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Association study of *IL2RA* and *CTLA4* gene variants with type-1 diabetes mellitus in children in the northwest of Iran

Mohammad Reza Ranjouri[1$], Parisa Aob[1$], Sima Mansoori Derakhshan[2], Mahmoud Shekari khaniani[2], Hossein Chiti[3], Ali Ramazani[4*]

[1] Student Research Committee, Zanjan University of Medical Sciences, Zanjan, Iran
[2] Medical Genetics Department, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Zanjan Metabolic Disease Research Center, Zanjan University of Medical Sciences, Zanjan, Iran
[4] Biotechnology Department, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran

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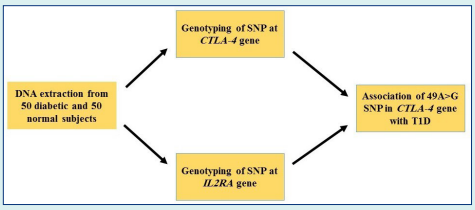
Abstract



Introduction: A variety of genetic predisposing factors and environmental factors are known to influence the pathogenesis of type-1 diabetes (T1D). This study intended to investigate the association of cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) and interleukin 2 receptor subunit alpha (*IL2RA)* gene polymorphisms with type 1 diabetes in children of northwest of Iran.

Methods: Genomic DNA was extracted by salting-out method. PCR amplification and direct sequencing methods were used for genotyping of *CTLA4* (exon 1) and IL2RA (intron 1) genes in all patients and controls. SNPStats was used to calculate odds ratios (ORs), 95% confidence intervals (CIs), and *p* values.

Results: In this study, the frequency of G allele and GG genotype of *CTLA-4* (+49A/G) polymorphism in T1D patients were significantly different from those in the controls (26% vs. 11%, $p = 0.006$). Moreover, a significant difference was observed between patients and control group in the allele frequencies of the new SNP (chr2:203868145) that was identified in exon one of *CTLA4* (14% vs. 3%, $p = 0.006$). The results showed that the GG homozygous genotype of +49 A>G was associated with increased glycemic level in T1D patients in the study population (95% CI = 10.47, $p = 0.0067$). However, no significant association was found between IL2RA (ss52580101C>A) polymorphism and T1D patients (2% vs. 4%, $p = 0.41$).

Conclusion: The results further support the association of T1D with +49A>G SNP in the CTLA4 gene in the population of northwest of Iran. However, no significant relationship was observed between ss52580101C>A polymorphism of IL2RA gene and T1D in this study.

Introduction

Type 1 diabetes mellitus (T1D) (OMIM 222100) is an organ-specific and autoimmune disease that primarily affects children and adolescents. It is caused by destruction of the beta cells of Langerhans of the pancreas that secrete insulin, which occurs through the autoimmune processes. Therefore, the affected patients have to use insulin throughout their lives.[1] The clinical symptoms of this disease include frequent urination (polyuria), excessive thirst (polydipsia), increased hunger (polyphagia), and weight loss.[2-5] T1D comprises 5%–10% of the total number of diabetes cases, and the risk for each individual in a specified population is estimated to be 4%. This risk would be further increased by more than 1%, if the mother suffers from T1D, and by more than 3%, if the father is also affected.[6] In recent decades, the prevalence of T1D has been increasing at a rapid rate, with an increase by about 40% from 1998 till 2010.[7] T1D is a heterogeneous and complex disease, and genetic and environmental factors are involved in increasing the risk of its incidence. In this regard, several studies have been conducted on family cases and monozygotic and dizygotic twins, which have reported the genetic factor as one of the important factors in this disease.[8,9] Diabetes is an epidemic disease and a major health risk in the world, and the situation is rapidly worsening. In the last three decades,

***Corresponding author:** Ali Ramazani, Email, ramazania@zums.ac.ir
[$] Mohammad Reza Ranjouri and Parisa Aob contributed equally to this study.

