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## Association of CTLA-4 Gene 49A/G Polymorphism with the Incidence of Type 1 Diabetes Mellitus in the Iranian Kurdish Population

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**Abstract:** Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) has an inhibitory function on T cells and is critical for the induction of peripheral tolerance. CTLA-4 +49 G allele affects the CTLA-4 function and has been reported to be correlated with a higher risk of various autoimmune diseases including type 1 diabetes (T1D). The present study was conducted to investigate the association between the polymorphism of the CTLA-4 exon 1+49 A/G and susceptibility to T1D and type 2 diabetes (T2D) in Kurds living in Iranian Kurdistan. The +49 A/G polymorphism was analyzed in 60 patients with T1D, 56 patients with T2D and 107 control subjects using PCR Single-strand Conformation Polymorphism (SSCP) and restriction fragment length polymorphism methods. All studied populations (T1D, T2D and Controls) were in Hardy-Weinberg equilibrium ( $p$ , 0.39, 0.94 and 0.89, respectively). Both +49 G allele ( $p = 0.015$ , OR = 1.86) and +49 A/G genotype frequencies ( $p = 0.012$ , OR = 2.31) were significantly higher in T1D patients than control. There was significant over-representation of the G allele in female T1D patients. No significant differences in +49 G allele and +49 A/G genotype frequencies were found between T2D and control subjects. SSCP analysis did not show new mutation in the amplified segment. The results of this study indicate that CTLA-4+49 A/G gene polymorphism confers genetic susceptibility to T1D but not T2D in the Kurdish population living in Iranian Kurdistan and women carrying the +49 G allele are at greater risk of getting T1D than men having the G allele.

**Key words:** Type 1 diabetes, CTLA-4, polymorphism, susceptibility, loci

### INTRODUCTION

Type 1 diabetes (T1D) is a chronic disease in which pancreatic islet  $\beta$  cells will be targeted for destruction by the immune system (Atkinson and Eisenbarth, 2001). Genetic and environmental factors are involved in the fast growing incidence of the disease (Gale, 2002). High concordance rates in monozygotic and dizygotic twins indicate the importance of genetic factors in the etiology of T1D (Hyttinen *et al.*, 2003). Based on genetic traits, those at high genetic risk can be diagnosed at birth or before islet autoantibodies are detectable in plasma. The best time for treatment of T1D in susceptible subjects is the period between progressing islet beta-cell destruction and the appearance of the symptoms of T1D, when there are enough functional beta cells remaining to be rescued through various intervention methods (Van der Auwera *et al.*, 2002; Van Belle *et al.*, 2011).

Human Leukocyte Antigen (HLA) is the most important genetic determinant of T1D. It has been

guessed that HLA-mediated susceptibility can be accounted for about half of the genetic risk of T1D (Meheers and Gillespie, 2008). Genome-wide association studies have resulted in the identification of a lot of non-HLA susceptibility loci for T1D including IDDM12 located on chromosome 2q33, that encodes for key lymphocyte co-receptor genes, including cytotoxic T lymphocyte antigen-4 (CTLA-4) and CD28 (Grant and Hakonarson, 2009). CTLA-4, also known as CD152, is a cell-surface transmembrane glycoprotein which is expressed mainly on CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets and plays a key role in the fine tuning of T-cell immunity by providing a negative signal for T-cell activation (Carreno *et al.*, 2000; Wells *et al.*, 2001). CTLA-4 indirectly, via competing with CD28 for CD80/86 binding attenuates stimulatory signal to T-cells.

There are contradictory results regarding the connection between the CTLA-4 gene polymorphisms and genetic susceptibility to some autoimmune diseases (Kristiansen *et al.*, 2000). Among CTLA-4 gene

polymorphisms, an adenine to guanine transition at position 49 of exon 1, rs231775, also known as +49A/G leads to an alanine to threonine amino acid substitution at codon 17 of the leader peptide (Nistico *et al.*, 1996). +49A/G leads to less efficient glycosylation and reduced expression of membrane CTLA4 protein (Anjos *et al.*, 2002). Reduced CTLA-4 expression and/or change in CTLA-4 activity result in uncontrolled T-cell-associated autoimmunity disease (Waterhouse *et al.*, 1995; Rudd and Schneider, 2003).

