

Association of Cytotoxic T Lymphocyte Antigen 4 Gene Polymorphism with Type 1 Diabetes in Children

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Abstract

Background: Type- 1 diabetes mellitus (T1DM) is an autoimmune disease in which combinations of environmental and genetic factors contribute to T-cell mediated destruction of insulin- secreting β - cells of the pancreas. The (CTLA- 4) encodes of the T-cell receptor involved in the control of T-cell proliferation.

Aim: The aim of this study was to investigate the association of CTLA- 4 gene exon 1 49 A/G polymorphism with T1DM in children and its relation to diabetic complications.

Subjects and methods: A total of 100 subjects were included in this cross sectional, case- control study. Fifty children with T1DM aged (10- 18) years (12.5 ± 2.0) years, and fifty children as a control group. All candidates were subjected to full clinical evaluation and anthropometric measurements. All the patients had the following laboratory investigation been done (RBG, average HbA1c, Quantitative determination of urinary microalbumin). CTLA- 4 gene polymorphism PCR- RFLP was done for all the subjects.

Results: CTLA- 4 genotyping among the diabetic group was: the mutant homozygous genotype GG in 15 (30%), the mutant heterozygous genotype AG in 29 (58%) and wild homozygous genotype AA in 6 (12%). However, among the control group: it was 3 (6%) with GG genotype, 19 (38%) with AG genotype and 28 (56%) with AA genotype with P value <0.001 which denoting a higher prevalence of AG and GG genotype in diabetic group with highly statistical significance. There was a significant association between CTLA- 4 mutant genotypes and patients with younger age of onset of diabetes ($P= 0.011$) and higher dose of insulin ($P= 0.002$). CTLA- 4 +49 mutant genes did not have any impact on complications of type 1 diabetes.

Conclusion: The results of the present study shows that the CTLA- 4 A/G +49 polymorphism was associated with type 1 diabetes in Egyptian children with a significant association between CTLA- 4 mutant genotypes and patients with younger age of onset of diabetes and higher dose of insulin.

ارتباط تعدد الأشكال في جين مستضد ٤ للمفاويات التائية السامة للخلايا بداء السكري من النوع الأول في الأطفال

الخلفية: ان مرض السكري من النوع الأول هو مرض مناعي وتحدث فيه العوامل الجينية والبيئية لتدمير خلايا بيتا المفرزة للإنسولين في البنكرياس بواسطة الخلايا التائية. ان جين مستضد ٤ للمفاويات التائية السامة للخلايا (ستلا ٤) يشفر احدى المستقبلات التي تظهر على الخلايا التائية والذي بدوره يتحكم في تكاثر الخلايا التائية وموت الخلايا التائية المبرمج.

الهدف: الهدف من هذه الدراسة هو دراسة ارتباط تعدد الأشكال في جين مستضد ٤ للمفاويات التائية السامة للخلايا بداء السكري النوع الأول في الأطفال واحتمالية ارتباطه بمضاعفات السكر.

الاساليب: دراسة حالة لمراقبة مستعرضة شملت مائة طفل، خمسون منهم مرضى بالنوع الأول من السكري تتراوح اعمارهم من ١٠- ١٨ سنة (12.5 ± 2.0) وخمسون اخرون اصحاء كمجموعة ضابطة. تم أخذ التاريخ الكامل والفحص السريري والقياسات الانثروبومترية وتحاليل معملية (قياس مستوى السكر بالدم مع اخذ متوسط القياسات، قياس مستوى الهيموجلوبين السكري، قياس مستوى الزلال المجهرى بالبول، قياس تعدد الأشكال في جين مستضد ٤ للمفاويات التائية السامة للخلايا بطريقة (بي سي آر) بليها طريقة (رفلب).

النتائج: جين مستضد ٤ للمفاويات التائية السامة للخلايا كان كالتالي GG في ١٥ (٣٠%)، AG في ٢٩ (٥٨%)، AA في ٦ (١٢%) في المرضى، وبالتنسيب للمجموعه الضابطة GG في ٣ (٦%)، AG في ١٩ (٣٨%)، AA في ٢٨ (٥٦%). مما يؤكد ان GG، AG اكثر بفرق ذو دلالة احصائية في المرضى. يوجد علاقة ذو دلالة احصائية بين جين مستضد ٤ للمفاويات التائية السامة للخلايا وصغر السن عند الاصابة بالمرض وكذلك جرعه الانسولين ولا يوجد علاقة من اي نوع بين جين مستضد ٤ للمفاويات التائية السامة للخلايا ونسبه الهيموجلوبين السكري ولا بين جين مستضد ٤ للمفاويات التائية السامة للخلايا ومضاعفات مرض السكري.

