ADRIANO NAMO CURY

ESTUDO DO POLIMORFISMO +49 A>G DO GENE CTLA-4

EM ADULTOS E CRIANÇAS COM DOENÇA DE GRAVES

Tese apresentada ao curso de Pós-Graduação da Faculdade de Ciências Médicas da Santa Casa de São Paulo para obtenção do título de doutor em Ciências da Saúde.

São Paulo

2008

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Área de Concentração: Ciências da Saúde

Orientador: Prof. Dr. Osmar Monte

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Dedicatória

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Sumário

JUSTIFICATIVA

As doenças tireoidianas auto-imunes (DTA), como a tireoidite de Hashimoto e a doença de Graves, são comuns na população em geral e apresentam prevalência estimada em 1%, de acordo com estudos norte-americanos (1). A patogênese das DTA, especialmente a doença de Graves, envolve uma complexa interação entre fatores ambientais e genéticos. Classicamente, a doença de Graves caracteriza-se pela tireotoxicose, bócio difuso e produção de auto-anticorpos estimuladores do receptor do TSH (TRAb), manifestações extra tireoidianas, como a oftalmopatia e, mais raramente, o mixedema pré-tibial. Os eventos patogênicos que justificam o quadro clínico podem ser enumerados de maneira simplificada a partir da susceptibilidade genética, primeiro evento na patogênese, seguido pelo segundo evento que se caracteriza pela interação genética com moduladores ambientais que serviram de gatilho para as doenças auto-imunes (infecções, tabagismo ou estresse). Como resultado, células T tireóide específicas são ativadas e infiltram o tecido glandular desencadeando a ativação dos linfócitos B, geração do TRAb, proliferação dos tireócitos e hipersecreção dos hormônios tireoidianos, com as manifestações típicas do hipertireoidismo.

A presença de genes que, supostamente, facilitam e predispõem ao interagir com fatores ambientais, ao desenvolvimento de uma das mais freqüentes doenças auto-imunes órgão específico (modulada por células T), como a doença de Graves (DG), dita *per se* a importância da pesquisa de genes de susceptibilidade para doenças auto-imunes. A genética de predisposição para DG é complexa pela natureza poligênica que, certamente, ocorre de maneira comum, como pano de fundo, para a maioria das doenças auto-imunes, envolvendo genes do sistema HLA e genes específicos que influenciam qualquer etapa na regulação da resposta imune, ou seja, ativação e supressão das células T.

Além dos genes do sistema MHC classe II, como HLA-DR3 e DQA1*0501 no cromossoma 6p21 (2-4), no cromossoma 2q33, encontramos o *loci* com genes relacionados com a regulação dos linfócitos T: CD28, CTLA4 e *ICOS,* nos quais, em particular, os polimorfismos do *cytotoxic T lymphocyte antigen-4 (*CTLA-4) estão associados a diversas doenças auto-imunes (5, 6). As proteínas CD28 e CTLA4 são moléculas co-estimuladoras presentes na superfície das células T que se ligam à família de receptores B7, expresso nas células apresentadoras de antígenos (7). A resposta imune depende da geração de dois sinais: o primeiro, proveniente da interação de peptídeos antigênicos com os receptores nas células T, no contexto do MHC; e o segundo sinal (co-estimulador) que ativa, amplifica e promove a proliferação das células T com a produção de citoquinas (como a IL-2), onde o complexo CD28/B7 funciona com regulador positivo das células T e o CTLA4/B7, expresso exclusivamente nos linfócitos T ativados, fornece um sinal inibidor, necessário para limitar a proliferação das células T e regular a resposta auto-imune (8, 9).

Em estudos caso-controle, na última década, o polimorfismo +49 A>G do exon 1 do gene CTLA4 foi associado a doenças auto-imunes, como a doença de Graves, diabetes Tipo 1, tireoidite de Hashimoto, artrite reumatóide, doença de Addison auto-imune e a esclerose múltipla (10-21); em estudos familial, foi associado com a doença de Graves (15, 22) em populações caucasiana, japonesa, chinesa e coreana.

Especificamente, o polimorfismo +49 A>G, que promove a troca de aminoácido treonina pela alanina na posição 17, surgiu como candidato natural, entre os polimorfismos do gene CTLA-4, com possibilidade de promover alterações funcionais da proteína CTLA-4, principalmente quando Kouki *et al.* (23) demonstraram a maior freqüência do genótipo GG ou AG na posição 49 do gene CTLA-4, em pacientes com doença de Graves, e o menor controle sobre proliferação das células T e, em 2002, quando Maürer *et al.(24)* observaram diferenças no *pool* de proteína CTLA-4 intracelular, promovendo desequilíbrio na expressão e competição entre o CTLA-4 e o CD28 na superfície das células

T, interferindo na ativação e supressão de células T e produzindo maior quantidade de citocinas inflamatórias.

Outros autores também correlacionaram o alelo G na posição 49 do gene CTLA-4 com o maior tempo de uso dos fármacos antitireoidianos para a remissão da doença de Graves(25) como fator preditor de recorrência após o término do tratamento(26) ou com a produção de anticorpos antitireoidianos (27, 28).

A doença de Graves na infância ocorre com menor freqüência quando comparada com sua incidência entre os adultos, mas corresponde à principal causa de hipertireoidismo entre as crianças. São poucos os estudos que avaliaram polimorfismos genéticos e a doença de Graves na infância. Existem apenas dois estudos publicados na literatura médica, referentes à população chinesa e japonesa, que correlacionam o CTLA-4 com a doença de Graves na infância (29, 30) pela maior freqüência do alelo 49G de susceptibilidade ou menor freqüência do genótipo de proteção AA. Porém, estudos de associação do polimorfismo +49 A>G do gene CTLA-4 em pacientes com doença de Graves, pertencentes à população adulta, já foram realizados em japoneses, chineses, caucasianos, americanos e europeus, com resultados distintos e por vezes opostos.

A população brasileira apresenta composição étnica única e heterogênea pela influência de imigrantes caucasianos, europeus (portugueses, italianos, alemães), africanos e asiáticos (japoneses, chineses e coreanos), sendo necessária a realização de estudos de associações genéticas entre as doenças mais prevalentes, como a doença de Graves, para elucidar o risco conferido pelo o fator genético e sua contribuição para o desenvolvimento das doenças auto-imunes.

O primeiro artigo, **Graves' Disease in Brazilian Children and Adults: Lack of genetic association with CTLA-4 +49A>G polymorphism,** mostra como um dos principais polimorfismos do gene CTLA-4 apresenta-se em pacientes com doença de Graves em duas faixas etárias distintas. Verifica

como este *single nucleotide polymorphism* (SNP) ocorre em crianças e adultos, comparando os resultados entre os dois grupos e entre os controles normais para auto-imunidade. Analisa, por outro lado, o polimorfismo do +49A>G em adultos e crianças e suas possíveis correlações com os marcadores bioquímicos e clínicos da doença de Graves, ou seja, associação com oftalmopatia, perfil hormonal ao diagnóstico e necessidade de radioiodoterapia como opção terapêutica definitiva. De maneira objetiva, analisa e compara a freqüência do polimorfismo +49A>G do gene CTLA-4 em adultos e crianças, e as possíveis correlações clínicas e laboratoriais ao diagnóstico com o genótipo.

