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ORIGINAL ARTICLE

The association between the *HLA-G* 14-bp insertion/deletion polymorphism and type 1 diabetes

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Type 1 diabetes (T1D) is a multifactorial disease that has a strong genetic component. The *HLA-G* is a nonclassical *HLA* class I locus that is associated with immunomodulatory functions, including downregulation of innate and adaptive immune responses and induction of immune tolerance. However, there is currently limited information about the involvement of *HLA-G* in T1D susceptibility. This case-control study aims to investigate the T1D susceptibility association of alleles and genotypes of a widely investigated 14-bp insertion/deletion polymorphism in the *HLA-G* and to provide further evidence of the frequency distribution of class II *HLA-DR-DQ*-risk genotypes in T1D children and adolescents in the Brazilian population. The deletion allele and the homozygous deletion genotype are associated with susceptibility to T1D and the insertion allele and the heterozygous deletion/insertion genotype are associated with protection from T1D. We also confirm that genetic susceptibility to T1D is associated with the *DRB1*03:01-DQA1*05:01-DQB1*02:01* and *DRB1*04-DQA1*03:01-DQB1*03:02* haplotypes in Brazilian northeast region. The DR3-DQ2/DR4-DQ8 genotype conferred the highest detected risk for T1D. Our results identify a novel association of the 14-bp deletion allele and the homozygous deletion genotype with T1D development and provide additional evidence of the importance of *HLA* class II heterozygous DR3-DQ2/DR4-DQ8 genotype in T1D susceptibility.

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INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disorder that results from β-cell destruction, usually leading to absolute insulin deficiency.[1] Numerous studies have demonstrated that the human leukocyte antigen gene (*HLA*) harbors genetic risk factors for T1D. The age of onset for TD1 is typically between 8 and 12 years.[2] However, there has been a marked increase in T1D incidence in children under age 5 in the last decade, and there is an alarming prediction that this will double by 2020.[3]

The contribution of *HLA* appears to be the most substantial when T1D is diagnosed before age 5 years, especially if the high-risk DR3-DQ2/DR4-DQ8 genotype is present.[4–6] This heterozygous genotype, which is composed of DRB1*03:01-DQA1*05:01-DQB1*02:01 and DRB1*04:01/02/05-DQA1*03:01-DQB1*03:02 haplotypes, has been linked to the formation of transdimers, which can bind and present unique sets of auto-antigen-derived peptides, that may ultimately lead to pancreas β-cell destruction and development of T1D.[7,8]

HLA contains an unusually high density of genes involved in immune functions, many of which are potential candidates for T1D susceptibility. Among these, a nonclassical *HLA* class Ib gene, *HLA-G*, is quite promising.[9] The *HLA-G* gene is composed of 8 exons, 7 introns, a 5′-promoter region (5′-URR) and a 3′-untranslated region (UTR).[10] The *HLA-G* primary transcript generates seven alternative mRNAs that encode membrane-bound (HLA-G1, -G2, -G3, -G4) and soluble (HLA-G5, -G6, -G7) protein isoforms.[11]

The HLA-G proteins possess the ability to bind to inhibitory receptors that are expressed by B and T cells, natural killer cells, and dendritic cells,[12,13] which are selectively located in immunologically protected sites such as the thymus[14] and pancreatic islets.[15] In pancreatic islets, HLA-G proteins were detected within insulin secretory granules, and the HLA-G proteins surface expression is regulated in response to growth, inflammatory and secretory stimuli.[15] As insulin granules and sites of insulin exocytosis may represent targets where a high density of potentially immunogenic ligands become exposed, local HLA-G could prevent the activation of low-affinity cytotoxic T cells, thus acting as a mediator in T1D immune tolerance.[15]

