ORIGINAL ARTICLES

Investigation of *CTLA-4* and *CD28* Gene Polymorphisms in Patients with Diabetes Mellitus Type 2 Using PCR-RFLP in a Turkish Population

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ABSTRACT

Objective: *The aim of this study is to investigate whether specific polymorphisms in the CTLA-4 and CD28 gene are associated with Type 2 diabetes mellitus (T2DM).*

Methods: *Blood samples were collected from 241 individuals (72 patients with T2DM and 169 healthy individuals) and DNA was isolated. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to detect the frequencies of CTLA-4 NM_005214.3:c.49A > G and c.-319C > T, and CD28 NM_006139.1:c.534+17T > C polymorphisms in T2DM patients in the Sanliurfa Population.*

Results: *The data suggested that body mass index (BMI), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-c) and haemoglobin A1c (HbA1c) were significantly higher in T2DM patients than in the control individuals ($p < 0.05$). No significant differences were observed for the frequencies of c.49A > G, c.-319C > T genotype and allele of CTLA-4 gene and c.534+17T > C of the CD28 gene in T2DM patients compared to healthy individuals (*$p > 0.05$*).*

Conclusion: *The CTLA-4 gene c.49A >G and c.-319C >T and CD28 gene c.534+17T > C polymorphisms did not represent an important risk factor for this disease in a group of the Turkish population.*

Keywords: *CD28*, *CTLA-4,* T2DM, polymorphism

Investigación de Polimorfismos en los Genes CTLA-4 y CD28 en Pacientes con Diabetes Mellitus Tipo 2 Usando PCR-RFLP en una Población Turca

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RESUMEN

Objetivo: *El objetivo de este estudio es investigar si los polimorfismos específicos en los genes CTLA-4 y CD28 se hallan asociados con la diabetes mellitus tipo 2 (DMT2).*

Métodos: *Se recogieron muestras de sangre de 241 individuos (72 pacientes con DMT2 y 169 individuos sanos) y se aisló el ADN. Se usó un método de polimorfismo de la longitud de los fragmentos de restricción-reacción en cadena por la polimerasa (PCR-RFLP), con el fin de detectar las frecuencias de CTLA-4 NM_005214.3:c.49A > G y c. -319C > T, y los polimorfismos CD28 NM_006139.1:c.534 + 17T > C en pacientes con DMT2 en la población de Sanliurfa.*

Resultados: *Los datos sugirieron que el índice de masa corporal (IMC), el colesterol total (CT), el triglicérido (TG), el colesterol de lipoproteína de baja densidad (CLBD) y la hemoglobina A1c (HbA1c) eran significativamente más altas en los pacientes con DMT2 que en los individuos del grupo control ($p < 0.05$). No se observó diferencia significativa alguna en relación con las frecuencias de c.49A >*

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*G, c. -319C > el genotipo de T y el alelo del gene CTLA-4 y c.534+17T > C del gene de CD28 en pacientes con DMT2 comparados a los individuos sanos (*p > *0.05).*
Conclusión: *Los polimorfismos del gene CTLA-4 c.49A > G y el gene de c. -319C > T, y los polimorfismos del gene CD28 c.534 + 17T > C no representaron un factor de riesgo importante para esta enfermedad en un grupo de la población turca.*

Palabras claves: *CD28*, *CTLA-4,* DMT2, polimorfismo

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INTRODUCTION

Diabetes mellitus (DM) is a heterogeneous group of disorders characterized by increased amounts of plasma glucose. The two major types of diabetes, called Type 1 diabetes (T1DM) and Type 2 diabetes (T2DM), display a markedly different pathophysiological disease process (1). T2DM is a multifactorial disorder caused by interactions between genetic and environmental factors (2). The Finland – United States Investigation of NIDDM Genetics (FUSION) (2), the Diabetes Genetics Initiative (DGI) (3) and the Wellcome Trust Case Control Consortium (WTCCC) (4) studies represent, in aggregation, more than 32 000 individuals which confirmed the impact of *PPARγ, KCNJ11, TCF7L2, HHEX/IDE, SLC30A8, FTO,CDKAL1, CDKN2A/B*, *IGF2BP2, KCNQ1* on the risk of development of T2DM.

