Type 1 diabetes susceptibility determined by *HLA* alleles and *CTLA-4* and insulin genes polymorphisms in Brazilians

Suscetibilidade ao diabetes tipo 1 determinada por alelos de *HLA* e polimorfismos nos genes *CTLA-4* e insulina em brasileiros

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ABSTRACT

Introduction: Type 1A *diabetes mellitus* (T1ADM) is a multifactorial disease in which genetic and environmental aspects are important to its development. The association of genetic variations with disease has been demonstrated in several studies; however, the role of some gene loci has not yet been fully elucidated. **Objective:** To compare the frequency of *HLA* alleles and polymorphism in *CTLA-4* and insulin genes in Brazilians with T1ADM and individuals without the disease, as well as to identify genetic markers that are able to discriminate between diabetic and non-diabetic individuals. **Methods:** The presence of *HLA* DQB1, DQA1 and DRB1 alleles, as well as the -2221 MspI polymorphism in the insulin gene and 49 A/G in the *CTLA-4* gene were identified by the "Time-resolved fluorometer" technique after hybridization with probes labeled with Eu (III) / Sm (III) and Tb (III). **Results:** The DQB1 *0302 and DQA1 *03 alleles were identified as predisposed to T1ADM, and the DQB1 *0301 allele presented a protective effect against the disease. The DQA1 label proved to be able to differentiate between 71.13% of the diabetic and non-diabetic individuals. This value increased to 82.47% when the DQB1 label was added. No significant difference in the frequency of polymorphisms in the insulin and *CTLA-4* genes was observed between the two groups. **Conclusions:** The genetic markers that best characterized and discriminated diabetic and non-diabetic individuals were the *HLA* DQA1 and DQB1.alleles. Arq Bras Endocrinol Metab. 2009;53(3):368-73.

Keywords
T1ADM; *HLA*; *CTLA-4*; insulin gene; discriminant factor analysis

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RESUMO

Introdução: O diabetes melito tipo 1 (T1ADM) é uma doença multifatorial em que os aspectos genéticos e ambientais são importantes para o seu desenvolvimento. A associação das variações genéticas com a doença tem sido demonstrada em vários trabalhos, no entanto, o papel de alguns locos gênicos não foi ainda completamente elucidado. **Objetivos:** Comparar a frequência de alelos do *HLA* e polimorfismos nos genes *CTLA-4* e insulina em brasileiros com T1ADM e indivíduos sem a doença, além de identificar marcadores gênicos que sejam capazes de discriminar indivíduos diabéticos e não diabéticos. **Métodos:** A presença dos alelos de *HLA* DQB1, DQA1 e DRB1, bem como dos polimorfismos -2221 *Msp*I no gene da insulina e 49 A/G no gene *CTLA-4*, foram identificados por meio da técnica *Time-resolved fluorometer*, após hibridização com sondas marcadas com Eu (III)/Sm (III) e Tb (III). **Resultados:** Os alelos DQB1*0302 e DQA1*03 foram identificados como sendo de predisposição ao T1ADM, e o alelo DQB1*0301 mostrou um efeito protetor à doença. Analisando somente o marcador DQA1, este mostrou ser capaz de diferenciar 71,13% dos indivíduos entre diabéticos e não diabéticos, cujo valor aumentou para 82,47% quando adicionado o marcador DQB1. A frequência dos polimorfismos nos genes da insulina e *CTLA-4* não mostrou diferença significativa entre os dois grupos estudados. **Conclusões:** Os marcadores genéticos que melhor caracterizaram e discriminaram diabéticos e não diabéticos foram os alelos de *HLA* DQA1 e DQB1. Arq Bras Endocrinol Metab. 2009;53(3):368-73.

Palavras-chave
T1ADM; *HLA*; *CTLA-4*; gene da insulina; análise fatorial discriminante

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INTRODUCTION

Type 1A *diabetes mellitus* (T1ADM) results from the cell-mediated autoimmune destruction of pancreas β-cells (1). Although T1ADM has always been recognized as a childhood disease, recent epidemiological studies have indicated that the incidence is comparable in adults (2). There is an enormous variation in the worldwide incidence of T1ADM. In Finland, the incidence is 30 to 40 per 100,000 inhabitants; but in Japan this incidence is 1 per 100,000 inhabitants (3-5). The incidence in the Brazilian population was estimated at 7.6 per 100,000 inhabitants (6,7).