Compared with the classical class I loci *HLA-A* and *HLA-B*, the *HLA-G* gene has less polymorphism.[12] Several genetic variations in the 5′-URR and 3′-UTR have been identified that could potentially affect the expression of *HLA-G*, as variation in these regions can alter binding affinity for transcription factors, mRNA stability and microRNA targeting.[16] The 14-nucleotide deletion polymorphism, referred to as the 14-bp insertion/deletion (Ins/Del) polymorphism, in the 3′-UTR region of *HLA-G* has been shown to regulate alternative splicing of the mRNA isoforms, *HLA-G* mRNA expression levels, and HLA-G protein levels for most of the membrane-bound and soluble isoforms.[17]

Various *HLA-G* expression profiles for the 14-bp Ins/Del polymorphic site are associated with pathological conditions, including transplantation,[18,19] autoimmune and inflammatory diseases,[20,21] viral/parasite infections[22–24] and malignancies,[10]

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but there is no clear consensus about these results. Studies have suggested that the deletion allele and homozygous deletion genotype,[18–20,24,25] the insertion allele and insertion homozygous genotype[23,26,27] and the heterozygous genotype[21,28] are all associated with high risk of developing a pathological condition.

No literature information was found about the involvement of the *HLA*-G 14-bp Ins/Del polymorphism in T1D. Thus, the objective of the present study was to evaluate, for the first time, the association of alleles and genotypes of the 14-bp Ins/Del polymorphism from *HLA-G* with the susceptibility to T1D. Moreover, it provides additional data concerning the frequency distribution of class II *HLA-DR-DQ*-risk genotypes in T1D children and adolescents in the Brazilian population. Significant differences in the frequencies of alleles and genotypes of the 14-bp Ins/Del *HLA-G* polymorphism were detected. The deletion allele (Del) and the homozygous deletion genotype (Del/Del) were associated with the susceptibility to T1D.

RESULTS

Subject characteristics

Demographics and clinical data for each group are listed in Table 1. As expected, serum glucose and glycated hemoglobin values were significantly higher in the diabetic groups compared with NG controls ($P < 0.05$).

HLA-G allele and genotype association with T1D

Allelic and genotypic frequencies of *HLA-G* 14-bp Ins/Del polymorphism in T1D and NG groups are presented in Table 2.

HLA-G typing was performed for 81 of 92 T1D patients and 100 NG subjects. The genotypic frequencies were in accordance with Hardy–Weinberg equilibrium in both T1D and NG groups (Hardy–Weinberg equilibrium *P*-value = 0.585 and 0.142, respectively). The genotypic frequencies between the two groups were significantly different ($P = 0.0452$). The 14-bp Del allele was overrepresented in the T1D group when compared with the NG group ($P = 0.0263$, odds ratio; OR = 1.68). The homozygous genotype for the 14-bp Del/Del was overrepresented in the T1D group when compared with the NG group ($P < 0.0220$, OR = 2.07). The heterozygous 14-bp Ins/Del ($P = 0.1366$, OR = 0.63) and the homozygous 14-bp insertion allele (Ins/Ins) ($P = 0.2091$, OR = 0.48) genotypes were underrepresented in the T1D group when compared with the NG group, although the latter two was not statistically significant. After Bonferroni correction none of the associations remained with significant.

HLA-DR-DQ diplotype association with T1D

DR-DQ genotypes were categorized on the basis of susceptible diplotypes: combinations of *DRB1*03:01-DQA1*05:01-DQB1*02:01* (DR3-DQ2) and *DRB1*04-DQA1*03:01-DQB1*03:02* (DR4-DQ8) haplotypes (Table 3).

The DR3-DQ2/DR4-DQ8 heterozygote diplotype, which represents the high-risk diplotype, was overrepresented ($P < 0.0001$) in the T1D group (29.3%, 27/92) when compared with the NG group (1%, 1/100). In contrast, the low-risk diplotype, DRX-DQX/DRX-DQX, which was composed of haplotypes other than DR3-DQ2 or DR4-DQ8, was overrepresented ($P < 0.0001$) in the NG group (66%, 66/100) when compared with the T1D group (16.3%, 15/92). The comparisons of frequencies for DR3-DQ2/DR4-DQ8 and DRX-DQX/DRX-DQX diplotypes remained significant even after a Bonferroni correction. The moderate-risk diplotypes, which were composed of the homozygous genotypes DR3-DQ2/DR3-DQ2 and DR4-DQ8/DR4-DQ8 and the genotypes composed of only one of susceptibility haplotypes (DR3-DQ2/ DRX-DQX, DR4-DQ8/DRX-DQX), together were also significantly more frequent ($P = 0.0035$) in the T1D group (54.3%, 50/92) compared with the NG group (33%, 33/100), standing out, the DR3-DQ2/DR3-DQ2 and DR4-DQ8/DR4-DQ8 diplotypes. A statistically significant linkage disequilibrium between the 14pb Ins/Del *HLA-G* locus and *HLA-DR-DQ* loci was not evidenced.