O segundo artigo, **Establishment of a stringent control group for the characterization of CTLA-4 exon-1 +49A>G polymorphism in a Brazilian Southeast population**, foi necessário para a validação dos resultados do primeiro. Como se mencionou anteriormente, a população brasileira apresenta composição genética única e, portanto, pode-se dizer que, em qualquer estudo de associação genética, deve-se estruturar o grupo de controle de maneira criteriosa para a análise dos seus resultados. O polimorfismo do gene CTLA-4 já foi pesquisado por Guzman *et al.(31)*. Porém, esses autores consideraram apenas o critério raça na avaliação do polimorfismo. O objetivo do estudo foi verificar novamente o polimorfismo do gene CTLA-4 na população brasileira de acordo com a raça, mas, obrigatoriamente, em indivíduos normais para doenças auto-imunes, sem qualquer sinal clínico de tireoidopatia, diabetes auto-imune, lúpus eritematoso sistêmico, artrite reumatóide, comprovando o eutireoidismo e a ausência de anticorpos antitireoidianos, como o anticorpo antiperoxidase, antitireoglobulina e o TRAb. Neste trabalho, foram avaliados indivíduos normais para doenças auto-imunes com idade superior a sessenta anos, quando já teria ocorrido a manifestação da maioria das doenças auto-imunes. Este trabalho foi realizado na unidade de Geriatria da Santa Casa de São Paulo (Hospital Geriátrico D. Pedro II) e o polimorfismo do gene CTLA-4 foi pesquisado somente na ausência de histórico de doenças auto-imunes, eutireoidismo documentado (excluímos disfunções tireoidianas clínicas e subclínicas), com ausência de anticorpos antitireoidianos. De maneira complementar, comparou-se o resultado deste trabalho com o de outros

estudos já publicados na literatura médica envolvendo diferentes populações, como a japonesa, chinesa, afro-americana, caucasianos, europeus e norte-americanos.

ARTIGO 1.

Graves' Disease in Brazilian Children and Adults: Lack of genetic association with CTLA-4 +49A>G polymorphism

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Graves' Disease in Brazilian Children and Adults: Lack of Genetic Association with CTLA-4 +49A>G Polymorphisms

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Key Words
Cytotoxic T lymphocyte antigen-4 gene polymorphism · Graves' disease · Children, Graves' disease

Abstract

Background/Aim: In several populations, major histocompatibility complex and CTLA-4 (cytotoxic T lymphocyte antigen-4) gene polymorphisms are related to adult subjects with Graves' disease (GD). Our aim was to study the association of +49A>G polymorphism of the CTLA-4 gene in Brazilian children and adults with GD and its correlation with clinical and laboratory markers of disease severity. ***Methods:*** CTLA-4 +49A>G polymorphism was established by polymerase chain reaction-restriction fragment length polymorphism analysis in 44 children and 72 adults with GD and compared to a stringent control group consisting of octogenarians with no history of thyroid disease; free T_4 and T_3 levels and T_3/T_4 ratio, antithyroid antibodies, and Graves' ophthalmopathy were also evaluated according to genotype. ***Results:*** No significant difference was found in the frequency of CTLA-4 +49A>G polymorphism among children and adults with GD compared to controls and within groups. There was no significant correlation between the presence of G allele and Graves' ophthalmopathy, gender, age at diagnosis, and biochemical markers of disease severity. ***Conclusion:*** The frequency of CTLA-4 +49A>G polymorphism is not different in children and adults with GD compared to the normal control population and does not seem to contribute independently to the severity of the clinical presentation of GD.

Introduction

Graves' disease (GD) is an autoimmune organ-specific disease, mediated by T cells and characterized by hyperthyroidism, diffuse goiter, Graves' ophthalmopathy (GO), and, in some cases, pretibial myxedema [1, 2]. The presence of stimulating autoantibodies to the thyroid-stimulating hormone receptor (TSAb) represents the main event in the GD pathophysiology, determining hormone hypersecretion by the thyroid gland [3].

Susceptibility to autoimmune thyroid diseases involves a complex interaction of environmental and genetic factors. Among the genetic factors, several studies have shown the association of GD and class II major histocompatibility complex system genes, such as HLA-DR3 and DQA1*0501, on chromosome 6p21 [4–6]. The cyto-

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toxic T lymphocyte antigen-4 (CTLA-4) locus on chromosome 2q33 was widely studied in several populations, and CTLA-4 is currently considered to be one of the main genes (non-HLA) associated with GD [7–9]. Vaidya et al. [10] demonstrated that, together with major histocompatibility complex genes, CTLA-4 loci confer approximately 50% of the inherited susceptibility to GD [10].

The production of TSAb is regulated by T cells [11], and genes related to the antigen-specific activation of T cells are involved in the development of GD. CTLA-4 is one of the main genes associated with GD, containing polymorphisms that can modulate the costimulatory signal of T cells and, as a consequence, self-tolerance to developing autoimmune diseases.

CTLA-4 is an important molecule on the T cell surface, which takes part in the interaction between T lymphocytes and antigen-presenting cells, homolog to another surface receptor, CD28 [12]. The antigen-presenting cells activate T lymphocytes after interaction of T cell receptors with antigenic peptides. This is mediated by HLA class II, with a concomitant stimulatory signal generated after binding of CD28 to B7-1 and B7-2 receptors, expressed on the antigen-presenting cells. This activates T lymphocytes and unleashes the immune response with the production of cytokines and cellular proliferation [13]. Activated T cells produce CTLA-4 (CD152), a 149-amino-acid glycoprotein expressed exclusively in CD8+ and CD4+ T lymphocytes [14]. CTLA-4/B7 interaction directly modulates T cell homeostasis. Its suppressive effect on activating T cells has raised the hypothesis that mutations of the CTLA-4 gene can decrease both the expression and function of the CTLA-4 protein, allowing the development of an autoimmune disease [15].

The CTLA-4 protein is expressed on activated T lymphocytes, and its binding to B7 receptors promotes a downregulation in differentiation and T cell activation [12, 16]. CTLA-4 +49A>G polymorphism leads to a threonine-to-alanine change in the leading peptide, which suggests that reductions in CTLA-4 expression or function might contribute to autoimmunity in this condition. Specifically, the +49A>G polymorphism is related to differences in the intracellular pool of CTLA-4 protein and imbalances in the competition between CTLA-4 and CD28 on the cellular surface, thus interfering with T cell activation and increasing interleukin-2 secretion [17]. The G allele at position 49 was also related to a reduced T cell proliferation and a decreased CTLA-4 function, correlating with GD pathogenesis [18, 19].

In 1993, Yanagawa et al. [5] published the first study showing the association between the microsatellite marker located at the 3′ untranslated region of the CTLA-4 gene and GD in a Caucasian population. Since then, other researchers have confirmed their findings in case-control studies in Caucasian, Japanese, and Chinese populations [20–22]. Another polymorphism was detected at position –318 (C/T_{-318}) in the promoter region [13], but the CTLA-4 +49A>G polymorphism frequency and its association with GD were widely confirmed in different populations [23–27]. The +49A>G polymorphism represents a possible marker of dysfunctional CTLA-4 protein, related to GD and associated with antibody production [28], greater treatment period with antithyroidal drugs (ATD), persistence of TSAb [29], as well as recurrence of thyrotoxicosis after ATD withdrawal [30].

The CTLA-4 +49A>G polymorphism has already been investigated in several adult GD populations, but only two studies were performed in the pediatric population [31, 32]. Our study aimed a t determining the frequency of the +49A>G polymorphism and its association with GD in Brazilian children and adults. We also correlate the +49A>G polymorphism with the clinical presentation, such as gender, age at the time of diagnosis, anti-thyroperoxidase antibody (TPO-Ab) and anti-thyroglobulin antibody (Tg-Ab), freeT_4 and T_3 levels at the time of diagnosis, and T_3/T_4 ratio, and its association with GO.

Patients and Methods

Patients and Controls

Two groups of patients were studied: 44 pediatric patients (11 boys, 33 girls) with GD onset during childhood with a mean age at the time of diagnosis of 12.0 ± (SD) 4.2 (range 4.0–18.0) years and 72 adult patients (30 males, 42 females) with their GD diagnosis at a mean age of 35.2 ± (SD) 12.1 (range 19.0–79.0) years. The GD diagnosis was performed according to clinical and laboratory criteria, including the presence of suppressed thyroid-stimulating hormone levels and high levels of T_4, freeT_4, and T_3 and diffuse goiter, with or without OG. Other causes of hyperthyroidism were excluded. The control group was carefully selected and consisted of 78 individuals aged ≥60 (mean 72.9 ± 7.6) years, without any history of autoimmune disease; the control group also presented normal thyroid-stimulating hormone and free T_4 levels, absence of TPO-Ab and Tg-Ab, in addition to negative TSAb and no clinical evidence of autoimmune disease. The study was approved by the Institution's Ethics Committee, and informed consent was obtained from all patients and controls.

