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Cytotoxic T lymphocyte antigen 4 heterozygous codon 49 A/G dimorphism is associated to latent autoimmune diabetes in adults (LADA)

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Abstract

Autoimmune diabetes is an organ specific and multifactorial disorder with a classical onset as insulin dependent diabetes mellitus (IDDM) and with another form of onset as latent autoimmune diabetes in adults (LADA), which has a slower onset and a later progress to insulin dependency as a result of the beta cells destruction.

The cytotoxic T lymphocyte-antigen 4 (CTLA4) has been identified as a susceptible marker of the disease; it is considered a down regulator of T cell function, playing a key role in autoimmunity.

We analyzed CTLA4 codon 49 A/G polymorphism in 123 IDDM patients, 63 LADA patients and 168 healthy non-diabetic control individuals.

The frequency of the heterozygous A/G genotype in LADA patients was significantly increased compared to IDDM patients (55.6 vs. 39.8%, $p = 0.0415$). There was no statistical significant difference in the distribution of the A/G dimorphism between autoimmune diabetes patients (LADA or IDDM) and non-diabetic control individuals.

HLA DQ region is responsible for the genetic susceptibility to autoimmune diabetes in IDDM patients in about 50% and it has a lower effect in genetic susceptibility in LADA patients. Several other genetic loci are needed to develop autoimmune diabetes in adult patients. Therefore, LADA may be the result of a combined minor risk loci effect in a major risk haplotype.

Keywords: *Diabetes, CTLA4, LADA, polymorphism*

Introduction

Autoimmune diabetes is an organ specific and multifactorial disorder, in which the beta cells are selectively destroyed.

There are two forms of autoimmune diabetes, type 1 diabetes, in which the mode of onset is abrupt, it is presented frequently in childhood, with classical clinical as diabetic ketoacidosis and insulin requirement at onset (IDDM) [1]. The other form is the latent autoimmune diabetes in adults (LADA) with a slow onset, clinical presentation as type 2 diabetes, without insulin treatment needed at diagnosis, but with a later progress to insulin dependency [2].

Both autoimmune diabetes forms are characterized by the presence of circulatory islet cell autoantibodies, glutamic acid decarboxilase autoantibodies (GADA), insulin autoantibodies (IAA) and/or anti phosphatase autoantibodies (IA2A), indicating the beta cells damage produced by cytotoxic T lymphocyte [2,3].

The insulin dependency is the result of the beta cells destruction, presumably caused by the interaction of a large number of susceptibility genes and environmental factors. Several predisposing loci have been

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mapped through the entire human genome in IDDM [4]. The most important locus is IDDM1 which corresponds to HLA class II DQ-DR region, it accounts for 50% of the familial clustering [5]. It described more than 40 different alleles from which the *0201/*0302 genotype confers the highest risk to develop type 1 diabetes [6].

A second locus named IDDM2 is a variable number of tandem repeats in the promotor region of the insulin gene. It is formed by a tandem repeat of 14–15 bp (INS-VNTR). This polymorphism is classified into three alleles: Small class I alleles (21–44 repeat units or RUs), intermediate alleles (45–137 RUs) and large class III alleles (138–159 RUs) [7].

The class I alleles (~70% in Caucasians) are associated with increased susceptibility to IDDM and the class III alleles are dominantly protective [8].

Other susceptibility marker such as cytotoxic T lymphocyte-antigen 4 (CTLA4) gene in IDDM12 has been identified by whole genomic scan [9,10]. CTLA4 belongs to the immunoglobulin superfamily and it shares many features with the CD 28 molecule [11]. Both of them bind to the same ligand B7, but they carry out different functions: The complex CD28-B7 produces activation of T-cells by inducing the expression of several cytokines and the complex CTLA4-B7 leads to T-cell apoptosis. The CTLA4 gene is expressed on activated T cells and it is an important factor in T cell regulation: it is considered a down regulator of T cell function, playing a key role in autoimmunity [12]. The CTLA4 gene contains three major polymorphic sites: (1) at position −318 of the promotor region: a C-T polymorphism, (2) at position 49 of exon 1: a A-G polymorphism and (3) at 3′ untranslated region of exon 4: (AT)n fragment with 84 to 134 repeats [13].

