Evaluation of CTLA-4 Gene polymorphism SNP 49 G/ A Association with Diabetes Mellitus Type 1 in Egyptian Population

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Abstract

The (*CTLA-4*) encodes of the T cell receptor involved in the control of T cell proliferation and mediates T cell apoptosis. The contribution of CTLA-4 gene variants to type 1 diabetes has been analyzed in several ethnic groups. In this study, the association of CTLA-4 +49 A/G polymorphism with type 1 diabetes was investigated in Egyptian patients. Sixty type 1 diabetic patients (25 males and 35 females) and 60 healthy individuals (33 males and 27 females) subjects formed the studied populations. CTLA-4 A/G polymorphism at position 49 in exon 1 was identified using allele specific methods. Patient numbers with A/G, A/A and G/G genotypes were 45 (75.0 %), 6 (10.0 %) and 9 (15.0%) while in healthy controls, these were 48 (80.0%), 2 (3.3%) and 10 (16.7%), respectively. In conclusion, the results of this study showed that CTLA-4 +49 A/G polymorphism were not associated with susceptibility to type 1 diabetes in Egyptian population.

Keywords: Type 1 diabetes; CTLA-4 polymorphism; susceptibility

تقيم ارتباط التباين الجيني للجين CTLA-4 +49 A/G مع مرض السكري من النوع الاول في المجتمع المصري

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الخلاصة:

يشفر *CTLA-4* لمستقبل خلايا T ويشارك في السيطره وتنظيم التكاثر للخلايا التائية, وايضا موت الخلايا التائية المبرمج. وقد تم تحليل مساهمة *CTLA-4* لمرض السكري من النوع الاول في العديد من المجموعات العرقية. تم التحري في هذه الدراسة عن الارتباط بين*CTLA-4* +49 A/G ومرض السكري من النوع الاول في المجتمع المصري. تم فحص 60 عينة من المصابين بداء السكري من النوع الاول (25 ذكور و35 اناث) مقارنة بالمجموعه الضابطة المتكونة من 60 عينة (33 ذكور و 27 اناث). وقد تم تحديد التباين الجيني للجين *CTLA-4* باستخدام طريقة allele specific. وكانت توزيع عدد المرضى بالنسبة للتركيب الوراثي A/G, A/A و G/G , (10.0 %) 6, (75.0 %) 45 و(15.0%) 9 على التوالي ,بينما توزيع مجموعة الاصحاء بالنسبة للتركيب الوراثي كان كالاتي (3.3%) 2, (80.0%) 48 و (16.7%) 10 على

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التوالي. أظهرت نتائج هذه الدراسة أن *CTLA-4* +49 A / G تعدد الأشكال لا ترتبط مع احتمالية الاصابة بمرض السكري من النوع الاول في المجتمع المصري.

Introduction

Type 1 DM results from β-cell destruction, usually leading to absolute insulin deficiency. Testing for islet-cell antibodies (ICA) or other autoantibodies (antibodies to glutamic acid decarboxylase [anti-GAD], insulin, and to the tyrosine phosphatase IA-2) in serum may be helpful if establishing the diagnosis is important; a positive result is indicative of immune-mediated or type 1 diabetes [1].Genetic susceptibility to T1D is conferred by more than 20 putative loci [2-4] . The cytotoxic T lymphocyte-associated antigen 4 gene (CTLA-4) is considered one of the most important candidate genes for autoimmunity and it has been reported to be associated with several endocrine disorders including type 1 diabetes, Graves' disease and autoimmune hypothyroidism [5]. The CTLA-4 encodes the T cell receptor involved in the control of T cell proliferation and mediates T cell apoptosis [6]. The human CTLA-4 gene was mapped to chromosome 2q33 [7]. Polymorphisms have been identified in the *CTLA-4* gene and have been associated with different susceptibilities to a wide range of T-cell-mediated autoimmune disorders [8]. Most molecular epidemiology studies have evaluated the role of the + 49A/G (rs#231775) single nucleotide polymorphism (SNP) that causes a threonine-toalanine substitution in codon 17 and is associated with altered protein expression [9]. This polymorphism seems to be associated with the genetic susceptibility to type 1 diabetes in several populations, although conflicting data also exist in populations of different ethnic backgrounds [10].