CTLA-4 Polymorphism

Genomic DNA was extracted from peripheral blood mononuclear cells. CTLA-4 was amplified using PCR targeted to the exon 1. The PCR reaction was performed with sense 5′-GCTCTACTTCCTGAAGACCT-3′ and reverse 5′-AGTCTCACTCACCTTTGCAG-3′ primers, as follows: denaturation for 5 min at

Table 1. CTLA-4 +49A>G polymorphism in pediatric and adult patients with GD and in controls

Subjects	n	Genotype				Allele		
		A/A	A/G	G/G	p	G	A	p
GD								
Childhood	44	17 (39)	22 (50)	5 (11)	0.48	32 (36)	56 (64)	0.34
Adults	72	26 (36)	36 (50)	10 (14)	0.20	56 (39)	88 (61)	0.11
Total	116	43 (37)	58 (50)	15 (13)	0.19	88 (38)	144 (62)	0.10
Controls	78	39 (50)	32 (41)	7 (9)		46 (30)	110 (70)	

Values represent the number of individuals, genotypes, or alleles with the percentages in parentheses. Comparisons of adults and children with GD to the control group were performed using χ^2 or Fisher's exact test when appropriate.

94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 71°C, and final extension at 72°C for 7 min. The PCR product was digested with *Bbv*I restriction enzyme (Fermentas, Hanover, Md., USA) and resolved on 2.5% agarose gel. The G_{49} allele can be digested by *Bbv*I, yielding a band including two fragments of 88 and 74 bp, respectively. The presence of allele A solely does not have a *Bbv*I restriction site, retaining the 162-bp PCR product size. Genotyping was confirmed by GeneScan analysis (Applied Biosystems, Foster City, Calif., USA) performed in an automatic DNA sequencer (ABI-PRISM 310; Applied Biosystems).

Statistical Analyses

The allele frequencies among pediatric and adult patients and the control group were compared employing χ^2 test or Fisher's exact test, as required. Both clinical and laboratory variables were analyzed between subjects and controls using one-way ANOVA or t test performed with Minitab 14 software (Minitab, State College, Pa., USA). $p < 0.05$ was considered statistically significant.

Table 2. Comparison of CTLA-4 +49A>G polymorphism in children and adults with GD

	Chilhood-onset GD (n = 44; 88 alleles)	Adult-onset GD (n = 72; 144 alleles)	p
Genotype			
G/G	5 (11)	10 (14)	0.91
A/G	22 (50)	36 (50)	
A/A	17 (39)	26 (36)	
Allele			
G	32 (36)	56 (39)	0.7
A	56 (64)	88 (61)	

Values represent the number of individuals (genotype) or alleles with the percentage in parentheses. Comparisons between children and adults with GD to the control group were performed using χ^2 or Fisher's exact test when appropriate.

Results

The frequency analysis of the +49A>G polymorphism showed no significant differences when children and adult GD patients were compared with each other or to the control group individuals. Allele G occurred in 36% of the GD children, in 39% of the GD adults, and in 30% of the control individuals (tables 1, 2).

There was no significant difference in clinical and laboratory findings when GD patients with different allele compositions were compared (table 3). There were no correlations of +49A>G single-nucleotide polymorphism with gender, age at the time of diagnosis, race, presence of GO, free T_4 and T_3 levels and T_3/T_4 ratio, or antibodies measured at the time of diagnosis. Additionally, no relationship to disease severity was observed when the CTLA-4 polymorphism was considered.

Most patients required a therapeutic dose of radioiodine to control the disease; among ATD, methimazole was the most frequently used, and no positive association was found between genotype and the different GD treatment regimens. Some adult subjects have not used ATD, but have received radioiodine therapy immediately after the first medical evaluation, because they were part of another study on the therapeutic efficiency of radioiodine therapy without using predose ATD (table 3).

Discussion

Several autoimmune diseases have been related to the presence of CTLA-4 +49A>G polymorphism, including type 1 diabetes, Hashimoto's thyroiditis, rheumatic ar-

Table 3. CTLA-4 +49A>G polymorphism and physical and laboratory characteristics of the patients with GD

	Childhood-onset GD (n = 44)				Adult-onset GD (n = 72)			
	G/G (n = 5)	A/G (n = 22)	A/A (n = 17)	p	G/G (n = 10)	A/G (n = 36)	A/A (n = 26)	p
Age at onset, years (± SD)	13.6 ± 4.2	11.6 ± 3.9	12.1 ± 4.7	NS	35.5 ± 8.5	32.05 ± 12	31.3 ± 11	NS
Female/male ratio	4/1	15/7	14/3	NS	5/5	22/14	15/11	NS
Race: white/nonwhite	5/0	22/0	16/1	NS	7/3	23/13	22/4	NS
GO, positive/negative	1/4	8/14	9/8	NS	4/6	17/19	13/13	NS
Initial free T_4, ng/dl (± SD)	8.4 ± 2.8	5 ± 3	4.9 ± 2.4	NS	5.85 (3.74)	5.38 (2.6)	4.47 (2.75)	NS
Initial T_3, ng/dl (± SD)	471.7 ± 165.8	389.7 ± 201.9	383.8 ± 185.5	NS	449 ± 195	356 ± 199	346 ± 175.3	NS
T_3/T_4 ratio	17 (5.3)	19.1 (7.9)	16.8 (3.3)	NS	20.2 (4.4)	18.3 (5.9)	17 (6)	NS
Antibody, positive/negative	3/2	16/6	14/3	NS	8/2	25/11	18/8	NS
TPO-Ab positive	1	4	2		1	8	5	
Tg-Ab positive	1	0	2		0	3	0	
TPO-Ab and Tg-Ab positive	1	12	10		7	14	13	
ATD: MTZ/PTU/none	3/2/0	16/6/0	8/9/0	NS	2/4/4	22/6/8	18/4/4	NS
Radioiodine therapy	5	18	10	NS	8	27	16	NS

According to the variable chosen, we performed one-way ANOVA or t test, and to analyze treatment the χ^2 test with tables 3 × 2 or 3 × 3 was used.

MTZ = Methimazole; PTU = propylthiouracil.

thritis, multiple sclerosis, and GD [27, 33–35], and most studies have been performed in an adult population. GD is not common in infancy, and only two studies have shown the association of GD with CTLA-4 gene +49A>G polymorphism in children [31, 32]. Our study was the first to assess the CTLA-4 gene in a Brazilian population with GD, especially in children, also correlating it with markers of disease severity: TPO-Ab, Tg-Ab, age at the time of diagnosis, gender, and GO.

The modern Brazilian population consists of individuals of Portuguese, Italian, Spanish, and German ancestry who mixed their lineages with African, Indian, and Asian populations, resulting in a heterogeneous population with a complex race admixture that cannot be considered to be exclusively Caucasian or non-Caucasian.

The distribution of the CTLA-4 alleles in Japanese and Caucasian control subjects differed from that in previous studies [23, 24]. The control group of this study was carefully chosen to highly accurately represent the Brazilian population according to its race composition. This offers an adequate group for comparative genetic studies, especially those employing a single-nucleotide polymorphism case-control methodology.

Our results clearly show that the +49A>G polymorphism, when analyzing either the genotype or the frequency of the G allele, presented similarly among controls, adults, and children with GD. Despite the trend of a higher frequency of the G allele, there was no significant difference when patients with GD were compared with control individuals. Likewise, GD severity markers at the time of diagnosis, such as free T_4 and T_3 levels or T_3/T_4 ratio, were not correlated with CTLA-4 +49A>G. Also, we found no relation between such polymorphism and TPO-Ab or Tg-Ab, skin color, age, and GO.