The A/G polymorphism in exon 1 leads to a Thr–Ala substitution in the leader peptide of CTLA4, this change has been associated with autoimmune diabetes and other autoimmune disorders [14]. The CTLA4 G allele has been found to be associated with IDDM in Spanish, Italian and other Caucasian populations [15].

We therefore, decided to investigate the association of CTLA4 49 A/G with LADA in an Argentinean population compared with IDDM diabetic patients and normal subjects.

Materials and methods

From 160 Argentinean patients previously characterized by clinical features as LADA (over 35 years of age at diagnosis with a mean age at onset of 51.4 ± 2.6 years and with a developed insulin dependency less than 6 years from diagnosis); we selected 63 patients for the genotyping analysis, with an autoimmune humoral positive pattern (for at least one autoantibody of GADA, IAA or ICA 512).

We also studied 123 Argentinean IDDM patients with proven positivism for autoantibodies. The mean age at onset was 15 ± 9.2 years and insulin therapy was installed within 6 months of diagnosis. All IDDM and LADA patients were diagnosed according to the World Health Organization (WHO) criteria.

The control group was integrated by 168 unrelated volunteers selected mainly from a dataset of healthy blood donors based on normal fasting blood glucose level and without family history of diabetes.

Genomic DNA was purified from 10 ml of fresh or frozen whole blood by CTAB method [16]. A polymerase chain reaction (PCR) was carried out to genotype the CTLA4 exon 1 position 49 A/G polymorphism, using a forward primer: 5′ GCTCT-ACTTCCTGAAGACCT3′ and a reverse primer: 5′ AACCCAGGTAG GAGAAAACAC 3′. We followed the manufacturer's conditions for the amplification of genomic DNA (250 ng) with the Taq polymerase (T-plus DNA polymerase Inbio-Highway) in a PTC 100 thermocycler (MJ Research Inc.): 5 min at 94°C, 30 cycles: 30 seg at 94°C, 30 seg at 60°C and 1 min at 72°C and 10 min at 72°C for the final extension.

A single strand conformational polymorphism (SSCP) was performed with 200 ng of the product in a 10% poliacrylamide gel. The samples genotyped as A/G or G/G were confirmed by enzymatic digestion using 20 μl of the product, digested by 25 U of ItaI enzyme in 50 μl final volume. The digestion was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Allelic and genotype frequencies were estimated by χ^2 tests with 2×2 contingency tables. (Graph Pad InStat software).

Results

The frequency of the heterozygous A/G genotype in LADA patients was significantly increased compared

Table I. Frequency of CTLA4 A/G polymorphism.

	LADA (%)	IDDM (%)	Control (%)	p1	p2	p3
A/A	22 (34.9)	54 (43.9)	71 (42.3)	ns	ns	ns
A/G	35 (55.6)	49 (39.8)	76 (45.2)	ns	ns	0.0415
G/G	6 (9.5)	20 (16.3)	21 (12.5)	ns	ns	ns
Total	63	123	168			

p1, LADA/Control; p2, IDDM/Control; p3, LADA/IDDM; ns, not significant ($p > 0.05$).

to IDDM patients (55.6 vs. 39.8%, *p*: 0.0415). (Table I). There was no significant difference in the distribution of the A/G dimorphism between autoimmune diabetes patients (LADA or IDDM) and healthy subjects.

The presence of the homozygous G/G genotype was higher in type 1 diabetes subjects (16.3%) compared to LADA (9.5%) or normal group (12.5%), but the difference did not reach statistical significance (Table I).

The distribution of homozygous A/A genotype was similar between the normal and the type 1 diabetes individuals (42.3 vs 43.9%) (Table I).

Discussion

The association of CTLA4 A/G polymorphism and autoimmune diabetes was reported in several trials. However, this association is controversial, some authors describe it as strong while other authors refer it as weak. Case control and segregation analysis in multiethnic groups have yielded contradictory results, in particular the preferential transmission of the G allele to diabetic offspring.