Age at T1D onset and *HLA-DR-DQ* diplotypes

Table 4 lists the frequencies and OR values for selected *HLA-DR-DQ* diplotypes, which were stratified according to the age of T1D onset.

The DR3-DQ2/DR4-DQ8 diplotype was significantly increased in the T1D group with age at onset < 5 years when compared with the group with age at onset > 5 years ($P = 0.020$, OR = 3.05). Approximately 43% of individuals with T1D that were diagnosed before the age of 5 years carried this high-risk diplotype. In contrast, the moderate- and low-risk diplotypes were more frequent in children with age at onset > 5 years. The DR3-DQ2/DRX-DQX moderate-risk diplotype frequency was underrepresented in the T1D group with age at onset < 5 years when compared with the group with age at onset > 5 years ($P = 0.046$, OR = 0.14).

DISCUSSION

T1D is a multifactorial disease with a strong genetic component.[29] The class II *HLA* genes, which include *DRB1*, *DQA1* and *DQB1*, are well-known susceptibility loci for T1D.[30] Significant T1D associations have been reported for the classical *HLA-A*, *-B* and *-C* loci, which suggests that there may be additional susceptibility loci within the *HLA* region.[31,32] It has been proposed that class II *HLA*

Table 1. Demographics and clinical data for NG and T1D groups

	NG	*T1D*	*Age at T1D onset subgroups*	
			> 5 years	*< 5 years*
n	100	92	55	37
Gender (female/male)	58/42	58/34	40/15	18/19
Age (years)	11.8 ± 4.4	11.3 ± 4.1	12.8 ± 3.0	9.2 ± 4.6[a]
Time of diagnosis (years)	—	4.4 ± 3.8	3.6 ± 2.9	5.6 ± 4.6
Age at onset (years)	—	6.9 ± 3.3	9.1 ± 2.3[a]	3.6 ± 1.3[a, b]
Glucose (mg dl^{-1})	81 (48–99)	222 (59–540)[c]	243 (59–540)[c]	213 (79-492)[c]
Glycated hemoglobin (%)	5.4 (3.0–7.5)	9.5 (4.8–17.8)[c]	10.3 (4.8–17.8)[c]	9.0 (4.8-16.6)[c]

Abbreviations: *n*, number of individuals; NG, normoglycemic group; T1D, type 1 diabetic group. Results are expressed as mean ± s.d. or median (interquartile range). [a]Significant compared with the T1D group ($P < 0.05$) as determined using ANOVA and *post hoc* analysis with a Tukey's test. [b]Significant compared with the > 5 years age at onset group ($P < 0.05$) as determined using ANOVA and *post hoc* analysis with a Tukey's test. [c]Significant compared with NG ($P < 0.05$). Testing by Kruskal–Wallis and *post hoc* analysis with Dunn's test.

Table 2. Frequency distribution of *HLA-G* alleles and genotypes for 14pb Ins/Del polymorphism for the NG and T1D groups

HLA-G		*NG* (n = *100*)		*T1D* (n = *81*)[b]		P-*value (Overall)*	P-*value*[a]	*OR*	*95% CI*
		n	%	n	%				
Alleles	Del	120	60	116	71.6	**0.0263**[c]	—	1.68	1.08-2.62
	Ins	80	40	46	28.4				
Genotypes	Del/Del	32	32	40	49.4	**0.0452**[d]	< **0.0220**	2.07	1.13-3.80
	Ins/Del	56	56	36	44.4		0.1366	0.63	0.35-1.13
	Ins/Ins	12	12	5	6.2		0.2091	0.48	0.16-1.43

Abbreviations: CI, confidence interval; OR, odds ratio; *n*, number of individuals; NG, normoglycemic group; T1D, type 1 diabetic group. [a]Determined by comparing each genotype against the others using a Fisher's exact test. [b]We analyzed 81 of the 92 T1D patients. [c]Determined using a Fisher's exact test. [d]Determined using a χ^2-test. Statistically significant values (*P* values < 0.05) are highlighted in bold.