GD has a low incidence in childhood, especially among the Caucasian population [31]. Studying GD in children could be more effective in identifying influences of genetic factors than environmental factors. Studies performed in both Chinese and Japanese populations have shown the association of +49A>G polymorphism with GD [22, 24]. With a significantly high number of patients, Yung et al. [31] have shown that the G allele is most prevalent in the Chinese population, with a very high incidence of childhood GD in Hong Kong, and confers susceptibility to GD with a minimal odds ratio of 2.8. The distribution of genotype frequencies in Japanese children with GD differed significantly with a lower frequency of AA genotype [32].

In the Brazilian population we found divergent results, which can be justified by the population characteristics or possible regional variations in single-nucleotide polymorphism case-control studies. It should be noted that the power of our study is low (34%). The number of patients was small, but adequate for the purpose of this

study. To confirm genetic differences in the Brazilian population involving autoimmune disorders such as GD in adults and children, a substantial number of patients must be enrolled, which could be more feasible in a multicentric study.

In this study, we observed that Brazilian adults with GD behave similarly to children in relation to such CTLA-4 polymorphism. Also, other genetic or environmental factors may contribute to GD in our population, in addition to +49A>G polymorphism. It is important to point out that the +49A>G polymorphism was not related to GD in other populations, such as Korean, French, Tunisian, and African-American [36–39].

Considering the low prevalence of GD in childhood, the number of patients in this study was adequate and similar to that published recently by Iwama et al. [32]. In conclusion, our study shows that other polymorphisms must be studied in order to enlighten the genetic contribution of the CTLA-4 gene to GD in Brazilian children and adults. The +49A>G polymorphism does not seem to contribute separately to the development of GD in Brazilian children or adults and does not seem to contribute independently to the severity of clinical presentation. We believe that other polymorphisms, such as CT60, J030, J031, and J027-1 [7], must be further investigated with respect to GD in the Brazilian population.

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ARTIGO 2.

Establishment of a stringent control group for the characterization of CTLA-4 exon-1 +49A>G polymorphism in a Brazilian Southeast population

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Establishment of a stringent control group for the characterization of CTLA-4 exon-1 +49A/G polymorphism in a Brazilian Southeast population

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Title

Establishment of a stringent control group for the characterization of CTLA-4 exon-1 +49A>G polymorphism in a Brazilian Southeast population

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Summary

In several populations, case-control studies have shown that CTLA-4 (cytotoxic T lymphocyte antigen-4) gene polymorphisms are related to autoimmune disorders. Our aim was to establish an ideal control group for the study of polymorphisms of CTLA-4 in the Brazilian population, and compare the frequency of G allele with data from reports involving other ethnic groups. CTLA-4 exon 1 G_{49} allele was evaluated by PCR-RFLP in 78 carefully selected Brazilian subjects, without autoimmune diseases history, older than 60 y.o. in proven euthyroidism and without the presence of anti-thyroid antibodies. The frequency of G allele was compared to that reported in Asian and Caucasian populations. No significant difference was found in the frequency of CTLA-4 +49A>G polymorphism among Caucasian control groups nor in other Brazilian studies. However, we found significant differences when comparing our control group to Asian populations. Determining the association of CTLA-4 +49A>G polymorphism to autoimmune diseases is highly dependent on the profile of the control group. The absence of clinical signs of autoimmune diseases and the concomitant euthyroidism confirmed by normal hormone levels and absence of autoantibodies are necessary for the analysis of results in these studies. The presence of G allele, that may favor susceptibility to autoimmune diseases, differs in Asian populations compared to Caucasian and Brazilian population.

Introduction

The chromosome 2q33 region contains the *loci* with genes related to the regulation of T lymphocytes reactivity: CD28, CTLA-4 (cytotoxic T lymphocyte antigen-4), and *ICOS*. In this region peculiar polymorphism of the CTLA-4 gene have been associated with several autoimmune diseases (Chistiakov and Turakulov 2003; Vaidya and Pearce 2004). CD28 and CTLA-4 proteins are co-stimulatory molecules present in the surface of T cells, which connect to the B7 receptor family, expressed in antigen-presenting cells (APC) (Reiser and Stadecker 1996). The immune response depends on the generation of two signals. The first, derived from the interaction of antigenic peptides with T-cell receptors, in the MHC context. The second acts as a co-stimulatory factor which activates and fosters the proliferation of T-cells, and the production of cytokines such as IL-2. Therefore, CD28/B7 complex works as a positive regulator of T-cells, while the CTLA4/B7, expressed exclusively in activated T-lymphocytes, provides an inhibitory signal necessary to limit the proliferation of T-cells and regulate the autoimmune response (Oosterwegel, Greenwald et al. 1999; Sharpe and Abbas 2006).

The +49A>G polymorphism of the CTLA-4 gene is associated with the predisposition of autoimmune diseases (Vaidya and Pearce 2004) and functional changes of the CTLA-4 protein with imbalances of the inhibitory control of the differentiation and the proliferation of T cells (Kouki, Sawai et al. 2000; Ban, Davies et al. 2003), and the production of inflammatory cytokines, such as IL-2 (Maurer, Loserth et al. 2002). CTLA-4 +49A>G polymorphism leads to a threonine to alanine change in the leading peptide, with potential reductions in CTLA-4 expression or function related to the development of organ-specific autoimmune diseases.

For more than a decade, several authors have been searching for the association of polymorphisms in CTLA-4 gene, especially the +49A>G polymorphism, with autoimmune thyroid diseases (Tomer and Davies 2003; Ueda, Howson et al. 2003; Ikegami, Awata et al. 2006) type 1 diabetes (T1D) (Nistico, Buzzetti et al. 1996; Donner, Rau et al. 1997), rheumatoid arthritis (Yanagawa, Gomi et al. 2000; Han, Li et al. 2005), autoimmune Addison's disease (Donner, Braun et al. 1997; Vaidya, Imrie et al. 2000; Blomhoff, Lie et al. 2004), multiple sclerosis (Fukazawa, Yanagawa et al. 1999; Lorentzen, Celius et al. 2005), systemic erythematosus lupus (Heward, Gordon et al. 1999; Parks, Hudson et al. 2004), myasthenia gravis (Chuang, Strobel et al. 2005), celiac disease (Djilali-Saiah, Schmitz et al. 1998; Popat, Hearle et al. 2002), and autoimmune hepatitis(Djilali-Saiah, Ouellette et al. 2001). The +49A>G polymorphism is related to the

production of thyroid auto-antibodies (Tomer, Greenberg et al. 2001; Bossowski, Stasiak-Barmuta et al. 2005; Zaletel, Krhin et al. 2006); stimulating auto-antibodies for the TSH receptor (TSAb) levels and the clinical remission rate of Graves' disease (GD) during treatment with anti-thyroid drugs (Kinjo, Takasu et al. 2002; Wang, Liu et al. 2004). The same polymorphism has already been associated with the generation of the antiglutamic acid decarboxylase antibody (anti-GAD) and ICA 512 Ab, mode of onset (Abe, Takino et al. 1999; Abe, Yamaguchi et al. 2001) and early onset of Type 1 diabetes (Zalloua, Abchee et al. 2004). These studies employed both case-control (Yanagawa, Hidaka et al. 1995; Nistico, Buzzetti et al. 1996; Kotsa, Watson et al. 1997; Yanagawa, Taniyama et al. 1997; Awata, Kurihara et al. 1998; Fukazawa, Yanagawa et al. 1999; Heward, Allahabadia et al. 1999; Vaidya, Imrie et al. 2000; Ueda, Howson et al. 2003; Blomhoff, Lie et al. 2004; Young-Min and Vaidya 2004) and family-based association studies (Heward, Allahabadia et al. 1999; Vaidya, Imrie et al. 1999). Most studies have included Caucasian, Japanese, Chinese or Korean populations presenting sometimes conflicting results (Perez de Nanclares, Martin-Pagola et al. 2004; Han, Li et al. 2005; Lorentzen, Celius et al. 2005; Cho, Chung et al. 2006). Thus, the frequency of this single nucleotide polymorphism (SNP), and its association to autoimmune diseases varied according to studied population (Brookes 1999; Vaidya and Pearce 2004; Han, Li et al. 2005).