Some studies found an association between +49A/G and susceptibility to T1D (Marron *et al.*, 1997; Nistico *et al.*, 1996), whereas others found no evidence for such association (Owerbach *et al.*, 1997). Inconsistency in results illustrates the importance of further studies in various ethnicities. The Iranian Kurds, primarily living in the west of Iran, constitute approximately 7% of Iran's overall population. To our knowledge there is no existing data regarding the association between +49A/G and susceptibility to T1D or T2D in Iranian Kurdish population. The present study was conducted to investigate the association of CTLA-4 SNP +49A/G with a predisposition to T1D and T2D in Iranian Kurdish population.

## MATERIALS AND METHODS

The study subjects comprised 60 unrelated T1D patients, 56 T2D patients and 107 healthy controls with no previous history of T1D or other autoimmune diseases. Diabetic patients were recruited from Kurdistan province diabetes center located in Tohid hospital, Sanandaj. The diagnosis of diabetes mellitus in the center was based on the American Diabetes Association diabetes diagnostic criteria. The research protocol was approved by the ethics committee at the Kurdistan University of Medical Sciences. All subjects enrolled in the study signed consent and institutional ethical requirements were met.

**DNA Extraction and CTLA-4 Genotyping:** Genomic DNA was extracted from frozen whole blood using the Promega wizard DNA Purification Kit (Cat. #A1120). CTLA-4 +49A/G polymorphism was defined using a polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) method as described by Dilmec *et al.* (2008). The PCR was performed using DreamTaq PCR Master Mix (#K1071) in a 50  $\mu$ L reaction mixture containing DreamTaq PCR Master Mix (2X) 25  $\mu$ L, Forward primer 1.0  $\mu$ M, Reverse primer 1.0  $\mu$ M, Template DNA 50 ng, Water, nuclease-free to 50  $\mu$ L.

**PCR profile for CTLA-4 +49A/G polymorphism:** The PCR conditions comprised an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C

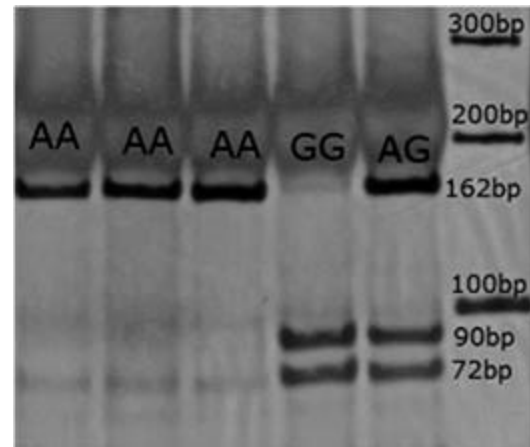


Fig. 1: Genotyping of CTLA-4 +49A/G polymorphism by Bbv I RFLP, From right: Lane 1: 100-300 bp DNA ladder, Lane 2: Heterozygous (AG, 162, 90 and 72 bp), Lane 3: Homozygous (GG, 90 and 72 bp) and Lanes 4, 5 and 6: Wild type (AA genotype, 162 bp)

for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 30 sec, with a final extension at 72°C for 6 min. 5  $\mu$ L PCR product (162 bp) was digested using 1.5 U of Bbv I (Bse XI, 100u #ER1451) in a 20  $\mu$ L volume overnight at 65°C. Digested A allele yielded a fragment of a 162 bp and G allele yielded 90 and 72 bp fragments (Fig. 1). The primers were 5'-GCT CTA CTT CCT GAA GAC CT-3' (forward) and 5'-AGT CTC ACT CAC CTT TGC AG-3' (reverse).