numerous studies have been conducted on T1D cases to assess complex multi-factors and identify susceptibility genes. These studies have resulted in the identification of more than 40 different genetic loci associated with this disease.[10,11] Several loci are more commonly associated with T1D, including human leukocyte antigen (HLA) region on chromosome 6p21, the *PTPN22* on 1p13, *IL2RA* on 10p15, the insulin gene (INS-VNTR) locus on 11p15, and the cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) locus on 2q33.[12] Although numerous genes have been associated with T1D, the frequency of each of these genes associated with this disease in various populations is very different.[7,9]

In 1996, the *CTLA4* gene was reported as one of the important susceptibility genes in T1D.[13] *CTLA4* is one of the members of the immunoglobulin family that has four exons and hence plays an important role in the pathogenesis of autoimmune diseases such as T1D.[14] This gene encodes a cell surface receptor that is expressed on activated T cells and functions as a negative regulatory factor.[15] A common polymorphism in the *CTLA4* gene coding region is +49 A>G, which is a conversion of alanine to threonine in exon one.[16]

In addition, it was reported in 2005 for the first time that interleukin 2 receptor subunit alpha (*IL2RA* gene or CD25) is strongly associated with T1D.[17] *IL2RA* gene has eight exons and encodes the α-chain of the interleukin-2 receptor complex. IL2 is a strong growth factor for lymphocytes. It is known that the expression of *IL2RA* in CD4+CD25+ regulatory T-cells is essential to control the immune response of T cells and to prevent autoimmune diseases.[18] The ss52580101 polymorphism located in intron 1 in this gene is associated with T1D.[19,20] Studies on different populations have reported different results regarding the association between *CTLA4* and *IL2RA* variants. Some of these studies have shown a significant correlation between *CTLA4* and *IL2RA* polymorphisms and T1D[16,21,22] while some studies reported no such relationship.[23-25] Therefore, these gene polymorphisms can be a new strategy for the prognosis of T1D and can offer improved treatment strategies.[2,6] Based on this evidence and because there is no information on the association of *CTLA4* and *IL2RA* variants with T1D in the northwest of Iran, the present study was conducted to confirm the association of these variants in children in the northwest of Iran.

Materials and methods

Subjects

A total of 50 unrelated T1D patients (23 females and 27 males; age at diagnosis: 5-15 years) were recruited from Children's hospital of Tabriz, Iran. T1D samples were diagnosed by the consultant endocrinologist according to clinical features and results of laboratory tests.

Fifty subjects (24 females and 26 males, age: 5-16 years) with normal glycemic levels and no family history of Diabetes or other autoimmune diseases were also studied as the control group. The control group were living in the same geographical area, and had the same ethnic origin as the patients.

DNA extraction

Three mL of blood samples was collected from all the subjects in EDTA anticoagulant tubes. Total genomic DNA was extracted from the whole peripheral blood of all subjects using the salting-out method, as described previously.[26] Finally, to ensure the quality of the extracted DNA OD_{260}/OD_{280} and concentration was measured with Nano Drop 2000 spectrophotometer (Eppendrof, Germany).

PCR-sequencing

The exon 1 of *CTLA4* gene and the intron of *IL2RA* gene fragments were amplified using gene-specific oligonucleotide primers. The primer sequences and PCR conditions are shown in Table 1.

Initially, each PCR was performed in 50 µL mixtures consisting of 300 ng of genomic DNA, 10 pM of each primer, and 25 µL of 2× Master Mix (Fermentas, USA) containing Taq DNA polymerase, $MgCl_2$, dNTPs, and reaction buffers. PCR products were analyzed in 1% agarose gel electrophoresis and visualized under UV light. Then, 25 µL of each PCR product with the forward primer were sent for direct sequencing (Macrogen, South Korea). Sequencing was performed by the Sanger method on an ABI 3730 sequencer (Macrogen, South Korea). The resulting sequences were analyzed using BLAST, Clustal X2, and Chromas V2.4 software packages.

