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Association of *HLA-G* 3′ untranslated region variants with type 1 diabetes mellitus

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ABSTRACT

Besides the well recognized association of *HLA-DRB1* and *DQB1* alleles with type 1 diabetes mellitus (T1D), linkage studies have identified a gene region close to the non-classical class I *HLA-G* gene as an independent susceptibility marker. HLA-G is constitutively expressed in the endocrine compartment of the human pancreas and may play a role in controlling autoimmune responses. We evaluated the genetic diversity of the 3′ untranslated region (3′UTR) of *HLA-G*, which have been associated with *HLA-G* mRNA post-transcriptional regulation, in 120 Brazilian T1D patients and in 120 healthy controls. We found the +3001 T allele was observed only in T1D patients. Notably, the +3001 T allele was in linkage disequilibrium with polymorphic sites associated with low production of *HLA-G* mRNA or soluble HLA-G levels. Moreover, T1D patients showed a low frequency of the *HLA-G* 3′UTR-17 (14bpINS/+3001T/+3003T/+3010C/+3027C/+3035T/+3142G/+3187A/+3196C). The +3010 CC genotype and the UTR-3 haplotype (14bp DEL/+3001C/+3003T/+3010C/+3027C/+3035C/+3142G/+3187A/+3196C), associated with low and moderate soluble HLA-G expression, respectively, were underrepresented in patients. The decreased expression of HLA-G at the pancreas level should be detrimental in individuals genetically prone to produce less HLA-G.

1. Introduction

Type 1 diabetes mellitus (T1D) is a multifactorial genetically complex autoimmune disease, in which the destruction of pancreatic beta cells is mediated by humoral and cellular immune responses. Genetic susceptibility to T1D has been primarily attributed to the genes of the major histocompatibility complex (MHC) at chromosome 6p21.3. MHC encompasses many genes actively involved in the coding of several molecules responsible for antigen presentation (HLA-A, -B, -C, -DR, -DQ and -DP) to T lymphocytes or molecules that modulate (HLA-E, F and G) the function of many leukocytes leukocytes [1,2]. HLA class II genes contribute up to 50% of the susceptibility to T1D, especially *HLA-DRB1**03 and *04 allele groups [3], and *DQA1**05:01-*DQB1**02:01 and *DQA1**03:01-*DQB1**03:02 allele combinations [4]. A study mapping the MHC region, performed on a large number of families with T1D patients, identified the region of the *HLA-G* gene as an independent locus for

Abbreviations: T1D, Type 1 diabetes mellitus; 3′UTR, 3′ untranslated region; SNP, single nucleotide polymorphism; MHC, major histocompatibility complex; HLA, human leukocyte antigen; NK, natural killer; CTL, cytotoxic T lymphocytes; HWE, Hardy–Weinberg equilibrium; EM, expectation-maximization; 5′URR, promoter; LD, linkage disequilibrium.

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disease susceptibility [5]. In addition, another reason to evaluate HLA-G in diabetes is that HLA-G soluble levels have been implicated on impaired glucose metabolism and is linked to a IL-6, a biomarker of insulin resistance [6].

HLA-G was first identified at the maternal-fetal interface, being expressed by cytotrophoblasts of the placenta, where it contributes to the lack of rejection of the fetus (semi-incompatible graft) by the mother. HLA-G is indeed a well-recognized tolerogenic molecule that interacts with the leukocyte receptors ILT2 (LILRB-1, CD85j), ILT4 (LILRB2, CD85d), CD160 (BY55) and KIR2DL4 (CD158d) inhibiting the function of natural killer (NK) and cytotoxic T lymphocytes (CTL) and modulating antigen-presenting cells [7,8]. ILT2 and ILT4 also interact with several HLA classical class I molecules, but have greater affinity for HLA-G [9], and KIR2DL4 is a specific receptor for HLA-G [10].

In adult tissues, HLA-G is expressed in the cornea, thymus and β pancreatic cells [11]. In the latter tissue, the expression of HLA-G is regulated in response to growth and inflammatory stimuli, and it may exhibit regulatory functions in human pancreatic islets, playing an important role in the progression to autoimmunity, as well as in the establishment of transplant tolerance to pancreatic islets [12]. In non-physiological conditions, such as autoimmune disorders, the expression of HLA-G has been associated with a lesser morbidity [13].

The coding region of the *HLA-G* gene shows similarity to the classical HLA class I loci, containing seven introns and eight exons; however, *HLA-G* polymorphism at the coding region is limited in relation to the classical HLA class I genes [14]. The first exon encodes the signal peptide, and exons 2, 3 and 4 encode the extracellular α1, α2 and α3 domains of the heavy chain, respectively. Exons 5 and 6 encode the transmembrane and cytoplasmic domains of the heavy chain, and exon 7 is always absent in the mature *HLA-G* mRNA. Due to the presence of a stop codon at exon 6, exon 8 is not translated and has been considered to be part of the 3′ untranslated region (3′UTR) of the gene [15].