**Single-strand conformational polymorphism (SSCP):** For confirmation of RFLP results and finding possible new mutations in the amplicon, SSCP was performed as described (Takahashi *et al.*, 1995). For this purpose 2 $\mu$ L aliquot of the product was diluted in 48  $\mu$ L of denaturing loading buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol FF), heated at 95°C for 10 min and then immediately cooled on ice for 10 min. Aliquots of 7  $\mu$ L were then loaded on a 12% polyacrylamide gel at 8 W constant power for 16-18 h at 4°C and finally silver stained.

**Statistical analysis:** The distribution of genotypes for 49 A/G SNP was assessed for deviation from the Hardy-weinberg Equilibrium (HWE) by chi square testing. Genotype and allele frequencies of the 49 A/G SNP analyzed in cases and controls with the De Finetti program (<http://ihg.gsf.de/cgi-bin/hw/hwal.pl>). The dominant/ recessive effect of single (AA, AG and GG) and combined (e.g. AG/GG vs. AA) effects of CTLA-4 genotypes on phenotype (disease) expression was examined in studied population using the Finetti program.

**RESULTS**

**Genotype analysis:** Samples from 60 patients with T1D, 56 patients with T2D and 107 healthy controls were successfully genotyped for CTLA-4 +49A/G polymorphism. The genotype frequency distribution of +49A/G was in Hardy-Weinberg equilibrium in T1D, T2D and control groups (p, 0.39, 0.94 and 0.89, respectively). Genotype and allele frequencies of +49A/G in T1D patients were significantly different from both T2D and healthy controls (Table 1). T1D patients had a higher frequency of heterozygous +49A/G (Odds Ratio (OR) = 2.308, C.I. = 1.18-4.48, p = 0.01), G allele frequency difference, (OR = 1.86, C.I. = 1.12-3.08, p=0.01), allele positivity (AA versus AG+GG), (OR= 2.34, C.I. = 1.23-4.47, p = 0.01) versus both control and T2D groups. The Cochran-armitage test for trend results showed the presence of strong association between G allele with T1D (common OR = 1.85, p = 0.01) but not with T2D (OR = 1.10, p = 0.85). CTLA-4 49 G allele frequency was not increased in diabetic patients compared to controls (21.4 vs. 20.6%, not significant). The distribution of GG, AG and AA CTLA-4 genotypes was similar in T2D and control groups.

**Gender analysis:** The gender distribution of alleles and genotype frequencies of CTLA-4 +49A/G in T1D and control groups is shown in Table 2. T1D female patients had a higher frequency of heterozygous +49A/G (OR = 2.47, C.I. = 1.07-5.69, p = 0.03) and allele positivity (OR = 2.35, C.I. = 1.04-5.29, p = 0.03) but not homozygous G allele than female control groups. G allele frequency

difference, (OR = 1.74, C.I. = 0.92-3.31, p = 0.08) was not significant among female T1D patients and female control groups. The Cochran-armitage test for trend results did not show an association between G allele with T1D (OR = 1.63, p = 0.07). Male T1D patients did not show significant differences for homozygous G allele (p = 0.07), heterozygous +49A/G (p = 0.18) and allele positivity (p = 0.11) with the male control group.

**DISCUSSION**

Type 1 diabetes is a chronic autoimmune disease in which destruction or damaging of the pancreatic islet beta-cells results in insulin deficiency and hyperglycemia (La Torre, 2012). There have been intensive efforts in recent years to reverse or ameliorate the diabetic state in T1D patients or animal models of T1D through various methods including, immune interventions (Lemmark and Larsson, 2013), restoration of the beta-cell mass (Cam *et al.*, 1999; Ahmadi *et al.*, 2010) or potentiation of peripheral insulin action (Dehghani *et al.*, 1997). Those strategies are more effective in the earlier stages of the autoimmune attack, when there are enough remaining healthy islet beta cells. In this study the contributions of CTLA-4 +49 A/G genotype to T1D and T2D susceptibility was tested in Iranian Kurdish population. Statistically significant differences were found in both G allele and heterozygous A/G genotype distribution among T1D patients with both T2D patients and control subjects but not between T2D patients and controls. The T1D risk was not increased in the homozygous form of G allele probably due to relatively