Statistical analysis

The linkage disequilibrium was measured by means of D, D′, and r^2. For the analysis of genetic data, SNPStats was used. A Hardy-Weinberg equilibrium test was conducted for each SNP in the control group, and logistic regression models were then applied to obtain odds ratios (ORs), 95% confidence intervals (CIs), and *p* values.[27]

Results

As described in methods, a total of 100 subjects (50 T1D patients and 50 healthy subjects) were selected in this

Table 1. Primer sequences and PCR conditions for specific regions of *CTLA4* and *IL2RA* genes

Loci	Sequence of primers (5′-3′)	PCR product size	PCR conditions
CTLA4	F: CAGTTGAGTGCTTGAGGTTGT	694 bp	ID: 95°C/2 min (1 cycle)
	R: TGAGCTCATCCTGAAACCCA		D: 94°C/1 min (35 cycles)
IL2RA	F: GGTACCTTTGTCTTCTGAGTGC	725 bp	A: 54°C/1 min (35 cycles)
	R: GATCTGATCACTGCACGTCA		E: 72°C/2 min (35 cycles)
			FE: 72°C/10 min (1 cycle)

F: forward, R: reverse, bp: base pair, ID: initial denaturation, D: denaturation, A: annealing, E: extension, FE: final extension.

study. The demographic data and clinical characteristics of the subjects are presented in Table 2.

At first, the aim of this study was to estimate the prevalence of common polymorphisms of the genes *CTLA4* (+49 A > G) and *IL2RA* (ss52580101 C > A) via Sanger sequencing, then two new variants chr2:203868145 A>C and chr10: 6072892 A>G were detected for *CTLA4* and *IL2RA* genes, respectively.

CTLA4 polymorphisms in T1D

The frequencies of +49 A>G genotypes and alleles were significantly different between T1D patients and control group (Table 3). Thirty-six (72%) patients were heterozygous for A>G, 2 (4%) were homozygous for A, and 12 (24%) were homozygous for G, while the numbers and frequencies of AG, AA, and GG genotypes in healthy subjects were 41 (82%), 7 (14%), and 2 (4%), respectively. Therefore, the homozygous GG genotype conferred a higher risk than the heterozygous genotype. The allele frequency of G was higher in T1D patients compared to controls (26% vs. 11%, $p = 0.006$) (Table 3).

Moreover, a significant difference was observed between patients and control group in the allele frequencies of the new polymorphism (chr2: 203868145 A>C) that was identified in exon one of *CTLA4*. The CC homozygous genotype was observed in three T1D patients, and the frequency of the C allele in patients was higher than that in healthy subjects (14% vs. 3%, $p = 0.006$).

Table 2. Characteristic of T1D patients and control subjects

Total numbers	T1D (n=50)	Controls (n=50)
Gender	F: 23 (46%) M: 27 (54%)	F: 24 (48%) M: 26 (52%)
The mean age	10±2	10.5±1.6
Family history	None	None
Insulin dependent	All	None
Glycemic level	135±4.1	96±3
BMI (kg/m²)	20.2±2.1	20.71±2

F: female, M: male, BMI: body mass index.
* The difference is statistically significant.

IL2RA polymorphisms in T1D

The AA homozygous genotype for variant ss52580101 C > A was not observed in patients and healthy controls. Significant difference was not also observed in AC heterozygous genotype between T1D patients, and the frequencies of A allele were 2% in patients and 4% in the control group ($p = 0.41$). In addition, the frequencies of the new polymorphism (ch10: 6072892 A>G) that was found in intron one of *IL2RA* of GG homozygous genotype were estimated to be 12% in patients and 6% in healthy controls. Although the frequency of G allele in the patients was more than that in the control group, there was no significant difference between them (14% vs. 7%, $p = 0.11$) (Table 4).

Relationship between genotype and phenotype

In this study, we compared the relationship between the genotypes of four polymorphisms mentioned above and the glycemic level and body mass index (BMI) of the subjects. Table 5 shows the relationship between the glycemic level and the above mentioned four polymorphisms in patients and controls. The results suggest that the GG homozygous genotype of +49 A>G is associated with increased glycemic levels in T1D patients in the study population ((95% CI) = 10.47, $p = 0.0067$). However, the results did not reveal a significant correlation between the BMI values of patients and the four polymorphisms (Table 6).

Haplotype analysis

Finally, the haplotypes of *CTLA4* and *IL2RA* genes were studied in the study population (Table 7). Based on the findings, we can conclude that the GA and GC haplotypes of CTLA4 gene are associated with T1D.