The aim of this study was to assess the contribution of this *CTLA4* (SNP 49 G / A rs#231775) polymorphism to the susceptibility to type 1 diabetes in the Egyptian children.

MATERIALS AND METHODS

Study populations

A total of 60 type 1 diabetic patients (25 males and 35 females) with an age mean ± SD 11.2±3.7, and 60 healthy individuals (33 males and 27 females) with an age mean ± SD 27.2± 6.4, family history (25 positive/ 35 negative to family history, disease onset (years) mean±SD 5.3±3.5 , were enrolled in this study and recruited at the El-Shatby University Hospital, Faculty of Medicine Alexandria University. Egypt. Patients diagnosed according to WHO criteria [11]. Patients were diagnosed on the basis of classical clinical presentation, first-degree family history of diabetes, history of chronic diabetes complications, and treatment of diabetes. Healthy controls had no personal or first-degree history of diabetes and were free from T1DM. The Ethics Committees of participating universities and university hospitals approved the study, and informed consent was obtained from all participants.

Blood sampling (one ml of venous blood) was collected in EDTA tubes from each individual (patient or healthy control) and was stored as whole blood at -20^{o}C for subsequent DNA isolation. Genomic DNA was isolated from whole blood according to Sambrook *et al* 1989 [12].

Genotyping of *CTLA-4* gene polymorphism

One SNP (SNP 49 G/A rs#231775) in CTLA-4 gene was genotyped among the participants groups in this study. The CTLA-4 A/G polymorphic region (rs#231775) was amplified by polymerase chain reaction (PCR) using allele specific PCR technique as shown in Table 1. Four primers (two allele specific primers, forward control and common reverse primer) were designed based on the nucleotide sequence of a partial fragment (retrieved from the online dbSNP) of the gene containing the target SNP. The polymorphism was visualized by separating the DNA fragments in a 2% agarose gel that was stained with ethidium bromide and illuminated by UV. To validate the PCR- allele specific results to validate the PCR- allele specific results. All primers used in this study were newly designed using Primer Blast online programme (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table.1- Primers sequences, PCR conditions, length of PCR products.

SNPs	Primers sequences	PCR Conditions	Size of PCR Products digestion products
CTLA-4 SNP49 G/A (rs#231775)**	**A-allele specific primer:** **F1:** 5-GCTCAGCTGAACCTGGCT **A** -3 **G-allele specific primer:** **F2:** 5-GCTCAGCTGAACCTGGCT **G-**3 **Forward control primer**: 5-GCTTTCTATTCAAGTGCC TTCT-3 5 **Common reverse primer:** **5-CTTTAACTTCTGGCTTTGCTAT-3**	**An initial denaturation** at 95°C for 5 min -Then, **30 cycles** each cycle consisted of denaturation at 94°C for 60s, annealing at 50 °C for 30s and extension at 72°C for 30 s. -**A final extension** at 72°C for 10min.	**Allele A:** 189 bp **Allele G:** 189 bp **Control fragment:**390 bp

Statistical analysis of data

Statistical analysis of data was done to correlate genotype distribution and allele frequencies were performed by SPSS package version 11. The frequencies of alleles, genotypes in different groups were compared using the Chi-squared test (X^2), t-test and Mann Whitney test were used to test the significance of results of quantitative variables. Odds ratio and 95% confidence interval (95% CIs) were calculated for different studied parameters. The confidence interval (CI) at 95% was used to describe the amount of uncertainty associated with the samples [13, 14]. A 95% confidence level means that 95% of the intervals would include the parameter. The significance of the results was taken at the $P < 0.05$ level of significance.

Results And Discussion

This study demonstrates that *CTLA-4* gene polymorphisms are associated with a risk of T1DM in Egyptian population. The Results revealed that the allele and genotypic distributions did not significantly differ between the two groups (P>0.05) for the SNP 49 G /A (rs#231775) as shown in table 2.