Table 3. Frequency distribution of high-risk, moderate-risk and low-risk *DR-DQ* diplotypes in the NG and T1D groups

Diplotypes HLA DR-DQ		*NG* (n = *100*)		*T1D* (n = *92*)		P-*value*[a]	*OR*	*95% CI*
		n	%	n	%			
High-risk	DR3-DQ2/DR4-DQ8	1	1.0	27	29.3	< **0.0001**[b]	41.1	5.5-310.2
Moderate-risk	DR3-DQ2/DR3-DQ2[c]	0	0.0	7	7.6	**0.01**	17.6	1.0-313.4
	DR3-DQ2/DRX-DQX[d]	6	6.0	10	10.9	0.29	1.91	0.67-5.49
	DR4-DQ8/DR4-DQ8[c]	0	0.0	6	6.5	0.01	15.1	0.8-272.2
	DR4-DQ8/DRX-DQX[d]	27	27.0	27	29.3	0.75	1.12	0.60-2.11
Low-risk	DRX-DQX[d]/DRX-DQX[d]	66	66.0	15	16.3	< **0.0001**[b]	0.10	0.05-0.20

Abbreviations: CI, confidence interval; OR, odds ratio; *n*, number of individuals; NG, normoglycemic group; T1D, type 1 diabetic group. [a]Determined using a Fisher's exact test. [b]Remained significant even after a Bonferroni correction. [c]Haldane correction was applied. [d]DRX-DQX = those other than DR3-DQ2 or DR4-DQ8. Statistically significant values (*P* values < 0.05) are highlighted in bold.

Table 4. Frequency distribution of high-risk, moderate-risk and low-risk *DR-DQ* diplotypes in the age at T1D onset groups

Diplotypes HLA DR-DQ		*Age at T1D onset subgroups*				P-*value*[a]	*OR*	*95% CI*
		> 5 years (n = *55*)		*< 5 years* (n = *37*)				
		n	%	n	%			
High-risk	DR3-DQ2/DR4-DQ8	11	20.0	16	43.2	**0.020**	3.05	1.21-7.70
Moderate-risk	DR3-DQ2/DR3-DQ2	5	9.1	2	5.4	0.698	0.57	0.10-3.11
	DR3-DQ2/DRX-DQX	9	16.4	1	2.7	**0.046**	0.14	0.02-1.12
	DR4-DQ8/DR4-DQ8	3	5.5	3	8.1	0.682	1.53	0.29-8.03
	DR4-DQ8/DRX-DQX[b]	19	34.5	10	27.0	0.499	0.70	0.28-1.75
Low-risk	DRX-DQX[b]/DRX-DQX[b]	8	14.5	5	13.5	1.000	0.92	0.28-3.06

Abbreviations: CI, confidence interval; OR, odds ratio; *n*, number of individuals; NG, normoglycemic group; T1D, type 1 diabetic group. [a]Determined using a Fisher's exact test. [b]DRX-DQX = those other than DR3-DQ2 or DR4-DQ8. Statistically significant values (*P* values < 0.05) are highlighted in bold.

genes determine the initiation of autoimmunity, whereas class I *HLA* genes regulate the progression of β-cell damage.[33]

Unlike the classical *HLA* loci, the nonclassical *HLA-G* locus has been linked to immunomodulatory activities, including down-regulation of innate and adaptive immune responses and induction of immune tolerance.[17] As T1D is an autoimmune disease where the breakdown of tolerance is directly implicated in disease development, the *HLA-G* locus might have an important role in this process.