Linkage disequilibrium analysis

As shown in Table 8, identified polymorphisms in *CTLA4* and *IL2RA* genes were in linkage disequilibrium.

Discussion

Diabetes is a relatively common chronic disease in

Table 3. Genotype and allele frequencies of *CTLA4* (+49 A>G and chr2:203868145 A>C) in patients with T1D and controls

Genes	SNP types	Patients (%)	Controls (%)	Odd ratio (95% CI)	*P* value
CTLA4	+49 A>G				
	AA	36 (72)	41 (82)	1.00	0.24
	AG	2 (4)	7 (14)	1.00	0.08
	GG	12 (24)	2 (4)	2.17 (0.67-6.96)	0.004*
	A	74 (74)	89 (89)	1.2 (0.19-8.72)	0.006*
	G	26 (26)	11 (11)	1.03 (0.26-1.93)	0.006*
	chr2:203868145				
	AA	39 (78)	47 (94)	1.00	0.02*
	AC	8 (16)	3 (6)	1.00	0.11
	CC	3 (6)	0 (0)	0.00	0.08*
	A	86 (86)	97 (97)	2.03 (0.61-6.46)	0.005*
	C	14 (14)	3 (3)	1.22 (0.06-8.47)	0.005*

Chr2: chromosome 2, SNP: single nucleotide polymorphism.

Table 4. Genotype and allele frequencies of *IL2RA* (ss52580101 C>A and chr10: 6072892 A>G) polymorphisms in patients with T1D and controls

Genes	SNP types	Patients (%)	Controls (%)	Odd ratio (95% CI)	*P* value
IL2RA	ss52580101				
	CC	48 (96)	46 (92)	0.30 (0.05-6.23)	0.40
	AC	2 (40)	4 (8)	0.32 (0.37-2.71)	0.40
	AA	0 (0)	0 (0)	-	-
	A	2 (2)	4 (4)	1.00	0.41
	C	98 (98)	96 (96)	1.00	0.41
	chr10: 6072892				
	AA	42 (84)	46 (92)	1.00	0.22
	AG	2 (4)	1 (2)	1.00 (0.21-4.00)	0.56
	GG	6 (12)	3 (6)	0.94 (0.06-6.21)	0.30
	A	86 (86)	93 (93)	1.02 (0.17-7.99)	0.11
	G	14 (14)	7 (7)	1.06 (0.49-20.05)	0.11

Chr10: chromosome 10, SNP: single nucleotide polymorphism.

Table 5. *CTLA4* and *IL2RA* association with glycemic level

Genes	SNP	Genotype	n	Case		n	Control		*P* value
				Response mean	Difference (95% CI)		Response mean	Difference (95% CI)	
CTLA4	+49A/G	AA	36	123.36	0.00	41	94	-29.36	0.0067*
		AG	2	126.5	3.14	7	96.71	-26.65	
		GG	12	133.83	10.47	2	92.5	-30.86	
	chr2:203868145	AA	39	125.95	0.00	47	94.4	-31.54	0.96
		AC	8	124.75	-1.20	3	93	-32.95	
		CC	3	130	4.05	0	-	-	
IL2RA	ss52580101	CC	48	125.79	0.00	46	94.46	-31.34	0.17
		CA	2	131	5.21	4	92.75	-33.04	
		AA	0	-	-	0	-	-	
	chr10: 6072892	AA	42	125.33	0.00	46	94.41	-30.92	0.22
		AG	2	124.5	-0.83	1	94	-31.33	
		GG	6	131.17	5.83	3	93	-32.33	

Chr2: chromosome 2, Chr10: chromosome 10, SNP: single nucleotide polymorphism.
* The difference is statistically significant.