Both the cytotoxic T-lymphocyte-associated protein 4 (*CTLA-4*) and CD28 molecule (*CD28*) genes are located on chromosome 2 (2q33) and consist of 4 exons, spanning 6175-bp and 31359-bp, respectively (5). CTLA-4 and CD28 co-stimulatory molecules are key regulatory elements in T-cell/antigen-presenting cell interaction and in retaining peripheral tolerance (6, 7). *CTLA-4* gene mediates antigen-specific apoptosis and progressive pancreatic β-cell failure which is a typical feature of T2DM (8). Recent data suggested that apoptosis mechanisms might explain insulin deficiency through a reduction in absolute pancreatic β-cell number (9). T lymphocyte receptors CD28 and CTLA-4 bind co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) on antigen-presenting cells and regulate T cell activation. CD28 promotes T-cell expansion on CTLA-4 and limits the immune response by inhibition of T cell activity (6). Because CTLA-4 and CD28 proteins mediate antigen-specific apoptosis (10), they might be candidate genes conferring susceptibility to T2DM. T2DM has also been postulated as a disease of the innate immune system (11). There is increasing evidence that an ongoing T-lymphocyte activation and cytokine-induced inflammatory response are closely related to the pathogenesis of T2DM (12).

The population of the Sanliurfa province in the Southeastern Anatolia Region of Turkey is about 383 870 and the total fertility rate is 4.83 in this province, whereas, the mean in Turkey is 2.59. In addition, the rate of population growth is 3.6 per cent per year, much higher than the national average of 1.8 per cent (13). Obesity, consanguineous marriage, blood pressure, total cholesterol, HDL-cholesterol and triglycerides were more prevalent in T2DM patients (14).

Several studies indicated that the *CTLA-4* gene c.49A > G polymorphism was associated with several autoimmune diseases, such as systemic lupus erythematosus (15), Hashimoto thyroiditis (16), Graves' disease (17) and T1DM (18–20). Furthermore, no association with *CD28* gene polymorphism has been observed for T1DM (21). Conflicting data exist as to whether the *CTLA-4* gene c.49A > G polymorphism is associated with T2DM (7, 19, 22–24). The allele frequencies of A and G of *CTLA-4* c.49A> G polymorphisms in patients with T2DM were not significantly different from controls in different populations (7, 19, 23). Plasma concentrations of soluble CTLA-4 and CD28 were not significantly lower in T2DM patients compared to healthy controls (12). These findings suggest that *VDR* is a novel candidate gene for both types of diabetes.

However, *CD28* gene c.534+17T > C polymorphism has not been studied in T2DM. The aim of the current study is to investigate whether or not the *CTLA-4* and *CD28* polymorphisms are important genetic factors associated with T2DM.

SUBJECTS AND METHODS

Study Population and DNA extraction

This study was conducted in Sanliurfa in the southeastern Anatolia region of Turkey during the period September 2007 – September 2008. This was a case-control study, designed to determine the relationship between T2DM and *CTLA-4/CD28* gene polymorphisms.

Seventy-two patients with T2DM (39 females, mean ages: 59 ± 3 and 33 males: mean ages: 55 ± 5) were newly-diagnosed with fasting plasma glucose (FPG) levels ≥ 126 mg/dL after 12 hours fasting and confirmed by repeated testing on a different day according to the World Health Organization criteria (25) from the Regional Reference Hospital in Harran University, Turkey. Healthy controls with no clinical evidence or family history of diabetes mellitus in first-degree relatives or other autoimmune disorders, were arbitrarily selected from the consecutive blood donors in the blood-donation unit located in the same hospital. One hundred and sixty-nine healthy controls (79 females, mean age: 57 ± 4; and 90 males mean age: 56 ± 3) who did not have any

disease, were tested for FPG. Control individuals with FPG levels more than 100 mg/dL and with a family history of diabetes were excluded from the study. In addition, all patients and healthy controls were tested for body mass index (BMI), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c) and Haemoglobin A1c (HbA1c). All study subjects came from Sanliurfa province in southeastern Turkey and were of the same ethnic origin. The T2DM patients were matched with healthy controls for age and gender. EDTA-blood was taken from these individuals and genomic DNA was extracted from peripheral blood leukocytes by using standard salting-out procedure (26).

