Genetic evaluation of CD28 rs3116496 in Egyptian children with Type 1 Diabetes (T1D)

Yasser BM Ali[1*], Dina R Elkera[2], Basima A El-Akhras[1], Heba Said El-Mahdy[2], Mohamed A. Osman[3]

[1]Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt.
[2]Pediatric Department, Faculty of Medicine, Tanta University, Egypt.
[3] Department of Bioinformatics, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt.

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***Corresponding author: Dr. Yasser Ali**
E-mail: yasser.ali@gebri.usc.edu.eg
Fax. +2 048 260 1266/68 **Tel.** +2 048 260 1264/65
RRCID number: 0000-0003-1153-2806

ABSTRACT

The genetic contribution of Cluster of Differentiation 28 (CD28) toward type 1 diabetes (T1D) was investigated in several ethnic groups. The genotyping of CD28 rs3116496 (+17 T/C) polymorphism was performed in the Egyptian population (101 T1D patients and 100 healthy controls) by using a single-stranded-polymorphism-PCR (SSP-PCR). Despite the comparable genetic distribution of CD28 between the two groups, patients had significantly higher CT/TT against CC than healthy individuals ($P<0.05$ and OR=3.63). Sodium levels were decreased in patients with the CC genotype (CD28 C/T), whereas urea levels were raised in TT genotype patients. Our present study found that there may be a link between Egyptian children's T1D risk and CD28 variants.

Keywords: Diabetic, Single nucleotide polymorphisms, PCR, CD28.

1. INTRODUCTION

Type 1 diabetes (T1D) is the most common confirmed autoimmune endocrinopathy disease. It represents a significant healthcare onus on both children and adults. T1D is marked by insulin deficiency (El Wakeel *et al*., 2017; Sabry *et al*., 2018; Dayan *et al*., 2021; Syed, 2022). El-Ziny *et al*. (2014) reported that although several studies documented the prevalence of T1D in children worldwide, there is still a shortage of epidemiological studies on childhood T1D in Egypt, which failed to decide the correct incidence of childhood. According to Mobasseri *et al.* (2020), there were 8 cases of T1D for every 100,000 people in Africa, and Egypt accounts for the largest proportion of the estimated total number of childhoods T1D cases. Primavera *et al.* (2020) also stated that among Egyptian children under the age of 15, the incidence of T1D is 8 cases/100,000 annually.

The complications of T1D and its etiological factors are not fully understood. Still, the Centers for Disease Control and Prevention (CDC) made an effort to recognize some risk factors for disease, including exalted hemoglobin A1c (HbA1c) with chronic medication, female gender, race, age, and the disease duration (Benoit *et al*., 2018; Nichols *et al*., 2021). Additionally, genetic predisposition to T1D is donated by around twenty putative loci (Mojtahedi *et al*., 2005). These loci have been labeled Insulin-dependent diabetes mellitus

(IDDM). Cluster of Differentiation 28 (CD28) is one of the most prominent critical genes from IDDM regions and is positioned on chromosome 2q33. Previous research have shown that the co-stimulatory receptor CD28 participates in regulating T cells (Ihara *et al*., 2001; Carreno and Collins, 2002; Gunavathy *et al*., 2019).

During T cell activation by antigen presentation, CD28 binds with the molecules of the B7 family, causing the growth of T cells. The functioning CD28 single nucleotide polymorphism (SNP) may increase the disease pathogenicity, correlating with abnormal T cell responses. Intron 3 of the CD28 gene contains the +17 T/C (rs3116496) SNP, linked to T1D and other autoimmune disturbances. (Ihara *et al*., 2001; Alegre *et al*., 2001; Baek *et al*., 2014).

Considering this evidence, we decided to evaluate the theory that the SNP of CD28 (rs3116496) is associated with the foretelling of childhood T1D disease within Egyptians.