Donner et al. found that patients with type 1 diabetes had more often the CTLA4 G allele than controls, but this difference was less significant than in Graves' disease [17]. Although, Fajardy et al. did not find any association between CTLA4 G allele and type 1 diabetes susceptibility, they found a significant sex ratio effect of A/G genotype exclusively in diabetic women [18].

A significantly higher incidence of CTLA4 A/G genotype has been observed in other autoimmune disease such as rheumatoid arthritis [19]. These results were similar to other studies in rheumatoid arthritis patients: A significantly higher rate of A/G heterozygosity was found among HLA DR4 positive (risk alleles for rheumatoid arthritis) [20] and a significantly higher rate of A/G heterozygotes among rheumatoid arthritis female patients [21].

The major finding in the present study was the significant statistical difference in the heterozygous A/G genotype CTLA4 gene frequency between LADA and IDDM subjects. It must be considered that we studied an Argentinean population, which is mainly a Caucasoid population (European descendents) with a minor proportion of admixture with Native Americans. Cosentino et al. found that heterozygous A/G genotype is increased in LADA subjects compared with the healthy control group [22]. Ueda et al. studied the allele specific transcription of CTLA4 gene in three heterozygous A/G individuals through the relative amounts of the mRNA of two known isoforms of CTLA4: full length isoform (flCTLA4) and soluble isoform (sCTLA4) [23]. sCTLA4 is expressed in activated T cells, secreted to serum, bound to T cell antigen receptor and in this way, it suppresses the T cell response. The sCTLA4 mRNA level from the disease protective allele A, was higher than mRNA level from susceptible allele G in heterozygous A/G individuals. The homozygous susceptible genotype G/G expressed a 50% less sCTLA4 mRNA compared with homozygous protected genotype A/A [23,24].

We found that the prevalence of heterozygous genotype is higher in LADA than in IDDM, so it could be hypothesized that the protective A allele in LADA individuals express higher levels of sCTLA4 mRNA producing a major effect of down regulation in T cell mediated by the high levels of the CTLA4. Anjor et al. found that although the susceptibility G allele of the CTLA4 gene has a lower expression, it also has a defective Endoplasmic Reticulum process of a significant portion of the CTLA4 molecule, resulting in an aberrantly glycosylated product and decreased cell surface expression. The protective A allele has a correct Endoplasmic Reticulum processing leading to functional expression at the cell surface [25]. We found a higher frequency of the G/G genotype in IDDM patients, this could determine a low functionality of the CTLA4 molecule due to an aberrantly glycosylated product, decreased cell surface expression and lower sCTLA4 mRNA levels.

We have previously described in other studies that the presence of the shorter class I INS-VNTR is a higher risk genetic factor in LADA patients than IDDM patients, and that the frequency of HLA DQB1 *0201/*0302 is lower in LADA patients when compared to IDDM patients [26]. Our findings indicate that there are remarkable differences in the genetic background of IDDM and LADA. The presence of the shorter class I VNTR allele, the high frequency of heterozygous CTLA4 A/G polymorphism, plus the finding of a lower frequency of highly predisposing HLA DQB1 *0201/*0302 in LADA than in IDDM patients could be the responsible factors for the development of the LADA disease.

We concluded that CTLA4 A/G polymorphism has a demonstrated effect in the autoimmune pathway of diabetes, such effect is different in abrupt onset (IDDM) or latent onset (LADA).

In summary, although HLA DQ region is responsible for 50% of the genetic susceptibility to autoimmune diabetes in IDDM patients, presents a lower effect in genetic susceptibility in LADA patients. Other predisposition loci should play the main role in LADA, indicating that several other genetic loci are needed to develop autoimmune diabetes in adult patients [27–29]. Therefore, LADA may be constituted by the combined effect of minor risk loci in a major risk haplotype [30].

The identification of autoimmune diabetes susceptibility genes will enhance the prediction of individuals that are at high risk of developing the disease, as well as to understand the pathogenesis in order to establish the prevention and the best treatment of the disease.

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