الاستنتاج: يوجد علاقة ذو دلالة احصائية بين جين مستضد ٤ للمفاويات التائية السامة للخلايا ومرض السكري من النوع الأول وخاصة عند صغر السن عند الاصابة بالمرض وكذلك جرعه الانسولين المرتفعه.

الكلمات المفتاحية: داء السكري النوع الأول، مستضد ٤ للمفاويات التائية السامة للخلايا، مستضد، تعدد الأشكال.

Introduction:

Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both (ISPAD, 2009). The chronic hyperglycemia of diabetes is associated with long term damage, microvascular complications (e.g. retinopathy, nephropathy and neuropathy) and macrovascular complications (e.g. myocardial infarction, angina pectoris and stroke). (Grandy and Fox, 2008).

Type 1 diabetes (T1D) is considered as one of the most common chronic diseases in children, the cause of the disease is an autoimmune process directed against the beta- cells of the islets of Langerhans leading to progressive and irreversible destruction of these cells causing complete and definitive cessation of endogenous insulin production. Accordingly, these patients should be treated with insulin since the beginning of the disease. (Egyetem et.al., 2010). At present, the incidence of Type 1 diabetes is on the rise, while its age of onset decreases (Haliloğlu et.al., 2011).

An effective treatment that leads to improved metabolic control is essential to prevent severe diabetes related complications and minimize long- term ones (Araujo and Mazza, 2008).

Type 1 diabetes mellitus is a T-cell mediated organ specific autoimmune disease (Nistico et.al., 1996).

There are over 20 regions in the human genome that are associated with T1DM, but most of it make only small contribution to the susceptibility of type 1 diabetes (Radha et.al., 2003). Cytotoxic T-lymphocyte antigen 4 gene (CTLA- 4), is one of the genes associated with T1DM (SiMonds et.al., 2005).

CTLA4 is a protein that plays an important regulatory role in the immune system. In humans, the CTLA4 protein is encoded by the CTLA4 gene (Dariavach et.al., 1988).

Cytotoxic T lymphocyte- associated antigen 4 (CTLA- 4) gene is a member of the immunoglobulin super family (Dariavach et.al., 1988). It is located on chromosome 2q33 (Teft et.al., 2006).

There is evidence to suggest that CTLA- 4 is an important negative regulator of T-cell responses involved in the maintenance of peripheral T-cell tolerance (Kosmaczewska et.al., 2001). T-cells are regulated by their surface receptors such as T-cell receptor (TCR), and co- stimulatory molecules, optimizing T-cell activation. Some of these co- stimulatory molecules such as CD28 have a positive co- stimulatory signals which complete T-cell activation, but others such as the cytotoxic T- lymphocyte antigen 4 (CTLA- 4), induce inhibitory effects on T-cells (Olive et.al., 2011). Because of its inhibitory role, CTLA- 4 gene is a strong candidate gene for involvement in autoimmune diseases (Kosmaczewska et.al., 2001).

Several diseases of presumed autoimmune etiology, including Graves' disease, Hashimoto's thyroiditis (Awata et.al., 1998), insulin dependent diabetes mellitus (Abe et.al., 1999), and Addison disease have been associated with CTLA- 4 gene polymorphism (Vaidya et.al., 2000).

Polymorphism of the CTLA- 4 gene could thus have effects upon the immune response. Of the several CTLA- 4 gene polymorphisms, three have been most frequently studied, namely a dinucleotide microsatellite (AT) n marker at position 642 of the 3'- untranslated region of exon 4: CTLA- 4 3' (AT) n18 in the promoter region CTLA- 4 C (- 318) T3, and the single nucleotide polymorphism in exon 1 CTLA- 4 A (49) G (Harper et.al., 1991). There are many studies revealed a significant association of CTLA- 4 exon 1 49 A/G polymorphism with T1DM and a recent study was done on Maduria population in India indicates the same results. (Philip and Isabel, 2011)

Additionally, CTLA4 has become a focus of research interest since it is a useful therapeutic target for immunotherapy in cancer and autoimmune diseases (Gough et.al., 2005).

Aim of the present study was to investigate the association of CTLA- 4 gene exon 1 49 A/G polymorphism with T1DM in children and its possible relation to diabetic complication.

