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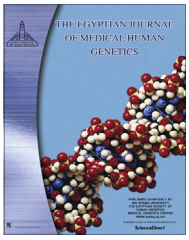

ORIGINAL ARTICLE

Lack of association of CTLA-4 +49 A/G polymorphism with predisposition to *type 1 diabetes* in a cohort of Egyptian families

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KEYWORDS

Type 1 diabetes;
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Abstract *Background: Type 1 diabetes* is one of the most common chronic childhood illnesses. Interplay between genetic susceptibility and environmental factors is thought to provide the fundamental element for the disease. Apart from the Major Histocompatibility locus which is the main contributor to risk susceptibility, more than 40 loci are recognized. One among these is the CTLA-4, however data from the literature are controversial. The aim of our study was to investigate the role of CTLA4 49 A/G as a risk susceptibility factor for the development of *type 1 diabetes* in a cohort of Egyptian families.

Subjects and methods: This is a case control study including 88 Egyptian families with one or more index cases (< 18 years). The control group comprised 369 healthy unrelated subjects with no family history of diabetes or autoimmune disease.

Using PCR-RFLP methodology, CTLA4 49 A/G was analyzed in 738 samples representing 88 families (88 patients, 125 siblings and 156 parents) and 369 control.

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Results: The age of onset was 6 days-12.5 years with a mean of 5.3 ± 3.6 and a median of 5 years. The mode of presentation was classic symptoms in 51 and diabetic ketoacidosis in 37 cases. Twenty-two cases had a history of viral infection or exanthematous disease and four had associated autoimmune diseases. No significant differences were encountered between the different groups with regard to CTLA4 +49 A/G genotype or allele frequencies. Neither was there a relation between the various genotypes and age of onset or the mode of presentation.

Conclusions: CTLA4 49 A/G polymorphism was not recognized as a risk susceptibility factor in our cohort. This may be attributed to the low co-incidence of autoimmune diseases. Up to our best knowledge, this is the first study involving families. We recommend that all studies performed on risk susceptibility to *type 1 diabetes* should include proper investigation for other autoimmune diseases to exclude their confounding effect on data analysis.

1. Introduction

Type 1 diabetes is one of the most common chronic childhood illnesses; its incidence is characterized by extensive differences between populations [1–3]. In Egypt prevalence rate varying between 0.7 and 1.9 was reported [4–6]. *Type 1 diabetes* develops in individuals who are genetically susceptible; interplay between genetic susceptibility and environmental factors is thought to provide the fundamental element for the disease [7].

Alleles or genetic variants associated with *type 1 diabetes* provide either susceptibility to or protection from the disease within a given environmental background [8]. The genetic makeup with the balance between susceptibility and protection alleles determines the age of onset of *type 1 diabetes* [7]. The risk for siblings of affected individuals is approximately 15 times as great as the risk in general population [9]. The offspring of a mother with *type 1 diabetes* mellitus is 4–10 times while that for offspring of a diabetic father is 15–22 times [10–12].The gender bias in transmission rate has not been fully explained [13–15]. The major genetic determinant of susceptibility to *type 1 diabetes* lies within the major histocompatibility complex (IDDM1) [16,17]. However, within diabetic families HLA identical siblings, though at a higher risk, do not always develop diabetes. This brought into the focus of interest other possible genetic loci and currently more than 40 DDM loci are recognized [18]. Among these is the cytotoxic T lymphocyte antigen-4 (CTLA-4) known as insulin-dependent diabetes mellitus 12 (IDDM 12). An A/G polymorphism in the first exon of CTLA-4 results in an amino acid change (Thr/Ala). The presence of an alanine at codon 17 of CTLA-4 has been associated with susceptibility to *type 1 diabetes*, autoimmune thyroiditis, systemic lupus erythematosis, celiac disease and biliary cirrhosis [19–21]. However there is a marked controversy about its effect on *type 1 diabetes*. On one side the effect of IDDM12 is claimed to be independent of other genetic markers of *type 1 diabetes* [22]. On the other side it is claimed to be weak or even questionable [23]. Ethnic heterogeneity has been reported with strong effects in some populations and weak transmission in others [22].