2. SUBJECTS AND METHODS

2.1. The subjects

Our study investigated the CD28 gene in 101 Egyptian children with T1D from families with one or more index diabetic children or adolescents, ages 2 to 19 years, attending an outpatient clinic in the Diabetes Endocrinology and Metabolism Pediatrics Unit (DEMPU), Kafr EL-Sheik. The study's protocol complies with the guidelines of the Declaration of Helsinki, as they were updated in Brazil in 2013. After obtaining the parents' informed agreement, the children with T1D were qualified to participate in the study. The American Diabetes Association's 2017 diagnostic criteria were used to determine the presence of diabetes mellitus. As normal controls, 100 healthy individuals were enlisted with normal random plasma glucose levels and no clinical indications of a family history of T1D or other autoimmune illnesses. Data was collected from each child by using a questionnaire approved by physicians. Table (1) shows the clinical characteristics of controls and patients. The study did not include those with type 2 diabetes or those suffering from other illnesses. All subjects provided informed consent.

2.2. Sampling and Biochemical investigations

The blood from the venous system was gathered from each person and placed into three sterile vacutainer tubes. Two tubes contained tri-potassium ethylene diamine tetra-acetic acid ($EDTAK_3$), and the third had no anticoagulant. The separated samples were promptly kept at -20°C until they were needed for genomic DNA extraction and biochemical analysis. Also, serum specimens were drawn to measure random blood sugar levels (RBS), creatinine, urea, potassium, and sodium (Biomerieux, Paris, France). Following the manufacturer's instructions, the anion-exchange chromatographic technique (Teco Diagnostica, Anaheim, USA) was used to test glycated hemoglobin (HbA1c). Sampling, serological, and biochemical analyses were conducted in the Clinical Pathology Department at Kafr EL-Sheik University.

2.3. CD28 Genotyping & Statistical analysis

Genomic DNA was isolated by the procedures outlined in Ali *et al*. (2021). The genotyping of CD28 (rs3116496) SNP was done by a single-stranded polymorphism-PCR (SSP-PCR) (Welsh and Bunce 1999). The primer sequences of CD28 (+17 C/T) SNP and PCR conditions are shown in Table (2) (Kusztal *et al*., 2010). Each forward primer was put in two different tubes for the PCR that we performed for each sample. The PCR mixture contained

DreamTaq PCR Master Mix (2X) (Fermentas, Thermo Fisher Scientific Inc.), 10 pmol of each primer, and 150 ng of template DNA with a total reaction volume of 25 µl. The PCR was done in the 2720 PCR thermal cycler (Thermo Fisher Scientific Inc.). The product of PCR was visualized by electrophoresis on 2% agarose gel and was estimated by comparing it with a 50bp DNA Ladder (Fermentas, Thermo Fisher Scientific Inc.). All statistical analyses for the current investigation were calculated using version 11 of SPSS in the same manner as Ali *et al*. (2021).

3. RESULTS

3.1. Characteristics of Subjects with T1D and controls

Our study analyzed the polymorphism of CD28 in 101 T1D subjects and 100 controls. Table (1) showed that both T1D patients and controls had an almost identical age distribution (11.13±3.37 and 10.34±3.03, respectively). Compared to the control group, the patients' mean HbA1c was exalted (P<0.001). Furthermore, among all studied variables, only RBS, potassium level, urea, and urine acetone varied significantly between two groups (P<0.001). The atrial blood gases test was performed only on patients and showed the mean of PH at presentation (7.28±0.11), PCO2 (33.34±9.71), and HCO3- (18.74±6.72), confirming that all T1D patients were diabetic ketoacidosis (DKA).

3.2. Genetic Analysis

Table (3) summarizes the genotypes' and alleles' distribution of CD28 rs3116496. Although the genetic variation of different alleles and genotypes did not reach statistical significance, we found an elevation of CC genotype was detected in patients. Also, by comparing patients harboring CT and TT genotypes against CC genotypes, we observed the value of OR was 3. 63 (P<0.05).

3.3. Biochemical parameters of T1D patients with different genotypes of Cd28 rs3116496.

As illustrated in Table 4, T1D patients with CC genotype rs3116496 had a slightly significant (P<0.05) association with a sodium level than the other genotypes (CT and TT genotypes). While patients harboring the TT genotype had a small significant association (P<0.05) with a higher level of urea than the different genotypes (CC and CT).