Subjects And Methods:

The present study was conducted at the Diabetes Clinic, Children's Hospital, Ain Shams University. Fifty (50) type 1 diabetic patients (with age range 10- 18 years) were collected from the regular attendants of the clinic for follow up during a full calendar year. All patients were diagnosed as diabetics according to Criteria for the Diagnosis of Diabetes; American Diabetes Association (ADA) Guideline for 2011 (ADA, 2011).

Inclusion Criteria:

1. Cases diagnosed with Type 1 diabetes mellitus.
2. Age: (10- 18) years.
3. Gender: Both Sexes
4. Patients receiving human insulin therapy.

Exclusion Criteria:

1. Cases diagnosed with Type 1 diabetes mellitus and associated with another chronic disease (e.g. chronic renal failure, cardiac diseases chronic chest disease... etc.).
2. Patients with other autoimmune diseases as Graves' disease, celiac disease... etc

Ethical aspect of the study:

Written informed consent was obtained from the parents after explanation of the aim of the study, its benefits and expected risks for their children if they participate in the study. Informed verbal assent was taken also from all the patients as their age exceeds eight years after a simplified explanation of the aim and benefits of the study for them. All the patients data were confidential, neither the data nor the collected samples were used in other researches. Approval was taken to conduct this research from the Ethical Committee of the Institute of Postgraduate Childhood Studies Ain Shams University, the Ethical Committee of the Faculty of Medicine Ain Shams University and the Ethical Committee of the National Research Center (NRC).

Methods:

All patients and controls were subjected to complete medical history

and thorough clinical examination. Anthropometric measurement in the form of height, weight and body mass index was done. All the patients had the following laboratory investigation been done:

1. Routine investigations for all cases: Routine home glucose monitoring using Bionime GS300 blood glucose monitoring system and mean value over two weeks was calculated.
2. Glycosylated Hb (HbA1c) by HPLC (high performance liquid chromatography) was done every three months and mean value all over the entire study was calculated (Rewers et al., 2009).
3. Quantitative determination of urinary microalbumin (Coonrod et al., 1993).
4. Molecular Analysis: CTLA- 4 (+49 A/G) gene polymorphism typing: This was done by PCR amplification followed by restriction fragment length polymorphism (RFLP) method (Abe et al., 1999).

Statistical Analysis:

Quantitative data were analyzed using SPSS version 16, with mean values for continuous variables compared using Independent t-test, and differences between proportions assessed using either the chi² test and McNemar test. Level of statistical significance for all tests was set at 0.05.

Results:

The results of the following study will be presented with the following tables and figures. This study was conducted on 50 children with type 1 diabetes 22 males (44%) and 28 females (56%) with a mean age of 12.5± 2.0 years and 50 healthy controls 23 males (46%) and 27 females (54%) with a mean age of (12.7± 2.8) years.

Diabetic patients and control group are well matched as regards age and gender (p>0.05). There was no statistically significant difference regarding BMI between diabetic patients and controls (p>0.05). Family history of DM was significantly more frequent among diabetic patients than among control group (p= 0.009), table (1).

Table (1) Comparison between diabetic patients and controls as regards age, gender, BMI and a family history of diabetes mellitus

Characteristics		Case (N= 50)	Control (N= 50)	P
Age (Years)	Mean± SD	12.5± 2.0	12.7± 2.8	^0.663
	Range	10.0- 18.0	10.0- 18.0	
BMI (Kg/M2)	Mean± SD	0.2± 1.0	0.4± 1.0	^0.166
	Range	- 2.2- 2.8	- 1.6- 2.5	
Sex	Male	22 (44.0%)	23 (46.0%)	#0.841
	Female	28 (56.0%)	27 (54.0%)	
Family History		32 (64.0%)	19 (38.0%)	#0.009*

^Independent t- test, #Chi square test, *Significant

Diabetic patients showed significantly higher RBG, HA1C and Microalbuminuria compared to controls (p<0.001) Table (2).

Table (2) Comparison between Diabetic patients and control groups regarding RBS, HA1C and Microalbuminuria.