Susceptibility to T1ADM involves both genetic and environmental components. Genetic associations with T1ADM were observed almost 30 years ago (8), but the full knowledge of genetically susceptible loci has not been fully elucidated. The *HLA* region accounts for approximately 50% of the genetic susceptibility to T1ADM, suggesting that the sum of the effects of other susceptible loci is nearly as great as the *HLA* effect (8-10).

There are several candidate gene regions for T1ADM susceptibility. However, many of these regions were associated with T1ADM in only a portion of the populations studied (11). One of them is the cytotoxic T lymphocyte-associated antigen-4 (*CTLA-4*) gene, localized on the 2q33 chromosome region. It encodes the T cell receptor involved in the control, proliferation and T cell apoptosis. This gene is a strong candidate for T cell-mediated autoimmune diseases like T1ADM (12,13). In this gene, the main polymorphism is localized in exon 1 at position 49 (A→G), which encodes threonine (Thr) or alanine (Ala), respectively (14,15). The *CTLA-4* G allele has been associated with T1ADM in many populations (16-19). The insulin (*INS*) gene, located on human chromosome 11p15.5, is another gene in which some polymorphisms in the promoter region have been reported to possess a strong association with T1ADM (20,21). The association of the C/T polymorphism of the insulin gene -2221 *Msp*I with the 49 A/G *CTLA-4* polymorphism may vary among populations, but the studies support the hypothesis that insulin CC and *CTLA-4* GG genotypes are an independent and clearly significant risk for developing type 1 diabetes (22-25).

In the Brazilian population, which is characterized by its great heterogeneity (24), only *HLA* has been studied in association with T1ADM (25-31). Volpini and cols., using the affected-family-based control (AFBAC) method to study 56 Southeastern Brazilian families, showed increased frequencies of the DRB1*03-DQA1*0501-DQB1*02 and DRB1*0401-DQA1*03-DQB1*0302 haplotypes in the patient group and a lack of a significant protective effect of the DRB1*1501-DQA1*0102-DQB1*0602 haplotype. An apparent protection conferred by the DRB1*13-DQB1*0301, DRB1*11-DQB1*0301, and DRB1*01-DQB1*0501 haplotypes was reported (28).

In view of the evidence that in some types of polymorphism in insulin, *CTLA-4* genes and *HLA* haplotypes are associated with the development of T1ADM, in spite of the fact that only one study evaluated these polymorphisms in the Brazilian population (29), the aim of the present study was to expand the analysis of these genes in the Brazilian population and to find a marker or a set of markers that would best discriminate the T1ADM susceptibility.

METHODS

Subjects

A total of 49 Brazilian patients with clinical and laboratory diagnoses of type 1 diabetes were screened for the presence of IA2 and GAD autoantibodies. The individuals had a mean age of 15.76 ± 1.91 and a diabetes diagnosis age of 9.75 ± 4.15. For the non-diabetic control group, 48 individuals without diabetes and no family history of the disease were included. These individuals had a mean age of 31.2 ± 5.12. Brazilian individuals over 25 years of age were evaluated for the control, so we were more confident that they would not develop type 1 diabetes. All of subjects provided a written informed consent, and this study was approved by the local ethical committee (COEP-UFMG).

Genotyping of *HLA* DQB1, DQA1 and DRB1 alleles

DNA was extracted from peripheral blood using the PUREGENE® kit. The alleles were detected using the DELFIA® technology (Walac Oy®). One microgram of genomic DNA was used as a substrate for the PCR (Polymerase Chain Reaction). For the amplification of the *HLA* region, the following primers were used: 5' GCA TGT GCT ACT TCA CCA ACG, 3'bio-CCT TCT GGC TGT TCC AGT ACT for DQB1; 5' bio-TAT GGT GTA AAC TTG TAC CAGT, 3' GGT AGC AGC GGT AGA GTT G for DQA1 and 5' GTT TCT TGG AGC AGG TTA AAC A, 3'bio-CTC GCC GCT GCA CTG TGA for DRB1. The time-resolved fluorimetric detection of the alleles was performed on the

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1420 VICTOR™ Time-resolved fluorometer after hybridization with a panel of labeled chelated lanthanide probes – Eu(III)/Sm(III) and Tb(III) (32,33)

Genotyping of *CTLA-4* and *INS* gene polymorphisms

The same technology described for the *HLA* alleles was used for detection of the 49 A/G polymorphism at exon 1 of the *CTLA-4* gene and the polymorphism at 2221 upstream from the *INS* gene (-2221 *Msp*I). The following primers were used for the PCR reaction: 5' TTC CTG AAG ACC TGA ACA CC, 3' bio-AAT GAC TGC CCT TGA CTG CT for CTLA4 and 5' ACC CCA CTA CAC GCT GCT G and 3' bio-CCC TTC AGA GAC ACC CCC A for the -2221 *Msp*I polymorphism in the *INS* gene. The time-resolved fluorimetric detection of the alleles was performed on the 1420 VICTOR™ Time-resolved fluorometer after hybridization with a panel of labeled chelated lanthanide probes – Eu(III)/Sm(III) and Tb(III) (33,34).