Table 1: Allele and genotype frequencies of CTLA-4 +49A/G in T1D, T2D and control groups

Group	Allele	No. (percent of total)			p-value	
		T1D (n = 60)	T2D (n = 56)	Control (n = 107)	T1D	T2D
Allele	A	81 (67.5)	98 (78.6)	170 (79.4)	0.01*	0.85
	G	39 (32.5)	24 (21.4)	44 (20.6)		
Genotype	A/A	25 (41.7)	35 (62.5)	67 (62.6)	0.01*	0.90
	A/G	32 (53.3)	18 (32.1)	36 (33.6)		
	G/G	3 (5.0)	3 (5.0)	4 (3.7)		
	(AG+GG)	35 (58.3)	21 (37.5)	40 (37.4)		

\*Significant difference from the control group p-value<0.05, T1D: Type 1 diabetes, T2D: Type 2 diabetes

Table 2: The gender distribution of alleles and genotype frequencies of CTLA-4+49A/G in T1D and control groups

Group	Gender	Allele	No. (percent of total)				p-value	
			T1D		Control		Female	Male
			Female (n = 40)	Male (n = 20)	Female (n = 63)	Male (n = 44)		
Allele	Gender	A	55 (69)	26 (65)	100 (79.4)	70 (79.5)	0.08	0.08
		G	25 (31)	14 (35)	26 (20.6)	18 (20.5)		
Genotype	Gender	A/A	17 (42.5)	8 (40)	40 (63.5)	27 (61.4)	0.03*	0.18
		A/G	21 (52.5)	10 (50)	20 (31.7)	16 (36.3)		
		G/G	2 (5.0)	2 (10)	3 (4.8)	1 (2.3)		
		(AG+GG)	23 (57.5)	12 (60)	23 (36.5)	17 (37.4)		

\*Significant difference from (female) Control group, T1D: Type 1 diabetes, T2D: Type 2 diabetes

low number of homozygous form of G allele in both cases and controls. The CTLA4 49 A/G genotype was found to be more frequently present in female T1D patients rather than male patients as compared to related control groups.

The incidence and prevalence of T1D are rapidly increasing worldwide (Maahs *et al.*, 2010). Both genetic and environmental factors contribute to the risk of T1D (Gan *et al.*, 2012). Gene polymorphisms in at-risk populations tilt the balance in their immune system toward self-destruction (Jahromi and Eisenbarth, 2007). Detection of the potential T1D candidates in an early stage of diabetes is required for on-time and effective therapeutic interventions. The most important genetic susceptibility locus for T1D is attributed to the HLA class II region but polymorphisms of individual genes outside the MHC also contribute to diabetes risk (Grant and Hakonarson, 2009). CTLA-4 is a co-stimulatory receptor and a vital negative regulator of T cell activation. CTLA-4 gene is a potential candidate for T cell-mediated autoimmune disorders (Benmansour *et al.*, 2010; Chen *et al.*, 2013). There are several polymorphic regions within the CTLA-4 gene, including a CTLA-4 -318C/T, CTLA-4 49A/G and CTLA-4 3' (AT)<sub>n</sub>. Adenine to Guanine substitution at position +49 in exon 1 and resulting threonine to alanine substitution in the CTLA-4 molecule leads to incomplete glycosylation (Anjos *et al.*, 2002). Such changes in CTLA-4 molecule may influence immunoregulation and may be used as an explanation of the predisposition to autoimmune diseases in +49 G allele carriers (Vaidya and Pearce, 2004).