Table 2- *CTLA-4* gene polymorphism and allele frequencies among diabetic patients and control

Gene polymorphism	Cases No. =60		Control No. =60		Significance	OR (95% CI)
	No.	%	No.	%		
49 G/A						
AA	6	10.0	2	3.3	X^2=2.149 P> 0.05	--
GG	9	15.0	10	16.7		0.3 (0.03-2.4)
AG	45	75.0	48	80.0		0.3 (0.04-1.9)
allele frequencies						
A	60	0.18	60	0.12	------	
G		0.82		0.88		

X^2: Chi-Square test

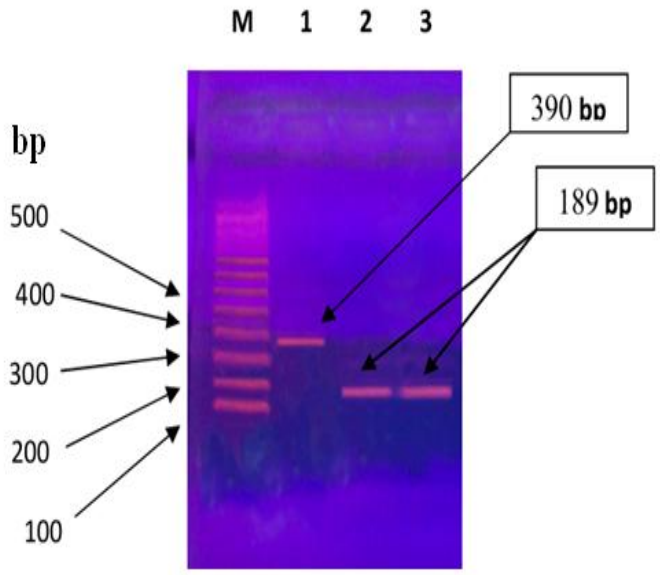

Figure 1-Shows 2% agarose gel electrophoresis for allele specific PCR for *CTLA-4* SNP 49 G>A (rs#231775). M: 100 bp DNA ladder from GeneDire*X*®. Lane1: PCR product upon using controls forward primer, Lanes 2 and 3: PCR products upon using allele specific G primer and allele specific A primer, respectively. Heterozygous genotype will give positive reaction upon using both allele specific primers. However, homozygous genotype will give positive reaction upon using only one of these allele specific primers.

Our results agree with Mosaad, *et al* . [15]. Proved that *CTLA-4* +49 GG homozygous genotype is associated with T1D in Egyptian children especially with younger age of onset and in younger patients, and not associated with grades of diabetic control or diabetic complication.

Kamel, *et al*. [16]. Proved that 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.

Saleh, *et al*. [17]. Found that an association of the C-819T and A+49G SNPs in the *CTLA-4* gene with T1D patients (*P*=0.0047) and (*P*=0.000575), respectively. Moreover, this association was stratified by gender and age to female patients with age at onset 0-5 years old (*P*=0.0186) and (*P*=0.00115) more than male patient with the age at onset 0-5 years old (*P*= 0.3120) and (*P*=0.345161), respectively.

Mochizuki, *et al*. [18]. In Type 1 Diabetes and Autoimmune Thyroid Disease in Japanese Children have demonstrated that a distinct association exists between the G allele of *CTLA-4* and high values of GAD Ab, residual β-cell function, and the absence of *HLA-DRB1**0405.

Present results disagree with Chistiakov, *et al*. [19]. In Russian population they found that the *CTLA-4* gene SNP 49 G/A is strongly associated with IDDM in a Russian population. While Lemos, M. C. *et al*. [20]. Investigated that no significant differences were observed, suggesting that this polymorphism is not strongly associated with T1DM in the Portuguese population.

Yanagawa, *et al.* [21]. Investigated the distribution of a *CTLA-4* gene SNP G>A polymorphism in 110 Japanese patients with IDDM and 200 control subjects. In 84 patients, they also investigated associations between this *CTLA-4 gene* polymorphism and GAD65 antibody positivity. An A/G transition at position 49 of exon 1, there was no significant difference in the distribution of *CTLA-4* alleles in patients and controls and no difference was observed in prevalence of *CTLA-4* alleles when GAD65 antibody-positive and -negative individuals in the IDDM groups were compared. This study did not support an association between the *CTLA-4 gene* and IDDM in the Japanese population.