The ethnic origin of the Brazilian population is extremely heterogeneous as a product of various immigration waves from Europe, Africa, Asia, as well as the local Indian population, and their miscegenation. Specifically in São Paulo, according to the official Brazilian census, between 1992 and 1999, ethnic composition in São Paulo is 71.8% white, 22.6% mulatto, 4.2% black, and 1.5% Asian and Indian. The Caucasian prevalence is explained by the large European immigration (Scarel-Caminaga, Trevilatto et al. 2002). Only one study has evaluated the frequency of +49A>G polymorphism in the Brazilian population relating the polymorphism exclusively to the ethnic group, and according to the phenotype characteristic, that is, the frequency of the polymorphism in white, mulatto, and black individuals (Guzman, Morgun et al. 2005).

Studies on genetic variations are important for understanding genetic influence in common diseases, such as +49A>G polymorphism of the CTLA-4 gene, and autoimmune diseases. These studies can also be useful as a genetic marker for predisposition in Graves' disease, Hashimoto's thyroiditis and type diabetes. The design employed in these studies is usually case-control studies, in which an appropriate control-group is essential for adequate recognition of the association of polymorphisms and pathologic conditions. However, the inclusion criteria for establishing the control group is not equally stringent among studies, which should be taken into account during data analyses.

Our study aimed to evaluate the frequency of the +49A>G polymorphism of the CTLA-4 gene in a stringent control group, representative of the São Paulo State population, and compare the frequency of the observed SNP with published data from other ethnic groups.

Methods

Subject Selection

The control group included individuals aged above 60 y.o., without a family history on autoimmune disease, with proven euthyroidism, without the presence of thyroid antibodies (TAb), previous diagnosis of autoimmune diabetes, rheumatoid arthritis, asthma, celiac disease, myasthenia gravis, systemic erythematosus lupus, or autoimmune hepatitis.

We selected subjects from the Geriatric Unit of Hospital D. Pedro II Santa Casa de São Paulo. Initially 90 healthy candidates were evaluated with laboratory tests: TSH and free T4, anti-thyroperoxidase antibody (TPO-Ab), anti-thyroglobulin antibody (Tg-Ab) and TSAb.

From the 90 subjects initially selected as possible candidates for the control group to study the polymorphisms of the CTLA-4 gene, 12 subjects (13%) were excluded because of abnormal TSH levels, presence of TPO-Ab or Tg-Ab. The remaining 78 (50 females, 28 males) subjects were considered healthy for autoimmune diseases, with proven euthyroidism. The average (SD) age was 72.9 (7.6) years. The following ethnic groups were established according to self-referred skin color: 65.5% white, 20.5 % mulatto, and 14.1% black or 65.5% white and 34.6% non-white.

The study was approved by the Internal Research Board, and Informed Consent was obtained from all subjects.

CTLA-4 Polymorphism

Genomic DNA was extracted from peripheral blood mononuclear cells. CTLA-4 was amplified using PCR targeted to the exon 1. The PCR reaction was performed with sense 5' GCTCTACTTCCTGAAGACCT3' and reverse 5'AGTCTCACTCACCTTTGCAG3' primers, as follows: denaturation for 5 min at 94 °C, followed by 35 cycles of 1 minute at 94ºC, 1 min at 60ºC, 1 min at 71ºC, and final extension at 72 °C for 7 minutes. The PCR product was digested with Bbv1 restriction enzyme (MBI Fermentas ®, USA), and resolved in 2.5% agarose gel. The G_{49} allele can be digested by Bbv1 enzyme yielding two fragments of 88 and 74 bp. The A allele did not have a Bbv1 restriction site, retaining the 162-bp PCR product size. Genotyping was confirmed by GeneScan analysis (Applied Biosystems), performed in an automatic DNA sequencer, ABI-PRISM 310 Genetic Analyzer (Applied Biosystems).

Comparison with other Ethnic Groups

After determining the genotype of the 78 subjects, we have compared the frequency of the +49A>G polymorphism of our population considered as normal for the main autoimmune diseases with the control populations from other ethnic groups reported in the literature.

Statistical Analysis

The frequency of the genotypes and alleles among the Brazilian population and other ethnic groups were compared using a chi-square test (x^2) using Minitab 14 software (Minitab, Inc., State College, PA). $P<0.05$ value was considered as statistically significant.

Results

The G Allele was identified in 29.5% of all subjects, 30% in white subjects, 28% in mulatto, and 27% in black subjects. When considering black and mulatto as a group, the G allele presented a frequency of 27.7%. Comparing our data to those reported by other Brazilian authors, there was no significant difference in the frequency of G allele. In contrast, Guzman et al (2005) observed the G allele in 30% of the white and in 35% of the non-white group. Omar et al (2005), observed the G allele in 37% of the controls without race distinction (table 1).

When comparing the frequency of the +49A>G polymorphism of CTLA-4 alleles of the Brazilian population with Asian populations, we observed a statistically significant difference, with a higher frequency of the G allele in the Japanese, Chinese, Korean and Russian individuals (table 2).

On the other hand, European and American control populations, considered essentially Caucasian, presented similar frequency of the +49A>G polymorphism to the Brazilian Southeast population, both in the frequency of genotype or G allele (table 3).

The comparative analysis of the frequency of G allele with other populations, such as the Lebanese, African-American showed no significant difference (table 3). However, when compared to the essentially Scandinavian (Norwegian) population, we found a significant difference, as seen in table 2. When comparing the Czech control group with our data, we found statistical significance only when compared for frequency of the G allele (table 3).

Discussion

The CTLA-4 +49A>G polymorphism was widely associated with autoimmune diseases in several ethnic groups (Vaidya and Pearce 2004). It is presented as a potential genetic marker for a functional change in CTLA-4 protein, reducing its inhibitory effect on T cells, becoming a determinant factor in the development of auto-immunity (Maurer, Loserth et al. 2002; Ban, Davies et al. 2003; Takara, Kouki et al. 2003).

Several authors structured control groups using different criteria such as ethnicity, and the absence of personal and family history of autoimmune diseases. In control groups, the absence of ATD is rarely confirmed, or even the presence of thyroid antibodies, conditions clearly related to the CTLA-4 polymorphism (Tomer, Greenberg et al. 2001; Zaletel, Krhin et al. 2006). Few authors planned effectively representative control groups such as Zalloua *et al.* (2004) who selected individuals older than 25 years and not presenting anti-GAD. Lee *et al.* (2000) considered as adequate controls, those adults older than 60 years, with clinical and laboratorial absence of diabetes. When studying the CTLA-4 gene in Chinese children with GD, Yung *et al.* (2002) proposed a control group with the local population, without a family history of autoimmune disease, and with negative TAb. However, most of the studies published used race as the criteria and the absence of autoimmune diseases in the clinical history.

In the present study, we selected a group of subjects that could be considered normal for most autoimmune diseases, in particular for the study of the association of a polymorphism, such as the CTLA-4 gene. We chose an elderly population, with an average mean (SD) age of 72.9 (7.6) years, considering that most of autoimmune diseases would already have manifested by this age.

In addition to the clinical history, we also studied the thyroid function for the presence of either hypothyroidism or hyperthyroidism, as well as for the presence of TAb. Therefore, only those who presented no clinical history of autoimmune diseases, with normal thyroid function and negative TAb were examined for +49A>G polymorphism of the CTLA-4 gene. From the 78 subjects studied, 23% presented Type 2 diabetes (T2D), which is not positively correlated with CTLA-4 as a risk factor for T2D (Rau, Braun et al. 2001).