Statistical analysis

Data analysis was performed using the GENEPOP software. An Odds Ratio was calculated according to Woolf's method and, by convention, presented as relative risk. A discriminant factor analysis was performed using the genotyping results to determine whether the two groups, diabetic and non-diabetic, could be distinguished.

RESULTS

Allelic frequency and relative risk were determined for all the alleles found (Tables 1, 2 and 3). For the polymorphisms of the *CTLA-4* (Table 2) and insulin gene (Table 3), the genotype frequencies were determined in addition to the allele frequencies.

It could be clearly observed that the main predisposition alleles in the samples were DQB1*0302 (RR = 3.84) and DQA1*03 (RR = 3.0). Similar results have been described in the Brazilian population and in other populations (27,28,29,30,31). A significant protective effect of the DQB1*0301 allele, which had been described in several populations, was also observed (4,27,28,29,30,31). Significant values were not obtained for DRB1*04 when the DRB*0401, DRB1*0402, DRB1*0403, DRB1*0404 and DRB1*0405 alleles were analyzed. Therefore, an analysis for the presence or absence of DRB1*04 was performed. A relative risk of 3.34 was found (p<0.0009). It should be emphasized that both the 49 A/G *CTLA-4* and the -2221 *Msp*I polymorphisms, which was previously shown to be an important risk factor for diabetes development in other populations (22,35-37), were not statistically significant for the genetic predisposition for type 1 diabetes in the subjects analyzed.

Table 1. Distribution of *HLA*-DQB1, *HLA*-DQA1 e DRB1 allele frequencies in diabetic and normal individuals (non-diabetic)

HLA alleles	% IDDM (2n = 98)	% Control (2n = 96)	Relative Risk	p value
DQB1*02	43.88	14.58	4.66	NS
DQB1*04	2.04	2.08	1.00	NS
DQB1*05	1.02	0.0	-	NS
DQB1*0301	5.10	25.0	0.16	< 0.05
DQB1*0302	25.51	8.33	3.84	< 0.05
DQB1*0303	0.0	2.08	0.0	NS
DQB1*0304	1.02	1.04	1.0	NS
DQB1*0501	15.31	19.79	0.75	NS
DQB1*0502	1.02	2.08	0.49	NS
DQB1*0503	0.0	5.21	0.0	NS
DQB1*0601	0.0	1.04	0.0	NS
DQB1*0602	1.02	9.38	0.1	NS
DQB1*0603	1.02	5.21	0.19	NS
DQB1*0604	3.06	4.17	0.74	NS
DQA1*03	27.55	9.37	3.0	< 0.009
DQA1*05	36.65	19.79	1.68	NS
DQB1*0201	5.10	11.45	0.46	NS
DRB1*0401	4.16	0.0	-	NS
DRB1*0402	6.12	3.06	2.03	NS
DRB1*0403	1.02	1.02	1.0	NS
DRB1*0404	6.12	3.06	2.03	NS
DRB1*0405	11.22	2.04	5.55	NS
DRB1*04	53.06	16.0	3.34	p < 0.0009

RR: relative risk; NS: non-significant; IDDM: type 1 *diabetes mellitus*.
The results were considered significant when p < 0.05.

Table 2. Distribution of allele and genotype frequencies for 49 A/G *CTLA-4* in diabetic and normal individuals (non-diabetic)

Allele/genotype	% IDDM	% Control	RR	p value
Allele frequencies				
A	73.47	67.71	1.34	NS
G	26.53	32.29	0.75	NS
Genotype frequencies				
AA	53.06	45.83	1.38	NS
AG	40.81	43.75	0.88	NS
GG	6.12	10.41	0.57	NS

RR: relative risk; NS: non-significant; IDDM: type 1 *diabetes mellitus*.
The results were considered significant when p < 0.05.