Characteristics		Case (N= 50)	Control (N= 50)	P
Rbg (Mg/Dl)	Mean± SD	195.8± 45.9	104.5± 3.0	<0.001*
	Range	111.0- 320.0	98.0- 112.0	
HbA1c	Mean± SD	8.0± 1.4	6.2± 0.2	<0.001*
	Range	6.0- 12.0	5.6- 6.7	
HbA1c> 7.5		28 (56.0%)	0 (0.0%)	<0.001*
Microalbuminuria		10 (20.0%)	0 (0.0%)	<0.001*

^Independent t- test, *Significant

Mutant alleles (GG/AG) were significantly more frequent among diabetic patients than among control group as well as G allele (P<0.001) table (3) and figure (1)

Table (3) Comparison between diabetic patients and control group regarding distribution of CTLA4 genotypes and alleles

Genes		Case (N= 50)	Control (N= 50)	#P	Or (95% Ci)
Types	Homo. GG	15 (30.0%)	3 (6.0%)	<0.001*	23.33 (5.10-106.08)
	Hetero. AG	29 (58.0%)	19 (38.0%)	<0.001*	7.12 (2.48- 20.45)
	Wild AA	6 (12.0%)	28 (56.0%)		Reference
Mutation	Mutant GG/AG	44 (88.0%)	22 (44.0%)	<0.001*	9.33 (3.38- 25.87)
	Wild AA	6 (12.0%)	28 (56.0%)		Reference
Alleles	G	59 (59.0%)	25 (25.0%)	<0.001*	4.32 (2.36- 7.89)
	A	41 (41.0%)	75 (75.0%)		Reference

#Chi square test, *Significant

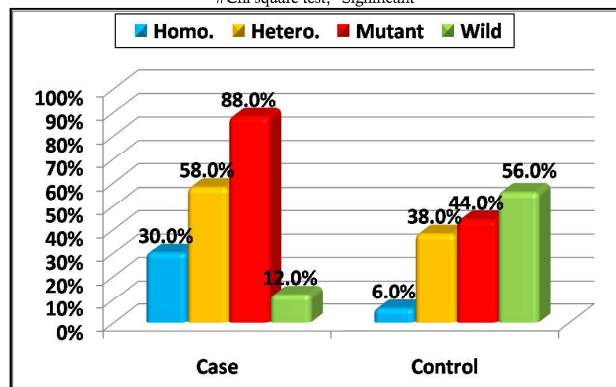


Figure (1) Comparison between diabetic patients and control group regarding genotypes of CTLA4 gene

Table (4) and figures (2), (3) show that: Mutant genes had significantly higher insulin dose and lower age of onset of diabetes.

Table (4) Comparison between mutant and wild genes regarding clinical characteristics among diabetic patients

Genes	Mutant (N= 44)	Wild (N= 6)	Mut./ Wild
Age at onset (years)	8.9± 3.1	13.0± 1.4	^0.011*
Insulin Dose (Unit)	52.8± 21.1	29.8± 4.7	^0.002*
Insulin Type	Interm.	4 (66.7%)	#0.756
	Long	2 (33.3%)	
Nephropathy	7 (15.9%)	1 (16.7%)	#0.962
Previous Hypoglycemia	12 (27.3%)	1 (16.7%)	#0.578
Previous Ketacidosis	29 (65.9%)	3 (50.0%)	#0.446

^Independent t- test, #Chi square test, *Significant

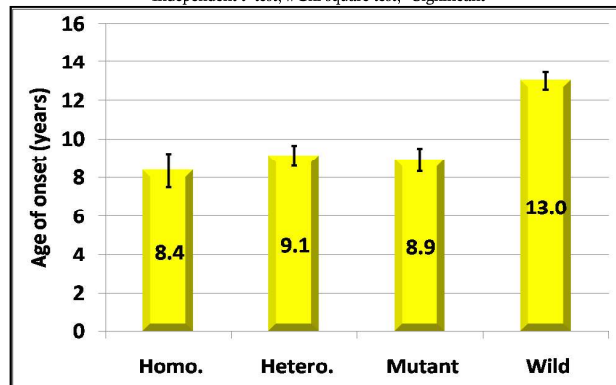


Figure (2) Comparison between different genotypes regarding age of onset among diabetic patients

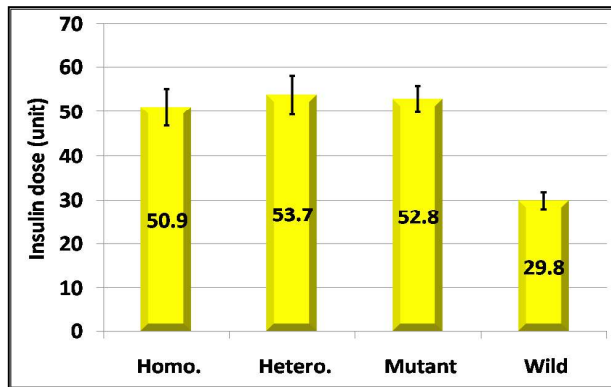


Figure (3) Comparison between different genotypes regarding insulin dose among diabetic patients

Discussion:

Type 1 diabetes (T1D) is an organ- specific autoimmune disease that results from T-cell- mediated destruction of insulin- producing pancreatic beta cells. This autoimmune disorder involves both genetic and environmental factors (Eizirik et al., 2009).

