE, as statistical significance for 2 x 3 table's calculation.

Statistically significant difference existed in the distribution of the C-819T SNP for the CTLA4 genotype between the 0-5 age group and healthy controls (P= 0.0096, $\chi2$= 9.2631) more than the between of the 6-14 age group and healthy controls (P= 0.0437, $\chi2$= 6.2563). Moreover, the statistically significant difference between the 0-14 age group and healthy controls presents (P= 0.0047, $\chi2$= 10.65); Table 2).

Similarly, statistically significant differences existed for the allele frequencies at position -819 in introns 1 between the 0-5 age group and healthy controls (p=0.005993, $\chi2$=7.5524) is more than the 6-14 age group and healthy controls (P= 0.02703, $\chi2$= 4.8889). Moreover, the statistically significant difference between the 0-14 age group and healthy controls (P= 0.001886, $\chi2$= 9.6573); Table 2).

Stratification of the data according to gender revealed statistically significant differences in genotype distribution for females between the 0-14 age group and controls (P= 0.0071, $\chi2$= 9.856) and females between the 0-5 age group and controls (P= 0.0186, $\chi2$= 7.952), whereas females between the 6-14 age group and controls (P= 0.041385, $\chi2$= 6.3697) stratified by gender differed less significantly from the respective control. While, the males between 0-14 age group and controls (P= 0.3542, $\chi2$= 2.0755), males between the 0-5 age group and controls (P= 0.312, $\chi2$= 2.329) and males between the 6-14 age group and controls (P= 0.6587, $\chi2$= 1.8358; Table 3) revealed statistically non-significant. Similarly, statistically significant differences existed for the allele frequencies at position -819 in introns 1 for females between the 0-14 age group and controls (P= 0.002777, $\chi2$= 8.9487), females between the 0-5 age group and controls (P= 0.007716, $\chi2$= 7.0982) and females between the 6-14 age group and controls (P=0.021734, $\chi2$= 4.190), whereas the difference for males between 0-14 age group and controls (P= 0.2250, $\chi2$= 1.4716) , males between the 0-5 age group and controls (P= 0.2323, $\chi2$=1.4262) and males between the 6-14 age group and controls (P= 0.4229, $\chi2$= 0.6422) were not statistically significant.

Table 1: Demographics of the Study Populations

Number of Patients		**Group I 0-5 yr old**	**Group II 6-14 yr old**	**All patients 0-14 yr old**	**Controls >24 yr old**
		198	**198**	**396**	**396**
Age (yr)±Mean Range		3.8 ± 0.3 female 3.9±0.2 male 3.7±0.4	9.6 ± 0.5 female 9.8±0.3 male 9.4± 0.7	6.7±0.4	32.5±3.7
Weight (kg) Range		10.3±1.2 2±15.6	29.7±1.7 17.2 - 44.8	20.5±1.45 2-44.8	73.4±2.56 65.5 - 83.5
Sex, n (%)	Female Male	99 (50) 99 (50)	99 (50) 99 (50)	198 (50) 198 (50)	198 (50) 198 (50)

Table 2: Distribution of the CTLA-4 gene in the Egyptian population

Genotype	Patient age groups			Controls
	(0-5) N=198	**(6-14) N=198**	**(0-14) N=396**	**(25-39) N=396**
	n (%)	**n (%)**	**n (%)**	**n (%)**
CC	88 (44.44)	92 (46.46)	180 (45.45)	214 (54)
CT	90 (45.45)	88 (44.44)	178 (44.95)	164 (41.67)
TT	20 (10.11)	18 (9.10)	38 (9.60)	18 (4.33)
Allele				
C	266 (67.17)	272 (68.7)	536 (67.68)	592 (74.75)
T	130 (32.83)	124 (31.3)	256 (32.32)	200 (25.25)
Statistical Analysisa				
			Patient age groups	
	Parameter	0-5	6-14	0-14
Genotype	χ^2,a	9.2631	6.2563	10.65
	P value	0.0096	0.0437	0.0047
	χ^2, b	7.5524	4.8889	9.6573
Allele	*P* value	0.005993	0.02703	0.001886
	Odds Ratio	0.690846	0.740277	0.708243