Inconsistent results have been reported regarding the association of CTLA4 +49A/G and susceptibility to T1D in different studies. Some studies conclude that the G allele of +49A/G is associated with a predisposition to T1D (Takara *et al.*, 2000; Tang *et al.*, 2012; Mosaad *et al.*, 2012; Douroudis *et al.*, 2009; Kavvoura and Ioannidis, 2005; Ueda *et al.*, 2003; Chen *et al.*, 2013) while others did not find such association (Lemos *et al.*, 2009; Fajardy *et al.*, 2002; Cinek *et al.*, 2002). The current data showed that AG genotype accounted for 53.3% of T1D patients in comparison to 33.6% in controls. An odds ratio of 1.85 indicates a strong association between G allele and T1D disease in Iranian Kurdish population. A similar finding has been reported by other researchers in different ethnicities (Baniasadi *et al.*, 2006; Turpeinen *et al.*, 2003; Cosentino *et al.*, 2002; Krokowski *et al.*, 1998; Genc *et al.*, 2004). Frequency of 49 G/G genotype was not significantly different between T1D patients and controls (5.0 vs. 3.7%), in this study, probably due to the small number of this genotype (n = 3 vs. n = 4, respectively). Mojtabedi *et al.* (2005) reported that +49A/G is associated with susceptibility to T1D in southern Iranian population. They found higher frequency of AG genotype in both

T1D patients and controls (71.5 vs. 45%) in comparison to what was found in this study (53.3 vs. 33.6%). Statistically speaking results were similar, in both studies significant difference was found in frequency of G allele between T1D patients and controls.

Even though, type 2 diabetes mellitus (T2D) has been considered to be a metabolic disease, new findings including latent autoimmune diabetes in adult patients (Pettersen *et al.*, 2010; Badaru and Pihoker, 2012) and the role of T cell modulation in the development of obesity-associated insulin resistance (Winer *et al.*, 2011) makes it difficult to distinguish the borders between T1D and T2D. To find out if +49 A/G polymorphism is associated with T2D, the distribution of alleles as well as the genotypic frequencies were analyzed in T2D patients. No significant association was found between G allele/genotype and T2D disease, indicating that +49 G allele/genotype is not a major risk factor for T2D. The result of this study is in agreement with what reported before in T2D patients in Estonian and German populations (Haller *et al.*, 2004; Rau *et al.*, 2001). It looks carrying G allele or GG genotype is not a risk factor for T2D, but it may affect other aspects of T2D, like age at disease manifestation (Rau *et al.*, 2001).

Based on the current data, higher numbers of female T1D patient are carriers of +49 G allele/genotypes compared to male T1D patients. Even though gene polymorphisms are linked to susceptibility to T1D, autoimmunity trigger factors appears to be specific for each affected individual (Peng and Hagopian, 2006). There may be epigenetic mechanisms that make females more susceptible to higher risk of carrying G alleles. Even though association of CTLA-4 +49 A/G with T1D was more profound in females, relatively small size of the studied population makes it difficult to get a firm conclusion. To confirm the gender dependence of the high risk CTLA-4 49A/G SNP in T1D patients and to avoid erroneous conclusion, more studies in a bigger sample size is needed. If the similar results can be repeated in larger population sizes, CTLA-4 49A/G SNP will find a more positive predictive value in risk-assessment of T1D in women.

In summary, the result of this study, supported by significant increase in frequency of +49 A/G genotype in T1D patients, show that CTLA-4 49 A/G is a genetic risk for T1D, but not T2D, in Iranian Kurdish population. Gender analyzes of data support the idea that women carrying +49 G allele are more likely to develop T1D but this proposal needs to be confirmed in a larger data set in follow-up studies. In conclusion, result of this study is in support of the engagement of CTLA-4+49 A/G in T1D pathogenesis and its potential application for screening potential T1D patients.