Ma, *et al*. [22] . In Han Chinese proved that *CTLA-4 49* AA is protective from diabetes mellitus, whereas, *CTLA-4 49* G allele (both as homozygotes and as heterozygotes) confers an increased risk of diabetes mellitus. In the same population Lee, *et al.* [23]. Noted that *CTLA4 49* A-G polymorphism is associated with T1DM in Han Chinese children. The *CTLA4 49* G allele confers an increased risk of type 1

diabetes. Mojtahedi, *et al*. [24]. Showed that *CTLA-4 +49* A/G polymorphism confers genetic susceptibility to T1DM, particularly in younger individuals in Iranians population.

Present findings provide no significant effect on the susceptibility to T1DM among the studied group. The results do not support the involvement of the *CTLA4* gene in the pathogenesis of type 1 diabetes in our population. The difference in the association of the aforementioned of above studies variants with T1DM among different populations may be attributed to the presence of multiple susceptibility alleles at the aforementioned genes variants, the genetic heterogeneity among the populations studied, different interactions with environmental factors involved in the pathogenesis of type 1 diabetes, racial/ethnic differences in the distribution of these variants and multiple hypothesis testing. Furthermore, the CTLA4+ 49A/G SNP may not be the true disease-associated variant, but rather a marker in linkage disequilibrium with the causal variant, and the discrepant findings may reflect variable strengths of linkage disequilibrium in different populations. In conclusion, our case–control study suggests that the + 49A/G SNP of the CTLA4 gene is not strongly associated with type 1 diabetes mellitus in the Egeptian population.

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References

1. Diagnosis and classification of diabetes mellitus .**2008**. *Diabetes Care 31 Suppl 1*, S55-60.
2. Raman, K., and Mohan, C. **2003**. Genetic underpinnings of autoimmunity--lessons from studies in arthritis, diabetes, lupus and multiple sclerosis. *Curr Opin Immunol 15*, 651-659.
3. Redondo, M.J., and Eisenbarth, G.S. **2002**. Genetic control of autoimmunity in Type I diabetes and associated disorders. *Diabetologia 45*, 605-622.
4. Field, L.L. **2002**. Genetic linkage and association studies of Type I diabetes: challenges and rewards. *Diabetologia 45*, 21-35.
5. Ueda, H., Howson, J.M., Esposito, L., Heward, J., Snook, H., Chamberlain, G., Rainbow, D.B., Hunter, K.M., Smith, A.N., Di Genova, G., *et al*. **2003**. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature 423*, 506-511.
6. Waterhouse, P., Penninger, J.M., Timms, E., Wakeham, A., Shahinian, A., Lee, K.P., Thompson, C.B., Griesser, H., and Mak, T.W. **1995**. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science 270*, 985-988.
7. Dariavach, P., Mattei, M.G., Golstein, P., and Lefranc, M.P. **1988**. Human Ig superfamily CTLA-4 gene: chromosomal localization and identity of protein sequence between murine and human CTLA-4 cytoplasmic domains. *Eur J Immunol 18*, 1901-1905.
8. Gough, S.C., Walker, L.S., and Sansom, D.M. **2005**. CTLA4 gene polymorphism and autoimmunity. *Immunol Rev 204*, 102-115.
9. Anjos, S., Nguyen, A., Ounissi-Benkalha, H., Tessier, M.C., and Polychronakos, C. **2002** . A common autoimmunity predisposing signal peptide variant of the cytotoxic T-lymphocyte antigen 4 results in inefficient glycosylation of the susceptibility allele. *J Biol Chem 277*, 46478-46486.
10. Kavvoura, F.K., and Ioannidis, J.P. **2005**. CTLA-4 gene polymorphisms and susceptibility to type 1 diabetes mellitus: a HuGE Review and meta-analysis. *Am J Epidemiol 162*, 3-16.
11. Report of the expert committee on the diagnosis and classification of diabetes mellitus .**2003**. *Diabetes Care 26 Suppl 1*, S5-20.
12. Sambrook, J., Frisch, E.F., and Maniatis, T. **1989**. Molecular cloning: A laboratory Manuel,2 nd edition. Cold spring Harbor laboratory press, New York.
13. Greenfield, B., Henry, M., Weiss, M., Tse, S.M., Guile, J.M., Dougherty, G., Zhang, X., Fombonne, E., Lis, E., Lapalme-Remis, S., et al. **2008**. Previously suicidal adolescents: predictors of six-month outcome. *J Can Acad Child Adolesc Psychiatry 17*, 197-201.
14. Szumilas, M. **2010**. Explaining odds ratios. *J Can Acad Child Adolesc Psychiatry 19*, 227-229.