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.

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 (Carreno et al., 2000& Wells et al., 2001)

In 1996, the cytotoxic T- lymphocyte antigen- 4 gene (CTLA- 4) encoded on chromosome 2q33 was identified as a further type 1 diabetes susceptibility gene. CTLA- 4 is a surface molecule found on activated T- cells which produces a negative signal by inhibiting the T-cell receptor signaling complex ligand interactions (blocks binding of CD80 and CD86). Two major splice forms exist- encoding membrane bound and soluble forms. When CTLA- 4 is knocked out, lymphoproliferative disorders result. An A49G polymorphism in exon 1 of CTLA- 4 changes the amino acid sequence resulting in reduced cell surface expression. It is thought that inherited changes in CTLA- 4 gene expression can increase T-cell self- reactivity and therefore play an important role in autoimmune diseases such as type 1 diabetes. (Gillespie K., 2014)

CTLA- 4 gene has been described with an A/G polymorphism at position 49 in exon 1 leading to three genotypes, GG and AA homozygotes and AG heterozygote

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 (Gale and Gillespie., 2001). This was denied in a more recent study that reported equal incidence in both genders with female predominance only in autoimmune diseases (Soltesz et al., 2007).

There was no difference of statistical significance between patients and controls as regards age and sex thus allowing us to compare both groups

Positive family history of diabetes was significantly more frequent in diabetic patients than control group in this study ($P= 0.009$). This is expected as the pathogenesis of diabetes mellitus type 1 is multifactorial. Both genetic predisposition and environmental factors are involved in the deregulation of immune system with subsequent breakage of self- tolerance (Krejsek et al., 2004).

In the present work, the mean random blood glucose was significantly higher in diabetic patients (195.8 ± 45.9 mg/dL) compared to control group (104.5 ± 3.0 mg/dL) ($P < 0.001$).

Patients in diabetic group exhibited significantly higher HbA1c and Microalbuminuria levels than did healthy subjects.

In the current study, CTLA- 4 genotyping among the diabetic group: was the mutant homozygous genotype GG in 15 (30%), the mutant heterozygous genotype AG in 29 (58%) and wild homozygous genotype AA in 6 (12%). However, among the control group: it was 3 (6%) with GG genotype, 19 (38%) with AG genotype and 28 (56%) with AA genotype with P value < 0.001 which denoting a higher prevalence of AG and GG genotype in diabetic group with highly statistical significance. The same finding was found by El wafai et al. (2011) who conducted the same study but on different ethnic groups and found the CTLA- 4 genotypes in diabetic patients as follow: GG genotype (23.1%), A G (53.8%) and AA (23.1%). However, among the control group: it was (0%) with GG genotype, (45.66%) with AG genotype and (54.34%) with AA genotype with P value= 0.0034.

In the current study, the frequency of G allele in the diabetic group 59 (59%) and that of A allele 41 (41%) however, among the control group the frequency of G allele 25 (25%) and that of A allele 75 (75%). Diabetic Patients have a higher number of mutant alleles than control group and it is statistically highly significant (p value < 0.001) so the G allele is associated with the type 1 diabetes. This finding was similar to Mosaad et al. (2012) who found that CTLA- 4 G allele was significantly increased in T1D patients than in control group ($P= 0.047$). In our study CTLA- 4 +49 mutant genes did not have any impact on complications of type 1 diabetes. Neither has it shown an impact on HbA1c. This result is correspondent with that reported by Mosaad, et al. (2012) who also reported that the CTLA- 4 GG genotype wasn't associated with grades of diabetic control or diabetic complication

This work showed that there was a significant association between CTLA- 4 mutant genotypes and patients with younger age of onset of diabetes ($P= 0.011$) and higher dose of insulin ($P= 0.002$). This result is correspondent with that reported by Mosaad, et al. (2012) who 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.

The lack of agreement between our results and those of other authors may be due to unrecognized differences in environmental exposures, may be possibly due to differences in age, ethnicity, atopic status and disease severity among the populations, Another possible explanation may be that

sample size has an effect on the contradictory result.

From this study we concluded that the CTLA- 4 A/G +49 polymorphism was associated with type 1 diabetes in Egyptian children with a significant association between CTLA- 4 mutant genotypes and patients with younger age of onset of diabetes and higher dose of insulin. However, this polymorphism did not have any impact on complications of type 1 diabetes.

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