PCR-RFLP Technique

The g.206A > G or c.49A > G (+49A > G) (rs231775) and c.-319C > T (-318C > T) (rs5742909) polymorphic sites of *CTLA-4* ((NM_005214); GI: 1493) and the g.23315T > C or c.534+17T > C (IVS3+17T > C) (rs3116496) of *CD28* ((NM_006139); GI: 940) genes were investigated by PCR-RFLP technique. To amplify the regions containing the polymorphic sites of both genes, PCR reactions were performed in a 10-µl reaction volume containing 1xPCR buffer, 2 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTPs, Fermentas, St.Leon-Rot, Germany), 40 ng of DNA, 0.5 unit of Taq DNA Polymerase (Fermentas) and 0.2 µM of each primer (Bio Basic Inc, Ontario, Canada). Primer sequences were designed according to the published papers (15, 27) and PCR programme settings are shown in Table 1.

Five-microliter PCR product of c.49A > G was digested with 1.5 Units of *Bbv*I (*Bse*XI) at 37˚C. The PCR product of c.-319 C > T was digested with 1.5 Units *Mse*I (*Tru1*I) at 65˚C. Finally, the c.534 + 17T > C of *CD28* gene was digested with 1.5 Units *Eco47*III (Fermentas, St. Leon-Rot, Germany) at 37˚C. The total reaction volume was 20-µl and the digestion was allowed to proceed for two hours.

The digested PCR products were separated on 3% agarose gel and analyzed using the Alpha Imager System (AlphaInnotech, San Leandro, California USA). The digested *CTLA-4*: c.49A allele yielded fragment of a 162 bp and G allele yielded two fragments of 90 and 72 bp (Fig. 1). The c.-319C allele yielded fragments of 226 and 21 bp and the T allele yielded 130, 96, 21 bp fragments (Fig. 2). On the other hand, *CD28*: c.534+17T allele yielded 126, 22 bp and C allele yielded a 148 bp fragment (Fig. 3).

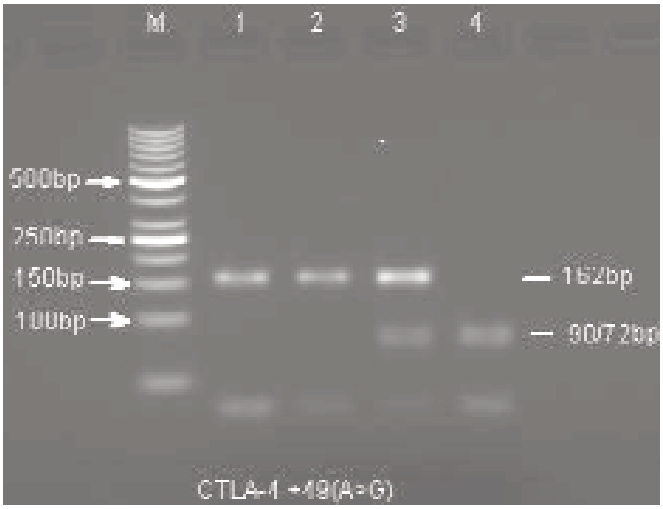

Fig. 1: The *Bbv*I (*Bse* XI) restriction profiles of the c.49A> G polymorphic site. Lane M shows DNA ladder (100-1500bp); lane 1 shows undigested PCR product (162 bp); lane 2 shows AA genotype (homozygous, wild-type); lane 3 shows AG genotype (heterozygous); lane 4 shows GG genotype (homozygous, polymorphic). (90 and 72 bp fragments could not be separated from each other on the gel).

Student's *t*-test was used to determine differences in means of demographic and clinical profiles by using the SPSS statistics programme (Table 2). Genotype and allele frequencies of *CTLA-4*: c.49A > G and c.-319C > T and *CD28*: c.534+17T > C were tested for Hardy-Weinberg equilibrium using the chi-square test. Genotype and allele frequencies of these polymorphisms were analysed by a *chi-square* test with continuity correction. Statistical signifi-