This study aimed to investigate, for the first time, the association of alleles and genotypes of the 14-bp Ins/Del polymorphism of the *HLA-G* gene with T1D susceptibility. In addition, we assessed the distribution of *HLA* class II-risk haplotypes and genotypes in a T1D population in the state of Rio Grande do Norte, Brazil.

To this point, the 3′-UTR of the *HLA-G* gene contains 16 polymorphisms that have been described in the literature and are suggested to be involved in the differential *HLA-G* expression profile.[16] The 14-bp Ins/Del polymorphism is among the most widely studied polymorphisms within this region. It is has been implicated in the regulation of protein expression, the modulation of *HLA-G* mRNA stability and in microRNA targeting.[34] However,

the exact mechanisms and functional significance of this polymorphism are not well understood.

Insertion of the 14-bp segment between positions +2961 and +2974 in exon 8 is suggested to result in an alternative splicing variant in which 92-bp are removed from the primary mRNA, and this truncated mRNA is more stable than the mRNA fragment that is generated by the 14-bp Del.[17] Despite this increase in mRNA stability, several studies have indicated that the 14-bp Ins decreases the protein levels of both soluble and membrane-bound HLA-G in blood plasma and serum; however, the 14-bp Del results in high HLA-G isoforms in blood plasma and serum.[11]

Cirulli *et al*.[15] demonstrated that HLA-G is constitutively expressed in the endocrine compartment of the human pancreas, specifically in insulin secretory granules. Moreover, it may be upregulated at the cell surface of primary islet cells that have been stimulated to secrete insulin. HLA-G may prevent the activation of autoreactive T cells, potentially by regulating immunogenic ligands present at sites of insulin exocytosis.

Significant differences in the frequencies of alleles and genotypes of the 14-bp Ins/Del *HLA-G* polymorphism were detected within the studied groups. The Del allele and the Del/Del genotype are associated with susceptibility to T1D given their higher frequencies in the T1D group when compared with the NG group. The Ins allele and the Ins/Del genotype are associated with protection from T1D, as they are present at higher frequencies in the NG group, although not statistically significant.

The allelic frequencies and the most frequent genotypes of polymorphic sites in the *HLA-G* regulatory regions, including the 14-bp Ins/Del polymorphism in 3′-UTR, have been investigated for individuals from different geographic Brazilian regions.[11,35,36] In general, a high frequency of the Del allele and the Del/Del genotype was detected in healthy subjects from the northeast, the southeast and southern Brazil. Therefore, the results here could be biased by the overrepresentation of the Del allele in the Brazilian population.

Studies involving the 14-bp Ins/Del polymorphic profile and the expressed HLA-G proteins present conflicting results. The expression of HLA-G1 (a membrane-bound isoform) was determined to be higher for the 14-bp Ins compared with the 14-bp Del in a study using human cell lines.[37] Studies in autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis have reported a higher frequency of the 14-bp Ins allele and the homozygous 14-bp Ins/Ins genotype, which are associated with lower HLA-G plasma levels.[38] In contrast, other studies have reported increased frequency of the 14-bp Del allele and homozygous Del/Del genotype that exhibit higher levels of HLA-G in blood plasma in affected patients compared with control subjects.[39,40]

A report involving type 2 diabetes, which is a disease characterized by obesity, inflammation and insulin resistance, indicated that there are no significant differences in the genetic distribution of the 14-bp Ins/Del *HLA-G* polymorphism in the type 2 diabetes group compared with a control group.[41] In contrast, a separate report provided evidence of higher expression of soluble HLA-G in type 2 diabetes patients when compared with a normal glucose tolerance group.[42]

Very little is known about the *HLA-G* gene as a risk factor for T1D development or protection. A conditional analysis using family-based haplotype estimates (T1DGC MHC data set) identified several T1D-associated SNPs in a region of *HLA* that is near *HLA-G*, but none was detected within the regulatory 3′-UTR region where the 14-bp Ins/Del polymorphism is located (rs1704).[43] Therefore, to the best of our knowledge, this is the first study evaluating the association between T1D and the 14-bp Ins/Del polymorphism of *HLA-G*.