Table 6. *CTLA4* and *IL2RA* association with response BMI

Genes	SNP	Genotype	n	Case		n	Control		*P* value
				Response mean	Difference (95% CI)		Response mean	Difference (95% CI)	
CTLA4	+49A/G	AA	36	19.89	0.00	41	20.24	0.35	0.78
		AG	2	18.84	-1.05	7	19.85	-0.04	
		GG	12	19.54	-0.34	2	20.04	0.15	
	chr2:203868145	AA	39	19.79	0.00	47	20.1	0.31	0.24
		AC	8	20.06	0.27	3	21.3	1.51	
		CC	3	18.64	-1.15	0	-	-	
IL2RA	ss52580101	CC	48	19.78	0.00	46	20.16	0.38	0.48
		CA	2	19.23	-0.56	4	20.32	0.54	
		AA	0	-	-	0	-	-	
	chr10: 6072892	AA	42	19.8	0.00	46	20.21	0.41	0.49
		AG	2	20.76	0.96	1	19.59	-0.21	
		GG	6	19.19	-0.61	3	19.87	0.08	

Chr2: chromosome 2, Chr10: chromosome 10, SNP: single nucleotide polymorphism.
* The difference is statistically significant.

the world that affects various races and populations. The prevalence of this disease in several communities especially in developing countries is increasing. Studies of the entire human genome with the aim of screening loci involved in the development of diabetes in families with more than one individual with T1D found different gene polymorphisms linked to T1D. The most important genes include *HLA*, *PTPN22, CTLA4, INF, IL2RA*, and *TGFB1*.[9,28] In recent years, several case-control studies have been performed to assess the relationship between polymorphisms of these genes with T1D.[16] As a result, it is reasonable to conduct a separate associative study on

Table 7. *CTLA4* and *IL2RA* haplotypes association with T1D

***CTLA4* haplotype**				
+49A>G	chr2:203868145	Frequency	Difference (95% CI)	*P* value
A	A	0.745	0.00	-
G	A	0.17	3.82 (2.34 -5.3)	<0.0001*
A	C	0.07	0.35 (-2.19 -2.89)	0.79
G	C	0.015	6.78 (2.35 -11.21)	0.0034*
***IL2RA* haplotype**				
ss52580101	chr10: 6072892	Frequency	Difference (95% CI)	*P* value
C	A	0.865	0.00	-
C	G	0.105	1.52 (-0.39 -3.43)	0.12
A	A	0.03	0.97 (-3.74 -5.68)	0.69

Chr2: chromosome 2, Chr10: chromosome 10.
* These haplotypes are associated with T1D.

Table 8. Linkage disequilibrium analysis of two SNPs for *CTLA4* and *IL2RA* genes

Genes	SNP	D	D′	*r*	*P* value
CTLA4	+49A>G- chr2:203868145	-7e-04	0.0463	-0.0067	0.9242
IL2RA	ss52580101- ss52580101	-0.0031	0.9833	-0.0592	0.4022

SNP: single nucleotide polymorphism; D: deviation; r: correlation coefficient.
Identified polymorphisms in *CTLA4* and *IL2RA* genes are in linkage disequilibrium.

any specific ethnic population.[7,12] In this regard, in the northwest of Iran, studies on the relationship between HLA[29] and PTPN22 gene polymorphisms[30] had been conducted on children with T1D, with no significant relationship observed between these genes and T1D.

We considered the most common SNP in *CTLA4* and *IL2RA* genes that was associated with T1D in children in the northwest of Iran. We observed that the frequency of G allele and GG genotype of +49A>G of *CTLA4* gene in these patients was significantly higher than that in the control group (the G allele frequency was 26% in patients and 11% in healthy subjects). Therefore, this study confirms the relationship of the polymorphism +49A>G with an increased risk of T1D in the study population. In addition, in the case of the new SNP (chr2: 203868145 A>C) that was identified for this gene in our population, it was found that the frequency of C allele was significantly different between two groups ($p = 0.005$). Moreover, we did not observe a significant relationship between ss52580101C>A polymorphism of *IL2RA* gene and T1D ($p = 0.4$). It is clear that the allele frequency of the new polymorphism of *IL2RA* gene (chr10:6072892A>G) was not significantly different between patients and healthy subjects ($p = 0.11$).