The +49A>G polymorphism of the CTLA-4 gene in the 78 subjects evaluated showed that the frequency of the G allele did not differ from data reported by two other Brazilian authors. Guzman *et al.* (2005) used race as a unique

criterion, and Omar *et al.* (2005) used the absence of autoimmune diabetes and the similar ethnic distribution, without antibodies measurement. Although this might represent a bias in these studies, since we have shown that even in a population that was believed to be normal for auto-immunity, 13% of the subjects were excluded due to abnormal thyroid function or the presence of TAb. We found no statistical difference in the frequency of the +49A>G polymorphism. This could be explained by a possible lack of association of G polymorphism and ATD in the Brazilian population.

When our control population was compared to other control groups published in the literature, we identified a variation according to the ethnic group and geographic region. Our ethnic origin, especially in the elderly population from the Southeast region of the country, is predominantly Caucasian. The smaller frequency of the G allele in the Brazilian population compared to Asian populations might be explained by the Asian immigration being a more recent event than the European immigration, and the proportion of Asian immigrants being smaller than the Caucasian population in the Brazilian Southeast (Alves-Silva, da Silva Santos et al. 2000).

Our data on Brazilian individuals is similar to the European, American (Kouki, Gardine et al. 2002) or UK (Heward, Allahabadia et al. 1999) Caucasian population. The frequency of G allele was also similar in Brazilian, Italian (Petrone, Giorgi et al. 2005) and Spanish (Marron, Raffel et al. 1997) control individuals. This could be explained by the influence of the Italian and Spanish immigrants in the Brazilian Southeast. When comparing the African-Brazilian population to African-Americans, we also did not find significant difference (Chen, Nadell et al. 2000), possibly a reflection of how the polymorphism of the CTLA-4 gene can behave in the genetic lines of African origin.

The frequency of the G allele observed in Asian populations differs significantly, which might explain the significant associations of ATD in Asians, and the polymorphisms of the CTLA-4. There are several studies showing the association of the CTLA-4 +49A>G polymorphism with ATD or T1D in Japanese and Chinese individuals. Chinese and Japanese controls also present higher frequency of G allele than the control Brazilian population. The G allele is present in 61% of the Japanese controls (Yanagawa, Taniyama et al. 1997), 65.4% of the Chinese in Taiwan(Lee, Huang et al. 2000), and 66.8% of the Chinese from Hong-Kong (Yung, Cheng et al. 2002), and the most pronounced frequency of the polymorphism in Koreans with 72.7% (Cho, Chung et al. 2006). Curiously, in the Korean population Cho *et al.* (2006) did not find any association of the +49A>G polymorphism with GD in adults, which differed from other Asians.

In the healthy control Norwegian population, there is a significant increase in G allele frequency (42%) when comparing with the Brazilian population

(Lorentzen, Celius et al. 2005), which can be related to a higher prevalence of T1D observed in the Scandinavian-origin populations. The frequency of CTLA-4 polymorphism in Brazilian subjects with individuals from different ethnic backgrounds is significantly different. These results reinforce the importance of studying population variation of the SNP CTLA-4 polymorphism according to region and ethnic groups.

Therefore, we concluded that in order to establish an appropriate control group it is essential that a stringent criteria must be defined to recognize its actual association with autoimmune diseases. The ethnic origin must obviously be considered in the analysis since we have shown that the CTLA-4 polymorphism differs between non-Caucasian, such as the Asian population, and the Brazilian population. We suggested the inclusion of individuals older than 60 y.o to avoid later detection of autoimmune disease in controls. We also demonstrated that it is important to confirm euthyroidism and the absence of anti-thyroid antibodies as inclusion criteria in the control group, improving the quality of the association studies with CTLA-4 polymorphism.

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Table 1- CTLA-4 +49A>G polymorphism in Brazilian control populations from the Southeast region

CTLA-4											
	Cury *et al* n (%) [A]					Guzman *et al* [1] n (%)					Omar *et al*[2] n (%)
	Total	**White**	**Mulatto**	**Black**	**Non-white**	**Total**	**White**	**Mulatto**	**Black**	**Non-White**	**Total**
Genotype	n=78	n=51	n=16	n=11	n=27	n=279	n=103	n=97	n=79	n=176	n=75
AA	39 (50)	24 (47)	9 (56)	6(54.5)	15(55.5)	124(45)	50(48.5)	47(48.5)	27(34)	74(42)	30 (40)
AG	32 (41)	23 (29)	5 (31)	4(36.4)	9(33.5)	127(46)	45(44)	37(38)	45(57)	82(46.5)	34 (45)
GG	7 (9)	4 (24)	2 (13)	1 (9.1)	3(11)	28(9)	8(7.5)	13(13.5)	7(9)	20(11.5)	11 (15)
Allele	N=156	n=102	n=32	n=22	n=54	n=558	n=206	n=194	n=158	n=352	n=150
A	110(70.5)	71 (70)	23 (72)	16 (73)	39(72)	375(68)	145(70)	131(67.5)	99(63)	230(65)	94 (63)
G	46 (29.5)	31 (30)	9 (28)	6 (27)	15(28)	183(32)	61(30)	63(32.5)	59(37)	122(35)	56 (37)

Literature reference data from [1] Guzman *et al.* (2005); [2] Omar *et al.* (2005)

[A] No statistically significant difference was found between control groups ethnically-matched in any of the comparisons

Table 2 - Comparisons of CTLA-4 +49A>G polymorphism allele and genotype frequencies in control groups from Brazilian, Spanish, Russian, Korean, Japanese and Chinese populations

	CTLA-4									
	Brazilian Population n (%)			Spanish[1] n (%) [A]	Norwegian[2] n (%) [B]	Russian[3] n (%) [C]	Korean[4] n (%) [C]	Japanese[5] n (%) [C]	Chinese[6] Taiwan n (%) [C]	Chinese[7] Hong-Kong n (%) [C]
	Total	White	Non-white							
Genotype	n=78	N=51	n=27	n=57	n=509	n=93	n=471	n=200	n=91	n=158
AA	39 (50)	24 (47)	15(55.5)	23(40.3)	168(33)	25(27)	30(6.37)	34(17)	9 (9.9)	23(14.6)
AG	32 (41)	23 (29)	9(33.5)	29(50.9)	249(49)	38(41)	197(41.83)	88(44)	45 (49.4)	59(37.3)
GG	7 (9)	4 (24)	3(11)	5(8.8)	92(18)	30(32)	244(51.8)	78(39)	37 (40.7)	76(48.1)
Allele	n=156	N=102	n=54	n=114	n=1018	n=146	n=942	n=400	n=182	n=316
A	110 (70.5)	71 (70)	39(72)	75(66)	585(57.5)	88(47.3)	257(27.28)	156(39)	63 (34.6)	105(33.2)
G	46 (29.5)	31 (30)	15(28)	39(34)	433(42.5)	98(52.7)	685(72.72)	244(61)	119 (65.4)	211(66.8)

Literature reference data from [1] Marron *et* al (1997); [2]Lorentzen *et al* (2005); [3]Chistyakov *et al.* (2000); [4] Cho *et al.* (2006); [5]Yanagawa *et al.* (1997); [6] Lee *et al.* (1999); [7]Yung (2002)

[A] No statistically significant difference was found between Brazilian control group and Spanish populations
Brazilian Genotype (Total) *vs* Spanish, P= 0,502
Brazilian Genotype (White) *vs* Spanish, P= 0,782
Brazilian Genotype (Non-white) *vs* Spanish, P= 0,319
Brazilian Allele (total) *vs* Spanish, P= 0,488
Brazilian Allele (White) *vs* Spanish, P= 0,651
Brazilian Allele (Non-white) *vs* Spanish, P= 0,511

[B] Statistically comparison between Brazilian control group and Norwegian controls
Brazilian Genotype (Total) *vs* Norwegian, p=0.008
Brazilian Genotype (White) *vs* Norwegian, P= 0,06
Brazilian Genotype (Non-white) *vs* Norwegian, P= 0,055
Brazilian Allele (total) *vs* Norwegian, P= 0,003
Brazilian Allele (White) *vs* Norwegian, P= 0,023
Brazilian Allele (Non-white) *vs* Norwegian, P= 0,045

[C] Statistically significant difference was found between controls group in all of the comparisons