Table 1: Primers sequences used in the study

SNPs	Primer sequences	PCR conditions	PCR product (bp)
CTLA-4 gene c.49A>G	5'-GCTCTACTTCCTGAAGACCT-3' 5'-AGTCTCACTCACCTTTGCAG-3'	94˚C for 3 min, 40 cycles: 94˚C for 3 min, 30 cycles: 72˚C 30s; and 72˚C for 5min	162
c.-319C>T	5'-AATGAATTGGACTGGATGG-3' 5'-TTACGAGAAAGGAAGCCGTG-3	94˚C for 30s, 60˚C for 30s, 94˚C for 45s, 58˚C for 45s, 72˚C 30s; and 72˚C for 5min	247
C28 gene c.534+17T>C	5'-TTTTCTGGGTAAGAGAAGCAGCGC-3' 5'-GAA CCT ACT CAA GCA TGG GG-3'	94˚C for 3 min, 30 cycles: 94˚C for 30s, 62˚C for 30s, 72˚C 30s; and 72˚C for 5min	148

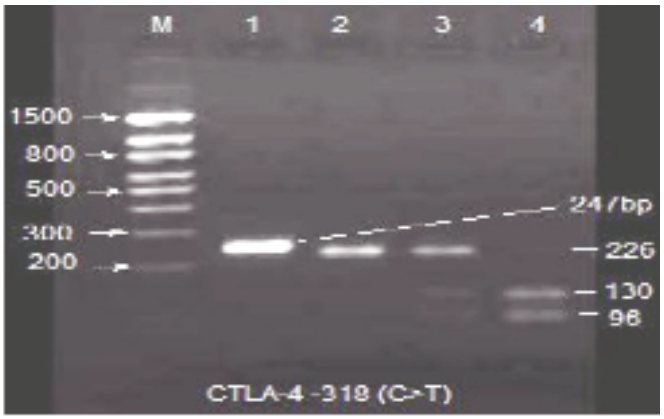

Fig. 2: The *Mse*I (*Tru1*I) restriction profiles of the c.-319C> T polymorphic site. Lane M shows DNA ladder (100–1500bp); lane 1shows undigested PCR product; lane 2 shows CC genotype (homozygous, wild-type); lane 3 shows CT genotype (heterozygous); lane 4 shows TT genotype (homozygous, polymorphic). (21-bp DNA fragment is not visible on the gel).

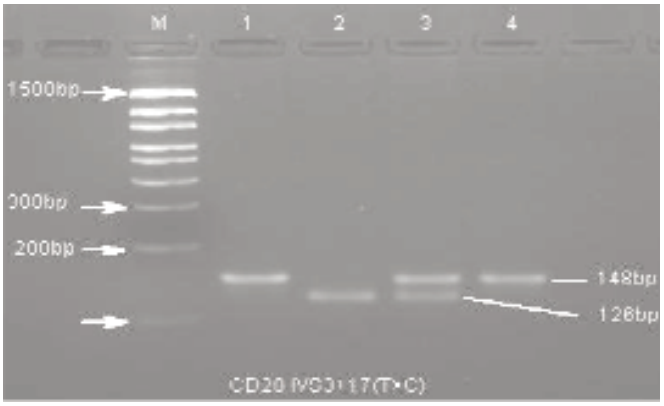

Fig. 3 The *Eco47* III restriction profiles of the c.534+17T> C polymorphic site. Lane M shows DNA ladder (100–1500bp); lane 1 shows undigested PCR product; lane 2 shows TT genotype (homozygous, wild-type); lane 3 shows TC genotype (heterozygous); lane 4 shows CC genotype (homozygous, polymorphic); (22-bp DNA fragment is not visible on the gel).

cance was defined as $p < 0.05$. The odds ratio (OR) was calculated to measure the strength of the association observed (Table 3).

The Harran University Medical Faculty Institutional Review Board approved the study and written informed consents were obtained from all patients. The study complied with the Helsinki Declaration.

RESULTS

The *CTLA-4*: c.49A > G (in exon 1) and c.-319C > T (in promoter), and *CD28*: c.534+17 T > C (in intron 3) polymorphisms were analysed in all 72 patients and 169 controls. The distribution of the genotypes for three polymorphic sites was consistent with the Hardy-Weinberg equilibrium in the T2DM and control groups ($p > 0.05$).