4. DISCUSSION

T1D (an autoimmune illness) is triggered by various genetic and ecological factors that interact to disrupt insulin-producing pancreatic cells (Mojtahedi *et al*., 2005). Despite being found inside HLA genes, the CD28 gene is unrelated to the HLA family. By preventing the cytotoxic T lymphocyte antigen-4 (CTLA-4) receptor from attaching to B7 receptors, CD28 contributes to the up-regulation of T lymphocytes. It is crucial for T1D since genetic changes within CD28 could affect whether the disease risk is increased or decreased (Gunavathy *et al*., 2019; Ihara *et al*., 2001and Mojtahedi *et al*., 2005;).

The current study was the first in the Egyptian population to find an association between childhood T1D and CD28 SNP. Our results showed that CD28 (+17 C/T) SNP allele and genotype frequencies did not show a significant association with T1D patients against controls. However, T allele carrier patients (CT/TT genotypes together) against C allele (dominant CC genotype) were significantly associated with disease risk. Also, we found that T1D patients harboring the CC genotype had a low sodium level in their blood, while serum urea was significantly raised in patients with the TT genotype. Hence,

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the CD28 polymorphism might be associated with T1D risk. Although there are minimal studies on CD28 (+17 C/T) polymorphism and T1D disease, this SNP was studied with T1D and other different types of cancer (Baek *et al*., 2015; Wood *et al*., 2002 and Mojtahedi *et al*., 2005). Like us, Ihara *et al*. (2001) observed that CD28 (+17 C/T) SNP was associated with T1D disease risk. Similarly, the meta-analysis study by Baek *et al*. (2015) found that in Asian nations but not in European countries, T1D patients with the TT and CT genotypes had lower cancer incidences than people who had the C allele (CC homozygotes). In disagreement with us, Gunavathy *et al*. (2019) showed that this SNP was not linked with the Indian T1D patients. Contrary, Wood *et al*. (2002) reported no association between CD28 polymorphism and T1D patients. Controversy, this SNP did not show any significant association with different diseases, such as autoimmune hepatitis (Djilali-Saiah *et al*., 2001) and several autoimmune disorders (Ahmed *et al*., 2001; Tomer *et al.*, 2001 and Van Veen *et al*., 2003).

5. CONCLUSION

The present study demonstrated that CD 28 SNP might be linked with T1D risk. The intricate relationship between CD28/CTLA-4 gene polymorphisms and T1D requires more research with a bigger sample size and functional examination of polymorphisms in different races.

FUNDING

This research was not supported financially.

COMPETING INTERESTS

No conflicting interests are stated by the writers.

ETHICAL APPROVAL

The University of Sadat City's Research Ethical Committee (USCREC) granted ethical approval for this investigation with approval number (GEBRI-USCREC-201833), September 2018. All samples were collected after obtaining parents' or guardians' consent was verbal and being verbally informed about the study objectives.

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Table (1). Demographic and biochemical data of T1D patients and healthy controls.

Variables	Control group (N=100) (M±SD)	T1D group (N=101) (M±SD)	P-value	Correlation with disease
Demographic data				
Age (years)	10.34 ±3.03	11.13 ±3.37	NS	r= 0.12 NS
Gender (F/M)	40/60	47/54	NS	r= -0.07 NS
Weight (Kg)	42.55±4.88	42.2±14.54	NS	r= 0.003 NS
Height (cm)	109.76± 10.96	129.75± 26.6	NS	r= 0.113 NS
Laboratory parameters				
RBS (mg/dL)	84.74±8.77	403.76±83.45	**P<0.001**	r= 0.94 **P<0.001**
HbA1C (%)	4.35±0.41	8.33±1.49	**P<0.001**	r= 0.80 **P<0.001**
Sodium (mmol/L)	148.19±10.34	137.47±8.9	NS	r= - 0.031 NS
Potassium (mmol/L)	3.10±0.31	2.19±0.94	**P<0.001**	r= - 0.54 **P<0.001**
Urea (mg/dL)	10.75±3.99	46.03±6.61	**P<0.001**	r=-0.95 **P<0.001**
Creatinine (mg/dL)	0.62±0.1	0.52±0.39	NS	r= - 0.02 NS
Urine acetone (mg/dL)	0.06±0.01	1.47±0.58	**P<0.001**	r= 0.89 **P<0.001**
PH at presentation	--	7.28±0.11		
PCO2 (mmHg)	--	33.37±9.71		
HCO3- (mmol/L)	--	18.74±6.72		