15. Mosaad, Y.M., Elsharkawy, A.A., and El-Deek, B.S. **2012**. Association of CTLA-4 (+49A/G) gene polymorphism with type 1 diabetes mellitus in Egyptian children. *Immunol Invest 41*, 28-37.
16. Kamel, A.M., Mira, M.F., Mossallam G. I., Ebid, G.T.A., Radwan, E.R., Aly Eldin, N.H., Mamdouh, M., Amin, M., Badawy, N., Bazaraa, H., et al. **2013**. Lack of association of CTLA-4 +49 A/G polymorphism with predisposition to type 1 diabetes in a cohort of Egyptian families. *Egyptian Journal of Medical Human Genetics 15*,, 25–30.
17. Saleh, H.M., Rohowsky, N., and Leski, M. **2008**. The CTLA4 -819 C/T and +49 A/G dimorphisms are associated with Type 1 diabetes in Egyptian children. *Indian J Hum Genet 14*, 92-98.
18. Mochizuki, M., Amemiya, S., Kobayashi, K., Shimura, Y., Ishihara, T., Nakagomi, Y., Onigata, K., Tamai, S., Kasuga, A., and Nanazawa, S. **2003**. Association of the CTLA-4 gene 49 A/G polymorphism with type 1 diabetes and autoimmune thyroid disease in Japanese children. *Diabetes Care 26*, 843-847.
19. Chistiakov, D.A., Savost'anov, K.V., and Nosikov, V.V. **2001**. CTLA4 gene polymorphisms are associated with, and linked to, insulin-dependent diabetes mellitus in a Russian population. *BMC Genet 2*, 6.
20. Lemos, M.C., Coutinho, E., Gomes, L., Bastos, M., Fagulha, A., Barros, L., Carrilho, F., Geraldes, E., Regateiro, F.J., and Carvalheiro, M. **2009**. The CTLA4 +49 A/G polymorphism is not associated with susceptibility to type 1 diabetes mellitus in the Portuguese population. *Int J Immunogenet 36*, 193-195.
21. Yanagawa, T., Maruyama, T., Gomi, K., Taniyama, M., Kasuga, A., Ozawa, Y., Terauchi, M., Hirose, H., Maruyama, H., and Saruta, T. **1999**. Lack of association between CTLA-4 gene polymorphism and IDDM in Japanese subjects. *Autoimmunity 29*, 53-56.
22. Ma, Y., Tang, X., Chang, W., Gao, L., Li, M., and Yan, W. **2002**. CTLA-4 gene A/G polymorphism associated with diabetes mellitus in Han Chinese. *Chin Med J (Engl) 115*, 1248-1250.
23. Lee, Y.J., Huang, F.Y., Lo, F.S., Wang, W.C., Hsu, C.H., Kao, H.A., Yang, T.Y., and Chang, J.G. **2000**. Association of CTLA4 gene A-G polymorphism with type 1 diabetes in Chinese children. *Clin Endocrinol (Oxf) 52*, 153-157.
24. Mojtahedi, Z., Omrani, G.R., Doroudchi, M., and Ghaderi, A. **2005**. CTLA-4 +49 A/G polymorphism is associated with predisposition to type 1 diabetes in Iranians. *Diabetes Res Clin Pract 68*, 111-116.