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## REFERENCES

- Ahmadi, S., S.M. Karimian, M. Sotoudeh, M. Bahadori and G.A. Dehghani, 2010. Pancreatic Islet beta cell protective effect of oral vanadyl sulphate in streptozotocin-induced diabetic rats, an ultrastructure study. *Pak. J. Biol. Sci.*, 13: 1135-1140.
- Anjos, S., A. Nguyen, H. Ounissi-Benkalha, M.C. Tessier and C. Polychronakos, 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.
- Atkinson, M.A. and G.S. Eisenbarth, 2001. Type 1 diabetes: New perspectives on disease pathogenesis and treatment. *Lancet*, 358: 221-229.
- Badaru, A. and C. Pihoker, 2012. Type 2 diabetes in childhood: Clinical characteristics and role of  $\beta$ -cell autoimmunity. *Curr. Diabetes Rep.*, 12: 75-81.
- Baniasadi, V., N. Narain, R. Goswami and S.N. Das, 2006. Promoter region-318 C/T and-1661 A/G *CTLA-4* single nucleotide polymorphisms and type 1 diabetes in North Indians. *Tissue Antigens*, 67: 383-389.
- Benmansour, J., M. Stayoussef, F.A. Al-Jenaidi, M.H. Rajab and C.B. Rayana *et al.*, 2010. Association of single nucleotide polymorphisms in cytotoxic T-lymphocyte antigen 4 and susceptibility to autoimmune type 1 diabetes in Tunisians. *Clin. Vaccine Immunol.*, 17: 1473-1477.
- Cam, M.C., B. Rodrigues and J.H. McNeill, 1999. Distinct glucose lowering and beta cell protective effects of vanadium and food restriction in streptozotocin-diabetes. *Eur. J. Endocrinol.*, 141: 546-554.
- Carreno, B.M., F. Bennett, T.A. Chau, V. Ling and D. Luxenberg *et al.*, 2000. CTLA-4 (CD152) can inhibit T cell activation by two different mechanisms depending on its level of cell surface expression. *J. Immunol.*, 165: 1352-1356.
- Chen, Z., M. Fei, D. Fu, L. Zhang and Y. Ma *et al.*, 2013. Association between cytotoxic T lymphocyte antigen-4 polymorphism and type 1 diabetes: A meta-analysis. *Gene*, 16: 263-270.
- Cinek, O., P. Drevinek, Z. Sumnik, B. Bendlova, S. Kolouskova, M. Snajderova and J. Vavrinec, 2002. The CTLA4 +49 A/G dimorphism is not associated with type 1 diabetes in Czech children. *Eur. J. Immunogenet.*, 29: 219-222.
- Cosentino, A., G. Gambelunghe, C. Tortoioli and A. Falorni, 2002. CTLA-4 gene polymorphism contributes to the genetic risk for latent autoimmune diabetes in adults. *Ann. N. Y. Acad. Sci.*, 958: 337-340.
- Dehghani, G.A., S. Ahmadi and G.R. Omrani, 1997. Effects of vanadyl sulphate on glucose homeostasis in severe diabetes induced by streptozotocin in rats. *Indian J. Med. Res.*, 106: 481-485.
- Dilmec, F., A. Ozgonul, A. Uzunkoy and F Akkafa, 2008. Investigation of CTLA-4 and CD28 gene polymorphisms in a group of Turkish patients with colorectal cancer. *Int. J. Immunogenet.*, 35: 317-321.
- Douroudis, K., A.P. Laine, M. Heinonen, R. Hermann and K. Lipponen *et al.*, 2009. Association of *CTLA4* but not *ICOS* polymorphisms with type 1 diabetes in two populations with different disease rates. *Human Immunol.*, 70: 536-539.
- Fajardy, I., A. Vambergue, C. Stuckens, J. Weill, P.M. Danze and P. Fontaine, 2002. CTLA-4 49 A/G dimorphism and type 1 diabetes susceptibility: A French case-control study and segregation analysis. Evidence of a maternal effect. *Eur. J. Immunogenet.*, 29: 251-257.
- Gale, E.A., 2002. The rise of childhood type 1 diabetes in the 20th century. *Diabetes*, 51: 3353-3361.
- Gan, M.J., A.A. O'Neill and M.J. Haller, 2012. Type 1 diabetes: Current concepts in epidemiology, pathophysiology, clinical care and research. *Curr. Problems Pediatr. Adolesc. Health Care*, 42: 269-291.
- Genc, S., K. Genc, O. Sercan, S. Kizildag and Z. Gulay *et al.*, 2004. Analysis of cytotoxic T lymphocyte antigen-4 (CTLA-4) exon 1 polymorphism in patients with type 1 diabetes mellitus in a Turkish population. *J. Pediatr. Endocrinol. Metab.*, 17: 731-735.
- Grant, S.F. and H. Hakonarson, 2009. Genome-wide association studies in type 1 diabetes. *Curr. Diabetes Rep.*, 9: 157-163.
- Haller, K., K. Kisand, V. Nemvalts, A.P. Laine, J. Ilonen and R. Uibo, 2004. Type 1 diabetes is insulin -2221 MspI and CTLA-4 +49 A/G polymorphism dependent. *Eur. J. Clin. Invest.*, 34: 543-548.
- Hytinen, V., J. Kaprio, L. Kinnunen, M. Koskenvuo and J. Tuomilehto, 2003. Genetic liability of type 1 diabetes and the onset age among 22,650 young Finnish twin pairs a nationwide follow-up study. *Diabetes*, 52: 1052-1055.