We also propose that the genetic susceptibility to T1D is strongly associated with the *HLA-DRB1*03:01-DQA1*05:01-DQB1*02:01* (DR3-DQ2) and *DRB1*04:01/02/05-DQA1*03:01-DQB1*03:02* (DR4-DQ8) class II haplotypes. In particular, both haplotypes combined into a single genotype (DR3-DQ2/DR4-DQ8) conferred the highest risk for T1D, and our results are consistent with previous studies that were conducted in Latin America[44,45] southeastern Brazil[46] and northeastern Brazil.[47] In addition, the presence of the *HLA-DR-DQ*-risk diplotypes in combination with the *HLA-G* 14-bp Ins/Del genotypes was assessed, and a higher frequency of individuals who carried the DR3-DQ2/DR4-DQ8 high-risk diplotype in combination with the Del/Del genotype or the Ins/Del genotype was detected in the T1D group when compared with the NG group.

Although no significant linkage disequilibrium has been evidenced between the 14pb Ins/Del *HLA-G* locus and HLA-DR-DQ loci in the present study, Hviid and Christiansen[48] identified a significant association between *HLA-DRB1*03* and the *HLA-G*01:01:02* alleles, which is included in the 14-bp sequence polymorphism, in women with recurrent spontaneous abortions. In recent studies using the T1DGC MHC data set, Eike *et al*.[9] identified SNPs and microsatellite regions[49] that are located in the vicinity of *HLA-G* (the closest is located 3.5 kb upstream of the *HLA-G* coding region) as additional risk factors for T1D, which are independent of the well-established *DRB1-DQA1-DQB1* loci.

The DR3-DQ2/DR4-DQ8 high-risk genotype was also significantly associated with age at T1D onset < 5 years. This genotype was accompanied by a threefold increased T1D risk in the younger age group. Our results are consistent with previous studies in which differences in genetic susceptibility for T1D are related to genetic acceleration of the disease process.[2,50,51] A decrease in the prevalence of the high-risk genotypes and an increase of moderate- and low-risk genotypes was detected in T1D patients with age at T1D onset > 5 years. These results suggest that there is a greater influence of environmental factors[52] as well as other *HLA*[53] and non-*HLA* loci[6,54] on susceptibility to T1D in this age at onset group when compared with the younger age at onset group.

In conclusion, our results suggest, for the first time, that there is an association of the 14-bp Del allele and the homozygous 14-bp Del genotype with T1D development. Considering the high frequency of this allele and genotype in the general population and taking into account the existence of other polymorphic loci in LD with the *HLA-G* variant, further studies are warranted to establish a definitive relationship of this genetic variation with T1D disease development and progression. Studies investigating the HLA-G protein levels should also be conducted to better understand the effects of phenotypes from this polymorphism and others in the 3'-UTR for T1D. In addition, our results provided evidence for an important role of *HLA* class II genes in susceptibility to T1D, particularly through the heterozygous DR3-DQ2/DR4-DQ8 genotype and its combination with the *HLA-G* 14-bp Del allele.

MATERIALS AND METHODS

Subjects

This study was conducted with 92 unrelated T1D patients (6–20-year old) that were recruited in the period of January 2010 to December 2011, from the Pediatrics Endocrinology Unit, Pediatrics Hospital of the Federal University of Rio Grande do Norte, in Natal, Rio Grande do Norte, Brazil. One hundred NG subjects (fasting serum glucose < 99 mg dl^{-1}), with the same age and sex of the T1D patients, were recruited from local public schools, during the same study period. T1D patients were categorized into two subgroups: those with age at onset < 5-year old and those with age at onset > 5-year old. Exclusion criteria included history of alcohol intake, smoking, other inflammatory diseases, infections and pregnancy. All T1D patients were on insulin treatment. The University Hospital Onofre Lopes Human Research Ethics Committee, in accordance with Brazilian law, which complies with the Declaration of Helsinki, approved the study (protocol number 207/09). Written free consent was obtained from participants and/or their parents. After taking medical history and performing physical

examination, fasting blood was obtained from all subjects for biochemical analysis and genotyping of peripheral blood mononuclear cells.