χ^2 and p values were calculated by Chi-square test on stratified 2 x 2 table
a Compared to controls
b Continuity corrected

Table 3: Gender-related Distribution of the CTLA-4 C-819 T SNP Polymorphism in the Egyptian population

Genotype	Females				Males			
	Patient age group			Controls	Patient age group			Controls
	0-5 (N=99)	**6-14 (N=99)**	**0-14 (N=198)**	**25-39 (N=198)**	**0-5 (N=99)**	**6-14 (N=99)**	**0-14 (N=198)**	**25-39 (n=198)**
CC	38(38.38%)	41(41.41%)	79 (39.9%)	104(52.52%)	50 (50.50%)	51(51.51%)	101(51.01%)	110 (55.56%)
CT	49(49.49%)	46(46.46%)	95 (48%)	84(42.42)	41 (41.41%)	42(42.42%)	83(41.92%)	80 (40.40%)
TT	12(12.13%)	12(12.13%)	24 (12.1%)	10(5.06%)	8 (8.09%)	6(6.07%)	14(6.57%)	8 (4.04%)
Allele								
C	125(63.13%)	128(64.65%)	253 (63.9%)	292(73.74%)	141(71.21%)	144(72.73%)	285(71.97%)	300(75.76%)
T	73(36.87%)	70(35.35%)	143 (36.1%)	104(26.26%)	57 (28.79%)	54(27.27%)	111(28.03%)	96 (24.24%)
Statistical Analysisa								
			Females				**Males**	
	parameter	0-5 (N=99)	6-14 (N=99)	0-14 (N=198)	0-5 (N=99)	6-14 (N=99)		0-14 (N=198)
Genotype	χ^2	7.952	6.3697	9.8560	2.3290	1.8358		2.0755
	P value	0.0186	0.041385	0.0071	0.3120	0.6587		0.3542
Allele	χ^2	7.0982	4.190	8.9487	1.4262	0.6422		1.4716
	P value	0.007716	0.021734	0.00277	0.2323	0.4229		0.2250
	Odds ratio	0.610184	0.651108	0.63211	0.788979	0.849592		0.822912

χ2 and p values were calculated by Chi-square tests
a Compared to controls
b Continuity corrected

Discussion

This study demonstrated an association of the C-819T SNP in introns 1 of the CTLA-4 gene with T1D. This association was stratified by onset age to the 0-5 age group. A similar association stratified by onset age to the 0-5 age group has been reported for other ethnic groups (20, 21). The association of the C-819T SNP of the CTLA-4 gene with T1D was also stratified to females in our study, supporting a role for the C-819T SNP in the pathogenesis of T1D by a gender-specific mechanism. The specific mechanism whereby the C-819T SNP of the CTLA-4 gene promotes autoimmunity in females and leads to the development of T1D is unknown. Steroid hormones are considered the most likely factors that trigger the onset of female gender-stratified, genetically based autoimmune diseases. This idea is reinforced by the increased prevalence of autoimmune diseases in women, the sexual dimorphism of the immune response, and the *in vitro* modulatory effects of sex steroids on immune functions. These modifiers could directly or indirectly target steroid receptors that act as transcription factors for the susceptibility genes associated with T1D, although no such regulatory role for sex hormones has been identified. However, this possibility is not without precedence, as sex hormones may act as critical modulatory factors that can induce disease expression (22). The observation that the T allele frequency was greater for T1D patients in the 0-5 onset age group suggests that this group experienced a stronger immune response than the 6-14 onset age groups in the Egyptian children.

This study was not properly powered to prove statistically significant associations between the SNP C-819T of the CTLA-4 gene and T1D patients among the different age groups and sex. Rather, the study was designed to evaluate an overall association present between the C-819T SNP of the CTLA-4 gene and patients with T1D, regardless of age group and sex. However, the findings suggest that specifically targeted follow-up studies could yield confirmatory outcomes in different subsets of interest. Regarding the issue of study power, at least 544 patients in total (272 per group) would need to be evaluated to confirm the results of the current study at 80% power.