- Jahromi, M.M. and G.S. Eisenbarth, 2007. Cellular and molecular pathogenesis of type 1A diabetes. *Cell. Mol. Life Sci.*, 64: 865-872.
- Kavvoura, F.K. and J.P. Ioannidis, 2005. *CTLA-4* gene polymorphisms and susceptibility to type 1 diabetes mellitus: A HuGE Review and meta-analysis. *Am. J. Epidemiol.*, 162: 3-16.
- Kristiansen, O.P., Z.M. Larsen and F. Pociot, 2000. *CTLA-4* in autoimmune diseases—a general susceptibility gene to autoimmunity? *Genes Immun.*, 1: 170-184.
- Krokowski, M., J. Bodalski, A. Bratek, P. Machejko and S. Caillat-Zucman, 1998. *CTLA-4* gene polymorphism is associated with predisposition to IDDM in a population from central Poland. *Diabetes Metab.*, 24: 241-243.
- La Torre, D., 2012. Immunobiology of beta-cell destruction. *Adv. Exp. Med. Biol.*, 771: 194-218.
- Lemos, M.C., E. Coutinho, L. Gomes, M. Bastos and A. Fagulha *et al.*, 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.
- Lermark, A. and H.E. Larsson, 2013. Immune therapy in type 1 diabetes mellitus. *Nat. Rev. Endocrinol.*, 9: 92-103.
- Maahs, D.M., N.A. West, J.M. Lawrence and E.J. Mayer-Davis, 2010. Epidemiology of type 1 diabetes. *Endocrinol. Metab. Clin. N. Am.*, 39: 481-497.
- Marron, M.P., L.J. Raffel, H.J. Garchon, C.O. Jacob and M. Serrano-Rios *et al.*, 1997. Insulin-dependent diabetes mellitus (IDDM) is associated with *CTLA4* polymorphisms in multiple ethnic groups. *Hum. Mol. Genet.*, 6: 1275-1282.
- Mehers, K.L. and K.M. Gillespie, 2008. The genetic basis for type 1 diabetes. *Br. Med. Bull.*, 88: 115-129.
- Mojtahedi, Z., G.R. Omrani, M. Doroudchi and A. Ghaderi, 2005. *CTLA-4+ 49 A/G* polymorphism is associated with predisposition to type 1 diabetes in Iranians. *Diabetes Res. Clin. Pract.*, 68: 111-116.
- Mosaad, Y.M., A.A. Elsharkawy and B.S. El-Deek, 2012. Association of *CTLA-4 (+ 49A/G)* gene polymorphism with type 1 diabetes mellitus in Egyptian children. *Immunol. Invest.*, 41: 28-37.
- Nistico, L., R. Buzzetti, L.E. Pritchard, B. Van der Auwera and C. Giovannini *et al.*, 1996. The *CTLA-4* gene region of chromosome 2q33 is linked to and associated with, type 1 diabetes. *Human Mol. Genet.*, 5: 1075-1080.
- Owerbach, D., F.J. Naya, M.J. Tsai, S.V. Allander and D.R. Powell *et al.*, 1997. Analysis of candidate genes for susceptibility to type I diabetes: A case-control and family-association study of genes on chromosome 2q31-35. *Diabetes*, 46: 1069-1074.
- Peng, H. and W. Hagopian, 2006. Environmental factors in the development of type 1 diabetes. *Rev. Endocr. Metab. Disorders*, 7: 149-162.