All data are presented as means ± standard deviations;
N, *number*; TID, *type one diabetes*; F, *female*; M, *male*; RBS, *Random Blood Sugar*; HbA1c, *Glycated hemoglobin A1c*; Na, *Sodium level*; K, *Potassium level*, PH *at presentation of patient's blood;* PCO2, *Partial Pressure of Carbon Dioxide*; HCO3-, *Plasma bicarbonate*; NS, *not significant*.

Table (2). Primers, PCR conditions of CD28 rs3116496 C/T.

SNPs	Primers	PCR conditions			Length of bands
rs3116496 T/C (SSP-PCR)	**F (T):** 5′- ATTTTCTGGGTAAGAGAAGCAGCAC**T**-3′ **F (C):** 5`- ATTTTCTGGGTAAGAGAAGCAGCAC**C**-3` **Common R:** 5′- ACCTACTCAATGCCTTCTGGAAATC-3′	**Pre-heating**	95ºC (5 min)	1 cycle	**For allele T and C** 223 bp
		Denaturation **Annealing** **Extension**	95ºC (30 sec) 60ºC (30 sec) 72ºC (30 sec)	40 cycles	
		Final extension	72ºC (7 min)	1 cycle	

Table (3). Genotype distribution and allelic frequency of CD28 rs3116496 C/T in T1D patients and healthy controls.

SNPs	Control N=100 (N%) (%)	Patients N=101 (N%) (%)	OR (95%CI)	P value
CD28 rs3116496T/C (Allele and Genotypes)				
C	103 (52)	94 (47)	0.88 (0.59-1.31)	NS
T	97 (48)	108 (53)	1.22 (0.82-1.80)	NS
CC	10 (10%)	3 (3%)	0.31 (0.08-1.17)	NS
CT	83 (83%)	88 (87.1%)	1.38 (0.63-3.03)	NS
TT	7 (7%)	10 (9.9%)	1.46 (0.53-4.00)	NS
CT and TT	90 (90%)	98 (97%)	**3.63 (0.96-13.60)**	**P<0.05**

N, *number*; NS, *not significant*.

Table (4). Association of T1D biochemical parameters with CD28 rs3116496 C/T in T1D patients.

Biochemical parameters	CD28 SNP (rs3116496 C/T)		
	CC	CT	TT
Weight	41.33 ±3.52	42.62 ±1.57	35.80 ±3.98
Height	130.33 ±2.66	131.60 ±2.79	114. 10±9.49
RBS	462.00 ±27.00	403.65 ±9.93	390.12 ±33.78
HBA1C	8.86 ±1.36	8.34 ±0.15	8. 06±0.42
Na	**122.43±4.24***	138.04±0.94	137.0±2.24
K	2.10 ±0.27	2.90 ±0.71	1.75 ±0.21
Urea	42.00 ±1.52	45.63 ±0.70	**50.70 ±1.83***
Creatinine	0.56 ±0.08	0.49 ±0.04	0.53 ±0.06
Urine acetone	2.00 ±0.57	1.47 ±0.08	1.00 ±0.0
PH	7.23 ±0.05	7.28 ±0.11	7.27±0.04
Pco2	28.73 ±5.58	33.13 ±1.04	36.90 ±2.80
HCO3-	16.26 ±3.93	18.76 ±0.68	19.35 ±2.95

All data are presented as means ± standard error; RBS, *Random Blood Sugar*; HbA1c, *Glycated hemoglobin A1c;* Na, *Sodium level*; K, *Potassium level*, PH *at presentation of patient's blood*; PCO2, *Partial Pressure of Carbon Dioxide*; HCO3-, *Plasma bicarbonate*; *, P<0.05.