Table 3 - Comparisons of CTLA-4 +49A>G polymorphism allele and genotype frequencies in control groups from Brazilian, United Kingdom (UK), Italian, Caucasian American, Canadian/German, African American, Lebanese and Czech population

	CTLA-4										
	Brazilian Population n (%) [4]			UK[1] n (%)	Italian[2] n (%)	Caucasian[3] American n (%)	Canadian[4] German n (%)	African[5] American n (%)	Lebanese[6] n (%)	Czech[7] n (%)
	Total	White	Non-white							
Genotype	n=78	N=51	n=27	n=363	n=301	n=80	n=466	n=47	n=96	n=289
AA	39 (50)[a]	24 (47)	15(55.5)[a]	164(45.1)	139(46)	30(37.5)	183(39)	23(48.9)	53(55)	106(37)
AG	32 (41)[a]	23 (29)	9(33.5)[a]	171(47.1)	138(46)	36(45)	215(46)	19(40.4)	39(40)	133(46)
GG	7 (9)[a]	4 (24)	3(11)[a]	28(7.8)	24(8)	17(17.5)	68(15)	5(10.6)	4(5)	50(17)
Allele	n=156	N=102	n=54	n=726	n=602	n=160	n=932	n=94	n=192	n=578
A	110 (70.5)[a]	71 (70)	39(72)[a]	498(68)	416(69)	96(60)	581(62)	65(69.1)	145(75.5)	345(60)
G	46 (29.5)[a]	31 (30)	15(28)[a]	228(32)	186(31)	64(40)	351(38)	29(30.9)	47(24.5)	233(40)

Literature reference data from [1]Heward *et al.* (1999); [2]Petrone *et al.* (2005); [3]Kouki *et al.* (2002); [4]Donner *et al.* (1997) Caucasians controls from Berlin (n=383) and Toronto (n=83); [5]Chen *et al.* (2000); [6]Zalloua *et al.* (2002); [7]Cinek *et al.* (2001);

[a] No statistically significant difference was found between control groups ethnically-matched in any of the comparisons

CONCLUSÕES

O polimorfismo +49A>G do gene CTLA-4 não demonstrou contribuição determinante para o desenvolvimento da doença de Graves em adultos e em crianças. De acordo com os resultados, esse polimorfismo não confere maior risco ou menor proteção ao desenvolvimento da doença de Graves. Também não foi demonstrada qualquer associação desse SNP com oftalmopatia de Graves em adultos ou com os marcadores bioquímicos de gravidade na apresentação clínica.

O SNP +49A>G do gene CTLA4, marcador de susceptibilidade para doenças auto-imunes, apresentou tendência de maior freqüência em pacientes com doença de Graves; ocorreu em 36% das crianças, 39% dos adultos e em 30% da população do grupo de controle, mas não atingiu diferença estatística relevante. Ao analisar a freqüência do alelo G entre as crianças púberes e pré-púberes, não se observou qualquer resultado estatisticamente significativo (resultados não publicados).

O fator limitante do estudo foi o número de pacientes analisados, com um poder estatístico baixo, ou seja, de 34%. Mas, pela baixa incidência da doença de Graves na infância, o número ideal de pacientes só seria possível com projetos do tipo multicêntrico. Portanto, o estudo alcançou o objetivo principal de analisar um importante SNP do gene CTLA-4 na doença de Graves, em brasileiros com faixas etárias distintas.

A natureza poligênica das doenças auto-imunes é evidente quando observamos a heterogeneidade das apresentações clínicas, sendo que o SNP +49A>G parece não conferir, isoladamente, maior risco em brasileiros com doença de Graves, mas desperta interesse quanto a outros marcadores genéticos que poderiam estar envolvidos, a outros polimorfismos do gene CTLA-4 como o CT60, ou a outros genes relacionados com o controle das células T, como o PTPN22.

A não associação do polimorfismo 49+A>G do gene CTLA-4 com marcadores bioquímicos ou clínicos da doença de Graves, como a oftalmopatia de Graves, está de acordo com outros estudos da literatura. Entretanto, alguns autores, principalmente em estudos com populações asiáticas, encontraram associação desse polimorfismo com a presença de auto-anticorpos tireoidianos, gravidade e recorrência da doença após interrupção do tratamento com fármacos antitireoidianos.

O segundo artigo valida os resultados do primeiro e confirma a necessidade da estruturação da população do grupo de controle com igual ou maior rigor do que se exige ao incluir pacientes em estudos de associação do tipo caso-controle. Ficou demonstrado que a freqüência do polimorfismo 49+A>G do gene CTLA-4 ocorre de maneira absolutamente distinta quando se comparam populações controles ocidentais (caucasiana e brasileira) com asiáticas (chinesa, japonesa e coreana), em que os genótipos GG e AG são freqüentes e podem esclarecer a forte correlação e associação das doenças auto-imunes com o polimorfismo do gene CTLA-4. Este estudo também verificou que, na população brasileira considerada normal para a auto-imunidade, o alelo 49G apresenta freqüência similar à dos indivíduos caucasianos europeus normais, possivelmente refletindo a origem étnica da sociedade brasileira.

Outro dado do segundo estudo foi a constatação da necessidade de afastar a presença de disfunções tireoidianas (hipotireoidismo e hipertireoidismo clínico ou subclínico), bem como a presença de auto-anticorpos tireoidianos, para o estabelecimento do grupo de controle adequado, visando ao estudo das doenças tireoidianas auto-imunes. Ao encontrar a presença dos auto-anticorpos tireoidianos ou de disfunção tireoidiana, foram excluídos aqueles que normalmente seriam candidatos a “grupo” de controle, se fosse considerada apenas a história clínica. Apesar da prevalência das disfunções tireoidianas aumentar com a idade, como a maior frequência dos anticorpos anti-peroxidase e anti-tireoglobulina, a maioria dos trabalhos publicados que analisam o polimorfismo do CTLA-4 não comprova o

eutireoidismo e não verifica a presença de anticorpos durante a composição do grupo de controle, o que pode comprometer a análise dos resultados de estudos de associação genética em qualquer etnia.

Outra consideração merece destaque e diz respeito à concordância dos resultados do segundo artigo, quanto à freqüência deste polimorfismo, com os resultados de outros autores brasileiros que estudaram o polimorfismo do gene CTLA-4 na população normal.

Portanto, os estudos realizados apontam para a necessidade da análise de outros polimorfismos genéticos relacionados com o controle da resposta imune na população brasileira com doença de Graves, os quais servirão como marcadores para as doenças tireoidianas auto-imunes e trarão maior entendimento sobre sua patofisiologia. Salienta-se a importância dos critérios usados para a estruturação da população controle a fim de validar ainda mais os trabalhos de associações genéticas para as doenças de maior prevalência mundial.

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ANEXOS

MINISTÉRIO DA SAÚDE
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PARECER Nº 163/2005

Registro CONEP: 11012 (Este nº deve ser citado nas correspondências referentes a este projeto)

Registro CEP: 036/04 **Processo nº 25000.151568/2004-51**
Projeto de Pesquisa: *"Mutações do Gene CTLA 4 e Associações com endocrinopatias auto-imunes"*
Pesquisador Responsável: Dr. Adriano Namo Cury (orientando)
Dr. Osmar Monte (orientador)
Instituição: Irmandade da Santa Casa de Misericórdia de São Paulo
Área Temática Especial: Genética Humana

Ao se proceder à análise do projeto de pesquisa em questão, em resposta ao Parecer nº 2411/04, cabem as seguintes considerações:

a) as informações enviadas relativas à adequação do Termo de Consentimento Livre e Esclarecido, atendem aos aspectos fundamentais da Res. CNS 196/96 sobre diretrizes e normas regulamentadoras de pesquisas envolvendo seres humanos;

b) o projeto foi aprovado pelo Comitê de Ética em Pesquisa – CEP da instituição supracitada.

Diante do exposto, a Comissão Nacional de Ética em Pesquisa – CONEP, de acordo com as atribuições definidas na Res. CNS 196/96, manifesta-se pela aprovação do projeto de pesquisa proposto.