- Pettersen, E., F. Skorpen, K. Kvaloy, K. Midthjell and V. Grill, 2010. Genetic heterogeneity in latent autoimmune diabetes is linked to various degrees of autoimmune activity results from the nord-trondegag health study. *Diabetes*, 59: 302-310.
- Rau, H., J. Braun, H. Donner, J. Seissler and T. Siegmund *et al.*, 2001. The codon 17 polymorphism of the *CTLA4* gene in type 2 diabetes mellitus. *J. Clin. Endocrinol. Metab.*, 86: 653-655.
- Rudd, C.E. and H. Schneider, 2003. Unifying concepts in CD28, ICOS and *CTLA4* co-receptor signalling. *Nat. Rev. Immunol.*, 3: 544-556.
- Takahashi, H., K. Behbakht, P.E. McGovern, H.C. Chiu and F.J. Couch *et al.*, 1995. Mutation analysis of the *BRCAl* gene in ovarian cancers. *Cancer Res.*, 55: 2998-3002.
- Takara, M., I. Komiya, Y. Kinjo, T. Tomoyose and S. Yamashiro *et al.*, 2000. Association of *CTLA-4* gene A/G polymorphism in Japanese type 1 diabetic patients with younger age of onset and autoimmune thyroid disease. *Diabetes Care*, 23: 975-978.
- Tang, S.T., H.Q. Tang, Q. Zhang, C.J. Wang, Y.M. Wang and W. Peng, 2012. Association of cytotoxic T-lymphocyte associated antigen 4 gene polymorphism with type 1 diabetes mellitus: A meta-analysis. *Gene*, 508: 165-187.
- Turpeinen, H., A.P. Laine, R. Hermann, O. Simell, R. Veijola, M. Knip and J. Ilonen, 2003. A linkage analysis of the *CTLA4* gene region in Finnish patients with type 1 diabetes. *Eur. J. Immunogenet.*, 30: 289-293.
- Ueda, H., J.M. Howson, L. Esposito, J. Heward and G.C. Snook *et al.*, 2003. Association of the T-cell regulatory gene *CTLA4* with susceptibility to autoimmune disease. *Nature*, 423: 506-511.
- Vaidya, B. and S. Pearce, 2004. The emerging role of the *CTLA-4* gene in autoimmune endocrinopathies. *Eur. J. Endocrinol.*, 150: 619-626.
- Van Belle, T.L., K.T. Coppieters and M.G. von Herrath, 2011. Type 1 diabetes: Etiology, immunology and therapeutic strategies. *Physiol. Rev.*, 91: 79-118.

- Van der Auwera, B.J., F.C. Schuit, I. Weets, A. Ivens, J.E. Van Autreve and F.K. Gorus, 2002. Relative and absolute HLA-DQA1-DQB1 linked risk for developing type I diabetes before 40 years of age in the Belgian population: Implications for future prevention studies. *Human Immunol.*, 63: 40-50.
- Waterhouse, P., J.M. Penninger, E. Timms, A. Wakeham and A. Shahinian *et al.*, 1995. Lymphoproliferative disorders with early lethality in mice deficient in *Ctla-4*. *Science*, 270: 985-988.
- Wells, A.D., M.C. Walsh, J.A. Bluestone and L.A. Turka, 2001. Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. *J. Clin. Invest.*, 108: 895-904.
- Winer, D.A., S. Winer, L. Shen, P.P. Wadia and J. Yantha *et al.*, 2011. B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. *Nat. Med.*, 17: 610-617.