The BMI, FPG, TC, TG, LDL-c, HbA1c and family history of diabetes were significantly different between T2DM patients and healthy subjects. However, no significant difference was observed in gender, age and HDL-c levels between both groups. In addition, out of the 72 T2DM patients, 48 had a family history of T2DM, but none had a family history of T2DM in 169 healthy controls (Table 2).

Table 2: The distribution of demographic and clinical variations in T2DM patients and control group.

Variations	T2DM patients	Healthy controls	p- value
Subjects (n)	72	169	
Gender (M/F)	33/39	90/79	
Age (years)	57.1 ± 10.8	56.1 ± 6.8	0.386
BMI (kg/m^2)	30.1 ± 4.3	25.1 ± 2.4	0.001
FPG (mg/dL)	232.9 ± 88	91.4 ± 5.3	0.001
TC (mg/dL)	213.2 ± 43.6	173.3 ± 13.3	0.001
TG (mg/dL)	179.6 ± 78.4	131.9 ± 15.5	0.001
HDL-C (mg/dL)	53.0 ± 7.6	53.48 ± 8.0	0.672
LDL-C (mg/dL)	140.2 ± 42.2	110.6 ± 17.2	0.001
HbA1c (%)	8.8 ± 1.9	5.0 ± 1.4	0.001
No. of family history	48	–	–

Values are mean ± standard deviation, M: male, F: female

Furthermore, there was no significant association between the *CTLA-4* gene c.49GG ($p = 1.000$, OR (95% CI) = 1.072 (0.359-3.204), and c.-319TT ($p = 0.760$, OR (95% CI) = 0.691 (0.185-2.590), and *CD28* gene c.534 + 17CC ($p = 1.000$, OR (95% CI) =1.181 (0.287-4.858) genotypes and T2DM patients compared with the healthy control group (Table 3).

DISCUSSION

T2DM, previously referred to as non-insulin-dependent diabetes or adult-onset diabetes, is a term used for individuals who have insulin resistance and usually have a relative (rather than absolute) insulin deficiency. However, the genetic factors underlying this form of diabetes are complex and not clearly established (1).

CTLA-4 and *CD28* genes have been mapped on chromosome 2q33 and represent a key regulatory element between the T cell and antigen-presenting cell interaction (5). These genes mediate progressive pancreatic β-cell failure which is a typical feature of T2DM (9, 10). In contrast to *CD28*, *CTLA-4* attributes negative signals to T cells (15). T2DM is an increasingly prevalent metabolic disease in which the amount of insulin produced by the pancreas is inadequate to meet body needs. T2DM has also been postulated as a disease of the innate immune system (11).

In the present study, though BMI (30.1 ± 4.3), FPG (232.9 ± 88), TC (213.2 ± 43.6), TG (179.6 ± 78.4), LDL-c (140.2 ± 42.2) and HbA1c (8.8 ± 1.9) levels in T2DM patients were significantly different from the healthy group (25.1 ± 2.4, 91.4 ± 5.3, 173.3 ± 13.3, 131.9 ± 15.5, 53.48 ±

Table 3: *CTLA-4* and *CD28* polymorphism in Turkish subjects with T2DM and controls

SNPs	T2DM patients (n = 72)	Healthy controls (n = 169)	X^2	OR (95% CI)	p-value
CTLA-4: c.49A>G					
Genotypes					
AA	43 (59.7%)	113 (66.9%)		Reference	
AG	24 (33.3%)	45 (26.6%)	0.807	1.378 (0.758-2.503)	0.350
GG	5 (6.9%)	11 (6.5%)	0.015	1.072 (0.359-3.204)	1.000
Alleles					
A	110 (76.4%)	271 (80.2%)		Reference	
G	34 (23.6%)	67 (19.8%)	0.661	1.250 (0.783-1.997)	0.392
CTLA-4: c.-319C>T					
Genotypes					
CC	55 (76.4%)	116 (68.6%)		Reference	
CT	14 (19.4%)	43 (25.4%)	0.702	0.707 (0.359-1.394)	0.402
TT	3 (4.2%)	10 (5.9%)	0.303	0.691 (0.185-2.590)	0.760
Alleles					
C	124 (86.1%)	275 (81.4%)		Reference	
T	20 (13.9%)	63 (18.6%)	1.283	0.704 (0.408-1.215)	0.257
CD28: c.534+17T>C					
Genotypes					
TT	47 (65.3%)	111 (65.7%)		Reference	
TC	22 (30.6%)	52 (30.8%)	0.000	0.990 (0.544-1.801)	1.000
CC	3 (4.2%)	6 (3.6%)	0.053	1.181 (0.287-4.858)	1.000
Alleles					
T	116 (80.6%)	274 (81.1%)		Reference	
C	28 (19.4%)	64 (18.9%)	0.000	1.033 (0.630-1.694)	0.997