Situação: Projeto aprovado.

Brasília, 28 de janeiro de 2005.

WILLIAM SAAD HOSSNE
Coordenador da CONEP/CNS/MS

IRMANDADE DA SANTA CASA DE MISERICÓRDIA DE SÃO PAULO
Comitê de Ética em Pesquisa em Seres Humanos
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PABX (11) 21767000 Ramal: 5502/5710 - Fax-2176-7041E-mail: eticamed@santacasasp.org.br

São Paulo, 26 de agosto de 2005.

Ilmo.(a). Dr.(a)
Sr.(a). Maria José Pedro Placeres
DD. Membro do Comitê de Ética em Pesquisa - SC

Projeto nº: 036/04
Título: "Mutações do gene CTLA 4 e associações com Endocrinopatias autoimunes."
Pesquisador Responsável: Dr. Adriano Namo Cury

Estamos encaminhando Adendo, relativo ao projeto supracitado, para proceder a sua análise e emitir parecer.

Atenciosamente,

Prof. Dr. Daniel R. Muñoz
Presidente do Comitê de Ética de Pesquisador
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De: **Zanzerl, Marc** (m.zanzerl@karger.ch)
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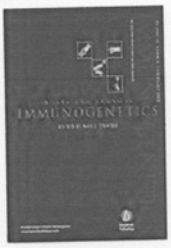

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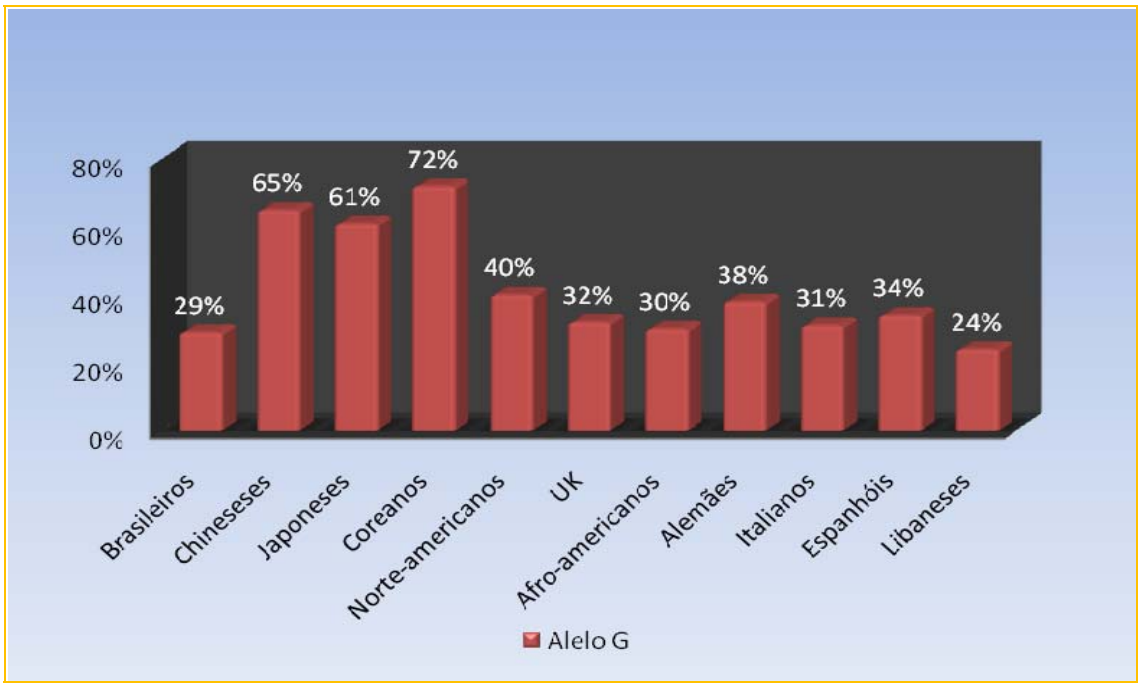


Gráfico 1- Freqüência do Alelo G entre populações controles de diferentes etnias

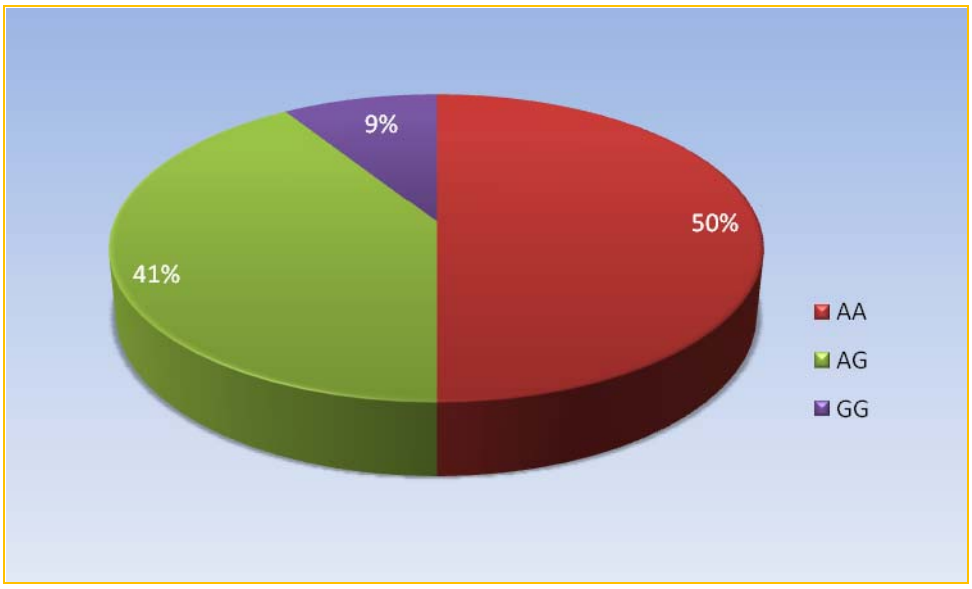
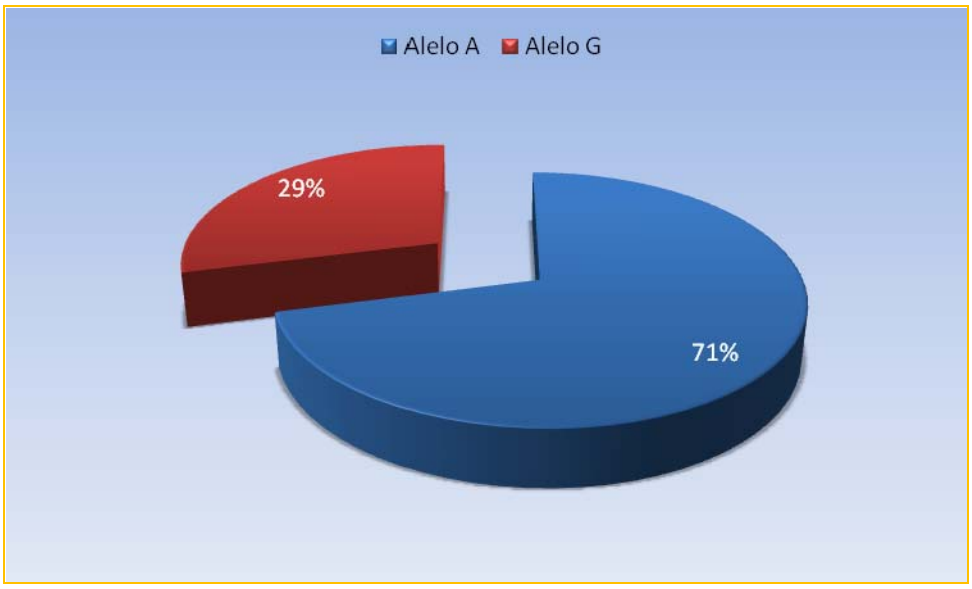


Gráfico 2- Genótipos do gene CTLA-4 na população brasileira controle para doenças auto-imunes



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