Considering the relatively low variability of the coding region of *HLA-G*, and that major biological functions of the molecule, such as dimerization and interaction with leukocyte receptors are apparently conserved for all described isoforms, it is likely that the magnitude of HLA-G expression in normal and pathological conditions may depend on the gene regulatory regions, i.e., 5′URR (promoter) and 3′UTR, as well as on microenvironment factors [15]. Both *HLA-G* regulatory regions have indeed a high degree of genetic variability and may influence the expression of HLA-G in complex processes modulated by several factors. At least 29 polymorphic sites have been described at the promoter region that may be target for transcriptional factors [16,17]. The *HLA-G* 3′UTR contains several post-transcriptional regulatory elements, and the most studied is the absence (deletion) or presence (insertion) of a fragment of 14 base pairs (14bp DEL/INS) that has been associated with the stability of *HLA-G* mRNA [18,19] and with soluble HLA-G levels [20]. In addition, the +3142C/G polymorphism has been associated with the magnitude of mRNA production, since the +3142 G allele may increase the affinity of this region for microRNAs (miR-148a, miR-148b and miR-152), decreasing the availability of *HLA-G* mRNA [19,21]. The +3187A/G polymorphism has been reported to affect mRNA stability due to its proximity to an AU-rich motif, which mediates the degradation of *HLA-G* mRNA [19,22]. Besides these polymorphic sites, other less studied single nucleotide polymorphisms (SNPs) at 3′UTR located at positions +3001C/T, +3003C/T, +3010C/G, +3027A/C, +3035C/T and +3196C/G may regulate *HLA-G* mRNA expression, and may represent potential targets for several microRNAs [23].

Given that: (i) the expression of HLA-G can influence the outcome of autoimmune diseases due to its tolerogenic properties, (ii) a previous linkage study has indicated that the *HLA-G* gene region confers susceptibility to T1D, (iii) the 3′UTR gene segment exerts post-transcriptional control of *HLA-G* mRNA production, in this study we evaluated the *HLA-G* 3′UTR variability in a cohort of T1D patients.

2. Patients and methods

2.1. Subjects

A total of 120 T1D patients (67 women) aged 6–63 years (mean 23.2 ± 12.1) followed-up at the Infant and Adult Outpatient Clinics of the Division of Endocrinology, Department of Medicine, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil, and 120 healthy individuals from the same geographical region of the patients and exhibiting no family history of T1D were studied. Controls were selected from a sample consisting of 155 individuals from a previous population genetics study [19], with case-control samples matched for age, sex, skin color in order to generate two homogeneous groups. The local Ethics Committee approved the protocol of the study (# 12542/2011) and all patients or their guardians gave written informed consent to participate.

2.2. Analyses of HLA-G 3′UTR variability

We collected 10 mL of peripheral venous blood from each individual into Vacutainer tubes (Becton Dickinson, Plymouth, England), containing EDTA K3 (0.054 mL/tube) for DNA extraction, using a salting out procedure [24]. The variability of the *HLA-G* 3′UTR was evaluated as previously described [18]. Briefly, DNA was amplified using the HLAG8R (5′-GTCTTCCATTTATTTTGTCTCT-3′) and HLAG8F (5′-TGTGAAACAGCTGCCCTGTGT-3′) primers. The amplification reaction was performed in a final volume of 25 μL, containing 1× amplification buffer (0.2 M Tris–HCl pH 8.5, 0.5 M KCl), 0.2 mM of each dNTP, 5 pmol of each primer, 1.5 mM $MgCl_2$, 0.5 U of Platinum DNA polymerase (Invitrogen, Carlsbad, CA) and 200 ng of genomic DNA. Cycling conditions included an initial step at 94 °C for 5 min, followed by 30 cycles at 95 °C for 45 s, 56 °C for 45 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min.

Each amplification product was directly sequenced using the HLAG8R primer in an automatic sequencer ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, CA), using the BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystems). In order to establish a quality control, some samples were cloned and sequenced.

The presence of a significant association between all *HLA-G* variation sites detected was evaluated by means of a likelihood ratio test of linkage disequilibrium (LD) [25] using the ARLEQUIN version 3.1 software [26]. The allele and genotype frequencies were estimated by direct counting, using the GENEPOP 3.4 program [27]. The adherence of observed genotypic frequencies to the proportion theory of Hardy–Weinberg equilibrium (HWE) was tested by the exact test of Guo and Thomson [28]. Given the positive association between pairs of SNPs but the unknown gametic phase, haplotypes of each individual were computationally inferred using the expectation-maximization (EM) [29] and PHASE [30] algorithms, by means of the PL-EM [31] and PHASE softwares [30], respectively. All cloned and sequenced samples confirmed the original haplotypes obtained by computational inference.

Allele, genotype and haplotype frequencies were compared between patients and controls using the Fisher exact test, and the odds ratio (OR) and 95% confidence interval (95%C.I.) were also estimated. Considering that the nine polymorphic sites observed at the *HLA-G* 3′UTR are included in a very small gene segment and considering that significant linkage disequilibria among pairs of