In view of the possible ethnic variations and the different environmental contributions in different societies, it is essential to study the main loci contributing to the susceptibility to or protection from *type 1 diabetes* in each community; results obtained from studying one population cannot be extrapolated to others.

Studies performed in Egypt on genetic background of *type 1 diabetes*, including ongoing studies of the research team, have so far addressed mainly the contribution of the HLA region. Recently two Egyptian studies have addressed the role of IDDM12 in risk susceptibility to *type 1 diabetes*; both were case-control studies not involving families.

2. Subjects and methods

The work is carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The protocol was revised and approved by the Institutional Review Board (IRB) of the National Cancer Institute, Cairo University. A written informed consent was obtained from all participants or their parents.

2.1. Subjects

The study cohort included 88 Egyptian families with one or more index diabetic children or adolescents who are attending outpatient clinic in the Diabetes Endocrinology and Metabolism Pediatrics Unit (DEMPU), at Cairo University Children Hospital. CTLA-4 was analyzed in 369 samples representing 88 families (88 patients, 125 siblings and 156 parents) and 369 controls. Seven siblings, belonging to 6 families, are diabetic and were excluded from the analysis; only the 118 non-diabetic siblings were analyzed.

Patient's age at the time of the study ranged from 1 to 18 years with a mean of 8.6 ± 4 and a median of 9 years. Thirty-five of our patients were males and 53 were females. The controls were chosen from blood bank donors. They were healthy unrelated subjects with no family history of diabetes or other autoimmune diseases. The choice of adults was to guarantee that they would not develop juvenile diabetes and to avoid ethical issues of using healthy children as controls.

2.2. DNA analysis and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) testing for CTLA-4 +49 A/G

Genomic DNA was extracted from EDTA peripheral blood samples using the salting out technique [24]. Identification of the loci was performed based on the PCR-RFLP protocol [25]. Oligonucleotide primers were synthesized (Pharmacia biotech). The amplification of the CTLA-4 gene was performed in

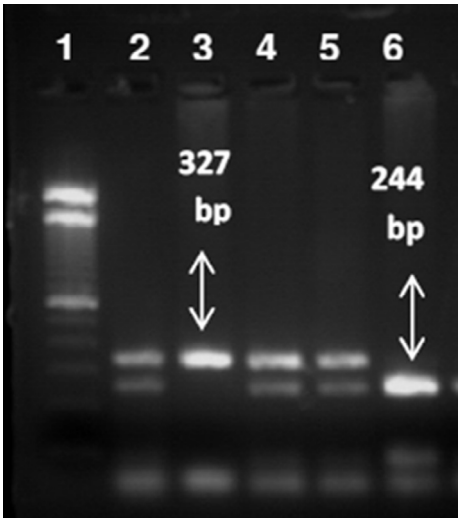

Figure 1 CTLA4–49 A/G genotypes after BbvI digestion. Lane 1: 100 bp Ladder. Lanes 2, 4 and 5: Heterozygous: AG: 327 bp, 244 bp and 84 bp. Lane 3: Wild type: AA: 327 bp. Lanes 6: Homozygous: GG: 244 bp and 84 bp.

a 25 µl PCR reaction containing 100 ng of genomic DNA, 25 pmol of each primer (forward primer 5′-CCACGGCTTCCTTTCTCGTA-3′ and a reverse primer 5′-AGTCTCACTCACCTTTGCAG-3′), 100 µM each dNTP, 1.5 mM $MgCl_2$ and 1.2 U Taq polymerase (Promega, Madison). PCR conditions consisted of initial denaturation at 95 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 50 °C for 45 s and 72 °C for 30 s and a final extension step at 72 °C for 10 min. This results in a fragment of 327 bp.