In summary, a strong association of the C-819T SNP in the CTLA-4 gene was found in the 0-5 onset age group for Egyptian female patients with T1D but not for male patients in the same age group. Currently, a gender preference is not evident in the Egyptian society, but future studies will clarify the demographics as well as the aetiology of T1D for the C-819T SNP of the CTLA-4 gene in this population.

Supplementary material

A 180 bp fragment in which the polymorphic site of the CTLA gene was located in a central position was amplified by PCR using the following primers: Forward, 5'-GGAGAGGGGCCTGGTTAGTTACA-3'; Reverse, 5'-AGAGAGGCAGCGGTG GTGTCA-3'. The sequence of C and T alleles published in NCBI with numbers EU103999 and EU104000 respectively. The reaction mixture contained 1 µl of target DNA (50-100 ηg/µl DNA), 1.5 mM MgCl2, thermophilic DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 0.2 mM of the four deoxynucleotide triphosphates, 0.2 unit of Taq polymerase and 10 ρmol of each primer in a total volume of 25 µl. PCR amplification was performed using a model PTC100 thermal cycler (M&J research, Watertown, MA, USA) as follows: Denaturation at 94 ºC for 5 min, followed by 35 cycles of 30 S at 94 ºC, 40 S at 62 ºC, and 40 S at 72 ºC, and a final extension step for 10 min at 72 ºC.

To identify single strand conformation polymorphism (SSCP), 5 µl of PCR product was mixed with 5 µl of loading dye (95% deionised formamide and 0.025% methylene blue), incubated for 5 min at 95 ºC, immersed in ice for 10 min, and loaded into an 8% polyacrylamide gel. Gel electrophoresis was performed at 240 volts over 4 h at room temperature. Silver staining revealed variant mobility of conformational fragments bands, corresponding to nucleotide substitution (23).

Temperature gradient gel electrophoresis (TGGE)

Was performed on a PCR product carrying the SNP-819C/T mutation and further amplified by

PCR using the previously described conditions and non-GC-clamped PCR primers (24). The PCR product (20 µl) was mixed with 20 µl of 4 M urea and 0.05% methylene blue, heated at 95 ºC for 5 min, and 50 ºC for 20 min. Samples were loaded into 1 mm polyacrylamide gels (5%) covalently bound to polyethylene gel support films (BioRad, Hercules, CA, USA) equilibrated with 1.25x TBE buffer. After electrophoresis, bands of heteroduplex and homoduplex DNA were electrophoresed overnight at 40 volts, ramping the temperature from 25-75 ºC (2±0.50 ºC/h) (25). DNA was stained with ethidium bromide (0.01 g/L for 45-60 S) and visualized using a UV transilluminator.

For PCR-RFLP, PCR products (10 µl) were incubated overnight at 37 °C with 2 units of the restriction enzyme CviKl-1 (New England Biolabs, UK). The digested PCR products were separated on a 2% agarose gel and visualized by EtBr staining. PCR products digested with the CviKl-1 restriction enzyme yielded two bands 69 bp and 111 bp in length for the CC allele, and three bands 180 bp, 111 bp, and 69 bp in length for the CT allele, whereas the TT allele was not cut by the restriction enzyme, and only one band 180 bp was evident.

The SNP C-819T of the CTLA4 genotype in the various populations was determined using the reverse dot blot (RDB) method as previously described (26, 27), with the exception that the length of the probe was increased to 23 bp in length (from 15-18 bp). This modification increased the specificity of the probe for the target and reduced the assay time, thereby permitting use of genomic DNA without prior amplification. Oligonucleotide probes with the 5'-terminal base coupled to an amino group and biotin were synthesized by Operon (Köln, Germany). The probe for the T allele was 5'-TGATG-CAAGTCTCTC-TGTATGGA-3', while the probe for the C allele was 5'-TGATGCAAGCCT-CTCT-GTATGGA-3'. Re-actions were performed at 43 °C using a 1:1000 dilution of DNA (~10 ηg/ml) in deionised water, conditions which minimized background and optimized specificity.

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