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Table 3. Distribution of allele and genotype frequencies of the –2221/MspI polymorphism at the 11p15.5 region in diabetic patients and normal individuals (non-diabetic)

Allele/genotype	% IDDM	% Controls	RR	p - value
Allele frequencies				
C	87.80	84.40	1.29	NS
T	12.2	15.6	0.77	NS
Genotype frequencies				
CC	79.59	68.75	1.8	NS
CT	20.40	31.75	0.56	NS
TT	0.0	0.0	-	-

RR: relative risk; NS: non-significant; IDDM: type 1 *diabetes mellitus*.
The results were considered significant when $p < 0.05$.

Discriminant factor analysis

The discriminant factor analysis showed that the markers that best characterized and discriminated between the two groups were DQA1 and DQB1, in this order. The remaining markers were not helpful in discriminating diabetic and non-diabetic individuals. It was possible to correctly distinguish diabetic from non-diabetic individuals in 71.13% of the subjects by analyzing only the DQA1 marker. This percentage increased to 82.47% when the DQB1 marker was included. The other markers (DRB*04, *CTLA-4* and -2221/*Msp*I) did not increase this parameter when they were added to the discriminant factor analysis.

DISCUSSION

The great variability in the incidence of type 1 diabetes in the world can be partly explained by differences in the frequencies of the alleles that predispose for the disease. The analysis of the *HLA* frequencies confirmed the importance of the DQB1*0302 and DQA1*03 alleles in the predisposition for type 1 diabetes. The importance of the DQB1*0301 in the protection against the development of the disease can be observed, since it was more common in non-diabetic individuals.

As for *HLA* DRB*04, when each allele (DRB*0401, DRB*0402, DRB*0403, DRB*0404 and DRB*0405) was analyzed separately, no significant values were observed. This observation is probably due to the limited number of individuals in the study. However, the determination of presence or absence of *HLA*DRB*04 led to significant results, suggesting that the analysis of DRB1*04, without differentiating the several alleles, is a good tool for determining the relative risk for development of diabetes.

The discriminant factor analysis was extremely important in this work. The objective of this analysis was to verify whether the two groups, diabetic and non-diabetic, were really distinct between themselves based on the distribution of alleles that predispose for diabetes, and also to verify, when defining the groups, which markers or set of marker among those markers studied could best discriminate between the groups.

The results showed that *HLA* DQA1 alone can correctly classify 71.13% of the individuals into diabetic and non-diabetic groups. This value is denominated a well-classified percentage. When the *HLA* DQB1 was introduced into the analysis, the well-classified percentage increased to 82.47%. The remaining markers analyzed did not increment this value, since there was an overlapping in the classification. Noticeably, since type 1 diabetes is a complex and multifactorial disease, where not only genetic but also environmental factors contribute for its development, there are individuals that exhibit the predisposition alleles and do not show the clinical symptoms, and there are those who do not show the predisposition alleles but are clinically diabetic. For these reasons, the above-mentioned well-classified percentage should not be absolute.

The analyses of the 49 A/G *CTLA-4* and the -2221/MspI polymorphism of the insulin gene, contrary to expectations, did not reveal any association with type 1 diabetes in the individuals analyzed. Allele and genotype frequencies of the two polymorphisms are similar in the affected and non-affected individuals. This fact is particularly surprising regarding the 49 A/G *CTLA-4* gene polymorphism since it is strongly associated with diabetes in several populations around the world (22,35-37). Although the *CTLA-4* gene is strongly associated with autoimmune diseases, specially those mediated by T-cells, the lack of this association has already been described in Brazilian patients with autoimmune hepatitis and primary biliar cirrhosis (38,39), contrary to the observations in several studies carried out in other populations (40,41).

It is worth noticing that, in the present study, the *HLA* DQA1 allele was more important for distinguishing diabetic and non-diabetic individuals than DQB1 and DRB1. The results confirmed the importance of the *HLA* region for the development of type 1 diabetes in Brazilian individuals and also suggest that 49 A/G at the *CTLA-4* gene and -2221/MspI polymorphisms at the *INS* gene, in principle, were not so important to the genetic predisposition to the disease. However,

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complementary studies are necessary for evaluating whether other previously-described polymorphisms at the *CTLA-4* and insulin genes collaborate for predisposition to the disease.

Many studies have presented new therapies for treating and avoiding the onset of type 1 diabetes in animal models and in humans (42-45). We believe that the correct identification of individuals with genetic predisposition for the disease can be a useful tool for prescribing new treatments in the near future.

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