Digestion of the PCR product was performed in a 20 µl mix containing 10 µl of the PCR product, 10 U BbvI (Fermentas) and 1 X buffer. The mix was incubated at 65 °C for 15 min. Digested products were separated on 2% agarose. This resulted in no digestion with a 327 bp single band in the wild type (AA) and two bands of 244 bp and 83 bp with G allele (Fig. 1) compared to 100 base pair ladder (Thermo-Fermentas).

2.3. Statistical analysis

SPSS version 17.0 was used for data management. Proportions were compared using the Chi square test and Fisher exact. Odds ratio of genotype(s) was calculated with 95% confidence interval. Parametric and non parametric tests compared the means of two or more than two independent groups (t test and ANOVA). P value was always two tailed and considered significant at 0.05 level.

3. Results

The age of onset ranged from 6 days to 12.5 years with a mean of 5.3 ± 3.6 and a median of 5 years. The age of onset was <1 year in 17 cases; 8 of them <6 months. The mode of presentation was classic symptoms in 51 and diabetic ketoacidosis (DKA) in 37 cases. Twenty-two cases had a history of viral infection or exanthematous disease before the onset of diabetes and 4 had associated autoimmune diseases namely, Hashimoto's thyroiditis, rheumatoid arthritis, rheumatic heart disease and Crohn's disease. Early introduction of cow's milk or cereals was reported in 45 cases while exclusive breast feeding in the first 6 months was adopted for the others.

The CTLA-4 +49 A/G genotype and allele frequency among the different groups are presented in Table 1 and Fig. 1. Distribution of CTLA-4 genotypes and alleles in patients compared to control did not show any significance (Table 2). Comparing patients with controls, non diabetic siblings and parents regarding AA versus AG + GG also did not show significance (p = 0.60, 0.49 and 0.68 respectively). Neither was there a relation between the various genotypes and age of onset nor the mode of presentation.

4. Discussion

At present, the development of *type 1 diabetes* mellitus is a life sentence to a difficult therapeutic regimen that is only partially effective in preventing acute and chronic complications of the disease. Knowledge of the genetics of *type 1 diabetes* mellitus in our community would allow better disease definition and improved ability to identify individuals at risk of diabetes and its associated disorders. In the current work, we studied 88 families with one or more children with *type 1 diabetes*. Our study showed female predominance which comes in agreement with the assumption that female predominance is encountered in regions with low incidence mainly populations of non-European origin [26]. This was denied in a more recent study that reported equal incidence in both genders with female predominance only in autoimmune diseases [27]. This explanation is

Table 1 Allele Frequency of CTLA4 49 A > G in *type 1 diabetes* Families.

Group	No	CTLA4 Genotype			G Allele frequency	
		Wild AA	Hetero AG	Homo GG	No.	%
Type 1 Diabetics	88	47 53.4%	36 40.9%	5 5.7%	46/176	26.1
Non-diabetic siblings	118*	66 55.9%	40 33.9%	12 10.2%	67/250	26.8
Parents	156	74 47.4%	68 43.6%	14 9.0%	96/312	32.8
Control	369	199 53.9%	150 40.7%	20 5.4%	190/738	25.7

* Seven more siblings were analyzed and excluded as they are diabetics.

Table 2 Distribution of CTLA4−49 A/G in *type 1 diabetes* as compared to control.

Allele/Genotype	Patients (88)	Control (369)	OR	95% CI	*P* value
A	130 (73.9)	548 (74.3)	1.03	0.65–1.51	0.93
G	46 (26.1)	190 (25.7)	1.02	0.7–1.48	0.92
AA	47 (53.4)	199 (53.9)	1.03	0.67–1.7	0.96
AG	36 (40.9)	150 (40.7)	1.02	0.63–1.65	0.95
GG	5 (5.7)	20 (5.4)	1.06	0.38–2.97	1.0
AG + GG	41 (46.6)	170 (46.1)	1.02	0.64–1.63	0.93

not applicable in our case as only four of our patients have a concomitant autoimmune disease.