CTLA4 gene is a cell surface molecule and a member of immunoglobulin family. It is believed that mutations in this gene increase the activity of T cells, and therefore it is associated with several autoimmune diseases, including T1D, Graves' disease, lupus, and celiac disease.[31-33] *CTLA4* gene is known as one of the important genes involved in T1D. Studies have reported a relationship between the polymorphism +49 A>G and T1D in different populations, including the Japanese,[32] Korean, Italian, Spanish, French,[33] Egyptian,[34] and Belgian.[35] Moreover, studies have been conducted in different parts of Iran in this regard. Mojtahedi et al reported that +49 A>C is linked to the risk of T1D in southern Iran. They showed that the frequencies of G allele were 45% in T1D patients and 33.4% in healthy subjects ($p = 0.0026$).[36] Ahmadi et al also reported that the frequencies of allele +49 A>G were 36.5% in T1D patients and 20.6% in healthy subjects, which confirms that this SNP in T1D patients is significantly different from that in the healthy subjects with Iranian Kurdish ethnicity.[37]

In this study, we observed a similar relationship in the northwest of Iran. Therefore, it can be confirmed that the most common gene polymorphism (+49 A>G) in *CTLA4* gene is important in increasing the risk of T1D in Iranian population. Of course, it should be noted that the results of studies in some populations, such as Turkish,[25] Chinese,[33] Japanese,[38] and Azerbaijan,[39] have reported the lack of such a relationship between *CTLA4* and this disease.

IL2RA gene has a key role in regulatory T (Treg) cell proliferation. Studies have shown that ss52580101 polymorphism located in intron 1 of IL2RA gene is involved in T1D. Low et al in a comprehensive study considered 288 SNPs of *IL2RA* gene and proved that ss52580101 is the most common SNP associated with T1D.[40]

Dendrou et al in a study on the population of Poland showed a significant increase in allele and genotype frequency of polymorphism of *IL2RA* in T1D patients compared to that in healthy people.[41] Maier et al reported that the allele frequency of ss52580101 in T1D patients is significantly higher than that in control subjects in USA.[19] In this study, based on a strong relationship between

polymorphisms in *IL2RA* and T1D that has been proven in different populations, we examined the genotype of *IL2RA*. However, the results observed for ss52580101 were in contrast to the above mentioned studies. We found that ss52580101 is the most common SNP of *IL2RA* gene and has no relationship with T1D in our population. However, our results were consistent with a study by Kawasaki et al in Japanese population who also showed that ss52580101 SNP is not associated with T1D.[22]

There are studies that have shown that polymorphisms of *CTLA4* are associated with clinical characteristics and pathological conditions of T1D. Abe et al reported that polymorphism of *CTLA4* is associated with the relationship between ICA512 antibodies and diabetic ketoacidosis.[42] Balic et al reported that patients who have G allele polymorphisms +49A > G have increased glycemic levels.[43] In this study, we found a significant relationship between GG genotype +49A > G and glycemic level of T1D patients (p = 0.0067). Glycemic level of diabetic patients has an important role in their survival, and we can thus say that this SNP in diabetic individuals can be important. In the present study, it was found that among these four SNPs, only *IL2RA* polymorphism follows the Hardy–Weinberg equilibrium. The reasons for the imbalance in the other three SNPs in the studied population could be the heterogeneous nature of T1D and the small sample size. Therefore, it is suggested that in such case-control studies, large sample sizes be selected to report results with greater confidence.

Conclusion

The results of this study confirmed the relationship between *CTLA-4* +49A>G polymorphism and the risk of T1D and increased blood glucose levels in patients in the northwest of Iran. In addition, a newly identified polymorphism of *CTLA4* (chr2: 203868145A>C) was found to be associated with T1D.

Ethical approval

All the experiments were performed according to the guideline provided by the Ethical Committee at Zanjan University of Medical Sciences. Informed consent was also obtained from the parents or guardians of all subjects who participated in this study.

Competing interests

Authors declare no conflict of interests.

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Research Highlights

What is current knowledge?

√ More than 40 different genetic loci are associated with TID.

√ The frequency of each gene associated with this disease is different in various populations.

What is new here?

√ The 49 A>G SNP in the *CTLA4* gene is associated with T1D in the population of northwest of Iran.

√ A Significant difference was observed between patients and control group in the allele frequencies of the new SNP (chr2:203868145) that was identified in exon one of *CTLA4* gene.

√ The ss52580101C>A polymorphism of *IL2RA* gene was not associated with T1D in this study.

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