Biochemical measurements

Glycemic controls were assessed using glycated hemoglobin from total blood and serum glucose. Measurements were performed using Labtest kits (Lagoa Santa, Minas Gerais, Brazil). Labmax Plenno (Labtest) was used to perform serum glucose measurements, and a RA 50 spectrophotometer (Bayer Diagnostics, Dublin, Ireland) was used to measure glycated hemoglobin.

HLA genotyping

Genomic DNA was obtained from peripheral blood mononuclear cells that were isolated using a discontinuous Ficoll-Hipaque (Sigma-Aldrich, St Louis, USA) gradient with a specific density of $1.070\,g\,ml^{-1}$, at room temperature. Extraction was performed using an Illustra Triple Prep kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer instructions. DNA was stored at − 20 °C until the time of analysis.

The *HLA-G* 14-bp Ins/Del genotypes in the 3′-UTR of exon 8 of the *HLA-G* locus (rs1704) were analyzed in 81 of 92 T1D patients and 100 NG subjects, according to the following protocol: 200 ng of genomic DNA was amplified in a 25 µl reaction mixture containing 0.20 mM dNTP (Ludwig, Alvorada, Rio Grande do Sul, Brazil), 0.2 mM of each primer, 0.5 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 1.5 mM $MgCl_2$ and a 1× PCR buffer (0.2 M Tris–HCl pH8.5, 0.5 M KCl). After an initial denaturation step at 94 °C for 5 min, samples were submitted to 30 additional cycles at 95 °C for 45 s, 56 °C for 45 s and 72 °C for 30 s, with a final extension cycle at 72 °C for 10 min with 5′-TGTGAAACAGCTGCCCTGTGT-3′ as the forward primer and 5′-GTCTTCCATTTATTTTGTCTCT-3′ as the reverse primer. The amplification products were evaluated using a 3.5% agarose gel. The presence of a 345-bp fragment corresponded to the deletion allele, while a 359-bp fragment corresponded to the 14-bp insertion allele.

HLA class II genes (*DRB1, DQA1* and *DQB1*) were genotyped using PCR sequence-specific oligonucleotides on a Luminex multianalyte system with a LABType sequence-specific oligonucleotides commercial kit (One Lambda, Inc., Canoga Park, CA, USA) as previously described.[55,56] The results were analyzed using HLA Fusion v 1.2.1. software.

Statistical analysis

Distribution of clinical and laboratory variables was analyzed using a Kolmogorov–Smirnov test. Differences between groups of normally distributed variables were calculated using the parametric test analysis of variance (one-way analysis of variance) followed by *post hoc* analysis with a Tukey's test. For a non-normally distributed variables was used the nonparametric test Kruskal–Wallis followed by Dunn's multiple comparisons test and this data were presented as median ± interquartile range. The allelic and genotypic frequencies for *HLA-DRB1*, *DQ* and *HLA-G* 14-bp Ins/Del polymorphisms were calculated using a direct count method for all groups. A Haldane's correction was applied where the allele frequency was zero to compute the corrected odds ratio by adding 0.5 to each cell.[57] The frequencies of the most probable *HLA-DRB1-DQA1-DQB1* haplotypes, adherences of genotypic proportions to Hardy–Weinberg equilibrium expectations and pairwise linkage disequilibrium were calculated using ARLEQUIN version 3.5.[58] The statistical significance for the allelic, haplotypic and genotypic frequencies between the T1D group and the NG group and with the age at onset groups was calculated with a two-sided simple χ^2-test or Fisher's exact test (accurate for small sample sizes) using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). GraphPad Prism 5.0 software was used to calculate the OR and its 95% CI. Statistical significance was defined as $P < 0.05$. For statistical haplotype and genotype association, the *P*-value was corrected using a Bonferroni test by dividing the previously established *P*-value by the number of haplotypes and genotypes tested.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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