In our study, early introduction of cow's milk or cereals was not a contributing factor to the development of *type 1 diabetes*. This is in agreement with one study from Finland [28] and another from Iran [29]. In the former study, it was even claimed that early introduction of cow's milk reduced the risk of development of *type 1 diabetes* before the age of 8 years; an effect that was abolished by the age of 15. In the latter, it was claimed that the total duration of cow's milk feeding rather than its early introduction would increase the risk of *type 1 diabetes* while longer duration of breast feeding is associated with protection. No effect of early introduction of cow's milk or cereals was also reported in Saudi Arabia [30]. In our study, history of viral infection or exanthematous disease was not associated with increased risk of *type 1 diabetes*. This is in agreement with Cherian et al. [30] who studied the impact of environmental factors on risk susceptibility to *type 1 diabetes* in the Eastern Province of Saudi Arabia. However, irregular vaccination and infection in the prior 6 months were reported as a trigger for the onset of *type 1 diabetes* [31].

Genotypes of CTLA-4 +49 A/G were in Hardy Weinberg equilibrium in patients, siblings, parents and control subjects ($p > 0.05$). In our studied cohort CTLA-4 +49 A/G did not have any impact on risk susceptibility to *type 1 diabetes*. Neither has it shown an impact on the age of onset of the disease. Interest in CTLA-4 was raised because of its role in T cell signaling as a negative regulator of T-cell activation and effector function [32,33]. The association of CTLA-4 polymorphism with *type 1 diabetes* was first reported in Italian subjects [34] and confirmed thereafter in several other studies [20,35–37]. However, the association was claimed to be Caucasian selective [38,39] as it was not detected in Korean [40], Portugese [41], [42] Chelian [43] or Azrbaijanian [44] populations. Our data are different from studies performed in populations from neighboring countries namely Lebanon, [45], Tunisia [46] and Iran [47]; a higher frequency of the G allele or GG genotype in *type 1 diabetes* mellitus in children was reported in all. Analysis of 24 CTLA-4 SNPs in the Type I Diabetes Genetics Consortium families did not include CTLA-4 49 A/G SNP [48].

Most important is that our findings are different from two case-control Egyptian studies [49,50]. They demonstrated that the G allele is overrepresented in *type 1 diabetes* patients compared to controls. Explanation of this controversy may be extracted from the study of Ikegami et al. [51]. These authors suggested that CTLA-4 is significantly associated with autoimmune thyroid disease (AITD) but not with *type 1 diabetes*; because of the strong association of AITD and *type 1 diabetes*, the association of CTLA-4 polymorphism with *type 1 diabetes* may be secondary to its association with AITD. To verify their theory, they divided their *type 1 diabetes* mellitus patients into two groups, those with and those without AITD. CTLA-4 polymorphism was found to be significantly associated with the *type 1 diabetes* with, but not with the group of *type 1 diabetes* without AITD indicating that the association is mainly with AITD not *type 1 diabetes*. Analyzing the Egyptian studies in view of these data, AITD was present in only four of our patients; the other two Egyptian studies did not comment on the associated autoimmune diseases in their cohorts.

In conclusion, CTLA-4 +49 A/G polymorphism was not recognized as a risk susceptibility factor in our cohort of Egyptian *type 1 diabetes* families. We recommend that all studies performed on risk susceptibility to *type 1 diabetes* should include proper investigation for other autoimmune diseases to exclude their confounding effect on data analysis.

Author Contributions

A.K. wrote the manuscript and researched data. M.M. researched clinical data. G.M. researched laboratory data, wrote the manuscript, G.E. researched laboratory data. E.R., researched laboratory data, N.A. Statistical analysis, M.M. researched clinical data, M.A. researched clinical data, N.B. researched clinical data, H.B. researched clinical data, A.I. researched clinical data, N.S. researched clinical data and J.H. revised the manuscript and researched data.

Conflict of Interest

The authors declare no conflict of interest. There is no financial or personal relationship with other people or organizations that could inappropriately influence their work.

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