X^2 = Chi-square = OR: Odds ratio, CI = Confidence interval, SNP = Single Nucleotide Polymorphism

8.0, 110.6 ± 17.2, and 5.0 ± 1.4, respectively) [Table 2], we did not observe any significant difference between the frequencies of c.49A > G and c.-319C >T alleles and genotypes of *CTLA-4* gene and c.534 + 17T > C of *CD28* gene in both groups (Table 3). The few studies conducted to date on the c.49A > G polymorphism of *CTLA-4* as a risk factor for T2DM have revealed controversial results. Recent studies suggested that the distribution of genotype and allele frequencies of *CTLA-4* gene c.49A > G polymorphism were similar among T2DM patients and controls (7, 19, 23). The results were consistent with the data reported by these authors. The allele frequencies of *CTLA-4* gene polymorphism G and A in T2DM patients who are positive for antiglutamic acid decarboxylase antibody were significantly different from healthy individuals in the Japanese population (22). Additionally, *CTLA-4* gene c.49 GG or AG genotypes are independent risk factors for developing latent autoimmune diabetes (LADA) in adults in the Estonian population (24). The *CTLA-4* gene is important in maintaining peripheral tolerance and its polymorphism might thus contribute to the failure in controlling beta-cell autoimmunity. Consanguineous marriages appear to be an important factor in causing the high frequency of this disease (14). There were consanguineous marriages in Sanliurfa population. In the same way, the total fertility rate in Sanliurfa province is higher than in Turkey overall. In addition, the rate of population growth is 3.6 per cent per year, much higher than the national average of 1.8 per cent (13). Genome-wide linkage scans studies have confirmed an association between variants in or close to different genes and T2DM. Variants in these genes show varying degrees of association to T2DM in different populations, most probably due to differences in allele frequencies. In complex diseases, most of the genetic variants contributing to disease are common susceptibility alleles contributing only small, but important, effects on disease. These small genetic effects, in combination with environmental factors, make it hard to locate true susceptibility genes for complex diseases. Due to the multifactorial nature of complex diseases, efforts to limit the genetic heterogeneity in studies of such diseases are undertaken, in the hope of increasing the probability of finding true disease susceptibility genes (2–4).

Up to now, six studies, including this one, have been conducted worldwide on *CTLA-4* c.49A> G between T2DM and healthy controls but the others were not in Turkey (7, 19, 22–24). This is, to our knowledge, the first study investigating whether *CTLA-4* and *CD28* polymorphisms are associated with T2DM patients in Turkey. Additionally, we demonstrated that the distribution of genotype and allele frequencies of *CD28* gene c.534+17T > C was not signi-

ficantly association in T2DM compared with controls (Table 3). Soluble CTLA-4 molecule can act as a competitor of CD28 to bind with CD80 or CD86, thereby interfering with T-lymphocyte activation. In the same way, it has been shown that plasma concentration of sCTLA-4 was significantly lower, whereas sCD28 was significantly higher in T2DM patients with nephropathy when compared to controls (12). However, the association between *CD28* gene polymorphism and T2DM has not been reported previously. Wood *et al* suggested that the *CD28* intron 3 (c.534+17T > C) polymorphism does not appear to be associated with susceptibility to T1DM, whereas the *CTLA-4* was found to be associated with the same disease (21).

Further studies are necessary to examine the immunological function and polymorphism of the *CTLA-4* and *CD28* genes and their roles in the pathogenesis and progression of T2DM. Both of these genes appear to be good candidates for influencing risk for T2DM, since it is possible that they may be mediators of progressive β-cell failure. Studies of larger numbers of cases and controls will be required in order to determine the influence of these genes and other candidate genes and single nucleotide polymorphisms on risk of T2DM.

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